Sequential separation and characterization of cellulose, xylan and lignin from alkali pretreated sugarcane tops and utilization of cellulose for higher scale bioethanol production by recombinant enzyme saccharification and fermentation

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Partial Fulfilment of the Requirements for the degree of
Doctor of Philosophy

By

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Dedicated to

my Parents

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

School of Energy Science and Engineering

STATEMENT

I do hereby declare that the content embodied in this thesis entitled "**Sequential separation and characterization of cellulose, xylan and lignin from alkali pretreated sugarcane tops and utilization of cellulose for higher scale bioethanol production by recombinant enzyme saccharification and fermentation"** is the result of investigations carried out by me in the School of Energy Science and Engineering, Indian Institute of Technology Guwahati, Guwahati, India, under the guidance of Prof. Arun Goyal and Prof. Vijayanand S. Moholkar. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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Certificate

It is certified that the work contained in the thesis entitled "**Sequential separation and characterization of cellulose, xylan and lignin from alkali pretreated sugarcane tops and utilization of cellulose for higher scale bioethanol production by recombinant enzyme saccharification and fermentation"**, by **Mr. Kaustubh Chandrakant Khaire (Roll No. 176151005),** has been carried out under our supervision and that this work has not been submitted elsewhere for degree.

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Date: *Mr. Kaustubh Chandrakant Khaire*

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SYNOPSIS

Introduction

Unabated exploitation of non-renewable energy resources and their depletion has become a major concern today. The production of renewable fuels from agricultural waste is sustainable and economically feasible. In the tropical regions, *Saccharum officinarum* or Sugarcane is one of the major cultivated crops (Gopitha *et al.,* 2010). Worldwide approximately, 1.91 billion tonnes of sugarcane are produced [Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), USA, 2018]. Sugarcane trash/tops (SCT) is one of the most abundant agricultural wastes produced in India (Ensinas *et al.,* 2009). More than 199 million metric tons of sugarcane were reported to be produced in different regions of India (Sukumaran *et al.,* 2010). Owing to the huge quantity of production of SCT and the high cost involved in its collection and transportation, the trash is burnt in the field.

Sugarcane top is the field residue remaining after the harvesting of the sugarcane stalk, while sugarcane bagasse is the fibrous residue left after the milling of the sugarcane stalk. Chemical composition analysis of SCT showed 40% cellulose, 33% hemicellulose and 17% lignin content, making it an attractive feedstock for bioethanol production (Bhardwaj *et al.,* 2019). SCT can serve as the cheap and potential renewable raw substrate for biofuels (Sukumaran *et al.,* 2010). From 1 ton of sugarcane plant, about 140 kg of bagasse (Dias *et al.,* 2012) and 250 kg of SCT (Chandel *et al.,* 2012) are produced. Approximately, 50% of SCT can be removed from the agricultural field and used for bioethanol production without compromising the nutritional status of the soil (Hassuani *et al.,* 2005). Up to 66% (w/w) of SCT could be used in the bioethanol industries, if collected as dry leaves. This post-harvest SCT is a low-cost and readily available substrate for bioethanol production (Sukumaran *et al.,* 2010). The SCT can also be used as a raw material for pyrolysis to gas, char and oil production (Pighinelli *et al.,* 2014), gasification (Jorapur and Rajvanshi, 1995), methanol synthesis and anaerobic methane (Canilha *et al.,* 2012) and bioethanol fermentation (Sukumaran *et al.,* 2010). SCT is also used as a ruminant feed (fresh or dried) for cows and buffalo (Akinbode *et al.,* 2017). Hemicellulose separation before the hydrolysis of the cellulosic part of feedstock is the new approach to increasing the value of biomass. This

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concept can add value to the hemicellulose part and also can provide hemicellulose-free solids (cellulosic part) which can be a suitable feedstock for bioethanol production. Separated hemicellulose can be used in the papermaking, pharmaceutical and food industries (Sporck *et al.,* 2017). The use of both cellulosic and hemicellulosic fractions can also increase the economic value of agricultural waste. In this study, the cellulose was separated from SCT and investigated for its purity. There is no report on the separation and characterization of cellulose from sugarcane trash. This approach of separation of cellulose and xylan from SCT may increase the commercial value of both cellulose and hemicellulose components for their use in respective industries. The technique involving the recombinant cellulosic enzymes in the saccharification of cellulose separated from SCT is reproducible and is cost-effective as compared with the saccharification by commercial enzymes. The structural properties of separated cellulose demonstrated that it can be used as a commercial substrate as well as an alternate substrate for bioethanol production.

Present work

The present work entitled "Bioconversion of sugarcane tops to bioethanol and other value-added products" has been divided into 6 chapters.

Chapter 1 is a General Introduction, which focuses on the brief review of literature dedicated to the bio-economy, the lignocellulosic waste and the structural component of the plant cell wall and their applications. The increasing use of non-renewable resources and their continuous depletion has become a major concern nowadays. The production of renewable fuels from agricultural lignocellulose waste is sustainable and economically feasible. Sugarcane top is one of the most abundant lignocellulosic biomasses in India. More conveniently, it is burned in the field being in large quantity and the high cost involved in its collection and transportation. The pretreatment process is one of the cost-intensive steps in bioethanol production, owing to the manpower and expensive chemicals to separate cellulose, hemicellulose and lignin from lignocellulosic biomass. In recent times, the advent of novel recombinant carbohydrate-active enzymes from different microbial sources by genetic manipulation has led to great advances in saccharification of pretreated biomass leading to higher production of bioethanol. The multi-enzyme complexes called cellulosomes are capable of hydrolyzing cellulose and hemicellulose. This study is aimed at the

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production of xylan, bioethanol and other value-added products from sugarcane tops. Isolation, separation and characterization of xylan, cellulose and lignin from sugarcane tops can be the most efficient industry-oriented approach due to their xylooligosaccharides or alternative substrate, bioethanol and oil-based petrochemical applications. The saccharified hydrolysate can be used for bioethanol production by fermenting with *Saccharomyces cerevisiae.* Xylooligosaccharides can also be produced by enzymatic hydrolysis of xylan extracted from agro-waste and have applications in controlling blood sugar levels, boosting immunity, improving intestinal function and reducing fatigue. Xylan can be utilized for the production of xylooligosaccharides, food coating, packaging films and other pharmaceutical applications. Lignin is mainly used as a rubber intensifier, polyolefin and rubber packing. It is also used in making cement, composite materials, unsaturated polyester and vinyl ester as filler and co-monomer.

Chapter 2 describes the separation of cellulose from delignified sugarcane tops (SCT) by following the alkaline pretreatment and its characterization. Raw SCT is composed of cellulose (43%), hemicellulose (27%) and lignin (19%). As per the mass balance calculations, about 40 g of cellulose was recovered from 100 g of raw SCT after delignification followed by alkaline pretreatment. The monosaccharide analysis of SCT cellulose by HPLC showed 91% D-glucose, 7.5% D-xylose and 1.5% D-arabinose residues. A surface morphology study of dried cellulose by FESEM exhibited the fibrous structure. The FTIR analysis of separated cellulose displayed the peaks corresponding to the peaks obtained from commercial cellulose, confirming its purity. The crystallinity index (*CrI*) of separated cellulose increased to 49% after delignification and xylan extraction from 36% of raw SCT. The typical TGA curve of separated SCT cellulose showed decomposition and mass reduction at 327 °C resulting in a single decomposition peak in TGA analysis, confirming its purity. CHNS analysis supported the purity of separated cellulose by confirming the absence of nitrogen and sulfur. The separated cellulose was hydrolyzed by recombinant endo-β-1,4-glucanase (*Ct*Cel8A), and cellobiohydrolase (*Ct*CBH5A) from *Clostridium themocellum* and β-1,4-glucosidase (*Ht*Bgl) from *Hungateiclostridium thermocellum* at pH 5.8, 50 °C for 24 h, resulting in the production of total reducing sugar (TRS), 188 mg/g of SCT cellulose. The separated cellulose from SCT can be utilized as an alternative substrate for commercialization and bioethanol production.

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Chapter 3 elaborates the extraction of xylan from liquid alkaline hydrolysate produced after the alkaline pretreatment of delignified SCT (as described in chapter 2) and its characterization. About 25 g of xylan was extracted from 100 g of raw SCT. Compositional analysis of xylan extracted from SCT (SCTx) displayed the presence of 74% of d-xylose residues, 16% of D-glucuronic acid residues and 10% of L-arabinose. High performance size exclusion chromatographic analysis of SCTx displayed a single peak corresponding to a molecular mass of ~57 kDa. The Fourier transform infrared spectroscopic analysis of SCTx displayed the peaks corresponding to those obtained from commercial xylan. FESEM analysis of SCTx showed the granular and porous surface structure. Differential thermogravimetric analysis (DTG) of SCTx displayed two thermal degradation temperatures (T_d) of 228°C, due to the breakdown of the side chains of glucuronic acid and arabinose and 275°C, due to the breakdown of the xylan backbone. The presence of arabinose and glucuronic acid as a side chain was confirmed by the DTG and thermogravimetric analysis. The CHNS analysis of SCTx showed the presence of only carbon and hydrogen supporting its purity. The recombinant xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* displayed a specific activity of 1394 ± 51 U/mg with SCTx, which was higher than those with commercial xylans. The thin layer chromatography and electrospray ionization mass spectroscopy analyses of *Ct*Xyn11A hydrolyzed SCTx showed a series of linear xylooligosaccharides ranging from degree of polymerization 2-6 and no substituted xylooligosaccharides because of the endolytic activity of the enzyme. The extracted xylan from SCT can be used as an alternative commercial substrate and for oligo-saccharide production.

Chapter 4 describes the sequential extraction of xylan and lignin by precipitation from the alkali pretreated sugarcane tops (apSCT) hydrolysate after alkaline pretreatment of water soluble extractives free SCT without the delignification. The xylan extracted from apSCT hydrolysate (apSCTx) was 26.5 g and the alkali lignin (apSCTal) was 8.5 g from 100 g of raw SCT. The composition analysis of apSCTx by high performance liquid chromatography showed 74% D-xylose, 15% D-glucuronic acid and 11% L-arabinose residues. The analysis of apSCTx by high performance size exclusion chromatography showed a molecular size, ~58 kDa. The first derivative of thermogravimetric analysis of apSCTx showed a Td (thermal degradation temperature)

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at 275ºC. The elemental (CHNS) analysis of apSCTx displayed the presence of only carbon and hydrogen, supporting its purity. The specific activity of recombinant clostridial endoxylanase (*Ct*Xyn11A) was 1823 ±51 U/mg. Liquid Chromatography-Mass Spectroscopy and Thin-layer chromatography analyses of *Ct*Xyn11A hydrolyzed apSCTx displayed a series of linear xylooligosaccharides ranging between 2-5 degrees of polymerization. apSCTx can serve as a potential commercial substrate and also for xylooligosaccharides production. The characterization of apSCTal by Field-Emission Scanning Electron Microscopy showed the exposed spherical structures of alkaline lignin. Fourier Transform Infrared spectroscopic analysis of apSCTal displayed the peaks corresponding to those obtained from commercial alkaline lignin. CHNS analysis of apSCTal confirmed the absence of protein impurities. The first derivative of the thermogravimetric analysis of apSCTal showed T_d at 362ºC. The extracted apSCTal can be used as dispersant, emulsifier, binder and sequestrant. The biorefinery approach of sequential extraction of apSCTx and apSCTal from alkali pretreated SCT hydrolysate waste can enhance the economics of the extraction process.

Chapter 5 includes the optimization of saccharification of alkali pretreated SCT by using recombinant cellulolytic enzymes. The water-soluble extractives removal was carried out prior to alkali pretreatment of SCT without its delignification. The solid alkali pretreated SCT (apSCT) recovered on Field-emission scanning electron microscopy (FE-SEM) analysis showed the exposure of cellulosic fibres as compared with raw SCT. The analyses of apSCT by Fourier Transform Infrared (FT-IR) spectroscopy, X-ray diffraction (XRD) and High performance liquid chromatography (HPLC) analysis also confirmed the enhanced cellulose content in apSCT. Optimum conditions for response surface methodology based saccharification of apSCT at 40 °C, 150 rpm were 2.14% (w/v) apSCT loading in citrate-phosphate buffer (50 mM, pH 6.0), recombinant hydrolytic enzymes (from *Clostridium thermocellum*) loading for *endo*-1,4-β-glucanase (*Ct*Cel8A) = 213.2 U/g, cellobiohydrolase (*Ct*CBH5A) = 272.5 U/g and β-glucosidase (*Ht*Bg1) = 299.8 U/g for 49.2 h. Under optimized saccharification conditions, the TRS yield was 265 mg/g (7.95 g/L) of apSCT. The glucose yield determined in the TRS was 214 mg/g of apSCT. The fermentation of produced glucose by S. *cerevisiae* in 50 mL reaction volume gave 0.19 g/g glucose (1.2 g/L) of bioethanol.

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Chapter 6 describes about the optimization of saccharification of solid apSCT produced after alkaline pretreatment of water soluble extractives free SCT (as described in chapter 5) by commercial cellulolytic enzyme and its fermentation at a higher scale and economic evaluation. The saccharification of apSCT biomass was optimized for commercial cellulolytic enzyme loading, apSCT loading and saccharification time. The saccharification at the flask level in 20 mL reaction volume gave the optimized conditions of 3% (w/v) apSCT, 35 FPU/$g_{of\ apSCT}$ commercial enzyme loading and 48 h at constant pH 4.5 and 45°C which resulted in 67.2% (w/w of apSCT) cellulose conversion from apSCT with 18.6 g/L (672 mg/g) total reducing sugar (TRS). These optimized conditions used for the higher scale saccharification, at 3.6 L taking 120 g of apSCT resulted in 68.7% (w/w of apSCT) cellulose conversion with 18.8 g/L (687 mg/g) TRS. From the optimized saccharified hydrolysate, containing 18.8 g/L TRS, the fermentation of hydrolysate containing 15 g/L TRS (15 g/L) supplemented with 3 g/L yeast extract and 5% (v/v) consortium of fermenting microbes (*Saccharomyces cerevisiae, Pichia stipitis and Pachysolen tannophilus*, 1:1:1 from 10^7 CFU/mL) in 20 mL was carried out at initial pH 5, 100 rpm and 35°C for 48 h which resulted in 7.5 g/L of bioethanol. Under the same fermentation conditions, the higher scale bioethanol production in a 5 L bioreactor at 3 L working volume gave 7.43 g/L of bioethanol within 24 h with 96.9% fermentation efficiency. As per the mass balance calculations, water soluble extractives removal followed by alkaline pretreatment of 1 kg of raw SCT around 143 g of bioethanol was produced. The cost analysis of the process showed that around 114 USD is required to process 1 kg of raw SCT biomass and approximately, 291.6 USD can be generated from extracted commercial grade xylan and alkali lignin. Therefore, the calculated overall profit is 178.6 USD, without considering the profit from the bioethanol sale. These saccharification and fermentation conditions can be scaled up for further higher scale bioethanol production from SCT.

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Chapter 1

General Introduction

1.1 Introduction

The Indian economy depends on agriculture. Over half of the Indian populace legitimately relies upon agriculture for their business. Various crops are grown in a year in India attributable to the assorted variety in the atmosphere. Significantly, maize, sorghum, sugarcane, rice, wheat and other crops are grown in India (Birthal *et al.*, 2014). The consumable portion of the plant is utilized to draw energy for the metabolic working of living beings, while the non-consumable part is known as agro-waste. Every year around, 200 billion tons of lignocellulosic wastes from agriculture are produced worldwide (Gou *et al.*, 2010), while 200 million tons of lignocellulosic wastes are consistently produced in India (Singh and Gu, 2010A). The huge amount of agricultural waste produced each year is neither adding any financial advantages to the farmer nor it is useful for nature. The development of any nation, for the most part, depends on three columns, i.e., advancement at the economic, environmental and social levels. The financial improvement of any nation whether it is developing or developed relies intensely on the usage of non-renewable or fossil fuels (Chiranjeevi *et al.*, 2018). These resources perform an important role in making lifestyle simple at

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different levels of transportation, in-home appliances, in different industrial procedures for the fulfillment of our day to day needs. As a result of the restricted accessibility with unsustainable utilization of fossil fuels, the research shift from a linear economy (fossil-based) to a circular economy (bio-economy/ bio-based) is expanding (Chiranjeevi *et al.,* 2018). The transformation of renewable resources into economically important products like animal feed, human food, bioenergy/biofuel and bio-based industrial products production by biotechnological mediation is named as bioeconomy (Chiranjeevi *et al.,* 2018). Farming practices are the principal component of bioeconomy. The requirement for food and energy supply is expanding with the increase in population and it implies an incredible pressure on agribusiness cultivating and its processing industries (Rosenau-Tornow *et al.,* 2009).

Organic waste biomass produced from crops and food processing industries is knowned as lignocellulosic waste. Around $1.3x10^{10}$ metric tons of lignocellulosic waste is produced every year and its management is turning out to be a thriving issue (Liu *et al.,* 2019). In the last few years, the burning of lignocellulosic biomass by farmers to clean the cultivating land has contributed immensely towards global climate change, air and water pollution. The lignocellulosic waste biomass is rich in carbon and other important crucial supplements and consequently, it can be utilized for the production of bio-based products and bioenergy (Liu *et al.,* 2019). To satisfy the huge energy demand, the most ideal route is to use lignocellulosic wastes for energy production. The energy demands are rapidly expanding. In 2010, the energy demand was equivalent to the 13 billion tons of oil and it should rise to 45 billion tons of oil by 2050 (Zhang and Konan, 2010). The utilization of nonrenewable energy

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sources, for example, coal, oil and petroleum gas to meet the energy demand will cause exhaustion of their reserves. This additionally also prompts to increase in fuel cost with the expanding demand. To achieve energy necessity, renewable sources are focused such as atomic energy, bioenergy, hydro energy, solar energy and wind energy. Bioenergy contributes the most elevated share of 14% (18% considered as a100%) out of 18% of the total sustainable energy supply (Berndes *et al.,* 2003). Bioenergy is produced either from agricultural residues or the forest residues. The lignocellulosic waste collected from the agricultural field can be converted into the source of energy e.g. biodiesel, biohydrogen, bioethanol, biobutanol and methane, etc. In most of the countries, biofuels are produced as a sustainable alternative for reducing the consumption of rapidly depleting conventional fuels and harmful gas emissions (Bote *et al.,* 2020A).

The world is searching for an option of conventional and sustainable fuels such as biofuels are getting consideration from the recent two decades. Biofuels production includes three stages: pretreatment, saccharification and fermentation. The pretreatment delignifies the agricultural waste biomass and subsequently makes it available for enzymatic hydrolysis to release C5 and C6 monosaccharides. The released monosaccharides are fermented to produce biofuels with the assistance of fermenting microorganisms. Contingent upon the feedstock utilized for biofuel production, biofuels are arranged in the following generations: first generation, second generation, third generation and fourth generation biofuels. The first generation biofuels rely upon animal fats, edible oil seeds and crops. First generation biofuel production procedure is a basic, financially less costly (no requirement of pretreatment) and ecofriendly. Major disadvantage of biofuel production from edible sources is that, this competes with the

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food meant for human consumption. From cottonseed oil, 77% biodiesel alongwith 20% methanol production was accomplished by pretreatment with 0.5% (w/v) sodium hydroxide (Nabi *et al.,* 2009). In the second-generation biofuels nonedible lignocellulosic biomass such as grain straw, sugarcane bagasse and forest residual wastes are utilized. Lignocellulosic biomass is waste produced during the crop harvesting process and can serve as an alternative promising source for biofuels production (Berndes *et al.,* 2003). Bioethanol has advantages over other biofuels such as high heat of vaporization, high octane number (108), low boiling point and similar energy content (Sindhu *et al.,* 2019). Up to 85% (v/v) bioethanol blend in gasoline can be utilized in vehicles without alteration of the existing engine (Sindhu *et al.,* 2019). The use of petroleum as well as greenhouse gas emission can be significantly reduced by the bioethanol blending in gasoline. The main issue with the bioethanol is the high cost associated with its pretreatment strategies. Other significant cost requirements are the expense on collection and transportation of feedstock, chemicals, enzymes, detoxification process and ethanol recovery (Sindhu *et al.,* 2019). The enzymatic hydrolysis is one of the significant steps towards reducing the production cost (Sun *et al.,* 2002). The third-generation biofuel is centered around the usage of algal biomass for biodiesel production. Algae develops at a higher growth rate and has no food crop rivalry and can also utilize wastewater and seawater. The serious issues with the third generation biofuel are higher energy prerequisites for algal development, lower lipid content and more noteworthy odd is the contamination (Singh and Gu, 2010B). In fourth-generation biofuel feedstock utilized are green algae and different organisms with high growth rate, high lipid content and more CO_2 catching capacity. The obstacle in fourth-generation biofuel is a greater expense for the basic research.

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1.2 Types of waste

Waste is a wide term that involves anything useless. The accumulation of waste causes damage to the land, pollution and several health issues. Waste can be arranged on the accompanying premise:

I. The state of waste

a) Gaseous waste (Lim *et al.*, 2016)

b) Liquid waste (Chen *et al.*, 2016)

c) Solid waste (Chen *et al.*, 2016)

II. Waste source

a) Agricultural waste (Saini *et al.*, 2015)

b) Commercial waste (Andritsos *et al.*, 2016)

c) Industrial waste (Chandra and Chowdhary, 2015)

d) Municipal waste (Aslani *et al.*, 2018)

III. The physical, chemical and biological properties

a) Biodegradable waste (Saveyn *et al.*, 2014)

b) Non-biodegradable waste (Nielfa *et al.*, 2015)

To conquer the energy shortage and to dispose of the waste aggregation, methodology for the conversion of waste into energy was recommended (Kothari *et al.*, 2010). The agricultural waste is biodegradable, solid and lignocellulosic. The plant cell wall is made up of carbohydrates, lignin and proteins. Carbohydrates are made up of cellulose and hemicellulose. Cellulose is the most abundant polysaccharide made up of glucose and hemicellulose is heterogeneous having various side chains and its backbone is mainly made up of arabinose, xylose, glucose, mannose, etc. Other than cellulose and hemicellulose, pectins are also present in the cell wall.

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1.3 Lignocellulosic waste

Lignocellulosic waste is referred to as the plant dry matter or biomass. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin molecules (Harmsen et. al., 2013). The principle polysaccharides of primary cell walls are cellulose, hemicellulose and pectin. The hemicelluloses interact with celluloses to form a hemicellulose network with the cellulose micro-fibrils. The pectin contains nearly 30 % of dry weight in the primary cell wall (Chen, 2014). The secondary cell wall has more cellulose as compared to the primary cell wall but it lacks pectin, due to this the plants secondary cell wall is much harder. The basic component of the secondary cell wall is hemicellulose (Chen, 2014). The lignin is situated in the external cell wall of ligocellulosic biomass. The structure of lignocellulosic biomass is shown in Fig. 1.1.

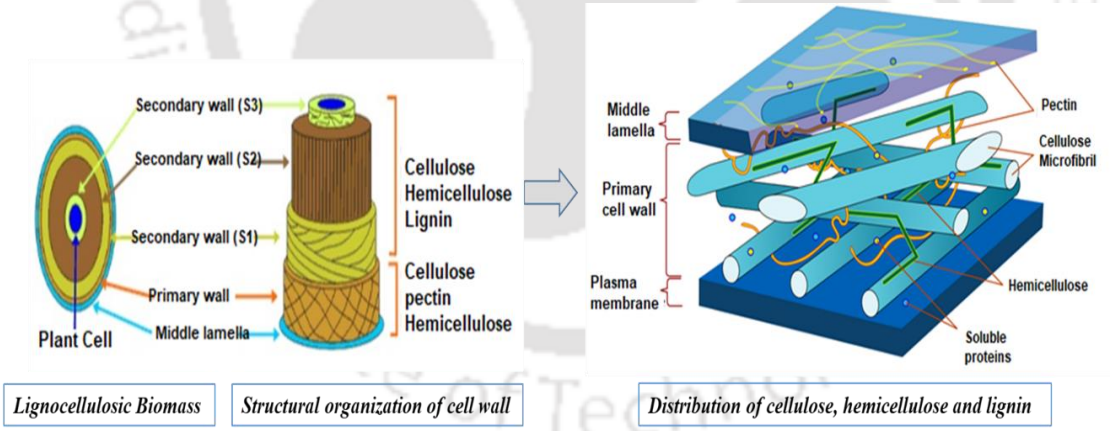

Fig. 1.1. Structure of lignocellulosic biomass (Ilnicka *et al.*, 2015).

Generally, cellulose is situated inside a lignin shell, while the hemicellulose with an irregular and undefined structure is situated within the cellulose and between the lignin and cellulose. The hydrogen bonding is present between the linin and cellulose, as well as the hemicellulose and cellulose. Moreover, covalent linkages, predominantly

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ether bonds, have been proposed to be present between lignin and cellulose (Hosoya *et al.,* 2007). Therefore, the lignin extracted from natural lignocellulose always contains some carbohydrate impurities (Hosoya *et al.,* 2007). The cell walls of plants mainly consist of hemicelluloses, cellulose and lignin in a ratio of 3:4:3 (Chen *et al*., 2014). The chemical structure of cellulose, hemicellulose and lignin is shown in Fig. 1.2. This ratio varies from source to source, for example, softwood, hardwood and grasses. Besides these molecules, lignocellulose also contains a small number of proteins and pectin. The elemental content of lignocellulosic material is around 50% Carbon, 44% Oxygen, 6% Hydrogen, and 0.05–0.4% Nitrogen (Chen, 2014). These lignocellulosic structural components have a great ability to prevent water loss from a plant leaves. The intercellular layer in the plant is mainly made up of pectic polymers, which are amorphous and have strong plasticity and hydrophobicity (Wu *et al.*, 2018). Pectin structure can easily break by acidic or enzymatic treatment leading to the separation of plant cells (Chen *et al*., 2012).

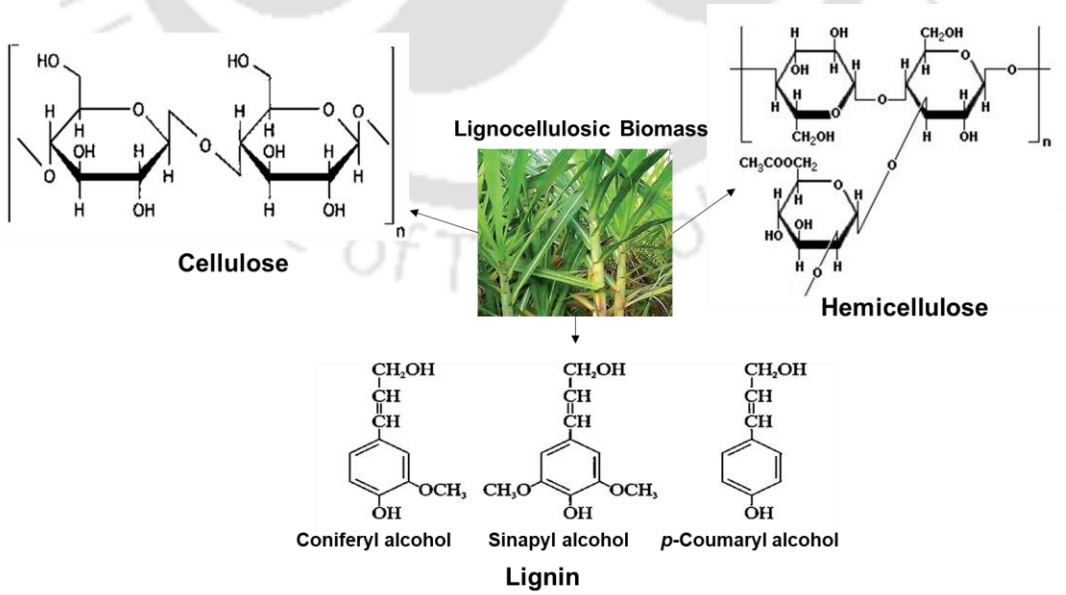

Fig. 1.2. Chemical structure of lignin, cellulose and hemicellulose.

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1.3.1 Cellulose

One of the principal structural components of the plant cell wall is cellulose. Cellulose accounts for about 15-30% dry weight of the total primary cell wall. Cellulose is a high molecular weight polysaccharide made up of repeating units of glucose monomers. Cellulose is strong, crystalline and hydrolysis resistant and hemicelluloses have an amorphous and random structure with little strength as compared to the cellulose (Balat *et. al*., 2009). The cellulose micro-fibrils form a parallel structure of the cell wall with a diameter of about 10–25 nm and the elementary fibril diameter is approximately, 2 and 4 nm. This systematic arrangement of cellulose fibers in micro-fibrils is called a micelle (Chen *et al.,* 2014). Cellulose is an organic compound $(C_6H_{10}O_5)_n$ is not soluble in dilute acid/alkali solutions and water at room temperatures. Cellulose contains 30–50% of the total lignocellulosic biomass with a worldwide production evaluated to be 120-140 billion tons per year (Wang *et al.,* 2016). Cellulose is principally present in plant cell walls. The composition of cellulose is reliant on the type of plant, species and age (Limayem and Ricke, 2012). The primary cell walls of all angiosperms and gymnosperms are dominantly made up of cellulosic polysaccharide. The cottonseed hair contains 91% of cellulose, which confirms its pure structure in contrast with wood cellulose. The cellulose is a polymer consisting of a straight chain of several thousand β-1→4 linkage of D-glucose units (Kulkarni *et. al.,* 2012). Every repeating β-D-glucopyranose unit is turned 180° to its next β-D-glucopyranose unit (Ten and Vermerris, 2013), which bring about the arrangement of the cellulose chain. These cellulose chains further aggregate or crosslink by framing intra- and intermolecular van der Waals and hydrogen bonding to create the cellulose microfibrils. The normal level of polymerization in the primary cell wall is

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approximately 6000 units and up to 14000 units of glucose in the secondary cell walls (Chan, 2014). Cellulose microfibrils are profoundly crystalline and hydrophobic, which contributes significantly to the recalcitrance of biomass (Harris *et al.,* 2009). Cellulose is a principle structural component of higher plants, bacteria, algae, other biomasses (the oomycetes and some bacterial species) can secrete it to produce biofilms (Chan, 2014).

1.3.2 Hemicellulose

Hemicellulose also called as polyose (Li et al., 2013). It is made up of several heteropolysaccharides such as arabinoxylans, which are present along with the cellulose in all plant cell walls. Hemicellulose polymers include arabinoxylan, xylan, glucomannan, glucuronoxylan and xyloglucan. These polymers contain various types of sugar monomers besides glucose. Other monosaccharide sugars in hemicelluloses include arabinose, mannose, xylose, galactose and rhamnose. In hemicellulose, a high amount of the D-sugars (C5) and sometimes small amounts of L-sugars (C5) are present. In most cases, the abundant monomeric sugar present in hemicellulose polymer is xylose although mannose can be the most abundant sugar present in softwoods. The modified form i.e. acidified sugars such as glucuronic acid and galacturonic acids are also be found in the hemicelluloses besides the regular sugar residues (Chen, 2014).

Hemicelluloses are easily hydrolyzed by dilute acid or base as well as the hemicellulolytic enzymes. Around 15-35% of total lignocellulosic biomass is made up of hemicellulose (Limayem and Ricke, 2012). Hemicelluloses are branched, single chain and amorphous heteropolysaccharide in nature. Hemicelluloses are composed of monomeric sugars, for example, pentose (arabinose and xylose), hexoses (glucose and mannose), uronic acids and acetylated sugars with degree of polymerization of 500-

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3000 monomeric units (Davison *et al.*, 2013). These D-pyranol sugars, for example, mannose and glucose are connected through β-1,4-glycosidic linkage at O4 position (Naidu *et al.*, 2018). The side chains of hemicelluloses are small in length, which makes hemicellulose non-crystalline in nature (Cunha and Gandini, 2010). The different functional groups present in branches and their number vary depending on the type of the biomass (Limayem and Ricke, 2012). Hemicelluloses are subdivided into four primary categories relying upon the 1→4 linked monosaccharides present in the main chain of polysaccharides, which are arabinans, xylans, mannans and xyloglucans (Scheller and Ulvskov, 2010). The most abundant hemicellulose is the xylan and its composition fluctuates among the species (Limayem and Ricke, 2012). The most significant role of hemicellulose is to add the strengthening of the plant cell wall by associating with the lignin and cellulose molecules (Scheller and Ulvskov, 2010).

1.3.3 Lignin

Lignin is a class of complex organic polymers present in the tissues of vascular plants and some algae that forms the important structural materials for their support. Lignin plays a significant role in the plant cell wall formation (bark and wood) that leads to the rigidity of plants. Chemically, lignins are cross-linked phenolic polymers (Hatfield *et. al.*, 1999). Lignin is an amorphous, highly branched and heterogeneous aromatic polymer that lacks primary structure. Lignin is made up of three fundamental phenylpropane monomers as repetitive units viz. *p*-coumaryl alcohol, sinapyl alcohol and coniferyl alcohol, which are commonly named as "monolignols." The softwood lignin is mostly made up of coniferyl alcohol, the hardwood lignin is made up of sinapyl alcohols and coniferyl alcohol, while the lignin present in grasses contains all the three previously mentioned monolignols (Duval and Lawoko, 2014). Lignin is present in the

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secondary cell wall of plants and contributes to the transportation of water and nutrients to the xylem in vascular plants by offering mechanical support to the cells. Its recalcitrance nature grants the resistance against plant pathogens (Baurhoo *et al.*, 2008). The composition of lignin differs from species to species. Lignin contains 63.4% Carbon, 30% Oxygen, 5.9% Hydrogen, 0.7% ash. A lignin molecule is unusual because of its heterogeneity and lack of definition to its primary structures. The lignin is fundamentally utilized as a fuel, yet it very well may be artificially altered to be utilized as a chelating agent (Gonçalves and Soto-Oviedo, 2002) for the expulsion of heavy metals (Stewart, 2008), or as a precursor for adhesives (Benar *et al.*, 1992), activated carbon (Fierro *et al.*, 2008) and surfactants (Chum *et al.*, 1985) like value-added product synthesis. Worldwide, the yearly commercial production of lignin polymer is around 1.1 million metric tons. It is used in a broad range of applications where the lignin purity is not required (Chen, 2014). Lignin is covered with the bundle cells, for example, sclerenchyma cells and wood fibers. It increases the rigidity of the cell wall. Lignin content in the plant cell wall varies from woody plants (27–32%) to herbaceous plants (14–25%) (Chen, 2014). The protective tissue of plants is also made up of wax, suberin, cutin and other polymers. The epidermis cell present on the plant surfaces is made up of cutin. The cork cells in plants secondary protecting tissue contain a suberin and cutin and sometimes it is covered with wax (Albersheim *et. al.*, 2010).

1.4 Selection of feedstock for second generation bioethanol production

Unabated exploitation of non-renewable energy resources and its continuous depletion is become a biggest concern for now a day. The production of renewable fuels from agricultural waste is sustainable and economically achievable. Worldwide, approximately 1.9 billion tonnes of sugarcane is produced [Food and Agriculture

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Organization Corporate Statistical Database (FAOSTAT), USA, 2016]. In tropical regions, Sugarcane (*Saccharum officinarum)* is one of the majorly cultivated crops (Gopitha *et al.*, 2010). Annual agro wastes produce in India is shown in Fig. 1.3.

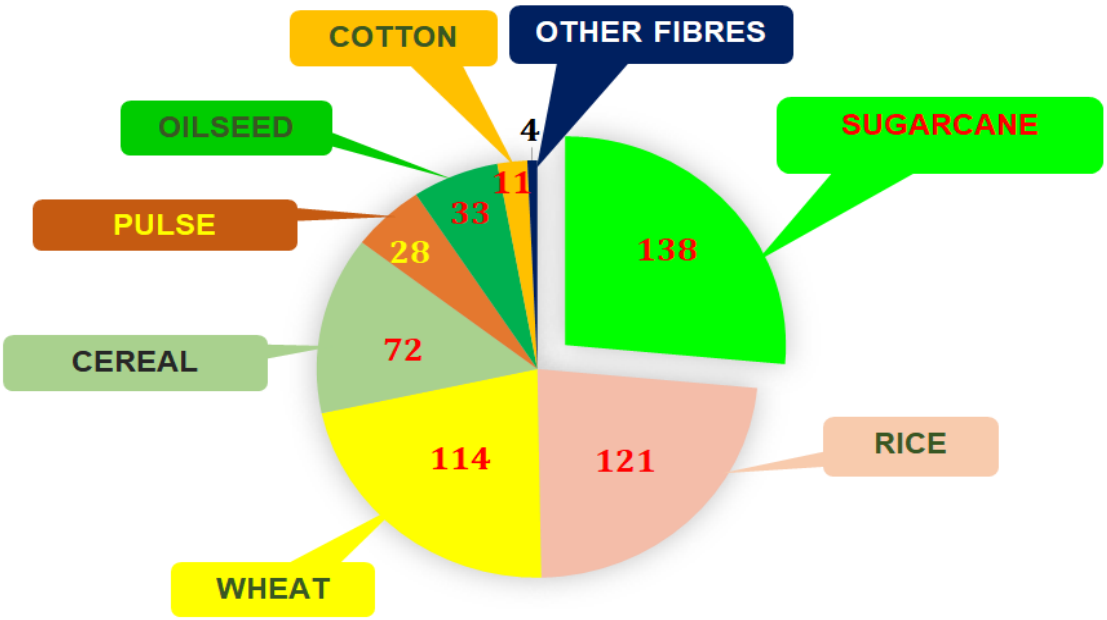

Fig. 1.3. Annual agriculture waste production in India (in Million Tons) (Report on State of Indian Agriculture 2015-16).

Sugarcane tops (SCT) is one of the most abundant agricultural waste produced in India (Chotirotsukon *et al.*, 2019). More than 199 million metric tons of sugarcane was reported to be produced in different regions of India in 2009 (Sukumaran *et al.*, 2010). The sugarcane plant is showed in Fig. 1.4A and the dried leaves and tops (Fig. 1.4B) are often called as sugarcane top/trash (SCT).

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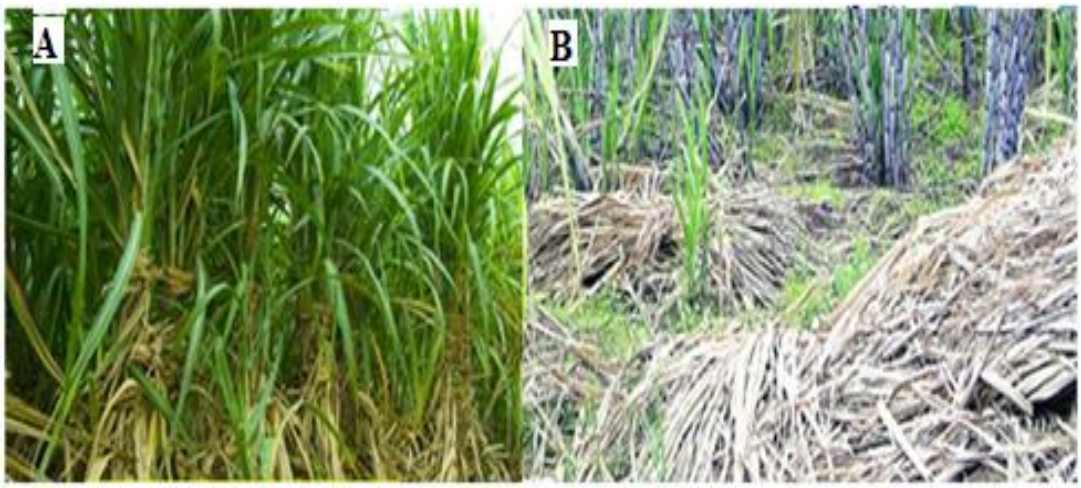

Fig. 1.4. A) Sugarcane plant and **B)** Dried sugarcane leaf top (SCT).

SCT are produced during harvesting of sugarcane stem and are suitable as raw material for second generation bioethanol productions because of their high availability. A few waste management systems such as recycling, landfills, pyrolysis are used in disposal of the agrowaste. Some of these techniques can cause soil and air pollution in surrounding environment. Even though fertility of soil is mainly depending on the chemical and physical property of environment but it also affected by human management practise. One of these practices demonstrated by farmers is burning the SCT after the sugarcane harvest. Around 7-12 tons of SCT can be produced from 1 ha of the sugarcane field (Suma and Savitha, 2015). Owing to the huge production quantity and associated high-cost for collection and transportation, mostly trash is burnt in the field. In any case, when SCT is burnt, a large portion of the nutrients and organic matter are lost, prompting the soil pollution (Mitchell *et al.*, 2000). Farmers ordinarily burn the waste, to reduce work, but it reduces the germination efficiency of the soil (Suma and Savitha., 2015). Moreover, most of the nutrients and the lignocellulosic matter is lost while burning the tops, leading to the pollution of the environment (Figueiredo *et al.*, 2011).

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SCT is a cheap and potential renewable raw substrate for bioethanol production (Gómez *et al.,* 2014). From 1 ton of sugarcane plant, about 140 Kg of bagasse (Dias *et al.,* 2012) and 250 Kg of SCT (Chandel *et al.,* 2012) were produced. Approximately, 50% of SCT can be removed from the agricultural field and used for bioethanol production without compromising the nutritional status of soil (Hassuani *et al.,* 2005). Up to 66% (w/w) of SCT could be used in the bioethanol industries if collected as dry leaves (Franco *et al.,* 2011). This post-harvest SCT can serve as a low cost and readily available substrate for bioethanol production (Sukumaran *et al.,* 2010). The use of SCT for ethanol production is favored because its production process can be annexed to the sugar/ethanol units already in place for lower investment, infrastructure, logistics and energy supply (Niju *et al.,* 2020). In such scenario, more ethanol can be produced by also utilizing SCT from the same amount of sugarcane, without compromising the cultivation aera. The yield of ethanol is 6,000 L/hectare of the sugarcane crop (Niju *et al.,* 2020). It was estimated that the ethanol production could reach 10,000 L/hectare, if only half of the SB generated is harnessed for the production of biofuel (Niju *et al.,* 2020). In India, 7.3 billion litres of ethanol is needed and this is growing annually at the rate of 6.6%. If the ethanol cannot be produced domestically, the whole purpose of achieving energy security is defeated (Sujata and Kaushal, 2020). Depending upon taxonomical interpretation sugarcane is any of 6 to 37 species of tall enduring grasses of the genus Saccharum (family: Poaceae, Class: Andropogoneae). The Sugarcane plant is a local warm calm atmosphere, basically found in tropical regions like Asia Pacific, Africa, India and Brazil (Canilha *et al.,* 2012). The composition analyses of SCT determined by different studies is shown in Table 1.1.

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Table 1.1. The composition analyses of SCT (%, w/w, dry weight basis).

Cellulose	Hemicellulose	Lignin	Ash	Extractives	Others	Reference
36.1	28.3	26.2	2.1	5.3	-	Moriya, 2007
34.4	18.4	24.0	11.7	11.5	-	Pitarelo, 2007
36.1	26.9	26.2	2.1	5.3	-	Saad *et al.*, 2008
33.6	28.9	31.8	5.7	-	-	da Silva *et al.*, 2010
33.3	27.4	26.1	2.6	-	10.6	Luz *et al.*, 2010
33.5	27.1	25.8	2.5	-	-	Costa *et al.*, 2013
40.5	30.8	22.8	2.1	2.5	-	Gómez *et al.*, 2014
38.1	25.4	21.1	8.2	-	7.2	Chotirotsukon *et al.*, 2019
40.4	33.2	17.4	6.4	-	2.8	Bhardwaj *et al.*, 2019

1.4.1 Structure of sugarcane plant

The sugarcane plant is formed by the straw (tops) and stem. The morphology of the sugarcane plant is shown in Fig. 1.5. Sugarcane stem is separated before the processing of sugarcane to extract a juice which is finally utilized for sugar or ethanol production. Sugarcane bagasse (SB) is the waste of sugarcane stem after the collection of juice. SCT is formed by dry or fresh leaves and tops accessible prior to collecting. Fresh leaves are yellow and green in shading and tops are the piece of sugarcane plant in between the terminal stalk node and the top end. Dry leaves are brownish (Neto, 2005). The sugarcane leaves are used as a fuel (combustion), crude feedstock for pyrolysis and gasification (methane to methanol biosynthesis). The tops are mainly used for animal feed, anaerobic methane fermentation or direct energy utilization (after drying) (Neto, 2005). Characterization of SCT, SB and stem, can be done on the basis of following perceptions. The sucrose collection is more prominent at the bottom of the sugarcane stem and the cellulose content and reducing sugars is prevalent in the upper part of stem and tops.

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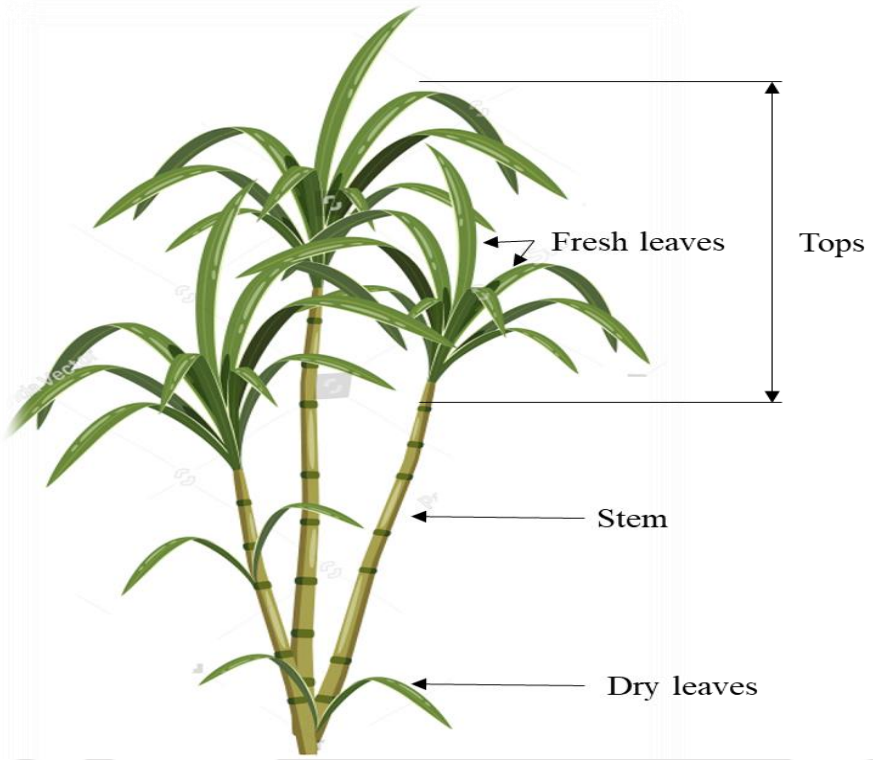

Fig. 1.5. Morphology of the sugarcane plant.

The length of the plants stem relies upon the assortment of the sugarcane plant and the cultural care given. So a grown-up stem can from under 2-4 m in size, influencing the length and quantity of internodes. The shading represent the chlorophyll content and anthocyanins present in the plant. The moisture content in sugarcane biomass varies from part to part, such as 13.5% (leaves) to 82.3% (SCT) (Canilha *et al.*, 2012). All composition present is experimentally same in sugarcane biomass, such as carbon (~45%), oxygen (~43%), hydrogen (~6%), sulfur (~0.1%) and nitrogen (0.5-1%) (Neto, 2005).

1.5 Pretreatments

Pretreatment of lignocellulosic biomass mainly involve the efficient separation of every component of complex feedstock enhancing their accessibility. A combination of pretreatments is beneficial as compared to conventional single pretreatment methods

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as this reduces the operational steps and overall cost of the bioethanol production process (Krap *et al.,* 2013). The lignin present in plant cell wall negatively impacts the pretreatment process (Rastogi and Shrivastava, 2017). Delignification of biomass can enhance the access of cellulosic and hemicellulosic components for the enzymatic saccharification. For the delignification of lignocellulosic waste, pretreatment is necessary. The process of pretreatment of lignocellulosic biomass is shown as schematic presentation in Fig. 1.6.

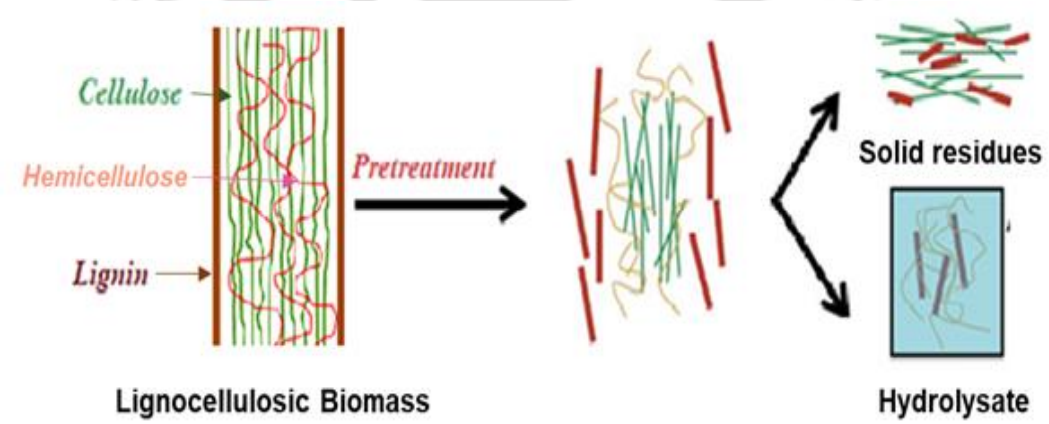

Fig. 1.6 Schematic presentation of pretreatment of lignocellulosic biomass process.

The task of hydrolysing the lignocellulose to monosaccharides is still a technical challenge, because of the indigestibility of cellulose structure. Different types of pretreatment and their advantages and disadvantages are listed in Table 1.2. Because of the structural characteristics of lignocellulosic biomass the pretreatment becomes an essential step for enhancing the cellulose accessibility for enzymes to release of fermentable sugars in the hydrolysis step and reducing the enzyme usage (Marulanda *et al.,* 2019). Biological, physical, chemical and physicochemical pretreatment strategies for lignocellulosic biomass are utilized before the enzymatic hydrolysis (Shafiei *et al.,* 2015). The pretreatment strategies can be utilized independently or in combination for upgrading the enzyme efficiency in the saccharification process

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(Jamaldheen *et al.*, 2018). Pretreatment methods such as liquid hot water, wet oxidations, alkali washing, steam explosion, dilute acid hydrolysis and ammonia fiber expansion, etc. are used to pretreat the agriculture waste (Shafiei *et al.*, 2015).

Table 1.2. Different types of pretreatment of lignocellulosic biomass and their advantages and disadvantages.

Pretreatment	Process	Advantage	Disadvantage	Reference
Thermo-mechanical	Grinding, milling and shearing	Reduce particle size and increase the surface/ volume ratio	High capital cost	Bhagwat *et al.*, 2016
Auto Hydrolysis	Supercritical CO_2 explosion	Enhances the yield	Mixture of monosaccharides	Vallejos *et al.*, 2015
Acid pretreatment	Dilute/conc. acid (H_2SO_4, HCl)	Enhance sugar production, dissolve hemicelluloses and lignin	Causes the sugar degradation, toxic and hazardous	Bhagwat *et al.*, 2016; Chotirotsukon *et al.*, 2019
Alkali pretreatment	NaOH, NH_3, alkaline H_2O_2	Increase surface area, reduce crystallinity, breakdown and separation of carbohydrate and lignin structure	Saccharification and fermentation processes, deacetylation of the structural units	Egüés et al., 2012; Shankarappa *et al.*, 2015
Physical pretreatment	Steam-explosion, microwave heating	No toxic by-products	High energy Consumption	Chen et al., 2012; Gupta *et al.*, 2014
Biological pretreatment	Brown, white and soft rot-fungi	Low energy requirement and mild operation conditions	Rate of this process very low, Require more time	Marulanda *et al.*, 2019

Combined pretreatment methods are considered more advantageous as it overcomes the challenges encountered in single pretreatments, such as inefficient monosaccharides production, the formation of inhibitors at high concentration and

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extreme pretreatment conditions. Therefore, the combined pretreatments result in higher productivity of bioethanol. Alkaline pretreatment of the biomass combined with dilute acid, dilute acid pretreatment combined with steam explosion and microwave assisted alkali pretreatements are effective for the removal of lignin and hemicellulose (Lu *et al.*, 2009). Rice straw pretreated with 2% (v/v) H_2SO_4 at 165°C for 2 min followed by a steam explosion at 180°C for 20 min gave higher xylose yield (Chen *et al.*, 2011). Alkali pretreatment followed by microwave irradiation can enhance the chemical reaction rate leading to an explosive effect among the biomass particles. The microwave-based heating in the presence of alkali was employed instead of the conventional heating for the pretreatment of switch grass (Li *et al.*, 2010). Microwave assisted alkali pretreated rice straw showed the effective removal of lignin and hemicellulose (Zhu *et al.*, 2005). The hydrolysate produced during pretreatment process contains hemicellulose, lignin and other plant extractive. This hydrolysate can be used for the production of other value added products such as furfural, hydroxy methyl furfural (HMF), acetic acid and lignin. Lignin separated from the hydrolysate after the enzymatic hydrolysis from Iogen process can be used for the production of electricity (Tolan 2006). Xylan extraction before the use of cellulosic portion for bioethanol production is an approach to increase the value of hemicellulose components. Extracted xylan can be utilized in papermaking, pharmaceutical and food industries (Sporck et al., 2017). The removal of hemicellulosic components also enhances the enzymatic hydrolysis of cellulose (Sporck et al., 2017). This biorefinery concept may add value to the hemicellulose portion and also can provide the hemicellulose free solids which could be suitable for bioethanol fermentation. It can also improve the economic value of the sugarcane tops.

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1.6 Extraction of xylan

Hemicellulose is the second most abundant biopolymer produced by green plants. Xylan is predominant hemicellulose found in secondary cell walls of SB. Xylan is made up of xylose backbone with a different degree of substitutions (such as arabinose and glucuronic acids), which directly affects the structural and functional characteristics (Naidu *et al.,* 2008). Based on the method of extraction and source of plant/tissue used, the structure and composition of extracted xylan will vary (Petzold-Welcke *et al.,* 2014). The backbone of xylan consists of β-1→4-linked xylose residues, which is often substituted with methylated α-D-gluco-pyranosyl uronic acid and α-L-arabino-furanosyl, ferulic acid, *p*-coumaric acid as the side chains (Ordaz-Ortiz and Saulnier, 2005). Apart from these substitutions, heteroxylan also contains D-galactose, D-xylose, L-rhamnose, D-glucose, acetyl groups and different dimeric and trimeric side chains (Moine *et al.,* 2007).

Xylan extracted from SB by the alkaline method showed low purity of isolated xylan and detected 16-18% of residual lignin (Chimphango *et al.,* 2012). However, the alkaline method for xylan extraction from SB showed a higher yield in de-lignified biomass (Chimphango *et al.,* 2012). Modifications in traditional alkaline hemicellulose extraction methods by including de-lignification with $NaClO_2$ followed by xylan extraction provides a high yield of the polymeric xylan (Höije *et al.,* 2005).

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Table 1.3. Xylan extraction from various agricultural wastes.

Raw material	Extraction method	Yield	Reference
Wheat straw	OrganoSolv	41% of the total hemicellulose	Sun *et al.*, 1998
Corn cob	5% (w/v) NaOH, 60°C, ultrasonication, 1h	36.1% of total xylan	Hromadkova *et al.*, 1999
Corn cob	Classical extraction, 5% (w/v) NaOH, 1h	31.5% of total xylan	Hromadkova *et al.* 1999
Corn cob	Ultrasonication, 10 min cycle, 1h, 25°C	52.7% of the total hemicellulose	Ebringerova & Hromadkova., 2002
Wheat straw	Ultrasonication, 0.5M KOH, 30 min	25.5% of total dried biomass	Sun & Tomkinson *et al*. 2002
Sugarcane bagasse	Ultrasonication, 10% (w/v) NaOH, 15h	97.3% of total hemicellulose	Ren *et al.* 2006
Wheat straw	Steam explosion	80% of the total hemicellulose	Hongzhang &Liying, 2007
Cotton stalks	24% (w/v) KOH + 1% (w/v) $NaHBH_4$, 3h	0.4g/2g of cotton stalk	Akpinar *et al.* 2007
Wheat straw	0.5M NaOH, 185°C, 30 min	49.3% of total xylan	Ruzene *et al.* 2008
Wheat straw	24% (w/v) KOH + 1% (w/v) $NaHBH_4$, 3h	20.6% of total dried raw biomass	Akpinar *et al.* 2009
Maize stem	8% (w/v) NaOH, 50°C, 3h	21% of total dried biomass	Sun *et al.* 2010
Rye Bran	1M NaOH, 95°C, 45 min	8.6% of total xylan	Sarossy *et al.* 2011
Corn stalks	10% (w/v) NaOH, 20°C	54% of the original xylan	Egüés *et al.*, 2012
Grape stalks	Hot water, 180°C, 30 min	8.09% of total dried biomass	Amendola *et al*, 2012
Rye Bran	Water extraction, 95°C, 45 min	3.2% of total xylan	Sarossy *et al.* 2013
Sweet sorghum stem	2.5% (w/v) KOH, 75°C, 3h	76% of the total hemicellulose	Sun *et al.*, 2013
Pear pomace	4M NaOH at 80°C for 18h	14% of total dried biomass	Rabetafika *et al*, 2014
Finger millet	1M $Ba(OH)_2$ +1M KOH, 25°C, 18h	6-10% of dried raw biomass	Prashanth & Murahkrishna, 2014
Rapeseed straw	0.5M NaOH, 105°C, 20 min	25% of total dried biomass	Svärd *et al*, 2015
Sugarcane bagasse	Hot water, 170°C, 1h	33% of total hemicellulose	Yao *et al*, 2015
Sugarcane bagasse	Hot water	69% of the total hemicellulose	Vallejos *et al*, 2015
Sugarcane bagasse	Twin screw extruder	54% of total hemicellulose	Vandenbossche, *et al.* 2016
Rice bran	Water extraction, 25°C, 2h	73.5% of total xylan	Palaniappan *et al*, 2017
Finger millet seed coat	Water extraction, 25°C, 2h	79.7% of total xylan	Palaniappan *et al*, 2017
Acacia sawdust	10% NaOH + 1% H_3BO_4, 60°C, 3h	23.5% of dried biomass	Sharma *et al*., 2020A
Neem sawdust	10% NaOH + 1% H_3BO_4, 60°C, 3h	22.5% of total biomass	Sharma *et al*., 2020B

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The xylan extraction before the enzymatic saccharification of cellulosic components can add value to the hemicellulose part and can provide the cellulosic solids which can be readily hydrolyzed and further used for bioethanol production. Separation of hemicellulose from lignocellulosic biomass can also enhance the hydrolysis by cellulolytic enzymes (Meng *et al.*, 2014). This concept can add value to the hemicellulose part and can also provide hemicellulose-free solids (cellulosic part) which can be suitable feedstock for bioethanol production (Khaire *et al.*, 2020). The extraction of xylan from different biomass and recovery yield listed in Table 1.3. Extracted xylans are suitable for biopolymer applications however some lignin fractions are also solubilized with xylan (Hutterer *et al.*, 2016). Another simpler traditional procedure of xylan extraction is based on alkaline pretreatment followed by ethanol precipitation of xylan, but it gives a lower xylan yield (Sporck *et al.*, 2017).

1.7 Saccharification of lignocellulosic biomass

The plentiful accessibility of cellulose in raw biomass makes it alluring for producing numerous industrially significant products. Unfortunately, a great part of the cellulosic waste is frequently discarded by burning (Bote *et al.*, 2020B). However, it is viewed as a global phenomenon. With the assistance of the cellulolytic framework, cellulose can be hydrolyzed to glucose which is a multi-utility substrate, and is lot less expensive (Gupta *et al.*, 2012). Polysaccharides are broken down gradually by the microbes. Microorganism secretes the collection of enzymes that degrade the polysaccharides to individual monosaccharides (Pandey *et al.*, 2000). The task of hydrolyzing lignocellulose to monomeric sugars is still a technical challenge, because of the hard digestibility of cellulose structure (Canilha *et al.*, 2012). The lignocellulosic biomass can be hydrolyzed by cellulase produced from both fungal and bacterial

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species. The fungal species e.g. *Aspergillus niger*, *Trichoderma reesei*, *Sporotrichum pulverulentum*, *Penicillium funiculosum* and *Myrothecium verrucaria* etc. can produce higher activity cellulase (Saratale *et al.,* 2009). From these fungal species, the *Trichoderma* has the potential to offer high enzyme yield of cellulase enzyme is mostly used (Singhania *et al.,* 2007). The enzymes like β-glucosidase (EC3.2.1.2), endoglucanase (E.C3.2.1.4) and exoglucanase (EC3.2.1.74) secreted from *T. reesei* act synergistically in the cellulose to glucose conversion process (Gray *et al.,* 2006). The bacterial species belonging to the genus *Bacillus*, *Clostridium*, *Cellulomonas*, *Ruminococcus*, *Acetovibrio*, *Streptomyces*, *Bacteroides*, *Microbispora*, *Thermomonospora* and *Erwinia* produce cellulase when grow on cellulose as a carbon source *(*Lakshmidevi and Muthukumar., 2010). The cellulase enzyme synthesized by bacterial species is not much efficient to degrade a cellulose crystallinity as compared with the fungal enzymes. The enzymes produced from bacterias are preferred compared to the enzymes from fungal sources due to the several advantages such as easy to handle, fast growth, cost-effective, consistency in production and genetically easy for the modification. The gene encoding the cellulase enzyme can be expressed in the *E. coli* to take advantage of a fast-growing model organism for enhancement of the enzyme productivity (Singh *et al.*, 2013). The enzyme expression is induced by the addition of IPTG (Isopropyl β-D-1- thiogalactopyranoside) (Amore *et al.,* 2015). In the saccharification process, the activity of the hydrolytic enzymes is measured by the amount of enzyme requires to produce 1 µmole of reducing sugar (D-glucose) per min under optimum condition. The saccharification of sugarcane biomass and its yield from various reports is listed in Table 1.4.

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Table 1.4. Total sugar yield from the sugarcane waste by cellulolytic enzymes produced from various sources.

Sugarcane waste	Source of Enzyme	Total Sugar Yield in mg/g dry substrate	Time (h)	References
SB	Commercial cellulase	400	72	Saska and Martin., 2006
SB	*Trichoderma sp*.	457	72	Mahamud and Gomes, 2012
ST	*Trichoderma reesei*	331	48	Zimbardi *et al.*, 2013
SB	*Trichoderma reesei*	365	48	Shankarappa *et al.*, 2015
SCT	Commercial cellulase	789	60	Sindhu *et al.*, 2012
SCT	Commercial cellulase	669	60	Sindhu *et al.*, 2013
SCT	*Trichoderma reesei*	329	48	Shankarappa *et al.*, 2015
ST	*Trichoderma reesei*	340	48	Shankarappa *et al.*, 2015
ST	cellulysin® cellulose	380	-	Dodo *et al.*, 2017

SB= Sugarcane bagasse; ST= Sugarcane trash; SCT= Sugarcane tops

1.8 Enzymes involved in saccharification

The plant biomass gets delignified after pretreatment and in this manner it gets available for enzymatic saccharification. For bioethanol production, the significant components in pretreated biomass are cellulose and hemicellulose, can be hydrolyzed by cellulases and hemicellulases respectively. Pectinases are likewise supplemented to decrease the cellulase and hemicellulase loading in the saccharification of plant biomass (Xiao and Anderson, 2013). A few microorganisms produce these enzymes. To enhance the production of these enzymes, the genes responsible for these proteins are cloned in a suitable vector and overexpressed (Taylor *et al.*, 2005, Chakraborty *et al.*, 2015, Verma *et al.*, 2016). Technologies such as site-directed mutagenesis (Escovar *et al.*, 2004) and protein engineering are additionally used to improve the catalytic efficiency of enzymes (Cao *et al.*, 2015). Change of amino acid phenylalanine to alanine (flexible residue) by the side-directed mutagenesis adjacent to the active site of endoglucanase (*Ct*GH5) results 2-fold enhancement of endoglucanase activity. Chimera of β-

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glucosidase (*Ct*GH1) and endoglucanase (*Ct*GH5-F194A) enzyme was enhanced both endoglucanase and β-glucosidase activities (Nath *et al.*, 2019).

1.8.1 Cellulases

The cellulases breakdown cellulose to glucose. Cellulases are the most requested enzymes /catalysts for their applications in biofilms and other industries. Cellulases are utilized in the feed (Spohner *et al.*, 2015), paper (Gupta *et al.*, 2015), cotton (Galante *et al.*, 2014) and the food industries (Meireles *et al.*, 2016). Plant biomass contains 40-50% of cellulose, consequently can be utilized for saccharification by cellulases for the production of biofuels. Cellulases are of three kinds i) endoglucanase; ii) exoglucanase (cellobiohydrolase) and iii) β-glucosidase dependent on their substrate specificities.

1.8.1.1 Endoglucanase

Endoglucanase (EC 3.2.1.4) arbitrarily acts against amorphous cellulase and produces cello-oligosaccharides of the fluctuating degree of polymerization (DP). Endoglucanases are available in glycoside hydrolase (GH) families 5, 8, 9, 26, 44, 48, 74 and 124 (http://www.cazy.org/). These oligosaccharides work as probiotics and have useful food applications (Qiang *et al.*, 2009). For biofuel production, monosaccharides are required by the fermenting microorganisms (Rastogi and Shrivastava, 2017). Endoglucanase isn't sufficient to produce glucose from cellulose and needs other types of cellulase enzymes for the complete hydrolysis of cellulose.

1.8.1.2 Exoglucanases

Exoglucanases (EC 3.2.1.91 and EC 3.2.1.176) is also called cellobiohydrolase, which acts against amorphous cellulose, crystalline cellulose and oligosaccharides. The exoglucanase acts on the reducing end are called as a cellobiohydrolase-I (EC 3.2.1.176) and are available in GH family 7 and 48. The cellobiohydrolase-II (EC

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3.2.1.91) severs at the non-reducing end of cellulose and is found in GH families 5, 9 and 48. The major product released by both enzymes is cellobiose, which is further broken down into two molecules of glucose.

1.8.1.3 β-Glucosidase

β-Glucosidase (EC 3.2.1.21) enzyme mainly acts on cellobiose and separates glucose from non-reducing end. The β-Glucosidases are belongs to the GH families 1, 2, 3, 5, 9, 30 and 39. β-Glucosidases cannot hydrolyze cellulose. Therefore, all the three enzymes endoglucanase, cellobiohydrolase and β-glucosidase are essential for the complete hydrolysis of lignocellulosic biomass.

1.8.2 Hemicellulases

In contrast to cellulose, hemicellulose is heterogeneous in structure and comprises 20-40% of plant biomass and is connected with cellulose, gelatin and lignin (Thakur *et al.,* 2019). Hemicelluloses have both C5 (arabinose, xylose) and C6 (glucose, galactose, mannose) sugars in the xylan backbone with different side chains. Xylan, glucuronoxylan, arabinoxylan, xyloglucan, galactan, arabinogalactan, mannan, glucomannan, galactomannan and arabinan are the major hemicellulose present in plant biomass (McMillan, 1993). Because of the multifaceted nature in the structure of hemicellulose, a variety of catalytic enzymes are required for its complete hydrolysis.

1.8.2.1 Xylanolytic enzymes

Xylan is the most plenteous hemicellulose that contains xylose in the backbone. Endoxylanase (3.2.1.8) cleaves xylan main chain and produces xylo-oligosaccharides. In carbohydrate-active enzymes database (CAZy) endoxylanase are reported from GH families 3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62, 98 and 141 (Lombard *et al.,*

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2013). The cellulases, hemicellulases and pectinases act explicitly and breakdown the polysaccharide into monosaccharides are listed in Table 1.5.

Table 1.5. Enzymes for saccharification of plant biomass.

Enzymes	EC number	Substrate	Product
Endoglucanase	3.2.1.4	Cellulose	Gluco-oligosaccharides
Endoxylanase	3.2.1.8	Xylan	Xylo-oligomers
Polygalacturonase	3.2.1.15	Polygalacturonan	Digalacturonate
β-Glucosidase	3.2.1.21	Cellobiose	Glucose
β-Galactosidase	3.2.1.23	β-Galactoside	D-galactose
Exo-β-1,4-mannosidase	3.2.1.25	Manno-oligosaccharides	Mannose
β-Xylosidase	3.2.1.37	Xylo-oligosaccharides	Xylose
α-Arabinofuranosidase	3.2.1.55	Arabinoxylan	Arabinose
Exopolygalacturonase	3.2.1.67	Polygalacturonan	Galacturonic acid
Ferulic acid esterase	3.1.1.73	Xylan	Ferulic acid
ρ-Coumaric acid esterase	3.1.1.-	Xylan	*ρ*-Coumaric acid
Acetyl xylan esterase	3.1.1.72	Xylan	Acetate
β-Mannanase	3.2.1.78	Mannan	Mannooligosaccharides
Endogalactanase	3.2.1.89	Galactan	Galactooligosaccharides
Cellobiohydrolase	3.2.1.91	Cellulose	Cellobiose
α-Glucuronidase	3.2.1.139	Glucuronoxylan	Glucuronic acid
Pectate lyase	4.2.2.2	Polygalactosyluronic acid	Pectin oligosaccharides
Exopectate lyase	4.2.2.9	Polygalactosyluronic acid	Digalacturonate
Pectin lyase	4.2.2.10	Methylated pectin	Oligosaccharides

Endoxylanase alone cannot release xylose from xylan and other auxiliary hemicellulases are essentially required for the complete hydrolysis of xylan to produce xylose. β-Xylosidase (EC 3.2.1.37) breaks down the xylan and xylobiose from the non-reducing end to release xylose. β-Xylosidase is necessary along with endoxylanase for the complete hydrolysis of xylan (Van *et al.*, 1997). β-Xylosidases are available in GH family 1, 3, 5, 30, 39, 43, 51, 52, 54, 116 and 120. Xylan backbone is substituted with 4-O-methyl glucuronic acid, arabinose, acetate and ferulic or *p*-coumaric acid as the

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side chains. These side chains meddle in the xylan hydrolysis. Side chain expelling enzymes, for example, ferulic acid esterase/*p*-coumaric acid esterase (EC 3.1.1.73), acetyl xylan esterase (EC 3.2.1.72), arabinofuranosidase (EC 3.2.1.55) and glucuronidase (EC 3.2.1.139) release the side chain and boost the xylan hydrolysis (Alvira *et al.*, 2011).

1.8.2.2 Mannan hydrolytic enzymes

β-Mannanase (EC 3.2.1.78) hydrolyzes the principle chain of glucomannan, galactomannan and galactoglucomannan in an endolytic way are produce monno-oligosaccharides. β-Mannanases are available in GH families 5, 9, 26, 44, 113 and 134. Manno-oligosaccharide is hydrolyzed to mannose from non-reducing terminal by exo-β-mannosidase enzyme. Exo-β-mannosidases belong to the GH families 1, 2 and 5.

1.8.3 Pectinolytic enzymes

The polysaccharide structure of pectin is extremely complex is composed of about 17 different monosaccharides and more than 20 linkages. Due to this, pectin degrading enzymes are also classified based on their site of action in the pectin polymer (Bonnin *et al.*, 2014). Pectin degrading enzymes are available in carbohydrate esterase (CE), glycoside hydrolase (GH) and polysaccharide lyase (PL) classes. The pectin degrading enzymes, for example, pectin acetylesterase (EC 3.1.1.6), pectin methylesterase (EC 3.1.1.11), polygalacturonase (3.2.1.15), rhamnogalacturonan I endohydrolase (EC 3.2.1.171), unsaturated rahmnogalactouronyl hydrolase (EC 3.2.1.172), rhamnogalacturonan I galactohydrolase (EC 3.2.1.173), rhamnogalacturonan I rhamnohydrolase (EC 3.2.1.174), exopolygalacturonase (EC 3.2.1.67), pectate lyase (EC 4.2.2.2), exopectate lyase (EC 4.2.2.9), pectin lyase (EC 4.2.2.10), and rhamnogalacturonan lyase (EC 4.2.2.23). These enzymes are found in

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CE (1,3,4,8 and 16) family, GH (4, 28, 78, 105 and 126) family and PL (1, 2, 3, 4, 9, 10, 11 and 26) family (Lombard *et al.*, 2013).

1.9 Xylo-oligosaccharides and their applications

The xylo-oligosaccharides (XOS) are produced by the breaking down the glycosidic bonds present in the main chain of xylan polysaccharides. This can be achieved by subjecting the xylan to higher temperature, chemical reagents or biological catalysts.

1.9.1 XOS production by chemical and physical treatments

The β-1,4 glycosidic bond in xylan can be broken down by acids. To avoid the direct hydrolysis of xylan into monosaccharides, the acid concentration used is low. At higher acid concentration, monomers formed can further undergo dehydration to give furfural/hydroxy-methyl-furfural. (Brienzo *et al.*, 2016; Hilpmann *et al.*, 2016).

1.9.2 XOS production by enzymatic hydrolysis

The xylan has to be isolated from lignocellulosic material by using a suitable pretreatment method before the XOS can be recovered by enzymatic hydrolysis. The characteristics of extracted xylan depend on the type of biomass and pretreatment used (Brienzo *et al.*, 2016). The extracted xylan can be hydrolyzed by endo β-1,4-xylanase having no β-xylosidase enzyme activity to produce XOS without the monomers. Xylanases with varying substrate specificities can produce various hydrolysis products and therefore, controlling the degree of polymerization of XOS can be difficult (Akpinar *et al.*, 2010). The specificity of the substrate varies in endoxylanases from GH10 and GH11 families. The GH10 family xylanases are less specific for xylan and can produce oligosaccharides with substituents at a non-reducing end of XOS. The true xylanase, GH11 family, are specific for xylan and cleave the unbranched region of

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xylan and can produce XOS with a higher degree of polymerization than the GH10 family xylanase (Moreira, 2016).

1.9.3 Applications of XOS

XOS can be used as a partial substitute for other sugars in the food industries. They also promote beneficial bacterial growth due to their prebiotic property. The food quality and consumer health can be improved by using these prebiotic XOS (Jain *et al.,* 2015). The quantity of monomeric units as well as the degree of polymerization of XOS depend on the source of extracted xylan. Xylan may also contain the side chains of acetyl and arabinofuranosyl residues. These xylans will lead to the synthesis of branched XOS (de Freitas *et al.*, 2019). Uronic acid, as a branch in XOS, is known for its anti-allergic and antioxidant properties (Jain *et al.*, 2015). The reduction of protein degradation in muscles for the production of energy is another biological function of XOS (de Freitas *et al.,* 2019). XOS also have applications in colon cancer prevention (Carvalho *et al.,* 2013), as a substitute in antibiotics, in agricultural and pharmaceutical industries (Moure *et al.,* 2006).

1.10 Microbial fermentation

The nature of the fermenting microbe and the characteristics of an enzyme are the deciding factors for choosing the mode of fermentation for bioethanol production. Different modes of fermentation used in the production of bioethanol have been described in the following sub-sections. The fermentation processes are classified as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP).

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1.10.1 Separate hydrolysis and fermentation

In separate hydrolysis and fermentation (SHF) process, the hydrolysis of pretreated biomass and the fermentation of monosaccharides to ethanol operate in two separate units. SSF can withstand high solid loading by intermittent feeding (Koppram. and Olsson 2014). This separation of the enzymatic hydrolysis process from fermentation allows enzymes to operate at a higher temperature (45-50°C) and fermentation at a slightly lower temperature (30-37°C) for better performance of individual process (Saha *et al.*, 2005). The SHF fermentation of hydrolyzate obtained from saccharification of pretreated SCT gave 11.4 g/L ethanol yield (Sindhu *et al.*, 2011). The 1.5% (w/v) sulfuric acid pretreated SCT gave 4.71 g/L or 8% (w/w) of ethanol yield after SHF by using *S. cerevisiae* for 24 h (Jutakanoke *et al.*, 2012).

1.10.2 Simultaneous Saccharification and Fermentation

The simultaneous saccharification and fermentation (SSF) process combines the hydrolysis and fermentation in a single operational unit. Comparison of SHF and SSF modes with different lignocellulosic feedstocks like corn stover and wheat straw showed that SSF contributes to higher ethanol productivity and yield than SHF (Öhgren et al., 2010). Moreover, no inhibitory compound was formed during SSF process. The SSF has the advantage of immediately utilizing the monosaccharides released during saccharification for ethanol fermentation (Krishna *et al.*, 2001). The major drawback of this process is the difference in operating conditions since the saccharification and fermentation operate at different optimal parameters. SSF requires compatible saccharification and fermentation conditions, with a similar pH, temperature and optimum substrate concentration. SSF process also produces the value-added products such as furfural, glycerol and furans, etc. which reduces the production costs in the

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industry (Das *et al.,* 2013). The use of single container for the saccharification and fermentation process is one of the major advantages of the SSF, which reduces both saccharification and fermentation process time and capital cost. Another noticeable advantage of SSF is the lower formation of inhibitory components from hydrolysis process, which improves the overall performance of the process (Marulanda *et al.,* 2019).

1.10.3 Simultaneous Saccharification and Co-Fermentation

Simultaneous Saccharification and Co-Fermentation (SSCF) is a type of fermentation where pentose in the hydrolysate is also fermented by using pentose fermenting microorganism along with the glucose fermentation. In co-fermentation process, pentose utilizing yeasts like *Pichia fermentans* and *Pichia stipites* are co-cultured with *S. cerevisiae* for glucose fermentation. *P. stipitis* is capable of performing both aerobic and oxygen limiting fermentations and has the natural ability to ferment xylose directly into ethanol (Weber *et al.,* 2010). The ability of *P. stipitis* to produce ethanol from xylose is advantageous as it can combat the inability of *Saccharomyces cerevisiae* to ferment pentose (Weber *et al.,* 2010). In the overall fermentation process by *P. stipitis,* 3 moles of xylose are converted to 5 moles of ethanol with a theoretical yield of 0.51 g ethanol/g xylose (Weber *et al.,* 2010). The SSCF system comprising enzyme cocktail of cellulase (GH5) and hemicellulase (GH43) with co-fermentation of *Saccharomyces cerevisiae* and *Candida shehatae* gave an ethanol titre of 23 g/L which was 2-fold higher than single enzyme (GH5, cellulase) with single culture (*Saccharomyces cerevisiae*) system employing 5% (w/v) pretreated wild grass. Since, the saccharification and fermentation have different operating conditions, the selection of suitable fermenting microbes for SSF is a challenging task (Das *et al.,* 2013).

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1.10.4 Consolidated Bioprocessing

Consolidated Bioprocessing (CBP) involves the use of a single microbial community for all three subsequent bioconversion process, *viz.* i) production of cellulolytic and xylanolytic enzyme ii) enzymatic saccharification of delignified biomass and iii) fermentation of monosaccharides to ethanol. Therefore, this mode of fermentation has the advantage of low capital and operational costs over other modes of fermentation. However, the crucial part in this mode of fermentation is the need for compatibility in the operating parameters of enzyme production, saccharification and fermentation. The native bacterial strains, *S. cerevisiae* and *Trichoderma* sp. have been currently in use for CBP. The disadvantage of the use of genetically engineered strain is the low yield of ethanol (Sun *et al.*, 2012). *Clostridium thermocellum* can produce proficient enzymes for lignocellulose hydrolysis and can do fermentation too in a CBP (Olson *et al.*, 2012). Around 99% of crystalline cellulose hydrolysis was accomplished by CBP approach (Singh *et al.*, 2018). The high ethanol yield is due to the SSF, was found when addition of various nutrients and *K. fragilis* is used, were able to ferment at higher temperature is closer to the cellulase optimal temperature (Krishna et al., 2001). Besides *S. cerevisiae* and *K. fragilis*, other microorganisms such as *Escherichia coli* and *Zymomonas mobilis* are also being selected through metabolic engineering for the production of bioethanol (Sarkar et al., 2020). The pretreatment used for sugarcane biomass and ethanol yield obtained are listed in Table 1.6.

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Table 1.6. Pretreatment for sugarcane biomass and ethanol yield.

Sugarcane biomass	Pretreatment	Fermentation Time (h)	Ethanol Yield	Reference
SB	H_3PO_4 + Steam explosion	12	19 g/L	Gupta *et al.*, 2014
SCT	6% Glycerol+ 5% NaOH+1% $FeCl_3$	72	31.9 g/L	Raghavi *et al.*, 2016
SS	1.5%(w/w) H_2SO_4	8	74.7%	Parera *et al.*, 2015
SCT	Acid pretreatment	8	65.5%	Parera *et al.*, 2015
SB	Acid pretreatment	8	54.7%	Parera *et al.*, 2015
SCT	1.6 M H_2SO_4	24	6.9 g/L	Dodo *et al.*, 2017
SCT	1.6 M H_2SO_4	72	11.7 g/L	Dodo *et al.*, 2017
SCT	Laccase enzyme pretreatement	24	27.2 g/L	Shepra *et al.*, 2019
SCT	Acid hydrolysis	72	13.7 g/L	Dodo *et al.*, 2017

SB= Sugarcane bagasse; SS= Sugarcane Straw; SCT= Sugarcane tops

The current trends of bioethanol production by using *S. cerevisiae* can be achieved from the different perspectives such as inhibitors and substrates reduction in biomass hydrolysates, co-culturing with different microorganisms, growth variables and various immobilization techniques (Tesfaw and Assefa., 2014).

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1.11 Significance of the investigation

The increasing use of non-renewable resources and its continuous depletion has become a major concern nowadays. The production of renewable fuels from agricultural lignocellulose waste is sustainable and economically feasible. Sugarcane tops are one of the most abundant biomasses in India. More conveniently, it is burned in the field being in large quantity and the high-cost involved in its collection and transportation. It is an inexpensive and renewable raw material available for biofuels. The sugarcane tops containing 40-45% cellulose, 25-30% hemicellulose and 15-19% lignin, can be used for bioethanol production. The pretreatment process is one of the cost intensive steps in the bioethanol production, owing to the manpower and expensive chemicals' requirement to separate cellulose, hemicellulose and lignin from lignocellulosic biomass. In recent times, the advent of novel recombinant carbohydrate-active enzymes from different microbial sources by genetic manipulation has led to great advances in saccharification of pretreated biomass leading to higher production of bioethanol. The cellulolytic and hemicellulolytic enzymes have received greater attention worldwide and got large market in the last few years. The production of efficient and engineered recombinant cellulolytic enzymes in laboratory is less expensive as compared with the commercial enzymes. The complex multi-enzyme complex called cellulosome is expressed by *Clostridium thermocellum*. These enzymes are capable of hydrolyzing cellulose and hemicellulose. The proposed study is aimed at producing bioethanol and other value-added products from Sugarcane tops (SCT) biomass. Isolation, separation and characterization of xylan, cellulose and lignin from SCT can be most efficient industry oriented approach due to their xylo-oligosaccharides or alternative substrate, bioethanol and oil based petro-chemical applications,

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respectively. The separated cellulose can be saccharified by recombinant cellulose hydrolyzing enzymes *viz.* endoglucanase (*Ct*Cel8A), cellobiohydrolase (C*t*CBH5A) and β-Glucosidase (*Ht*Bg1) cloned from *Hungateiclostridium thermocellum*. The saccharified hydrolysate will be used for bioethanol production by fermenting with *Saccharomyces cerevisiae*. Xylan can be utilized for the production of xylo-oligosaccharides, food coating, packaging films and other pharmaceutical applications. Extracted xylan can also be used for production of xylooligosaccharides (XOS) by hydrolysis by using recombinant xylanolytic enzymes. XOS have significant prebiotic potential being beneficial for human gut microbiota, can be used in numerous nourishing food products. Effective and economically adhesive properties of lignin can be improved through aldehyde and phenol or by lignin modification itself. Lignin is mainly used as a rubber intensifier, polyolefin and rubber packing. It is also used in cement, composite materials, unsaturated polyester and vinyl ester as filler and co-monomer. Several questions remain unanswered such as, how the effective separation method will be chosen for xylan and lignin extraction from SCT. Moreover, it should be cost effective and applicable on a large scale. Next is the selection of factors for enzyme saccharification and bioethanol fermentation. The proposed study involves the extraction and characterization of cellulose, xylan and lignin and their applications. The optimization of enzymatic saccharification and fermentation will be carried out by using response surface methodology. The specific objectives of the proposed study will be as follows.

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1.12 Specific objectives

1. Separation and characterization of cellulose from sugarcane tops and its saccharification by recombinant cellulolytic enzymes.
2. Extraction, characterization of xylan from delignified sugarcane tops and its application.
3. Extraction and characterization of alkali lignin from sugarcane tops and its applications.
4. Alkaline pretreatment and response surface methodology based recombinant enzymatic saccharification and fermentation of sugarcane tops.
5. Optimization of saccharification and fermentation of alkali pretreated sugarcane tops for higher scale bioethanol production
6. Economic evaluation for conversion of sugarcane tops to ethanol and value added products.

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Chapter 2

Separation and characterization of cellulose from sugarcane tops and its saccharification by recombinant cellulolytic enzymes

2.1 Introduction

Unabated exploitation of non-renewable energy resources and their depletion has become a major concern today. The production of renewable fuels from the agricultural waste is sustainable and economically feasible. In the tropical regions, *Saccharum officinarum* or Sugarcane is one of the major cultivated agricultural crops (Gopitha *et al.*, 2010). Worldwide approximately, 1.91 billion tonnes of sugarcane are produced [Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), USA, 2018]. Sugarcane trash/tops (SCT) is one of the most abundant agricultural waste produced in India (Ensinas *et al.*, 2009). More than 199 million metric tons of sugarcane were reported to be produced in different regions of India (Sukumaran *et al.*, 2010). Owing to the huge quantity of production of SCT and the high-cost involved in its collection and transportation, mostly the trash is burnt in the field.

Sugarcane top is the field residue remaining after the harvesting of sugarcane stalk, while sugarcane bagasse is the fibrous residue left after milling of sugarcane stalk. Chemical composition analysis of SCT showed 40% cellulose, 33% hemicellulose and

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17% lignin content, making it an attractive feedstock for bioethanol production (Bhardwaj *et al.,* 2019). SCT can serve as a cheap and potential renewable raw substrate for biofuels (Sukumaran *et al.,* 2010). From 1 ton of sugarcane plant, about 140 Kg of bagasse (Dias *et al.,* 2012) and 250 Kg of SCT (Chandel *et al.,* 2012) are produced. Approximately, 50% of SCT can be removed from the agricultural field and used for the bioethanol production without compromising the nutritional status of the soil (Hassuani *et al.,* 2005). Up to 66% (w/w) of SCT could be used in the bioethanol industries, if collected as dry leaves. This post-harvest SCT is low cost and readily available substrate for bioethanol production (Sukumaran *et al.,* 2010). The SCT can also be used as a raw material for pyrolysis to gas, char and oil production (Pighinelli *et al.,* 2014), gasification (Jorapur and Rajvanshi, 1995), methanol synthesis and anaerobic methane (Canilha *et al.,* 2012) and bioethanol fermentation (Sukumaran *et al.,* 2010). SCT is also used as a ruminant feed (fresh or dried) for cow and buffalo (Akinbode *et al.,* 2017). Hemicellulose separation before the hydrolysis of cellulosic part of feedstock is the new approach to increase the value of biomass. This concept can add value to the hemicellulose part and also can provide hemicellulose free solids (cellulosic part) which can be suitable feedstock for bioethanol production. Separated hemicellulose can be used in papermaking, pharmaceutical and food industries (Sporck *et al.,* 2017). The use of both cellulosic and hemicellulosic fractions can also increase the economic value of the agricultural waste. In this study, the cellulose was separated from SCT and investigated for its purity. There is no report on the separation and characterization of cellulose from sugarcane trash. This approach of separation of cellulose and xylan from SCT may increase the commercial value of both cellulose and hemicellulose components for their use in respective industries. The technique

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involving the recombinant cellulosic enzymes in saccharification of cellulose separated from SCT is reproducible and is cost effective as compared with the saccharification by commercial enzymes. The structural properties of separated cellulose demonstrated that, it can be used as commercial substrate as well as an alternate substrate for bioethanol production. This study focuses on the structure analysis of cellulose separated from SCT and its enzymatic hydrolysis by recombinant cellulolytic enzymes.

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2.2 Material and Methods

2.2.1 Material and feedstock collection and its processing

All chemicals (sodium chlorite, sodium hydroxide, boric acid etc.) were bought from Himedia, India. Ethanol, acetic acid and thin layer chromatography (TLC) plate were procured from Merck India Pvt. Ltd. SCT was collected from field nearby Guwahati, Assam, India. The feedstock was washed under tap water and chopped into one to two inch pieces. The chopped biomass was rinsed with deionized water and dried in the oven at 60-70 °C for 24 h. Dried biomass was ground by grinder (HL1606/03, Philips) and passed through a standard test sieve of 0.85 mm pore size.

2.2.2 Composition analysis of raw SCT

One gram of powdered SCT was dried in hot air oven at 105 °C for 24 h. Then biomass was weighed before and after drying for the determination of moisture content. 0.5 g of raw SCT biomass was used for holocellulose and hemicellulose estimation and 0.1 g of raw SCT was taken for acid soluble lignin (ASL)/Klason lignin estimation (Jamaldheen *et al.,* 2018). Holocellulose content was estimated by Browning method (Browning, 1967). Hemicellulose and Klason lignin were estimated by the standard TAPPI protocols (Jamaldheen *et al.,* 2018).

2.2.3 Delignification of the sugarcane trash

50 g of moisture free SCT powder was suspended in 500 mL distilled water in a 1000 mL reagent bottle. This was followed by addition of sodium chlorite (20 g) and glacial acetic acid (2.5 mL), mixed thoroughly and heated in water bath at 60°C for 3 h. The hydrolysate was then removed by filtration through Whatman no. 1 filter paper and biomass was neutralized by washing with distilled water (Rowley *et al.,* 2013). The de-lignified SCT was dried at 80°C for 24 h and used further for other experiments.

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2.2.4 Separation of cellulose from de-lignified sugarcane trash

The removal of hemicellulose is required to achieve the pure cellulose from SCT. The cellulose was recovered from SCT by following the modified alkaline separation method as reported earlier (Zilliox and Debeire, 1998). 20 g of de-lignified SCT was mixed with 200 mL of a mixture containing 10 (%, w/v) of sodium hydroxide and 1 (%, w/v) of boric acid. The higher concentration of sodium hydroxide to remove the hemicellulose has been also reported previously (Sundarraj and Ranganathan, 2018). The suspension was stirred at 2000 rpm, 60°C for 3 h and filtered through cheese cloth. The separated solid residues as cellulosic fibres were neutralized by washing under tap water and final washing with distilled water. The separated cellulosic fibres were dried at 80°C for 12 h and used for further studies. The dried weight of separated cellulose was noted for determining the cellulose yield. Cellulose yield was calculated by,

$$Cellulose\ yield\ (\%) = \frac{\text{Dried weight of cellulose fiber}}{\text{Total cellulose present in raw biomass}} X\ 100$$

Here, the total cellulose present in raw SCT biomass was calculated by the difference between total holocellulose estimated by Browning (Browning, 1967) method and total hemicellulose estimated by standard TAPPI (Jamaldheen *et al.*, 2018) protocols.

2.2.5 Monosaccharide analysis of cellulose separated from SCT

The monosaccharide composition analysis of cellulose separated from SCT was performed by treating 5 mg separated cellulose with1 mL of 2 M Tri-fluro-acetic acid (TFA) in a 1.5 mL Eppendorf tube and by heating in a boiling water bath for 3 h. The hydrolyzed cellulose was kept in hot air oven at 80°C for 12 h to remove the remaining residual TFA. The dried TFA hydrolysed cellulose sample was mixed in 500 µL

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degassed Milli-Q water and then filtered through a syringe filter using by PVDF membrane (Pore size: 0.22 µm). The filtered sample was transferred to the HPLC vials (Axiva Sichem Biotech, India) and analyzed for monosaccharides present by High Performance Liquid Chromatography (HPLC) (UFLC, Prominence, Shimadzu, Japan) using a RI detector. 10 µL of TFA treated SCT cellulose was loaded and passed through the guard column (50 mm x 7.8 mm), followed by passing through the connected monosaccharide separation column phenomenex Rezex ROA (H+) (300 mm x 7.8 mm). The elution was performed by 0.005 N H_2SO_4 solution at 70ºC at 0.5 mL/min flow rate for 90 min run time per sample. 1 mg/mL each of glucose, xylose, arabinose or glucuronic acid was used as standard for composition determination.

2.2.6 Field-Emission Scanning Electron Microscopy (FE-SEM) analysis of raw SCT and separated cellulose

The surface morphology of raw SCT and separated cellulose were analyzed by FE-SEM (Carl Zeiss, Model-Gemini 300, Germany). The carbon tape was fixed on the stab surface before placing the raw SCT and separated cellulose samples. Both the samples prepared on the stab was double gold coated and kept in the vacuum chamber to remove the moisture from the samples. Then sample image was taken by FE-SEM (Carl Zeiss, Model-Gemini 300, Germany) at 2000X magnification.

2.2.7 Crystallinity study of raw SCT and separated cellulose by X-Ray Diffraction

XRD analysis was done to compare the crystallinity indices of raw SCT and separated cellulose. The dried raw SCT and separated cellulose from SCT (1 g of each) was assessed for their respective crystallinity indices (*CrI*) by X-ray diffractometer (D8 Advance, Bruker, Germany). Each sample was scanned over a range of $2\theta = 10°–30°$

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with a step size of 0.05°. The *CrI* of SCT samples was calculated by using following formula (Segal *et al.,* 1959),

$$CrI\ (\%) = ((I_{crystalline} - I_{amorphous}) / I_{crystalline}) \times 100$$

Where, $I_{crystalline}$ is the intensity at degree, $2\Theta = 22^{o}$ and $I_{amorphous}$ is intensity at a degree, $2\Theta = 18^{o}$ (Jamaldheen *et al.*, 2018).

2.2.8 Fourier Transform Infrared (FT-IR) analysis of separated cellulose

The functional groups present in separated cellulose were determined by the FT-IR analysis. The SCT cellulose and cellulose (HiMedia Laboratories, India) samples were mixed with KBr in the ratio 1:100 (w/w) and ground by using mortar and pestle. The pellet was prepared from ground powder with the help of 15 tonne hydraulic press. The prepared pellet was scanned on FT-IR spectrophotometer (Perkin Elmer, Spectrum Two, USA) between wavenumber range, 4000-400 cm^{-1} (Akhtar *et al.,* 2017).

2.2.9 Thermal analysis of cellulose separated from SCT

The thermo-gravimetric and differential thermal properties of cellulose separated from sugarcane trash were analysed by thermal analyser (Netzsch, Model: STA449F3A00). For TGA analysis, 6 mg of SCT cellulose was taken in alumina crucible and heated at a rate of 10ºC min^{-1} from 30 to 500ºC. The nitrogen gas was used at a flow rate of 60 mL/min. The weight loss and heating rate of TGA analyser were continuously recorded throughout the experiment.

2.2.10 Elemental composition analysis of separated SCT cellulose

Ultimate analysis of biomass gives the information regarding the composition of carbon, hydrogen, nitrogen and sulphur (CHNS) of separated cellulose fibers. Carbon, nitrogen, sulphur and hydrogen present in cellulosic fibers were determined by

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the CHNS analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Italy), whereas oxygen was determined by calculating the difference in the percentage.

2.2.11 Production of recombinant hydrolytic enzymes for hydrolysis of SCT samples

The gene encoding endo-β-1,4-glucanase (*Ct*Cel8A) of family 8 glycoside hydrolase (Jamaldheen *et al.*, 2018) and β-1,4-glucosidase, (*Ht*Bgl) of family 1 glycoside hydrolase cloned and reported earlier (Jamaldheen *et al.*, 2018, Sharma *et al.*, 2019) were used in the present study. The plasmid containing gene encoding cellobiohydrolase, *Ct*CBH5A of family 5 glycoside hydrolase cloned from *Clostridium thermocellum* (renamed *Hungateiclostridium thermocellum*) was a gift from Prof. Carlos M.G.A. Fontes, NZYTech Ltd., Lisbon, Portugal. The plasmids containing the genes encoding endo-β-1,4-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-1,4-glucosidase (*Ht*Bgl) from *Hungateiclostridium thermocellum* were transformed in *Escherichia coli* BL21 (DE3) cells and grown as reported earlier (Jamaldheen *et al.*, 2018). In 5 mL of Luria-Bertani (LB) medium, 50 µg/mL of kanamycin were added 25 ml culture tubes and 50 µL of each from the glycerol stock was inoculated separately and incubated at 37°C, 180 rpm for 12 h. The revived *E. coli* cells expressing *Ct*Cel8A, *Ct*CBH5A or *Ht*Bg1 enzymes were inoculated at 1% (v/v) inoculum in 400 mL LB medium in 1 L Erlenmeyer flasks containing kanamycin (final conc. 50 µg/mL) and incubated at 37°C, 180 rpm for 2-3h till the OD at 600 nm reached 0.4. Then 1 mM IPTG was added to each culture flask to induce the respective enzyme production and incubated at 24°C, 180 rpm for 18 h. The cell cultures were centrifuged at 8000g, 4°C for 10 min. The cell pellet was re-suspended in 15 mL lysis buffer (50 mM sodium phosphate, pH 7.5) and the cell free extract obtained was subjected to sonication for 30

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min (5 s on/10 s off pulse, 33% amplitude, Sonics, Vibra cell). The sonicated cells were centrifuged at 16000g, 4°C for 1 h. The N-terminal hexa-His tag containing *Ct*Cel8A, *Ct*CBH5A and *Ht*Bgl enzymes were purified by 5 mL sepharose column (Hi-Trap Chelating, GE Healthcare). The purified *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1 enzymes were extensively dialyzed against 50 mM sodium phosphate buffer (pH 7.5). The concentration of enzymes was determined by Bradford method by taking absorbance at 595 nm (A_{595}) on a UV spectrophotometer (Varian, Cary 100). Bovine serum albumin (BSA) was used as a standard.

2.2.12 Enzyme activity assay of recombinant hydrolytic enzymes against SCT

The endo-β-1,4-glucanase (*Ct*Cel8A) enzyme activity was determined by incubating 5 µL (0.1 mg/ml) of enzyme with 1% (w/v) carboxymethyl cellulose (CMC) (Sigma, USA) in 50 mM citrate phosphate buffer (pH 5.8) in a 0.1 mL reaction mixture at 70 °C for 1 min. The enzyme activity was calculated by determining the reducing sugar release by the method reported earlier (Nelson, 1944, and Somogyi, 1945). The total amount of reducing sugar released was calculated using standard plot of D-Glucose. The 1 unit (U) of enzyme activity was defined as the amount of *Ct*Cel8A enzyme required in mg to produce 1 µmole of D-glucose per min under optimum condition.

The cellobiohydrolase (*Ct*CBH5A) enzyme activity was determined by taking 5 µL (0.1mg/ml) of enzyme with 1% (w/v) carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 6) in a 0.1 mL reaction mixture and incubating at 60°C for 2 min. The enzyme activity was calculated by determining the released reducing sugar concentration by method of Nelson (1944) and Somogyi (1945). The amount of reducing sugar released was calculated by using the standard plot of D-Glucose. The 1

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unit (U) of enzyme activity was defined as the amount of *Ct*CBH5A enzyme required in mg to produce 1 µmole of D-glucose per min under optimum condition.

The β-1,4-glucosidase (*Ht*Bgl) enzyme activity was determined by using cellobiose as substrate. The enzymatic hydrolysis was performed in 1 mL reaction mixture containing 1% (w/v) cellobiose in 50 mM citrate phosphate buffer (pH 6.5), 5 µL of the enzyme (0.1 mg/mL) by incubating at 65°C for 5 min. The enzyme activity was calculated by estimating the release of glucose by taking absorbance at 505 nm (A_{505}) on a UV–Visible spectrophotometer (Varian, Cary 100 Bio). One unit of enzyme activity for cellobiose was defined as the amount of *Ht*Bgl enzyme in mg was required to release 1 µmole of glucose per min (Brini *et al.*, 2010).

2.2.13 Enzymatic saccharification of SCT samples

The enzymatic hydrolysis of raw SCT and separated cellulose was carried out by taking 1% (w/v) biomass in 10 mL 50 mM citrate phosphate buffer, pH 6 in 15 mL centrifuge tube in triplicate sets. The recombinant enzymes, endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bgl) in a ratio 150:150:200 U/g were added to each of the reaction tube. Sodium azide 0.03% (w/v) was added to each reaction mixture containing tube to prevent the contamination. The reaction mixture was incubated at 50°C in a shaker incubator at 180 rpm for 24 h. The reaction was stopped by boiling the reaction mixture for 5 min in boiling water bath and then centrifuged at 13,000g, 25°C for 10 min, to collect the supernatant containing released sugar. Total reducing sugar (TRS) released was calculated by the method of Nelson (1944) and Somogyi (1945). The supernatant was used for the TLC analysis. The standard glucose and cellobiose samples were also loaded along with the hydrolysed samples of raw SCT and SCT cellulose in separate lanes on TLC plate and dried for 5

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min. The plate was kept in the TLC chamber within the mobile phase having methanol, acetic acid and water in a ratio 2:1:1. The migrated hydrolyzed products were analysed by immersing the plate in a solution containing 0.5% (w/v) α-naphthol in methanol and sulfuric acid (95:5) solution (Gupta *et al.*, 2017).

2.2.14 Mass balance of raw SCT to enzymatic saccharification process

The mass balance of untreated SCT biomass (100 g) for each processing step including delignification, alkali pretreatement and recombinant enzymatic saccharification was carried out. The recovery of biomass at each step on dry weight basis was calculated by formula (Guilherme *et al.*, 2017),

$$Biomass\ recovery\ yield\ (\%) = \frac{Recovered\ cellulose\ fiber\ (g)}{Initial\ weight\ of\ raw\ SCT\ (g)} X\ 100$$

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2.3 Results and Discussion

2.3.1 Analysis of structure composition of raw SCT

The moisture content of raw SCT was found to be 4.3% (w/w). Raw SCT contained 70.1% (w/w) holocellulose, 27.2% (w/w) hemicellulose and 14% (w/w) acid soluble lignin/Klason lignin. The total amount of cellulose present in raw SCT was estimated by calculating the difference between holocellulose (70.1%, w/w) and hemicellulose contents (27.2%, w/w) determined by Browning (1967) and TAPPI (Jamaldheen *et al.*, 2018) methods, which was found to be 43% (w/w).

2.3.2 Delignification of raw SCT

The raw SCT after delignification by sodium chlorite method gave 34.5 g of delignified SCT, resulting in 69% (w/w) yield of delignified SCT. The delignification was confirmed by standard TAPPI protocol.

2.3.3 Separation of cellulose from delignified SCT

Twenty grams of delignified SCT gave 11.6 g of cellulose, resulting in an overall 40% (w/w) yield based on raw SCT. The recovery yield of cellulose was 85% (w/w) of total cellulose. The cellulose recovered from SCT is shown in the Fig.2.1.

Fig.2.1. Digital image showing the **c**ellulose separated from sugarcane top (SCT).

2.3.4 Monosaccharide analysis of separated SCT cellulose

The monosaccharide composition analysis of separated cellulose by HPLC displayed the presence of 91% of D-glucose, 7.5% of D-xylose and 1.5% of D-arabinose residues (Fig. 2.2).

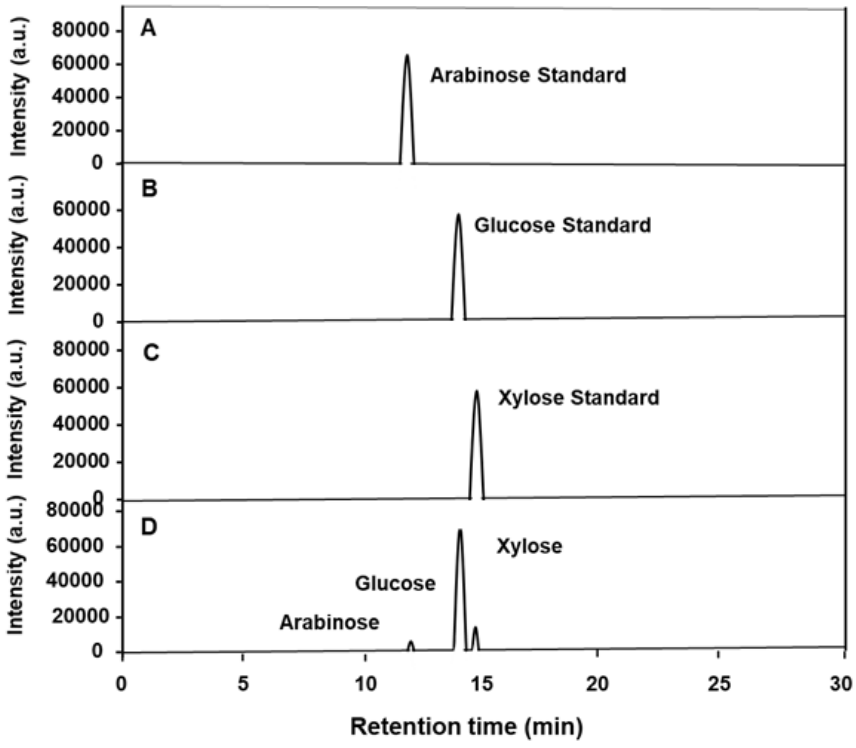

Fig. 2.2. The composition analysis of SCT cellulose by high performance liquid chromatography (HPLC). A) Arabinose standard B) Glucose standard, C) Xylose standard for HPLC and D) HPLC analysis of TFA treated SCT cellulose.

2.3.5 Surface morphology analysis of raw SCT and cellulose separated from SCT

The FESEM electromicrographs of the cellulose separated from SCT (Fig. 2.3B) showed clear fibrous structures as compared to the raw SCT (Fig. 2.3A) as also reported earlier (Kain *et al.*, 2018). This fibrous structure of separated cellulose could due to prior delignification and xylan removal leading to the exposure of cellulose fibres appearing as fibrous bundles, which are absent in raw SCT.

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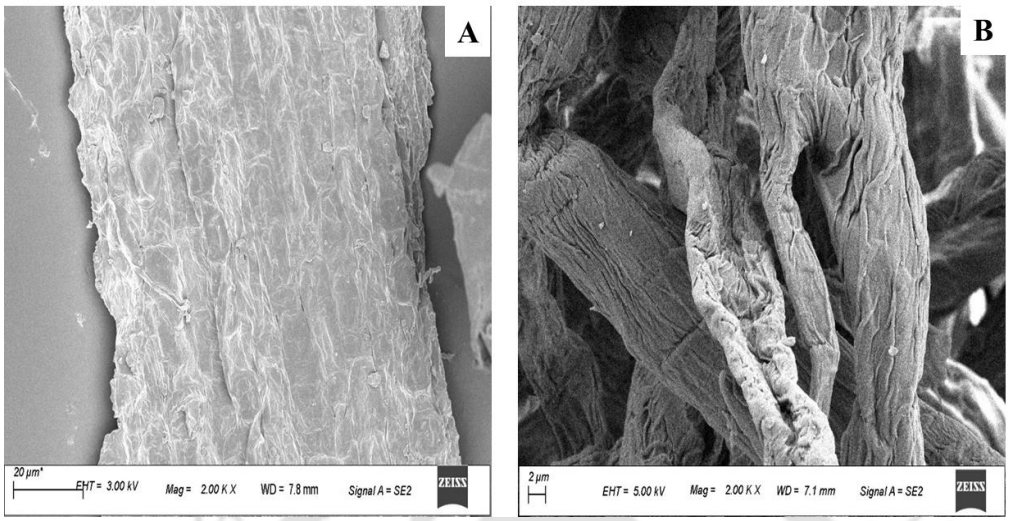

Fig. 2.3. FE-SEM image of raw biomass (A) and separated cellulose (B) from SCT.

2.3.6 X-Ray Diffraction analysis of raw SCT and cellulose separated from SCT

The powdered raw SCT and cellulose separated from SCT displayed 36% and 49% crystallinity index (*CrI*), respectively. These results explained that the increase in crystallinity index (Fig. 2.4) of separated cellulose was due to the prior delignification and xylan removal that resulted in the exposure of the crystalline cellulose, as also reported earlier (Fan *et al.*, 1980). The crystallinity index of commercial cellulose II reported was 61% (El-Sakhawy *et al.*, 2017). This difference between the crystallinity index of cellulose II and cellulose separated from SCT may be due to the difference in their purity.

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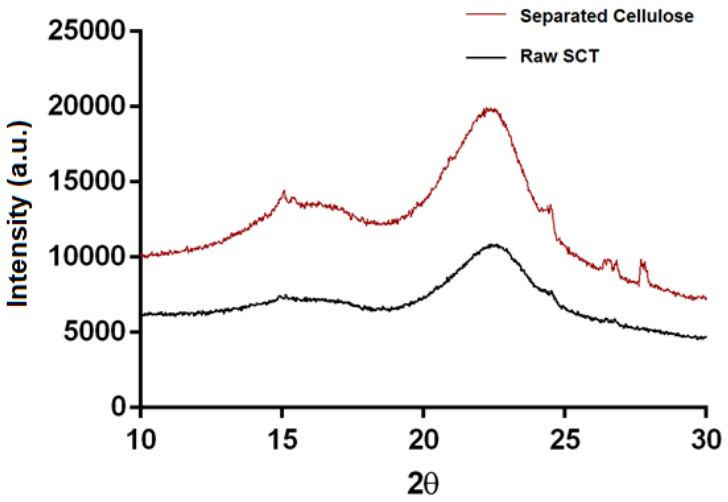

Fig. 2.4. XRD analysis of raw SCT and separated cellulose from SCT.

2.3.7 Functional group analysis of cellulose separated from SCT

The FTIR spectra of cellulose separated from SCT and commercial cellulose are shown in Fig. 2.5. The absorption peaks for cellulose were observed in two regions at wavenumbers ranges (3660 to 2800 cm^{-1} and 1650 to 800 cm^{-1}). The stretching vibrations of C-H and O-H bonds of the polysaccharide showed peaks in the range, 3660 to 2900 cm^{-1}. However, the stretching vibration of O-H group in cellulose showed a broad peak at 3331 cm^{-1} as also reported previously (Rosa *et al.*, 2010, Poletto *et al.*, 2011). The intra- and intermolecular vibrations (Popescu *et al.*, 2011) and C-H stretching vibration present in overall hydrocarbon constituents is attributed at 2894 cm^{-1} (Jamaldheen *et al.*, 2018). Bands assigned particularly for cellulose were found in the region, 1630 to 900 cm^{-1} (Poletto *et al.*, 2011). A peak at 1633 cm^{-1} represented the absorbed water vibration in separated cellulose. The bending and stretching vibrations of -CH_2 and -CH, -OH and C-O bonds in separated cellulose were observed at 1428, 1367, 1334, 1027 and 896 cm^{-1} as also reported earlier (Xu *et al.*, 2013). The crystalline structure of cellulose is associated with the peaks at around 1430 to 1420 cm^{-1}, while

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amorphous region in cellulose is associated with the band at 897 cm^{-1} as also mentioned earlier (Poletto *et al.*, 2011). The CH and CH_2 bond vibrations in cellulose showed the peaks at 1428 cm^{-1} and 1337 cm^{-1}, respectively (Ibrahim *et al.*, 2011). All above mentioned peaks in FTIR spectrum observed in the separated cellulose were also found in the commercial cellulose confirmed the purity of cellulose separated from SCT.

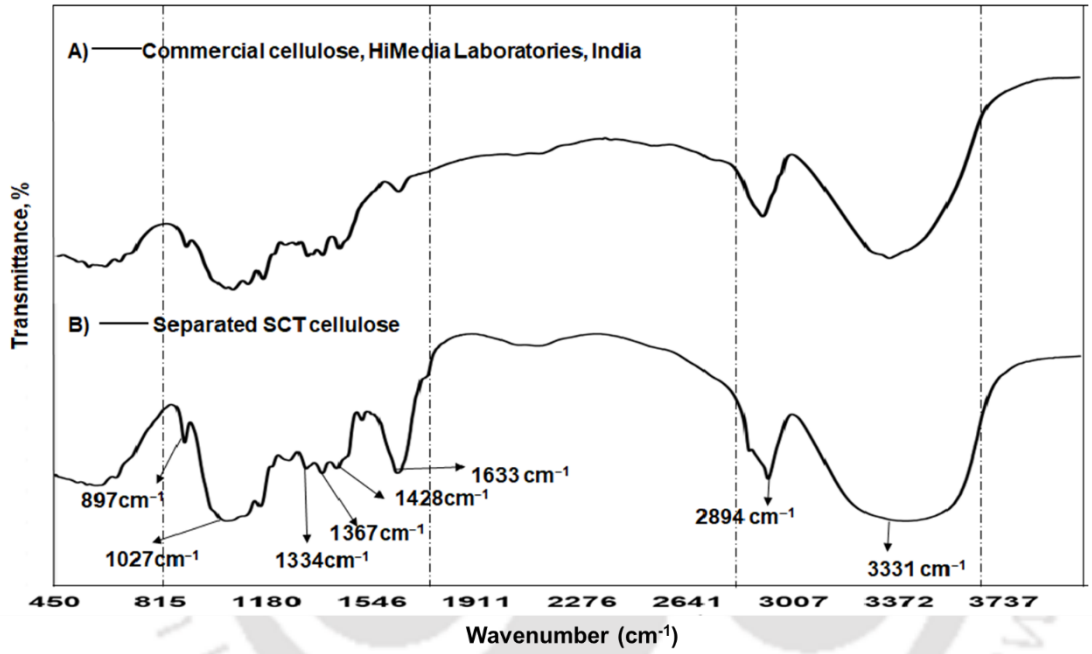

Fig. 2.5. FT-IR analysis of A) commercial cellulose (HiMedia laboratories, India) and B) separated cellulose from SCT.

2.3.8 TGA analysis of cellulose separated from SCT

TGA analysis of cellulose separated from SCT was used to estimate the amount of remaining inorganic impurities in the separated cellulose fibres (Fig. 2.6). Thermal analysis of separated cellulose showed weight loss between 80°C and 150°C, which are associated with the dehydration process. The decomposition process of separated cellulose initiated at 180°C. This decomposition process ended at 400°C and is strictly

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related to the thermal decomposition of cellulose (organic carbon) in the oxidizing atmosphere as also mentioned earlier (Huang, 2012).

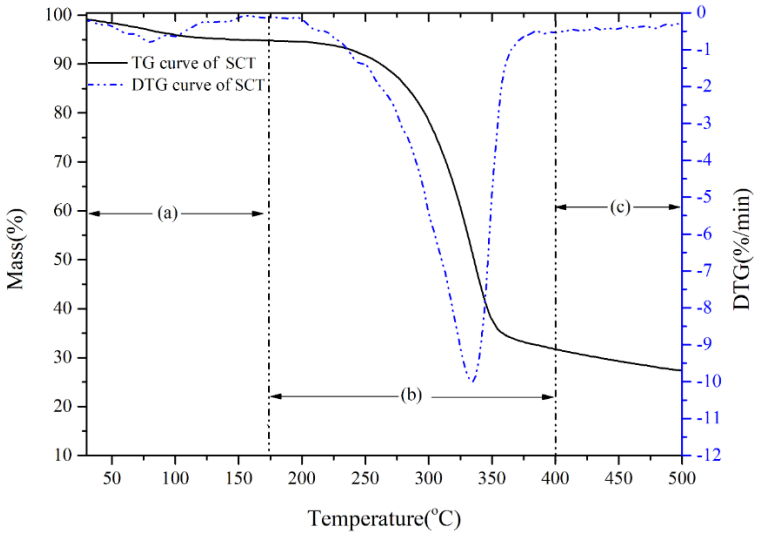

Fig. 2.6. TGA and DTG analysis of SCT cellulose. Region (a) showed the dehydration of water present in the separated cellulose from SCT. Region (b) indicated the thermal degradation/ decomposition of separated cellulose into organic compounds and region (c) denoted the decomposition of residual biomass.

The typical TGA curve for separated SCT cellulose showed 65% mass reduction and DTG decomposition at 327ºC. The microcrystalline cellulose from Sigma Aldrich, USA showed similar DTG decomposition curve at 322ºC and 70% mass reduction in TGA as also reported earlier (Taib *et al.*, 2020). After 400°C, the decomposition of residual biomass leads to the formation of CO, CO_2, H_2O and char as also reported earlier (Yang *et al.*, 2006). Breakage of the glycoside linkage after 400°, reduces the degree of polymerization within the cellulosic fibers, leads to the formation of organic compounds such as alkenes and other hydrocarbon derivatives as reported earlier (Poletto *et al.*, 2012). In the present study, the single decomposition peak obtained in DTG analyses confirmed the SCT cellulose is pure and there are no other impurities (xylan or proteins) in it.

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2.3.9 Elemental composition analysis of cellulose separated from SCT

The air dried cellulose separated from SCT was used to determine the elemental composition by CHNS analyzer. The element composition analysis of powdered cellulose displayed that the carbon, hydrogen and oxygen contents 41.45%, 4.4% and 55.85%, respectively. The absence of nitrogen and sulphur in the extracted cellulose showed that the cellulose is pure and devoid of protein (Oftedal *et al.,* 2014) and lignin cross linkages (Karunarathna *et al.,* 2019).

2.3.10 Production of recombinant cellulolytic enzymes for saccharification

The protein concentration of purified endo-β-1,4-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-1,4-glucosidase (*Ht*Bgl) enzymes was 4.2 mg/mL, 2.9 mg/mL and 2.5 mg/mL, respectively. The purified *Ct*Cel8A and *Ct*CBH5A enzymes showed endoglucanase activity, 108 U/mg and cellobiohydrolase activity, 30 U/mg, respectively, against CMC-Na. The purified *Ht*Bgl showed β-glucosidase activity, 58 U/mg against cellobiose.

2.3.11 Molecular mass and purity of recombinant proteins by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The expressed recombinant proteins *viz.* endo-β-1,4-glucanase (*Ct*Cel8A) of family 8 glycoside hydrolase, cellobiohydrolase (*Ct*CBH5A) of family 5 glycoside hydrolase and β-1,4-glucosidase (*Ht*Bgl) of family 1 glycoside hydrolase were run on SDS-PAGE using 10% (w/v) gel for analyzing their molecular mass and purity (Fig. 2.7A, B and C). The Biobharati marker, India were used for the molecular mass analysis in (kDa).

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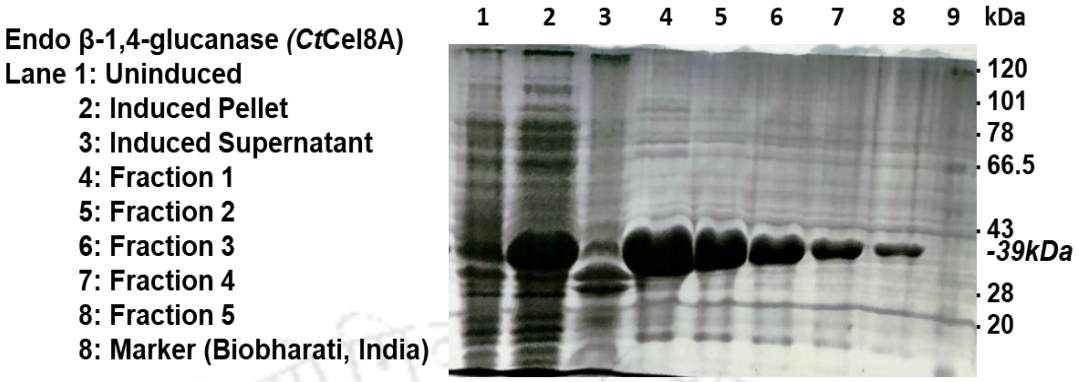

Fig. 2.7A. SDS-PAGE of endo-β-1,4-glucanase (*Ct*Cel8A).

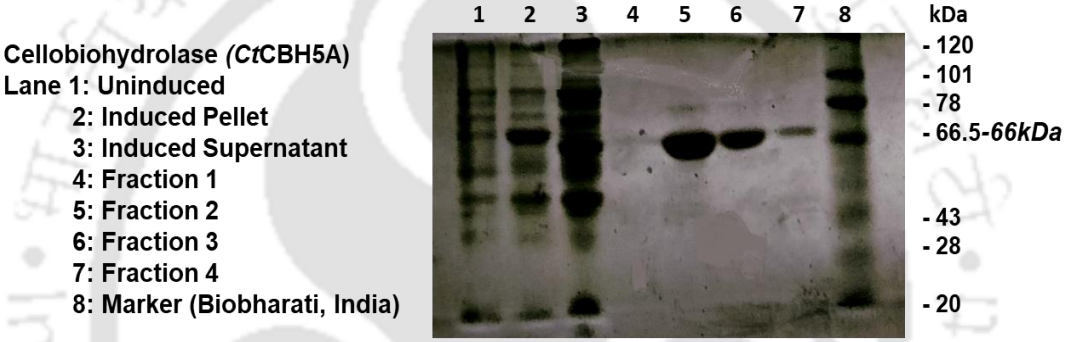

Fig. 2.7B. SDS-PAGE of cellobiohydrolase (*Ct*CBH5A).

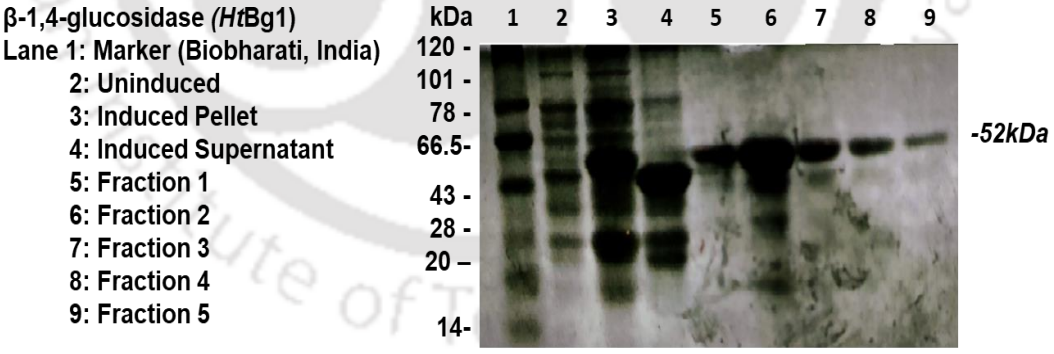

Fig. 2.7C. SDS-PAGE of β-glucosidase (*Ht*Bgl).

2.3.12 Optimization of enzyme loading for saccharification of SCT

The optimization of enzymatic saccharification of SCT cellulose was carried out by recombinant endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) enzymes from *Clostridium thermocellum* and β-glucosidase (*Ht*Bgl) from *Hungateiclostridium*

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thermocellum, respectively. The enzyme loading was varied from 100 U/g to 200 U/g of SCT cellulose for each enzyme and the total loading of all three enzymes was taken constant *i.e.* 500 U/g of SCT cellulose. Other parameters such as substrate, 1% (w/v) SCT cellulose, 50 mM sodium citrate buffer (pH 6), temperature, 50°C and saccharification time, 24 h were kept constant. The reaction conditions, reducing sugar and glucose yield obtained are listed in Table 2.1. The maximum TRS yield (188 mg/g: Nelson-Somogyi method) and glucose yield (115 mg/g: GOD-POD analysis) for SCT cellulose saccharification was obtained at 150 U/g, 150 U/g and 200 U/g loading of recombinant endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bgl), respectively.

Table 2.1. Optimization of enzyme activity (U/g) for saccharification of SCT.

Experiment	*Ct*Cel8A (U/g of SCT)	*Ct*CBH5A (U/g of SCT)	*Ht*Bgl (U/g of SCT)	TRS yield (mg/g)	Glucose yield (mg/g)
I	200	150	150	170±9.1	97±3.0
II	150	200	150	180±8.0	100±4.1
III	150	150	200	188±12.1	115±3.4
IV	100	200	200	180±13.2	110±5.5
V	200	200	100	150±8.1	70±2.8
VI	200	100	200	182±7.2	108±4.9

2.3.13 Enzymatic saccharification of raw SCT and cellulose separated from SCT

The enzymatic hydrolysis of raw SCT by using endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bgl) gave the TRS yield, 2.83 mg/g of raw SCT, whereas, separated cellulose yielded TRS, 188 mg/g of separated cellulose. The GOD-POD analysis of enzyme hydrolyzed separated cellulose yielded glucose, 115 mg/g of separated cellulose. The TRS (188 mg/g of separated cellulose) released from 1 g of separated cellulose gave 21% of glucose yield (showed by HPLC

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analysis) from total 91% glucose present in separated cellulose. The TLC analysis of enzymatic hydrolyzed raw SCT showed no hydrolyzed product in hydrolysate. However, the SCT cellulose clearly showed cello-oligosaccharides of various degrees of polymerization in the hydrolysate (Fig. 2.8).

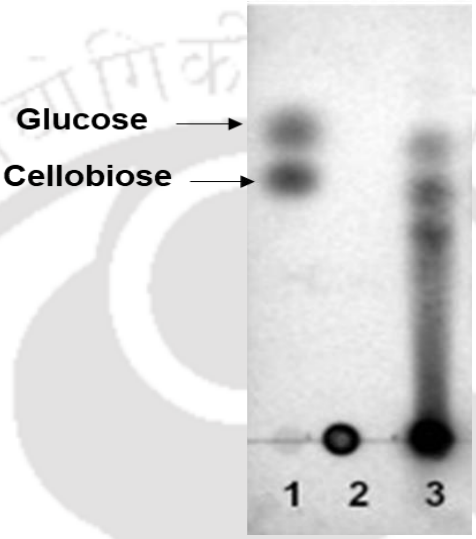

Fig. 2.8. TLC analysis of hydrolysed products of raw SCT and SCT cellulose by recombinant endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bgl) enzyme from *Clostridium thermocellum* at 50°C for 24 h. Lane 1, Standards (Glucose and Cellobiose), Lane 2, Unhydrolysed product of raw SCT, Lane 3, Hydrolysed products of separated SCT cellulose.

As seen in the FESEM image discussed above, prior delignification and xylan removal of raw SCT increases the accessibility of separated cellulose by disrupting the interlinkages between crystalline and amorphous cellulose that cause steric hindrance and exposure of cellulosic fibers towards the cellulolytic enzymes. However, much higher glucose yield was reported for other biomasses by using commercial enzymes (Dodo *et al.,* 2017). The lower TRS yield obtained here could be attributed to the difference in biomass, the type of the pretreatment and also the enzymes used. The saccharification of SCT cellulose by recombinant enzymes *Ct*Cel8A, *Ct*CBH5A and *Ht*Bgl can be further enhanced by optimizing the process parameters.

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2.3.14 Mass balance of raw SCT to enzymatic saccharification process

The mass balance of 100 g raw biomass to recombinant enzymatic saccharification is shown in the Fig. 2.9. In initial delignification step, 69 g of solid delignified SCT residue was recovered that contained 43 g cellulose and 26 g hemicellulose. After the alkali pretreatment of delignified SCT, 40 g of apSCT solid was recovered which contained 36.4 g glucose, 3.4 g xylose and 0.6 g arabinose. The enzymatic saccharification of 40 g delignified SCT gave 18.8 g of reducing sugar (or 11.5 g of glucose), thereby resulting 7.52 g (w/w) reducing sugar (or 4.6 g, w/w glucose) released from 100 g of raw SCT.

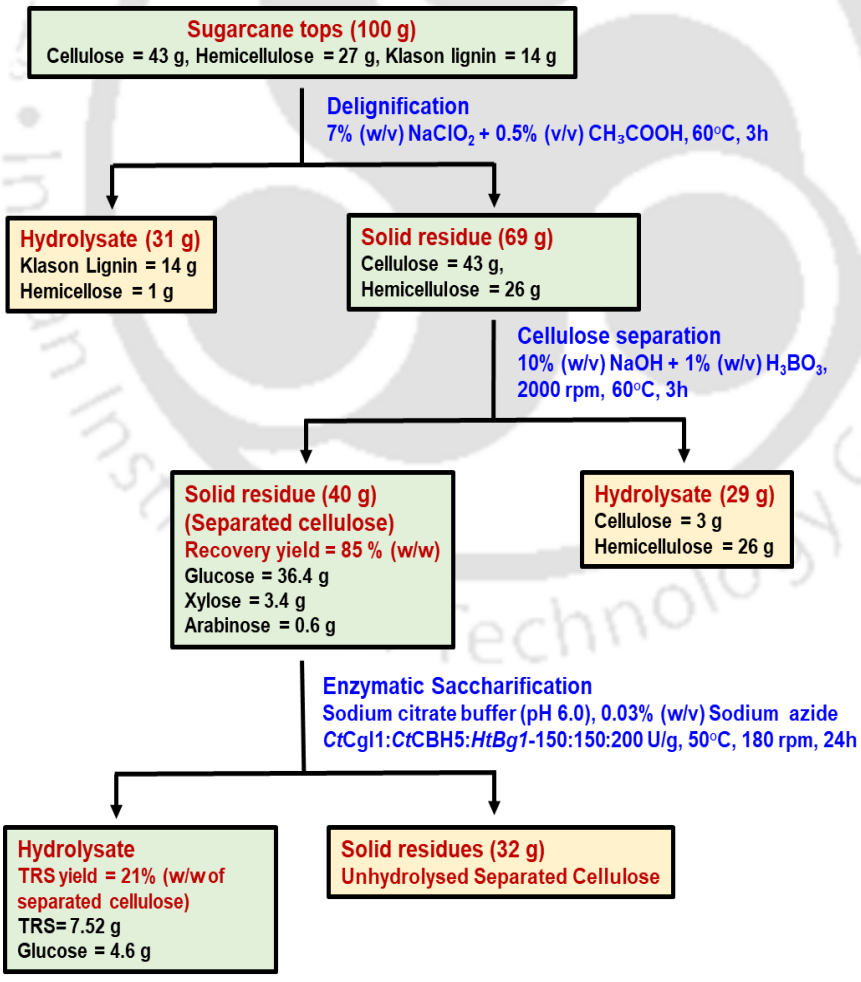

Fig. 2.9. Mass balance for saccharification process of raw SCT biomass by recombinant enzyme cocktail.

Conclusions

The agro-waste, SCT was explored for recovery of potentially a commercial cellulose. The separated cellulose was found to contain 70% (w/w) holocellulose content, which is a significant fraction for production of monosaccharide that can be used in bioethanol production. The monosaccharide analysis of SCT cellulose after TFA hydrolysis by HPLC displayed the presence of 91% D-glucose, 7.5% D-xylose and 1.5% D-arabinose residues. The 85% of recovery yield was obtained from the total cellulose present in SCT biomass. The exposed cellulose fibres observed by FESEM analysis and the enhanced crystallinity by XRD analysis supported the purity of separated cellulose. The presence of crystalline and amorphous cellulose was confirmed by FTIR spectrum. TGA of SCT cellulose gave single decomposition temperature at 327ºC and CHNS analysis confirmed absence of impurities. The enzymatic saccharification of 1 g cellulose separated from SCT by recombinant endo-β-1,4-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-1,4-glucosidase (*Ht*Bg1) yielded 188 mg/g (TRS) of separated cellulose of reducing sugar and 115 mg/g of glucose per gram of separated cellulose, respectively. This resulted the 21% of reducing sugar conversion from saccharification of separated SCT cellulose. The enzymatic hydrolysis using these enzymes did not show efficient saccharification yield, which might be due to the increase in crystallinity of SCT cellulose. However, there is a scope of enhancing the yield by optimization of enzyme hydrolysis parameters. The above results showed the purity of separated cellulose and this process can help in improving the bio-refinery concept for the bioethanol production and the pure separated cellulose can be also used for other commercial applications. The use of purified cellulose for bioethanol production can be expensive due its high commercial value. Therefore,

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another pretreatment strategy was developed (described in the chapters 5 and 6) in order to achieve the bioconversion of SCT biomass to bioethanol (described in the chapter 6) and other value added products (described in chapter 4).

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Chapter 3

Extraction and characterization of xylan from delignified sugarcane tops as a potential commercial substrate

3.1 Introduction

Sugarcane (*Saccharum officinarum*) is one of the major cultivated crops in tropical regions. Every one ton of processed sugarcane produces 140 kg of bagasse (Dias *et al.*, 2012) and 250 kg (dry mass) of sugarcane trash/ sugarcane top (SCT) residues (Chandel *et al.*, 2012). By means of the quantity, sugarcane was the world's largest crop produced in 2016 and was 1.9 billion tons as reported by Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), USA. The worldwide annual production of SCT is 279 million metric tons that could be used for cellulosic ethanol production (Chandel *et al.*, 2012). Around 50% of SCT can be removed from the field without affecting the nutritional properties of soil (Macrelli *et al.*, 2012). The dry leaves can be collected from agricultural field up to 66% and used as a feedstock in biofuel industries (Chandel *et al.*, 2012). These post harvested residues are abundant, readily available and are cheap source of lignocellulosic biomass (Sukumaran *et al.*, 2010). SCT contains three main components *viz.* cellulose,

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hemicellulose and lignin. SCT is the field residue remaining after the harvesting of sugarcane stalk, while sugarcane bagasse is the fibrous residue left after milling of sugarcane stalk. The SCT contains 43% cellulose, 27% hemicellulose and 17% lignin (Khaire *et al.*, 2020) while SB contains 34% cellulose, 24% hemicellulose and 25% lignin (Faruk and Sain., 2015). Cellulose is a straight chain polymer of glucose units having β-1, 4-glycosidic linkages. Hemicelluloses are a group of complex plant polysaccharides that are biosynthesized in large quantities in most trees and terrestrial plants. Hemicellulose is the second most abundant biopolymer produced by plants. Xylan is the major hemicellulose found in secondary cell walls of sugarcane bagasse. Xylan is made up of xylose backbone with a high degree of substitutions of arabinose and glucuronic acids, which directly affect its structure and functional characteristics (Costa *et al.*, 2016). Based on the method of extraction and source of plant/tissue used, the structure and composition of extracted xylan varies (Petzold-Welcke *et al.*, 2014). The backbone of xylan consists of β-1,4-linked xylose residues, which is often substituted with methylated α-D-glucopyranosyl uronic acid, α-L-arabinofuranosyl, ferulic acid and *p*-coumaric acid in the side chain (Ordaz-Ortiz *et al.*, 2005). Xylans also contain D-galactose, D-xylose, L-rhamnose, D-glucose, acetyl groups and different dimeric and trimeric side chains (Moine *et al.*, 2007).

A chosen extraction method for xylan extraction compromises among extraction yield, selectivity and degree of polymerization (DP) (Liu *et al.*, 2011). The extracted xylans can be used for biotechnological applications, however, some lignin fractions are also associated (Hutterer *et al.*, 2016). Another simple traditional procedure of xylan extraction is by alkaline method followed by ethanol precipitation of xylan, however, the yield is generally low (Sporck *et al.*, 2017). However, the alkaline method used for

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xylan extraction from delignified sugarcane bagasse showed a higher yield of low purity and contains 16-18% residual lignin (Chimphango *et al.*, 2012). The modification in traditional alkaline hemicellulose extraction methods by including de-lignification with sodium chlorite followed by the extraction gave high xylan yield (Höije *et al.*, 2005). The xylan can be recovered from pretreated biomass, before the enzymatic saccharification of the cellulosic component, and used for other value added products thus leaving the cellulosic solids for bioethanol production. It has been reported that the hemicellulose removal can also enhance the cellulolytic hydrolysis (Meng *et al.*, 2014). The extraction of xylan from SCT is not much explored. The present study elaborates the extraction of xylan from liquid alkaline hydrolysate produced after the alkaline pretreatment of delignified SCT (as described in chapter 2) and its characterization. The extracted and purified xylan was structurally and functionally characterized and demonstrated as a commercial xylan for laboratory and industrial purposes. The extracted xylan was used to produce xylo-oligosaccharides (XOS) by hydrolysis by using recombinant clostridial xylanase. XOS have prebiotic potentials and are used in numerous nourishment products.

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3.2 Material and Methods

3.2.1 Material and feedstock collection and its processing

SCT (residue remaining after the harvesting of sugarcane stalk) was collected from Machkhowa, Guwahati, Assam, India. Sodium chlorite, sodium hydroxide, boric acid and other chemicals were purchased from Himedia Pvt. Ltd. (Mumbai, India). Reagents (ethanol and acetic acid) and thin layer chromatography (TLC) plate were purchased from Merck India Pvt. Ltd. (Mumbai, India). Beechwood xylan, oat spelt xylan and 4-O-methyl D-glucurono D-xylan from Sigma Chemical Corporation (St. Louis, MO, USA) and xylan from Carbosynth, (Compton, UK) were purchased. The feedstock was washed before and after chopping with tap water and rinsed with distilled water. Chopped SCT were dried in oven at 60°C for 24 h and then ground to powder using a mixer grinder (HL1606/03, Philips, Amsterdam, Netherlands) and passed through a 0.85 mm sieve.

3.2.2 Delignification of Sugarcane tops

SCT was dried at 80ºC for 24 h to remove the moisture content. Delignification was done in 1 L reagent bottle (Borosil, Ahmedabad, India) by adding 500 mL of distilled water and 50 g of air-dried sugarcane top powder. Approximately, 35 g of sodium chlorite ($NaClO_2$) was added and mixed well followed by addition of 2.5 mL glacial acetic acid. The bottle was closed and incubated at 60ºC in a water bath in a fume hood for 3 h. After incubation, the hydrolysate was removed by filtering using Whatman no. 1 filter paper under tap water till de-lignified SCT was neutralized. De-lignified SCT was dried at 80ºC for 24 h (Rowley *et al.*, 2013). The delignification of SCT was confirmed by standard TAPPI protocol.

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3.2.3 Extraction of xylan from SCT

The extraction of xylan from SCT was carried out by alkaline xylan extraction method as shown in Fig. 3.1. The de-lignified SCT biomass recovered from previous step i.e. 34.6 g was mixed with 346 mL solution containing 10% (w/v) sodium hydroxide (NaOH) and 1% (w/v) boric acid (H_3BO_4) and stirred at 200 rpm on a magnetic stirrer at 60ºC for 3 h. The suspension was filtered through cotton cloth and xylan was precipitated with 3 volumes of ice-cold ethanol (95%, v/v) containing 10% (v/v) acetic acid and incubated it for 4ºC for 12 h. The impurities were removed by washing with 95% (v/v) ethanol and suspension was centrifuged at 10000g for 10 min to recover all the precipitated xylan. The extracted xylan (SCTx) was collected and lyophilized by lyophilizer (Labconco, Kansas City, MO, USA) and stored at 25ºC for further experiments. The total xylan yield was determined by considering the amount of hemicellulose in raw SCT biomass and extracted SCTx sample. The xylan yield was calculated as follows:

$$\text{Xylan yield (\%)} = \frac{\text{Dried weight of extracted SCTx}}{\text{Total hemicellulose present in raw SCT}} \text{X } 100$$

3.2.4 Field-Emission Scanning Electron Microscopy analysis of SCTx

The surface morphology of SCTx was analysed by using field-emission scanning electron microscopy **(**FE-SEM) (Gemini 300, Carl Zeiss, Jena, Germany). The SCTx sample was prepared on carbon tape and placed over the stub surface. A double gold coating of the sample was performed on a prepared stub and kept in the vacuum chamber before imaging by FE-SEM.

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3.2.5 Fourier Transform Infrared (FT-IR) spectroscopic analysis of SCTx

FT-IR spectroscopic analysis for SCTx was performed to determine the functional groups present. Extracted SCTx and potassium bromide (KBr) were mixed in ratio 1:100 (w/w) and ground with the help of mortar and pestle and a pellet was prepared under 15-ton hydraulic press. The sample was scanned on a FT-IR spectrophotometer (Spectrum Two, PerkinElmer, Waltham, MA, USA) and spectrum was recorded with in the wavenumber range, 4000-400 cm^{-1}.

3.2.6 Elemental analysis and specific optical rotation of SCTx

Carbon, nitrogen, sulphur and hydrogen present in SCTx were determined by CHNS analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Pavia, Italy), whereas, the oxygen was determined by using the following formula:

$$Oxygen\ (\%) = 100\ (\%) - (\text{Sum of present carbon, hydrogen and nitrogen})$$

The extracted xylan from SCT and commercial xylans, xylan (Carbosynth) and beechwood xylan (Sigma Chemical Corporation) at 0.5% (w/v) concentration were separately added in 2 mL of 50 mM NaOH and dissolved by warming at 60ºC. The leftover insoluble xylo-polysaccharide was separated by centrifuging the xylan sample at 13000g for 15 min. The clear solution was transferred to the polariscope tube. The specific optical rotation of extracted xylan from SCT and commercial xylans were recorded by the digital polarimeter (MCP150, Anton Paar, Graz, Austria) at 25°C maintained by the peltier temperature controller.

3.2.7 Analysis of average molecular mass of SCTx

The SCTx was analysed for average molecular mass by high performance size exclusion chromatography (HP-SEC) by using Phenomenex Polysep-GFC-P6000 column connected with a guard column (Phenomenex Polysep-GFC-P) of HPLC

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system (Shimadzu, kyoto, Japan) equipped with RI detector. 10 µL of SCTx (1 mg/mL) and dextran standards (1 mg/mL concentration of 10 kDa, 20 kDa, 40 kDa, 70 kDa, 100 kDa, 200 kDa, 270 kDa and 500 kDa) were loaded sequentially and eluted by 100 mM $NaNO_3$ (mobile phase) at a 0.5 mL/min flow rate. With the help of dextran standards, the molecular mass of SCTx was calculated.

3.2.8 Thermal degradation of SCTx

The changes in physical properties of SCTx were measured by observing the change in temperature, by the thermo-gravimetric analysis (TGA). TGA and differential thermogravimetric (DTG) analysis (DTG) of SCTx were performed by using a thermal analyzer (model STA449F3A00, Netzsch, Selb, Germany). For TGA and DTG analysis, 6 mg of SCTx was taken in an alumina crucible and heated from ambient temperature to 500°C at a rate of 10°C min^{-1} and the nitrogen gas was purged at a flow rate of 60 mL/min.

3.2.9 Monosaccharide composition analysis of SCTx

The carbohydrate composition of SCTx was determined by treating 5 mg with 1 mL of 2 M TFA (Tri-fluoro-acetic acid) for complete hydrolysis. The hydrolyzed xylan was kept at 80ºC in hot air oven for overnight to remove residual TFA. The dried sample was dissolved in 500 µL degassed Milli-Q water and filtered through a syringe filter using PVDF membrane (Pore size: 0.22 µm). The 10 µL of TFA treated SCTx sample was then transferred to 500 µL HPLC vials and analyzed for the different sugars with the help of RI detector by High Performance Liquid Chromatography (HPLC) (UFLC, Prominence, Shimadzu, Japan). TFA treated SCTx was loaded and passed through the guard column (50 mm x 7.8 mm) followed by the connected Phenomenex Rezex ROA (H+) column (300 mm x 7.8 mm). The samples were eluted by using

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0.005N H_2SO_4 as a mobile phase at 70ºC and at a 0.5 mL/min flow rate for 90 min run time. The standards, xylose, arabinose and glucuronic acid at 1 mg/mL of each, was used for composition determination.

3.2.10 Enzyme activity analysis of xylanase against SCTx and other xylans

The enzyme, endo-β-1,4-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* was expressed, purified and its enzyme activity was determined by following the method as reported earlier (Jamaldheen *et al.*, 2019). The enzyme, 5 µL (5 µg/mL) was added to 0.5% (w/v) SCTx, beechwood xylan, birchwood xylan, oat spelt xylan and 4-O-methyl D-glucorono D-xylan or xylan (from Carbosynth) in 50 mM sodium phosphate buffer (pH 7.5) in 100 µL reaction mixture and incubated at 65°C for 1 min. The enzyme activity was calculated by estimating the reducing sugar released, by Nelson-Somogyi method using D-xylose as standard. One unit (U) of enzyme activity was defined as the amount of *Ct*Xyn11A required to produce 1 µmole of reducing sugar (D-xylose) per min under optimum conditions.

3.2.11 Xylo-oligosaccharides production from SCTx by xylanase

SCTx (1%, w/v) was dissolved in 990 µL of 50 mM sodium phosphate buffer (pH 7.5) and 10 µL (5 µg/mL) of *Ct*Xyn11A was added in 2.0 mL microcentrifuge tube and the reaction mixture was incubated in a water bath at 50ºC for 1 h. After that, 3 volumes of 95% (v/v) ethanol was added to it and the mixture was centrifuged at 10000g for 10 min to separate the residual unhydrolyzed polysaccharide. The supernatant was separated and kept at 80ºC for 12 h to evaporate the ethanol. The yield of concentrated xylo-oligosaccharides released from SCTx was determined by using the phenol-sulfuric acid method (Sharma *et al.*, 2019). The qualitative analysis and identification of xylo-oligosaccharides released from SCTx was performed by TLC by using a mobile phase

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of chloroform: acetic acid: water (6:7:1). The TLC plate was stained with staining solution of (0.5%, w/v) α-naphthol in methanol and sulphuric acid (95:5) solution. XOS with DP 2-5 and alduronic acid mixture was used as standard for identifying the hydrolyzed products. The produced xylo-oligosaccharides species present in XOS mixture was detected by electrospray ionization mass spectroscopy (ESI-MS) analysis as reported earlier (Sharma *et al.*, 2020).

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3.3 Result and Discussion

3.3.1 Delignification of SCT and extraction of xylan from SCT

The raw SCT after delignification by sodium chlorite method gave 34.2 g of delignified SCT, resulting in 69.2% (w/w) yield of delignified SCT. The delignified SCT colour was white as shown in Fig. 3.1.

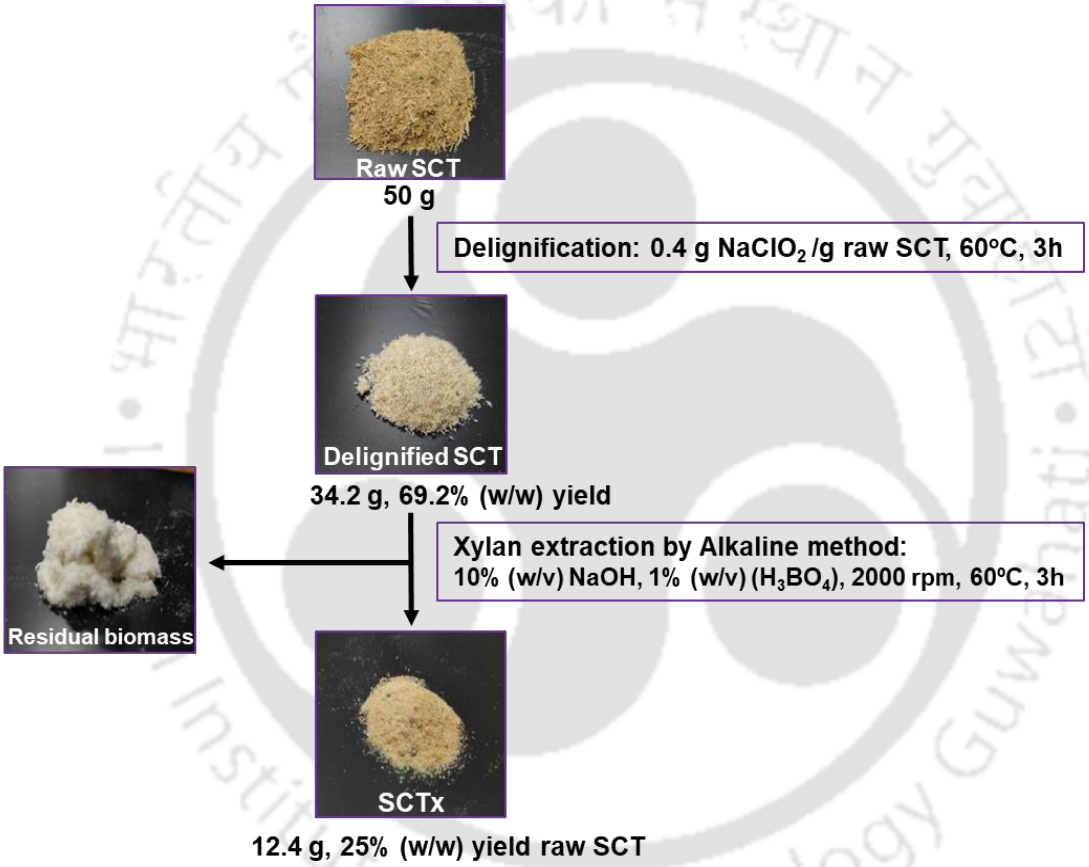

Fig. 3.1. Scheme showing the xylan extraction process from Sugarcane tops.

The delignification was confirmed by standard TAPPI protocol. This delignified SCT (20 g) was used for the xylan extraction by alkaline extraction method that gave 7.25 g of xylan from SCT (SCTx) after lyophilization, resulting an overall 25% (w/w) yield based on raw SCT (Fig. 3.1). The xylan extracted from SB by using alkali method gave about 19.6% (w/w) of recovery yield (de Figueiredo *et al.*, 2017). The xylan

extracted from acacia by using similar extraction process gave 23.5% (w/w) recovery yield (Sharma *et al.*, 2020). The xylan extraction from bleached corn stover by DMSO mediated extraction process gave a lower recovery yield of 8.7% (w/w) (Rowley *et al.*, 2013). The lower recovery yields of about 12.3% (w/w) from bambara and 13.6% (w/w) from cowpea were reported by alkali mediated xylan extraction method (Arumugam *et al.*, 2019).

3.3.2 FE-SEM analysis of SCTx

The surface morphology of dried SCTx on analysis by FE-SEM showed rough and highly porous surface with irregular granules (Fig. 3.2A). The previous reports on xylan extracted from rice bran and finger millet (Palaniappan *et al.*, 2017), from pineapple peel waste (Banerjee *et al.*, 2019) and from acacia saw dust (Sharma *et al.*, 2020), also showed irregular and rough surface morphology.

3.3.3 FTIR analysis of SCTx

The FT-IR spectrum of SCTx (Fig. 3.2B) showed a discrete peak at 3429 cm^{-1} for stretching vibrations of the hydroxyl (OH) group, which was similar to the peak for OH stretching as reported earlier (Shi *et al.*, 2014). The peak at 2922 cm^{-1} revealed symmetric CH stretching in extracted SCTx, which was similar to the peak previously reported (Luo *et al.*, 2015). The symmetric and asymmetric stretching modes of the carboxyl group (-COOH) of glucuronic acid in SCTx were found at 1569 cm^{-1} and 1413 cm^{-1}, respectively as also reported earlier (Biely *et al.*, 2015; Corradini *et al.*, 2018). A small peak at 1642 cm^{-1} in SCTx denoted the presence of moisture (water), as also earlier reported (Bian *et al.*, 2012; Zhang *et al.*, 2016). The β-glycosidic linkage in SCTx showed a small absorption peak at 897 cm^{-1} that is considered as the anomeric region as mentioned earlier (Kacurakova *et al.*, 2000; Zhang *et al.*, 2016).

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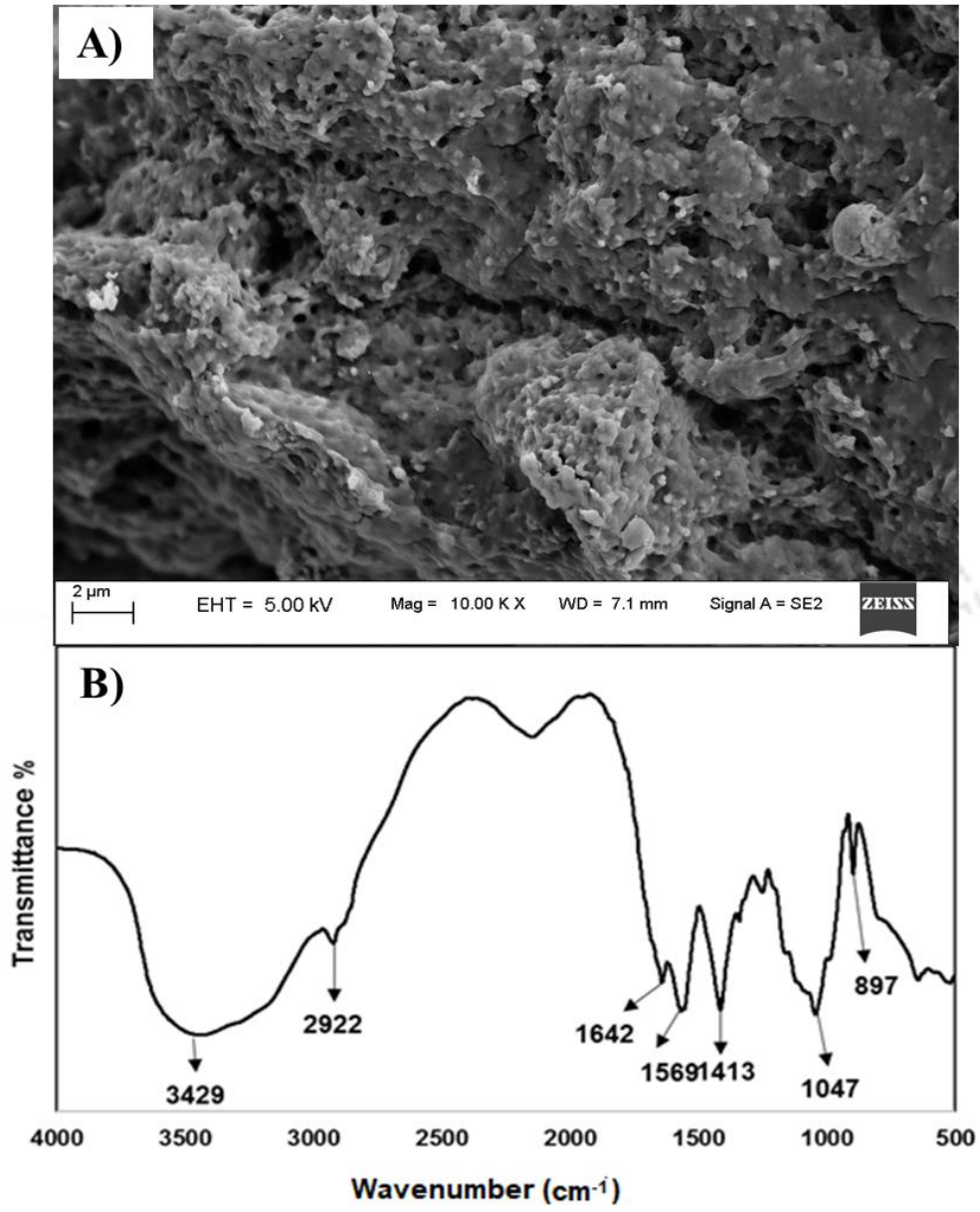

Fig. 3.2. A) Field emission scanning electron micrograph (FE-SEM) analysis of extracted xylan B) Fourier transform infrared (FT-IR) spectroscopic analysis of SCTx.

The stretching vibration of the OH side group and the C-O-C glycosidic group overlap in the region, 1200-1000 cm^{-1} in the FT-IR spectrum. The β-1,4 backbone of extracted xylan represented by the peak at 1047 cm^{-1} as reported earlier (Li *et al.*, 2015).

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3.3.4 Elemental composition analysis and specific optical rotation of SCTx

The elemental composition analysis of SCTx showed 35.2% carbon, 4.6% hydrogen and approximately, 60.2%, oxygen content. The absence of nitrogen and sulphur in the extracted xylan showed the purity of xylan that is devoid of protein and lignin as also mentioned earlier (Laurens *et al.*, 2018). The specific optical rotation (OR) estimated for extracted xylan from SCT was -72°. The commercial xylans, the xylan from Carbosynth and beechwood xylan (from Sigma Chemical Corporation) showed OR of -70.36° and -68.40°, respectively. Similar results, of 72.65° of specific optical rotation was reported for the xylan extracted from Acacia (Sharma *et al.*, 2020). The similar OR of extracted xylan with the commercial xylan confirmed that the SCTx is composed of β-linked D-xylose monosaccharides, α-linked D-glucuronic acid and L-arabinose residues.

3.3.5 Molecular mass determination of SCTx

The HPSEC analysis of SCTx showed a single peak that corresponded to the molecular mass of ~57 kDa (Fig. 3.3A). The molecular mass of SCTx was similar to 56.6 kDa of glucuronoxylan isolated from Eucalyptus (Biely *et al.*, 2013) and was higher than 49 kDa of xylan from seeds of *Cassia obtusifolia* (Feng *et al.*, 2018). The molecular mass of xylan extracted from acacia using the similar extraction method was ~70 kDa (Sharma *et al.*, 2020). However, much lower molecular mass of extracted xylan from beechwood (20.84 kDa) (Konduri and Fatehi., 2016) and from waste fibers (15 kDa) (Zhang *et al.*, 2016), has been also reported earlier.

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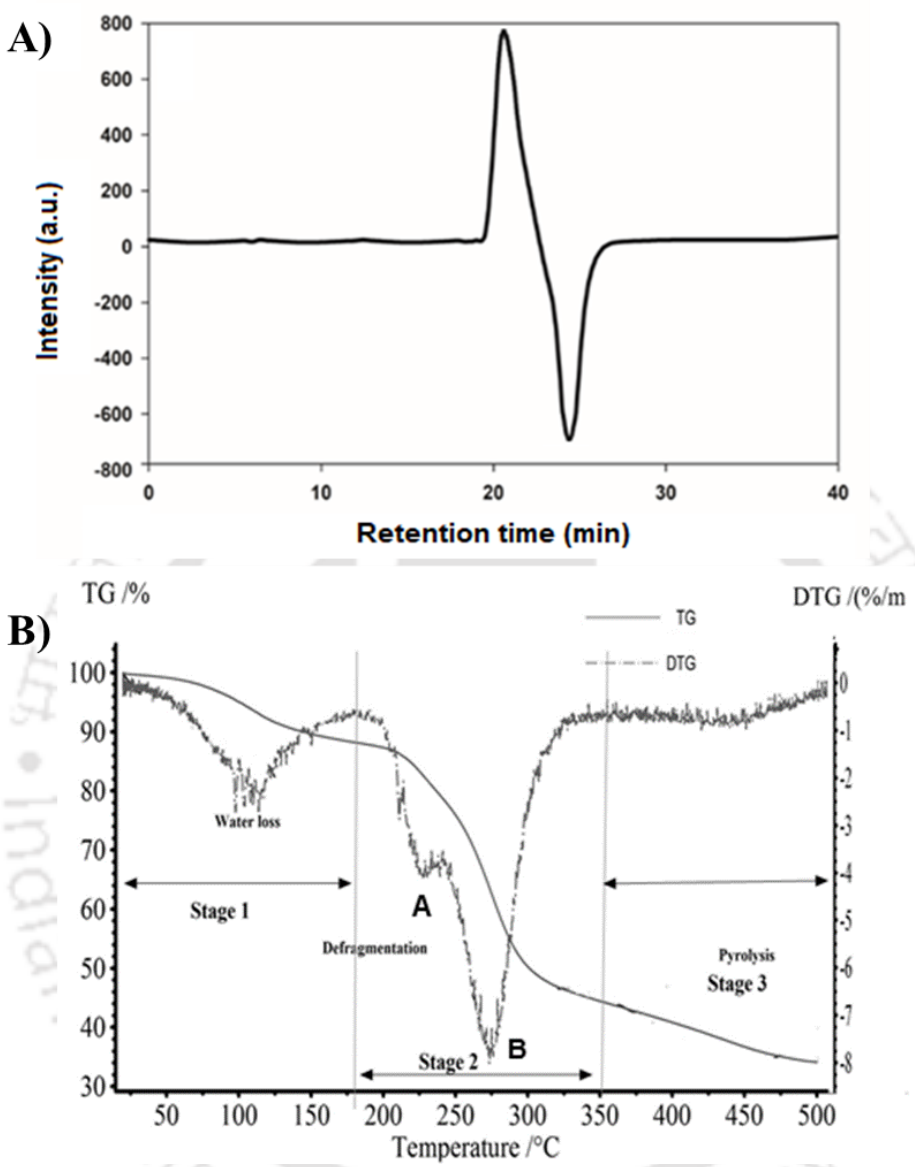

Fig. 3.3. A) HP-SEC profile of xylan extracted from SCT and B) Thermogravimetric analysis (TGA) showing the decomposition of SCTx (from 160°C to 350°C) and differential thermogravimetric analysis (DTG) of SCTx displaying the breakdown of arabinose and glucuronic acid side chains at 228°C (stage A) and thermal degradation temperature (Td) of xylose back bone at 275°C (stage B).

3.3.6 DTG and TGA analysis of extracted SCTx

The thermal stability and thermal decomposition analyses of SCTx were studied by DTG and TGA, respectively. The maximum thermal degradation (Td) temperature is designated as DTG_{max} and can be used for analyzing the sample stability. The reaction occurring at 100°C (Fig. 3.3B, Stage 1) are endothermic and mainly attributing to the removal of moisture, when SCTx is heated. The DTG profile of SCTx showed two peaks, one at 228°C (Fig. 3.3B, Stage 2A) and second at 275°C (Fig. 3.3B, Stage 2B), indicating that there are side chains present in SCTx. The DTG curve represented the breakdown of arabinose and glucuronic acid side chains at 228°C and xylose back bone at 275°C and these peaks corresponded to the Td or DTG_{max} (Fig. 3.3). Two-step degradation for beechwood xlan was reported in which, the first step was associated with the breakage of the side chain units and the second step was associated with the depolymerisation of xylan backbone (Dorez *et al.*, 2014). SCTx showed decomposition or weight loss in the temperature range, 200-350ºC. The xylan from rice bran, finger millet seed coat and beech wood showed weight losses at the temperature ranges, 132-334ºC, 220-380ºC and 247-340ºC, respectively (Palaniappan *et al.*, 2017). The birchwood xylan (commercial) and wheat straw xylan also showed the weight losses between 220°C and 350°C, similar to SCTx (Ruzene *et al.*, 2007).

The TGA analysis of SCTx displayed that it was stable up to 158°C, above, the thermal degradation started. The decomposition of SCTx occurred in three stages. The first stage of decomposition was between 70ºC and 160ºC and the weight loss was 8%, which denotes the moisture or water loss in SCTx as also reported earlier for commercial xylan (Zhang *et al.*, 2017) and hemicellulose from banana rachis (Deumaga *et al.*, 2020). In the second stage, weight loss (52%) was observed between 160ºC and

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350ºC due to the decomposition and fragmentation of SCTx into smaller molecules such as CH_3COOH, CH_4, CO, CO_2, and HCOOH as also reported earlier (Zhao *et al.*, 2014; Deumaga *et al.*, 2020). The third stage of decomposition represented the pyrolysis of residual biomass that started from 400ºC. In this process, the remaining residual xylan was pyrolyzed to ash.

3.3.7 Monosaccharide analysis of SCTx

The monosaccharide composition analysis of SCTx by HPLC displayed the retention time of xylose, D-glucuronic acid and L-arabinose sugar similar to those with the standards (Fig. 3.4).

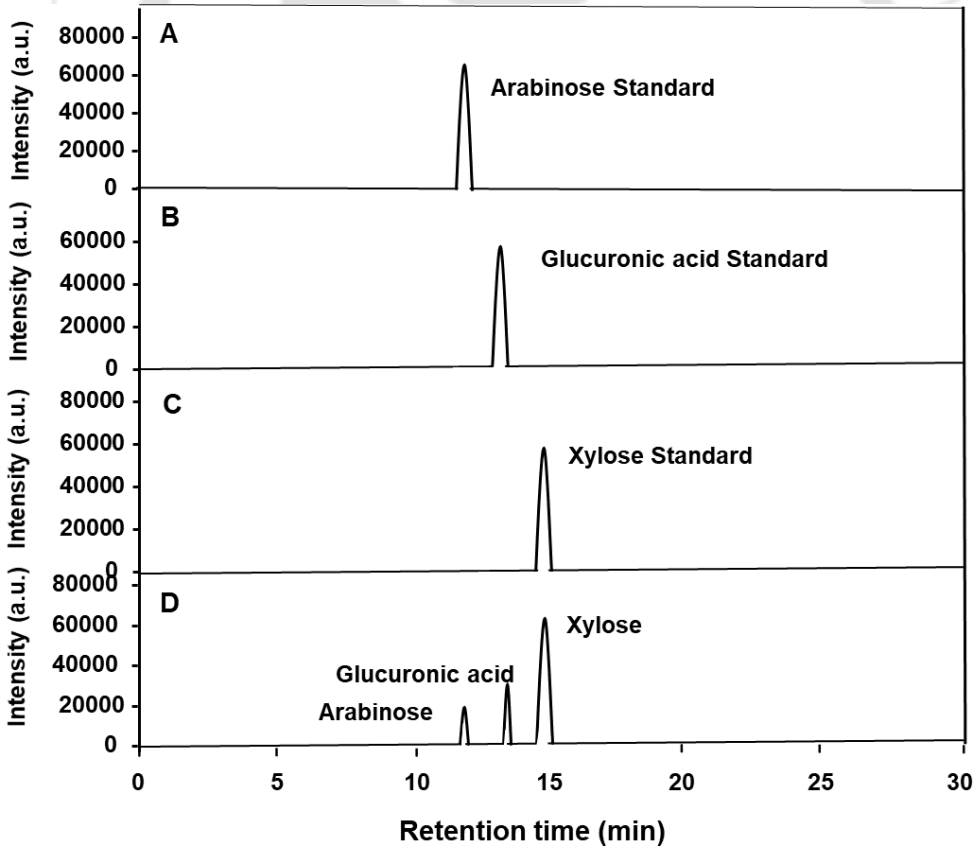

Fig. 3.4. A) High performance liquid chromatography (HPLC) analysis of TFA treated SCTx, B) Arabinose standard C) Glucuronic acid standard and D) Xylose standard for HPLC.

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The presence of 74% (w/w) D-xylose residues, 16% (w/w) of D-glucuronic acid residues and 10% (w/w) of L-arabinose content was determined by using standards**.** The presence of higher percentage of xylose in SCTx shows that the backbone is composed of D-xylose monomers and arabinose and glucuronic acid are present as side chains. The major side chain components in sugarcane bagasse were L-arabinofuranose, In the sugarcane bagasse, alkali (40%, w/v NaOH) mediated extracted xylan, the arabinose/xylose and D-glucuronic acid/xylose ratios reported were 0.12 and 0.09, respectively (Sporck *et al.*, 2017). The arabinose/xylose ratio in xylan extracted from SCT by 10% NaOH was 0.14, similar to the previously reported xylan extracted from sugarcane bagasse (SBx). However, the D-glucuronic acid/xylose ratio found was higher (i.e. 0.22) in SCTx than reported for SBx. These ratios suggested that the low NaOH load (10%, w/v) in xylan extraction method protected the side chains in SCTx, whereas the high NaOH load (40%, w/v) removed the side chain groups from SBx, especially the D-glucuronic acid (Sporck *et al.*, 2017).

3.3.8 Enzyme activity analysis of SCTx by xylanase

The enzyme activity of xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* against the xylans from different sources is listed in Table 3.1. The specific activity of *Ct*Xyn11A against SCTx (molecular mass: 57000) was 1394 ±51 U/mg, which was higher than the studied commercial xylans (beechwood xylan, oat spelt xylan and 4-O-methyl D-glucurono D-xylan purchased from Sigma chemical corporation and xylan from Carbosynth).

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Table 3.1. Comparison of xylanase (*Ct*Xyn11A) activity against SCTx and commercial xylans.

Xylan	Specific Activity (U/mg)
Xylan extracted from SCT (SCTx)	1394 ±51
Xylan (Carbosynth, UK)	1220 ±42
Beechwood xylan*	1218 ±13
Birchwood xylan*	894 ±58
Oat spelt xylan*	751 ±43
4-O-methyl D-glucorono D-xylan*	332 ±92

** Sigma Chem. Co., USA*

The xylanase enzyme activity depends on the xylan's structure and its solubility. Birchwood xylan and beechwood xylan contain similar percentage (87.7%) xylose and lower content of glucose, arabinose and galactose. However, the relative content of hexuronic acid is different (10.2% in birchwood xylan and 7% in beechwood xylan) and therefore differ in relative solubilities. Similarly, oat spelt xylan also contains mixture of xylose (84%) and arabinose, glucose and galactose and also very less soluble (Moraïs *et al.*, 2010). Owing to the higher water solubility, beechwood, xylan (Carbosynth, UK) and SCTx showed enzyme activity compared with the other studied xylan (Table 1). The 4-O-Methyl D-glucurono D-xylan (MGX) has high degree of substitution (70-80%) as compared with the other xylans (Bajpai, 2018). Xylanase (*Ct*Xyn11A) showed less activity against MGX, which may due to non-accessibility of the main chain of the substrate owing to the presence of high degree of side chain substitutions. These results amply demonstrated that the alkali extracted SCTx can serve as a commercial substrate.

3.3.9 Identification and production of xylanase mediated xylo-oligosaccharides

The total xylo-oligosaccharide yield from 10 mL hydrolysis of 1% (w/v) SCTx by 50 µl of *Ct*Xyn11A (5 µg/mL) was 41.2±1.7 mg as estimated by phenol sulfuric acid method (Dubois *et al.*, 1956). The TLC analysis of mixture of XOS produced by enzymatic hydrolysis of SCTx showed the presence of series of XOS of degree of

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polymerization (DP) ranging from DP2 to DP5 (Fig. 3.5A). The hydrolysis of 4-O-methyl glucuronoxylan by recombinant *Ct*XynGH30 (Verma and Goyal, 2016) and *Ps*Xyn10A (Sharma *et al.*, 2018) released identical types of XOS. The TLC analysis of hydrolysed products also showed higher range of unresolved XOS. Therefore, to obtain the precise information about presence of any substitution and degree of polymerization, the XOS mixture was further analysed by mass spectrometry.

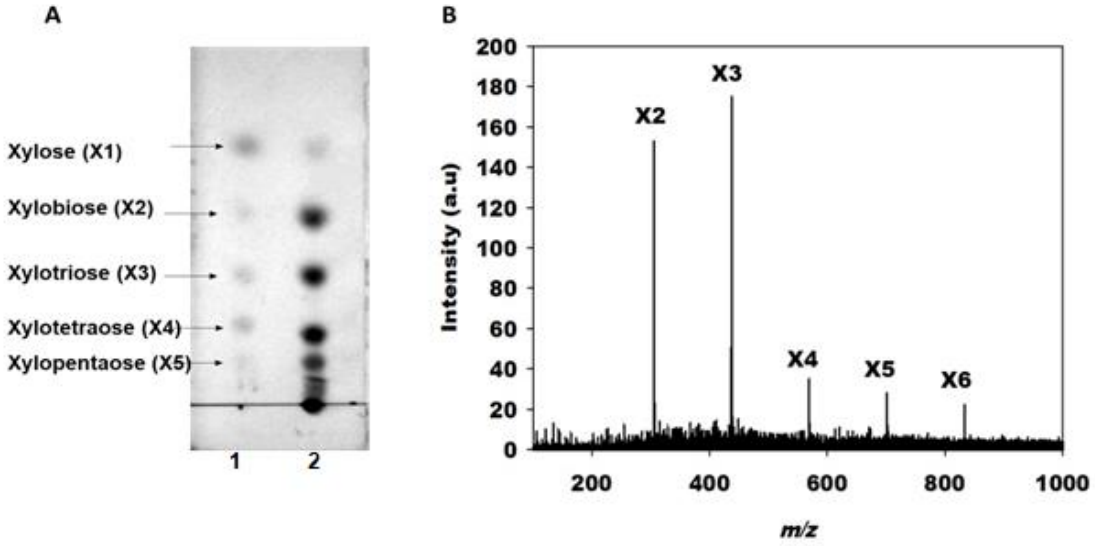

Fig. 3.5. A) TLC analysis of hydrolyzed products of SCTx by endo β-1,4-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum.* Lane 1, Standards (xylose, DP2 to DP5), Lane 2, *Ct*Xyn11A hydrolysed SCTx products and B) ESI-MS analysis of endo β-1,4-xylanase (*Ct*Xyn11A) hydrolysed products of SCTx.

The ESI-MS analysis of *Ct*Xyn11A hydrolysed XOS mixture revealed that the mixture contains a series of linear XOS ranging from DP2 to DP6 and no substituted XOS (Fig. 3.5B). The mixture includes xylobiose (m/z 305.01), xylotriose (m/z 437.0), xylotetraose (m/z 569.0), xylopentaose (m/z 701.0) and xylohexaose (m/z 833.0). The molecular mass of acidic and neutral XOS matches well with the earlier reports (Biely *et al.*, 2013). The ESI-MS analysis of XOS produced by alkali hydrolysis of extracted xylan from Acacia saw dust displayed the production of series of XOS (DP2-DP8) (Sharma *et al.*, 2020). Acetyl glucuronoxylan hydrolysed by acetylxylan esterase

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resulted in a mixture containing linear XOS from DP2 to DP6 reports (Biely *et al.*, 2013). The utilization of enzyme assisted production of XOS in various applications such as antioxidative agent, as antiproliferative agent in healthcare (Aachary *et al.*, 2015) and as food supplement (Reddy and Krishnan, 2016) has been extensively made. Therefore, the extracted xylan apart from its role as a substitute of commercial substrate, can also be used for diverse biotechnological applications.

Conclusions

The agro-waste, sugarcane tops was explored for xylan extraction. SCT biomass after delignification by sodium chlorite gave 69.2% (w/w) yield. The delignified SCT was subjected to a modified alkaline extraction method, that gave overall 25% (w/w) yield of the extracted xylan (SCTx) based on raw SCT biomass. FESEM analysis showed the granular surface morphology of SCTx. FTIR analysis also confirmed the presence of xylan in SCTx. The elemental composition analysis showed no impurity of lignin and protein in SCTx. The specific optical rotation of SCTx was -72^{o} and the molecular mass as analysed by HPSEC found was 57 kDa. The DTG and TGA analyses of SCTx also confirmed the presence of glucuronic acid and arabinose as side chains. The monosaccharide analysis of SCTx after TFA hydrolysis by HPLC displayed the presence of 74% (w/w) xylose, 16% (w/w) glucuronic acid and 10% (w/w) arabinose residues. The higher xylanase activity achieved on SCTx than the commercial xylans, established that it can be taken for commercial production and can be utilized by different industries such as food, energy and pharmaceutical. The xylo-oligosaccharides produced from SCTx can be used as a prebiotics and other healthcare applications.

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Chapter 4

A biorefinery approach for sequential extraction of commercial grade xylan and alkali lignin from alkali pretreated sugarcane tops hydrolysate

4.1 Introduction

The major part of Indian population relies on the agriculture and agri-based industries for their living. Barley, maize, rice, sugarcane and wheat are the major crops in India. Yearly around 200 billion tonnes of sugarcane tops (SCT) is produced in India (Khaire *et al.,* 2021a). This leftover residual biomass is usually burnt in agricultural land which leads to environmental pollution. The composition of SCT from different locations shows cellulose (40-45%), hemicellulose (25-30%) and lignin (15-19%) (Khaire *et al.,* 2020). SCT can be utilized as a substrate for the production of various value-added products such as acetic acid, biobutanol, bioethanol, cellulose, furfural, lactic acid, lignin and xylan (Canilha *et al.,* 2012, Khaire *et al.,* 2021a).

The hemicellulose is made up of a mixture of heteropolysaccharides such as glucomanan, galactomannan, xyloglucan, glucuronoxylan and xylan. The separation of xylan before enzymatic hydrolysis of biomass can enhance the enzymatic digestibility of cellulose in biomass (Meng *et al.,* 2014, Huang *et al.,* 2022). The characteristic

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structure of xylan is dependent on the plant source and the extraction method used (Petzold-Welcke *et al.,* 2014). The main chain of xylan is made up of xylose residues via β-1,4-glycosidic linkages with other side chain residues such as arabinose, glucose, galactose, acetyl groups, rhamnose as well as dimeric and trimeric side chains (Moine *et al.,* 2007). Traditionally, alkaline treatment followed by ethanol precipitation method is used for the extraction of xylan from various plant sources. However, this method generally shows low xylan yield. The optimization of extraction process can enhance the xylan yield (Banerjee *et al.,* 2019). Commercial grade xylan was extracted from sugarcane tops by alkaline treatment method (Khaire *et al.,* 2021c). Xylan is extensively used in industrial applications such as food packaging, bio-catalyst in pollutant removal (Ananpattarachai and Kajitvichyanukul, 2016), bioplastics (Brodin *et al.,* 2017), as additives in paper making (Sharma *et al.,* 2020a) and as drug carriers in pharmaceutical applications (Khaire *et al.,* 2022). The xylooligosacharides produced from xylan extracted from neem saw dust can have potential as a prebiotic in nourishing products (Sharma *et al.,* 2020c).

The most abundant aromatic biopolymer found on Earth is lignin accounting for almost 30% organic carbon (Kajaste, 2014). More than 70 million tons of lignin is produced annually which receives considerable attention as a bio-based precursor (Molinari *et al.,* 2013). Lignin is mainly recovered from sulfate and Kraft pulping processes in paper and pulp industry (Stewart., 2008). However, only 2% of the produced lignin is utilized for value-added products whereas the rest is burnt as low-energy value fuels. Lignin is highly branched, irregular three-dimensional and amorphous phenolic polymers which provides a cover for structural support and protection against biological and chemical attacks (Poovaiah *et al.,* 2014, Lu *et al.,*

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2017). The chemical structure of lignin is extremely complex and it is made up of three basic phenylpropanoid monomers, syringyl (S), guaiacyl (G) and *p*-hydrophenyl (H) units (Shigeto *et al.,* 2017). The lignin composition and content varies in different lignocellulosic biomasses because of radical coupling reactions of monomers forming a phenolic, cross-linked and racemic polymer using its biosynthesis (Shigeto, *et al.,* 2017). It also varies with the source of tissue and the age of the lignocellulosic biomass (Healey *et al.,* 2016). The percentage of lignin in lignocellulosic biomass varies from 10-40%, depending on the species, variety, maturity and developmental growth conditions (Shigeto, *et al.,* 2017). The typical lignin content in softwoods, hardwoods and grasses are 24-33%, 19-28% and 15-25%, respectively. The composition and structure of lignin are dependent on the functional group (carbonyl, carboxyl, hydroxyl and methyl etc.) linkage with aliphatic or aromatic moieties in different proportions (Kai *et al.,* 2016). Lignin contains a number of functional groups like aliphatic, phenolic, hydroxyl and the carbonyl groups, enabling it to undergo several modifications. Lignin can be utilized as complexing, binding, dispersing and emulsion-stabilizing agent for several applications (Liao *et al.,* 2020). Lignin can also be used for the sustainable production of aromatic chemicals such as ferulic acid, vanillin and phenol (Zhang *et al.,* 2022). Lignin also has some novel applications such as packaging material, 3D-printing materials, flame retardants, surfactants, biocompatible and slow releasing fertilizers, lignin-epoxy resins and biobased polyurethanes (Bertella and Luterbacher, 2020).

The hemicellulose and lignin separation during the pretreatment process and their utilization as a substrate by other industries for value addition is the ultimate goal of a biorefinery. The extraction of xylan and lignin from sugarcane tops is not yet

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explored. The alkali pretreated SCT hydrolysate is waste produced from the alkaline pretreatment of SCT biomass for bioethanol production (Khaire *et al.*, 2021b). The current study is based on the sequential extraction and characterization of xylan and alkali lignin from the alkali pretreated SCT hydrolysate. Therefore, the utilization of the waste SCT hydrolysate for production of commercial grade xylan and alkali lignin will enhance the economics of a biorefinery process.

4.2 Material and Methods

4.2.1 Materials and biomass processing

The sugarcane tops was collected from the nearby vicinity of Guwahati, India. After collection, the SCT biomass was washed under tap water and then cut into pieces (1-2 cm). Moisture was removed from the pieces by drying at 60°C for 48 h in the hot air oven (Labtech, Mumbai, India). Then the dried SCT pieces were milled to powder (particle size: 0.85 mm) with the help of mixer-grinder (Philips, HL1606/03) and stored at 25°C for future experiments. All reagent or analytical grade chemicals and reagents were procured from Himedia Pvt. Ltd. and Merck India Pvt. Ltd., respectively.

4.2.2 Pretreatment of SCT biomass

The water soluble extractives were removed from 100 g of raw SCT (in 1000 mL of distilled water) by incubating at 50°C for 12 h in a shaker incubator. Then the solid SCT biomass was separated from the hydrolysate by filtering through a muslin cloth and dried it at 80°C in hot air oven (Khaire *et al.*, 2021c). About 76 g of extractive free SCT biomass was further pretreated with 760 mL alkaline solution containing 10% (w/v) sodium hydroxide and 1% (w/v) boric acid in 1 L reagent bottle on a magnetic stirrer at 60ºC, 200 rpm for 3 h. The slurry was filtered by passing through muslin cloth and the 600 mL of alkali pretreated SCT (apSCT) hydrolysate was collected in a new

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reagent bottle for sequential extraction of xylan and alkali lignin. The separated solid biomass residue was previously studied for statistical optimization of enzymatic saccharification and bioethanol production (Khaire *et al.*, 2021b).

4.2.3 Sequential extraction of xylan and alkali lignin from alkali pretreated SCT hydrolysate

The xylan from apSCT (apSCTx) was precipitated in step one by adding 3 volumes (1800 mL) of solution containing ice-cold rectified spirit (ethanol: 95%, v/v) and glacial acetic acid (10%, v/v) to 1 volume (600 mL) of apSCT hydrolysate. The reagent bottle was kept for incubation at 4ºC for 12 h. The suspension was then centrifuged at 4ºC and 10000g for 10 min to collect all the precipitated apSCTx. Used ethanol was recovered by partial distillation process before lignin precipitation. In the second step, the alkali lignin (apSCTal) from the remaining supernatant was recovered by precipitating by lowering its pH to 1.5 by adding hydrochloric acid. The precipitated apSCTal was recovered by centrifugation at 10000g for 10 min. The sequentially extracted apSCTx and alkali lignin were stored at 25ºC after lyophilization (Lyophilizer: Labconco, Kansas City, MO, USA). Total apSCTx and apSCTal yield from SCT biomass were determined by considering the hemicellulose and lignin present in raw SCT and in extracted samples. The apSCTx yield was calculated by,

$$apSCTx\ yield\ (\%) = \frac{Dried\ weight\ of\ apSCTx}{Total\ hemicellulose\ present\ in\ raw\ SCT} X\ 100$$

The apSCTal yield was calculated by,

$$apSCTal\ (\%) = \frac{Dried\ weight\ of\ apSCTal}{Total\ lignin\ present\ in\ raw\ SCT} X\ 100$$

4.2.4 Composition analysis of apSCTx

The tri-fluro-acetic acid (TFA) hydrolyzed apSCTx sample was prepared as per the previously mentioned method (Khaire *et al.*, 2021c). The prepared apSCTx sample

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was solubilized in degassed water (Milli-Q: 500 µL) and passed through the 0.22 µm PVDF membrane by using a syringe filter to HPLC vials (Axiva Sichem Biotech, India). The 10 µL sample was then analyzed for the different monosaccharides by using High-Performance Liquid Chromatography (HPLC) (UFLC, Prominence, Shimadzu, Japan) and RI detector. The arabinose, glucuronic acid and xylose were taken as standard monosaccharides.

4.2.5 Analysis of average molecular mass of extracted apSCTx

The apSCTx was analyzed for the average molecular mass by using high-performance size exclusion chromatography method as described earlier (Sharma *et al.*, 2020b). The dextran standards markers (10, 20, 40, 70, 100, 200, 270 and 500 kDa) and apSCTx (1 mg/mL) were analyzed by using 100 mM $NaNO_3$ solution as a mobile phase at a flow rate 0.5 mL/min. The molecular weight of apSCTx was calculated with the help of dextran standards.

4.2.6 Ultimate elemental composition analysis of apSCTx and apSCTal

The CHNS analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Pavia, Italy) was used for the analysis of carbon, hydrogen, nitrogen and sulphur content present in apSCTx and apSCTal whereas the present oxygen content was calculated by using the formula,

$$Oxygen\ (\%) = 100\ (\%) - (\text{Sum of present carbon, hydrogen and nitrogen})$$

4.2.7 Specific optical rotation of apSCTx

The apSCTx sample was prepared by adding 0.5% (w/v) apSCTx in 2 mL sodium hydroxide (50 mM) solution and dissolved by warming at 60ºC. The insoluble apSCTx was removed from sample by centrifugation at 13000 g for 15 min. The clear supernatant was collected in the clean polariscope tube. The specific optical rotation of

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apSCTx was recorded at 25°C (Peltier temperature controller) by using the digital polarimeter (MCP150, Anton Paar, Graz, Austria).

4.2.8 Functional group and the surface morphology analysis of apSCTx and apSCTal

The functional group present in apSCTx and apSCTal were examined by using the Fourier Transform Infrared (FTIR) spectrophotometer (Perkin Elmer, Spectrum Two, USA) by scanning at 4 cm^{-1} resolutions for wavenumber ranges from 450 to 4000 cm^{-1} (Khaire *et al.*, 2021c). The morphological structure imaging of apSCTx was studied by using the Field-Emission Scanning Electron Microscopy (FESEM) (Carl Zeiss, Model-Gemini 300, Germany) (Sharma *et al.*, 2020c).

4.2.9 Thermal degradation analysis of apSCTx and apSCTal

Thermogravimetric (TG) and differential thermogravimetric (DTG) analyses were carried out for studying the changes in physical properties of apSCTx and apSCTal with increase in the temperature by using a thermal analyzer (Netzsch, Model: STA449F3A00). The sample was prepared as mentioned earlier and heated at a rate of 10ºC min^{-1} from 30 to 500ºC in a nitrogen environment (Khaire *et al.*, 2021c).

4.2.10 Enzyme activity analysis of xylanase (*Ct*GH11A)

Endo-β-1,4-xylanase (*Ct*GH11A) from *Clostridium thermocellum* was expressed in *E. coli* BL21(DE3) cells and purified as reported earlier (Jamaldheen *et al.*, 2019). The enzyme activity of *Ct*GH11A was estimated by taking 0.5% (w/v) substrate (apSCTx or SCTx: xylan extracted from delignified SCT) with 5 µL (5 µg/mL) of enzyme in 95 µL sodium-phosphate buffer (50 mM, pH 7.5) in a 2 mL Eppendorf tube and incubated at 65°C for 1 min (Jamaldheen *et al.*, 2019). The enzyme activity of *Ct*GH11A was estimated by the released reducing sugar that was measured by following the Nelson (1944) and Somogyi (1945) method by using D-xylose as a standard.

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4.2.11 Xylanase mediated xylooligosaccharides production

ApSCTx (0.1 g) was solubilized in 10 mL of sodium phosphate buffer (50 mM, pH 7.5) and partially digested with 10 µL of recombinant xylanase (*Ct*GH11A, stock conc. 5 µg/mL) at 50ºC for 1 h. The residual unhydrolyzed xylan was precipitated by adding 3 volumes of absolute ethanol followed by centrifugation at 10000g for 10 min. The separated supernatant was dried at 80ºC for 12 h and the concentration of xylooligosaccharides was estimated by following the phenol-sulfuric acid method (Khaire *et al.,* 2021c). The qualitative identification of xylooligosaccharides produced from apSCT was determined by TLC analysis by using mixture of standard xylooligosaccharides of degree of polymerization (DP) 2-5 (xylobiose, xylotriose, xylotetraose and xylopentaose). The degree of polymerization of produced xylooligosaccharides was also confirmed by LC-MS analysis following the method reported earlier (Sharma *et al.,* 2020b).

4.2.12 UV spectroscopic analysis of apSCTal

The content of alkali-soluble lignin, the purity and the components of the apSCTal was studied by UV spectroscopy (Thermo Fisher Scientific, MA USA) in the range 200 to 400 nm (Lu *et al.,* 2017).

4.2.13 Matrix-assisted laser desorption ionization coupled with time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of apSCTal

The MALDI-TOF-MS spectrometer (Bruker Daltonic, MA, USA) was used to record the high-resolution mass spectra of apSCTal at linear positive mode with a pulsed nitrogen laser (Ionization at 337 nm, the pulse rate: 10 Hz, acceleration voltage: ±20 kV). A 5.2 kV lens was used to focus the laser light and a total of 300 shots were provided for apSCTal sample.

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Sample preparation: Lignin sample at 1 mg/mL was prepared in water:acetonitrile:ethanol (10:50:40, v/v). The matrix, 2,5 dihydroxy benzoic acid was prepared at 1 mg/mL in a mixture of water: acetone (10:90 v/v). 1 µL of apSCTal sample was mixed with 10 µL of matrix solution and then 1 µL of the mixed sample was spotted on MALDI target and dried before the analysis. The spectrum was recorded in positive ionization mode for the lignin sample.

4.2.14 Mass balance and economic evaluation of sequential extraction of apSCT xylan and alkali lignin from raw SCT

The mass balance of sequential extraction of apSCT xylan and alkali lignin from 100 g of raw SCT for was carried out on the basis of the dry weight recovery. Economic evaluation of xylan and lignin extraction was carried out for 1 kg raw SCT biomass. The economic evaluation of each step was calculated by considering the commercial cost of all components used throughout the process (chemicals, water, electricity and instrumentation). Other costs involved such as manpower, transportation and storage were not considered in economic calculations for the laboratory scale process. The overall profit was calculated by

$$\text{Overall profit} = Total\ income - Process\ cost$$

4.3 Results and Discussion

4.3.1 Sequential extraction of xylan (apSCTx) from alkali pretreated SCT hydrolysate

One hundred gram of raw SCT contained 43 g of cellulose, 27.2 g of hemicellulose and 14 g Klason lignin. Alkaline pretreatment of 100 g of raw SCT biomass gave 42 g solid biomass (87% of glucose, 9% of xylose, 1.2% arabinose and 1.8% of glucuronic acid residues as reported earlier (Khaire *et al.*, 2021b). This pretreatment resulted in production of around 600 mL of alkali pretreated SCT hydrolysate which was used for sequential extraction of xylan and lignin. Around 26.5

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g (26.5%, w/w) of apSCTx (Figure 4.1A) was recovered from 600 mL alkali pretreated SCT hydrolysate which was 98.2% (w/w) of total xylan (27%, w/w) present in the raw SCT biomass. The xylan extracted from SCT biomass after delignification process using similar extraction process reported 25% (w/w) xylan yield from raw SCT (Khaire *et al.*, 2021c). The xylan extracted from sugarcane bagasse by following the alkali extraction method yielded 19.6%, w/w (de Figueiredo *et al.*, 2017). The delignification of acacia sawdust followed by xylan extraction by the alkali extraction process resulted 23.5% (w/w) yield (Sharma *et al.*, 2020b). A comparative account of xylan extraction methods and yield from different waste biomass is given in Table 4.1.

Table 4.1. Xylan extraction methods and yield from different waste biomass.

Biomass	**Xylan Extraction Method**	**Extraction Yield**	**Reference**
Sugarcane bagasse	40%, w/v NaOH, 60°C, 2 h	53% of total xylan	Spork *et al.*, 2017
Sugarcane tops	Delignification, (18%, w/v NaOH + 0.26 M $NaBH_4$), 18 h, room temperature + 24%, w/v KOH	83% of total xylan	Flórez-Pardo *et al.*, 2019
Pineapple peel	10%, w/v NaOH, 45°C, 16 h	87.6% of total xylan	Banerjee *et al.*, 2019
Acacia sawdust	Delignification, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60°C, 3h	23.5% of dry biomass	Sharma *et al.*, 2020b
Neem sawdust	Delignification, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60°C, 3 h	22.5% of dry biomass	Sharma *et al.*, 2020c
Sugarcane tops	Delignification, 10%, w/v NaOH + 1%,w/v H_3BO_4, 60°C, 3h	92.6% of total xylan	Khaire *et al.*, 2021c
Sugarcane bagasse	0.53%, w/v NaClO, 52ºC, 75 min	72.7% of total xylan	Gupta *et al.*, 2022
Sugarcane tops	Extraction, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60°C, 3h	98.2% of total xylan	This study

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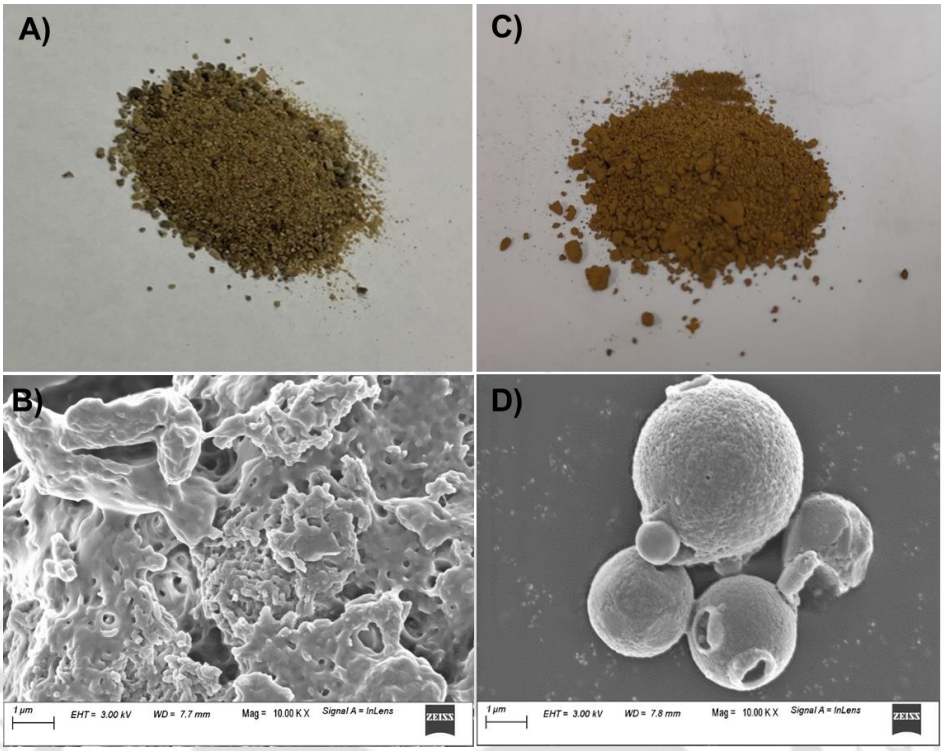

Fig. 4.1. A) SCT xylan separated from alkali pretreated SCT hydrolysate, B) Field emission scanning electron micrograph (FE-SEM) of apSCTx, C) Alkaline SCT lignin separated from alkali pretreated SCT hydrolysate and D) Field emission scanning electron micrograph (FE-SEM) of apSCTal.

4.3.2 Characterization of apSCTx

4.3.2.1 Monosaccharide analysis of apSCTx

ApSCTx was analysed for its monosaccharide composition after TFA hydrolysis by HPLC. The HPLC analysis showed 74% (w/w) xylose, 15% (w/w) Glucuronic acid and 11% (w/w) arabinose residues in apSCTx. Due to the xylan backbone, the higher xylose percentage was found in the apSCTx and glucuronic acid and arabinose are found as side chains.

4.3.2.2 Molecular mass estimation of apSCTx

In HPSEC analysis of apSCTx, the molecular mass was found to be ~58 kDa, which was similar to the previously reported xylan extracted from SCT biomass (~57

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kDa) (Khaire *et al.,* 2021c). This was also similar to 56.6 kDa of glucuronoxylan extracted from Eucalyptus (Biely *et al.,* 2013). The higher molecular mass of xylan (~70 kDa) was reported for acacia xylan extracted by using a similar extraction method (Sharma *et al.,* 2020b).

4.3.2.3 Elemental composition analysis and specific optical rotation of apSCTx

The air-dried apSCTx was used to determine the elemental composition by CHNS analyzer. The element composition analysis of apSCTx displayed the carbon, hydrogen and oxygen contents, 38.81%, 6.42% and 54.77%, respectively. The absence of nitrogen and sulfur in the extracted apSCTx displayed that the xylan is pure and without the protein and lignin cross-linkages as also reported earlier (Khaire *et al.,* 2020).

The specific optical rotation (SOR) determined for apSCTx was -70°, similar to the SOR (-72°) reported previously for xylan extracted from delignified SCT biomass (Khaire *et al.,* 2021c). The commercial xylans, beechwood xylan (from Sigma Chem. Co., USA) and xylan (from Carbosynth, UK) showed SOR of -68.40° and -70.36°, respectively as reported earlier (Khaire *et al.,* 2021c). The similar value of SOR of apSCTx to the commercial xylans confirmed that the apSCTx is also composed of L-arabinose, α-linked D-glucuronic acid and β-linked D-xylose monosaccharide residues.

4.3.2.4 Functional group analysis of apSCTx

The FTIR spectrum of apSCTx (Figure 4.2A) displayed a discrete peak at 3429 cm^{-1}, denoting the hydroxyl (OH) groups stretching vibrations which are similar to the peak obtained for OH group stretching in xylan extracted from roots of *Cudrania tricuspidata* (Shi *et al.,* 2014). The 2922 cm^{-1} peak demonstrated the symmetric CH stretching in xylan extracted from sugarcane bagasse as also reported earlier (Luo *et al.,*

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2015). The asymmetric and symmetric stretching mode of the carboxyl group (-COOH) of glucuronic acid was reported at 1413 cm^{-1} and 1569 cm^{-1}, respectively as studied previously (Corradini *et al.,* 2018). Water absorption in apSCTx was displayed at 1642 cm^{-1} as reported earlier (Zhang *et al.,* 2016). A small absorption peak of β-glycosidic linkage at 897 cm^{-1} was observed in the anomeric region (Kacuráková *et al.,* 2000; Zhang *et al.,* 2016). The stretching vibration of the C-O-C glycosidic group and OH side groups overlap the ring vibrations in a region between 1200-1000 cm^{-1} as reported earlier (Khaire *et al.*, 2021c). The extracted xylan β-1,4 backbone demonstrated a peak of 1047 cm^{-1}, as also reported earlier (Kacuráková *et al.,* 2000). This characteristic functional groups similar to previously reported commercial xylans confirms the purity of extracted apSCTx.

4.3.2.5 FESEM analysis of apSCTx

The highly porous irregular granular and rough surface morphology was observed in the FESEM image of apSCTx extracted from apSCT hydrolysate (Figure 4.1B). A similar morphological structure was also reported previously for xylan extracted from finger millet and rice bran xylan (Palaniappan *et al.,* 2017), pineapple peel waste (Banerjee *et al.*, 2019), acacia sawdust (Sharma *et al.*, 2020b), neem sawdust (Sharma *et al.,* 2020c) and sugarcane tops (Khaire *et al.*, 2021c).

4.3.2.6 DTG and TGA analysis of apSCTx

The thermal decomposition and stability of apSCTx were studied by TG and DTG analyses, respectively (Figure 4.2B). The TG analysis of apSCTx showed that xylan was stable up to 215°C and there after the thermal degradation started. The decomposition of apSCTx occurred in 3 steps. In the first step, the decomposition between 70ºC to 200ºC showed the 6% weight loss that denotes the water loss in

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apSCTx as also mentioned earlier for banana rachis xylan (Deumaga *et al.*, 2020) and commercial xylan (Zhang *et al.*, 2016). The decomposition between 200ºC and 400ºC temperature in the second step showed 54% weight loss due to fragmentation of apSCTx into CO, CO_2, CH_4, CH_3COOH and HCOOH as also mentioned in previous reports (Deumaga *et al.*, 2020, Khaire *et al.*, 2021c). In the final step of apSCTx decomposition, pyrolysis of residual xylan to ash occurred above 400ºC.

The DTG_{max} is the maximum thermal degradation (Td) temperature of apSCTx and was analyzed by DTG (Figure 4.2B). The overall thermal degradation gave a single peak at 275°C, which is similar to the depolymerization of xylan extracted from delignified SCT as reported earlier (Khaire *et al.*, 2021c). This endothermic reaction occurred due to the weight loss or decomposition of apSCTx between 230°C-350°C which is similar to the xylan from rice bran (132-334°C), from finger millet seed coat (220-380ºC), from beechwood (247-340ºC) (Palaniappan *et al.*, 2017), from birchwood and wheat straw (220-350ºC) (Ruzene *et al.*, 2007).

4.3.2.7 Enzyme activity analysis of apSCTx by xylanase

The specific activity of endoxylanase *Ct*Xyn11A against apSCTx (Molecular mass: 58000) was 1823 ±51 U/mg, which was similar to 1876 ±73 U/mg from the xylan extracted from delignified SCT and also the commercial xylans as reported earlier (Khaire *et al.*, 2021c). Owing to the higher enzyme activity, apSCTx can be considered as a commercial substrate.

4.3.2.8 Xylanase mediated production of xylooligosaccharides

The total xylooligosaccharide produced from 1% (w/v) apSCTx in 10 mL reaction mixture after hydrolysis by 50 µl of endoxylanase enzyme (*Ct*Xyn11A: 5 µg/mL) was found to be 27.6 ±2.5 mg by phenol sulfuric acid method. The TLC analysis

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of apSCTx hydrolysed products showed the presence of xylooligosaccharides of degree of polymerization (DP), from 2 to 5 (Figure 4.2Cb). The identical type of oligosaccharides was produced in xylan extracted from delignified SCT (Khaire et al. 2021c). The produced oligosaccharides were further analyzed by LCMS for the confirmation of the degree of polymerization. The ESI-MS analysis of endoxylanase treated apSCT hydrolysate sample showed a series of linear chain xylooligosaccharides of DP from 2 to 5 with no substitution of other residues. The xylooligosaccharides produced from apSCTx sample included xylobiose, xylotriose, xylotetraose and xylopentaose with m/z: 305.0, 437.0, 569.0 and 701.0, respectively (Figure 4.2Ca). The ESI-MS analysis of endoxylanase mediated xylooligosaccharides produced from acacia xylan (Sharma *et al.*, 2020b) and SCT xylan (Khaire *et al.*, 2021c) displayed a similar series of xylooligosaccharides (DP2-DP8 and DP2-DP6, respectively). These enzymatically produced xylooligosaccharides can be utilized in a variety of applications such as antiproliferative agents, as an antioxidative agent in healthcare (Aachary *et al.*, 2015) and as prebiotics (Sharma *et al.*, 2020c). Apart from a substitute from the commercial substrate, apSCTx can be also utilized for various biotechnological applications (Kumar *et al.*, 2021).

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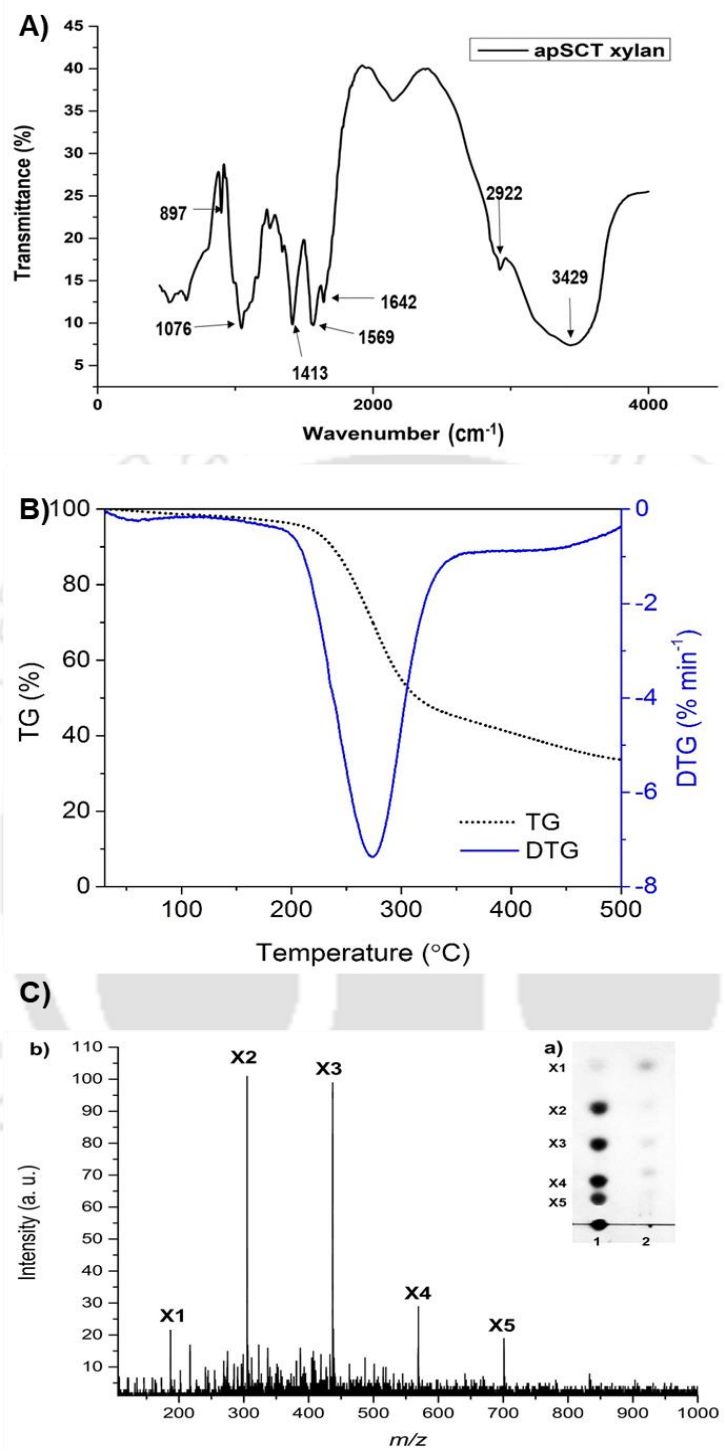

Fig. 4.2. A) Fourier Transform Infrared (FT-IR) spectroscopic analysis of apSCTx, B) Thermogravimetric analysis (TGA) and Derivative Thermogravimetric Analysis (DTG) of apSCTx displaying single thermal degradation temperature (T_d) of 275ºC and C) Endo β(1,4) xylanase (*Ct*Xyn11A) mediated oligosaccharides a) Thin-layer chromatography based analysis of. endo β(1,4) xylanase (*Ct*Xyn11A) hydrolyzed products of apSCTx: Lane 1, *Ct*Xyn11A hydrolyzed apSCTx products, Lane 2, Standards (xylose, DP2 to DP5) and b) ESI-MS analysis of endo β(1,4) xylanase (*Ct*Xyn11A) hydrolyzed products of apSCTx.

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4.3.3 Sequential extraction of apSCTal from alkali pretreated SCT hydrolysate

After the xylan (apSCTx) extraction (by precipitation) from 600 mL apSCT hydrolysate, the remaining liquid fraction was further used for apSCTal extraction by precipitation using HCl at pH 1.5. Around 8.5 g of apSCTal was recovered (Figure 4.1C) from 500 mL of liquid fraction (from 100 g initial biomass) resulting in an overall 60.7% (w/w) yield based on total alkali lignin present in the raw SCT biomass (14%, w/w). The lignin extracted by dioxane and hydrochloric acid after cyclohexane-ethanol (1:1, v/v) pretreatment gave 10% (w/w) recovery yield from raw sugarcane bagasse (Hoareau *et al.*, 2004). The lignin extraction from sugarcane bagasse by alkaline pulping with sodium hydroxide solution after hot water pretreatment gave 13% (w/w) yield (Wahba *et al.*, 2015). Table 4.2 shows a comparative analysis of different lignin extraction methods and yield from sugarcane waste.

Table 4.2. Lignin extraction methods and yield from sugarcane waste.

Biomass	**Lignin Extraction Method**	**Extraction Yield**	**Reference**
Sugarcane bagasse	0.5 M NaOH, Autoclaving at 120°C, 1h	7.5% of total dry biomass	Costa *et al.*, 2017
Sugarcane bagasse	95%, w/w acetic acid + 0.1%, v/v HCl, 187°C, 40 min	55% of Klason lignin	Pinheiro *et al.*, 2017
Sugarcane bagasse	Ionic liquid 1-ethyl-3-methyl imidazolium acetate, 140°C, 120 min	90% of total lignin	Saha *et al.*, 2018
Sugarcane bagasse	7.0%, w/v NaOH, 135°C, 48 min	79% of total lignin	Ratanasumarn *et al.*, 2020
Sugarcane bagasse	Acid catalyst (H_2SO_4, 0.5%, w/w) at 150 °C, 30 min	10% of total dry biomass	Kauldhar *et al.*, 2020
Sugarcane tops	Extractive removal, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60°C, 3h	61% of total alkali lignin	This study

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4.3.4 Characterization of apSCTal

4.3.4.1 Elemental compositional analysis of apSCTal

The air-dried apSCTal was used for determining its elemental composition by CHNS analyzer. The element composition analysis of powdered alkali lignin displayed that the carbon and hydrogen contents to be 40.52% and 5.58% respectively. The absence of sulfur and nitrogen in the separated alkali lignin from SCT showed that the lignin is pure (without lignin cross-linkages) and is devoid of the protein (Khaire *et al.*, 2020).

4.3.4.2 Surface morphology and Functional group analysis of apSCTal

The electron micrograph of apSCTal by FESEM showed spherical structures (Figure 4.1D). These spherical structures may have formed due to the surface tension during the acid precipitation of apSCTal (HCl) as also reported earlier for Kraft lignin extracted from oil palm (H_2SO_4) (Ibrahim *et al.*, 2011). This similar structural morphology of apSCTal can confirms the presence of lignin in the apSCT hydrolysate.

FTIR spectrum of apSCTal (Figure 4.3A) revealed that the functional groups present are similar to those previously reported lignin (Ibrahim *et al.*, 2011). A predominant absorption peak was found at 3404 cm^{-1} denoting the aliphatic and aromatic –OH bands stretching vibration present in the extracted apSCTal (Mousavioun *et al.* 2012). The strong absorption peak in alkali lignin at 2937 cm^{-1} indicated the stretching of C–H band vibration in -CH2, -CH3O, and -CH3 groups (Liu *et al.*, 2014). The C-H stretching in -OCH3 group of apSCTal was represented by 2845 cm^{-1}. C=O stretching of unconjugated ketone, carbonyl and ester groups were seen at 1717 cm^{-1} as mentioned in earlier reports (Sun *et al.*, 2013, Liu *et al.*, 2014). The C=C stretching of the aromatic skeleton of apSCTal was represented by a peak at 1610 cm^{-1} (Nan *et al.*,

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2020). A 1514 cm^{-1} absorption peak found in FTIR spectra of apSCTal represents the C–C stretching in the aromatic skeleton of lignin as mentioned previously (Sun *et al.*, 2013). A C–O stretching in apSCTal denotes the peak at 1328 cm^{-1}, which represents the syringyl group (Prado *et al.*, 2016). A plane deformation of Ar–CH group in syringyl was represented by a peak at 1120 cm^{-1}. An ether (C–O-C) was confirmed by the presence of a peak at 1038 cm^{-1} (Liu *et al.*, 2014). A dominant peak at 834 cm^{-1} denoting the C-H stretching out of plane symmetry in apSCTal as also reported earlier (Liu *et al.*, 2014). The characteristic functional groups similar to previously reported alkali lignin confirms the purity of apSCTal.

4.3.4.3 TG and DTG analysis of apSCTal

The TG curve of apSCTal (Figure 4.3B) showed the first decomposition curve between 70ºC to 100ºC with 6% weight loss representing the loss of water absorbed by lignin which was similar to the previous reports on oil plam showing 8% weight loss between 70ºC to 100ºC (Ibrahim *et al.*, 2011). During the second step of decomposition between 200ºC and 500ºC, almost 68.75% of weight loss was observed due to the fragmentation of alkaline SCT lignin as also mentioned previously (Liu *et al.*, 2014). In the final step, the decomposition of residual lignin occurred after 500ºC. The first derivative of TG (DTG) of apSCTal was analyzed for DTG_{max} (maximum thermal degradation (Td) temperature). The DTG curve showed 2 decomposition peaks, a small peak at 275°C for xylan as contaminant (Khaire *et al.*, 2021c) and a clear dominant peak at 358°C (Figure 4.3B) representing apSCTal, as reported earlier (Ibrahim *et al.*, 2011).

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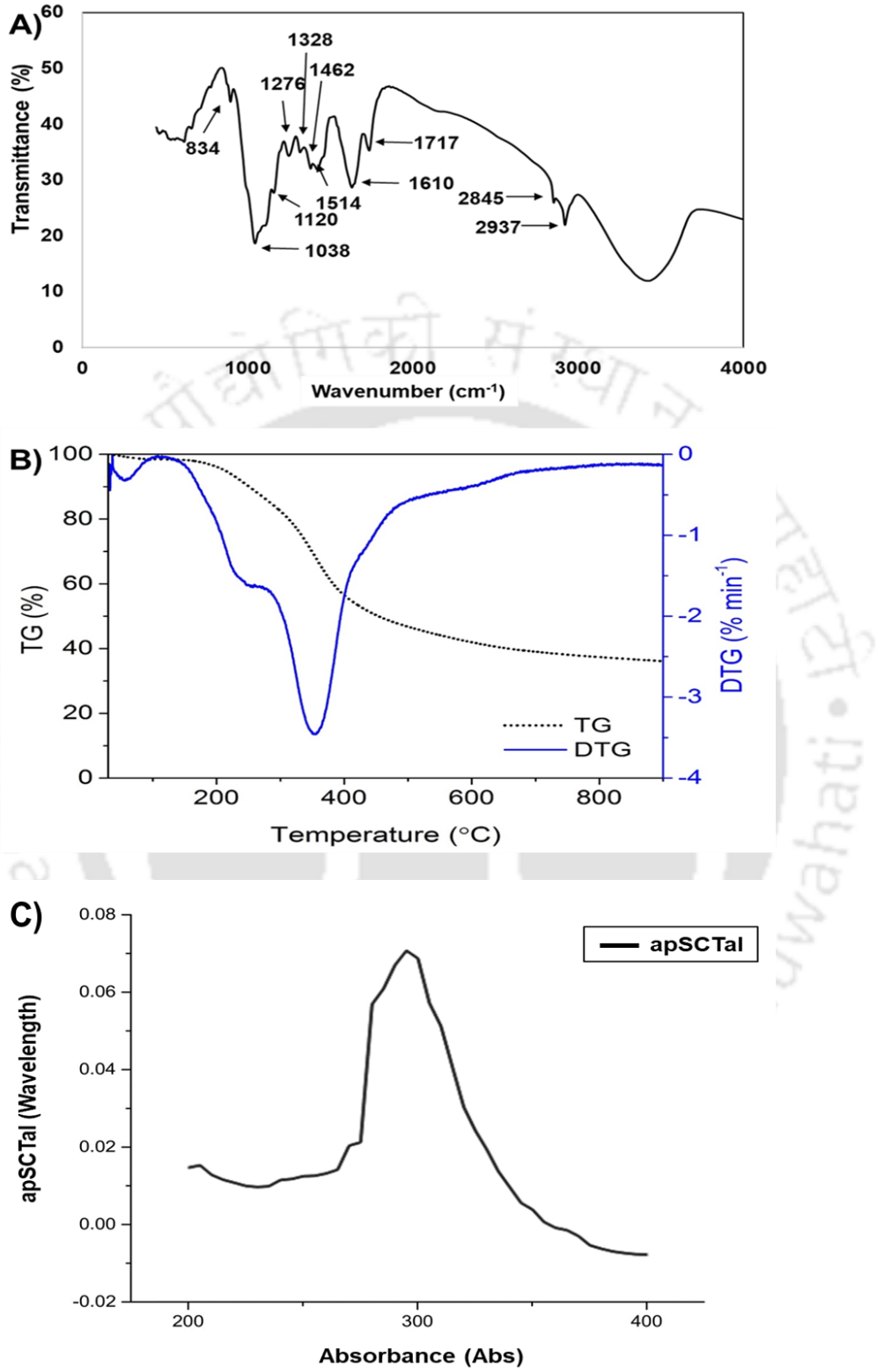

Fig. 4.3. A) Fourier Transform Infrared (FT-IR) spectroscopic analysis of apSCTal, B) Thermogravimetric analysis (TGA) and Derivative Thermogravimetric Analysis (DTG) of apSCTal displaying thermal degradation temperature (T_d) of 358ºC and C) Qualitative spectrometric analysis of apSCTal.

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4.3.4.4 UV spectrophotometric analysis of recovered apSCTal

The UV scan of apSCTal showed the a symmetrical syringyl unit in lignin at 295 nm (Figure 4.3C). The phenolic hydroxyl groups of alkali lignin in neutral solution at absorption maxima at 295 nm was also reported earlier (Lu *et al.,* 2017). No other absorption peak in UV spectrum of apSCTal also supported the purity of the extracted lignin.

4.3.4.5 Matrix-assisted laser desorption ionization (MALDI) coupled with time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of apSCTal

A series of alkaline SCT lignin (apSCTal) oligomers were characterized by MALDI TOF (Figure 4.4). The most abundant oligomers were found between the mass range of m/z 200-555 Da with maximum number of residues at m/z = 308 Da with highest covered area of 2959 square unit, which is similar to the previously reported MALDI TOF for switchgrass lignin (Richel *et al.,* 2012). Figure 4.4 is tentatively showing the molecular structure of different cations. MALDI-TOF MS analysis of apSCTal showed the series of the degree of polymerization and the modified monolignol subunits bellow m/z = 250 Da. These specific MS peaks recorded with variable intensities represented the syringyl (S), guaiacyl (G) and p-hydrophenyl (H) units derived from sinapyl, coniferyl, and p-coumaryl alcoholic precursors. The m/z ranges from 250 to 400 Da denotes the specific linkages between any two phenylpropane subunits or their precursors as mentioned previously for Switchgrass lignin (Rechel *et al.,* 2012). However, the autohydrolysis of lignin during extraction process makes it difficult to analyze the absolute molar mass of lignin as also reported earlier (Fernández-Rodríguez *et al.,* 2019). The most abundant ions over a mass range of m/z 308 Da with 243 S/N ratios displayed in Figure 4.4 represent the dimeric linkages between lignin monomers. The signals at m/z 474, 525 and 553 displayed the

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trioligomers of S, G or H subunits formed by α→5 and *β*→O→4 linkages with linear and regular subunit structures. This confirmed that the produced apSCTal is composed of sinapyl, coniferyl, and *p*-coumaryl alcoholic precursors which can be further used for the production of aromatic chemicals. The separated lignin can have applications in biomedical fields as nanofibril composite for antimicrobial property (Huang *et al.*, 2020), bioactive substances for anti-inflammatory or antioxidative property (Wang *et al.*, 2021) and therapeutic agents for degenerative orthopaedic diseases (Pei *et al.*, 2022).

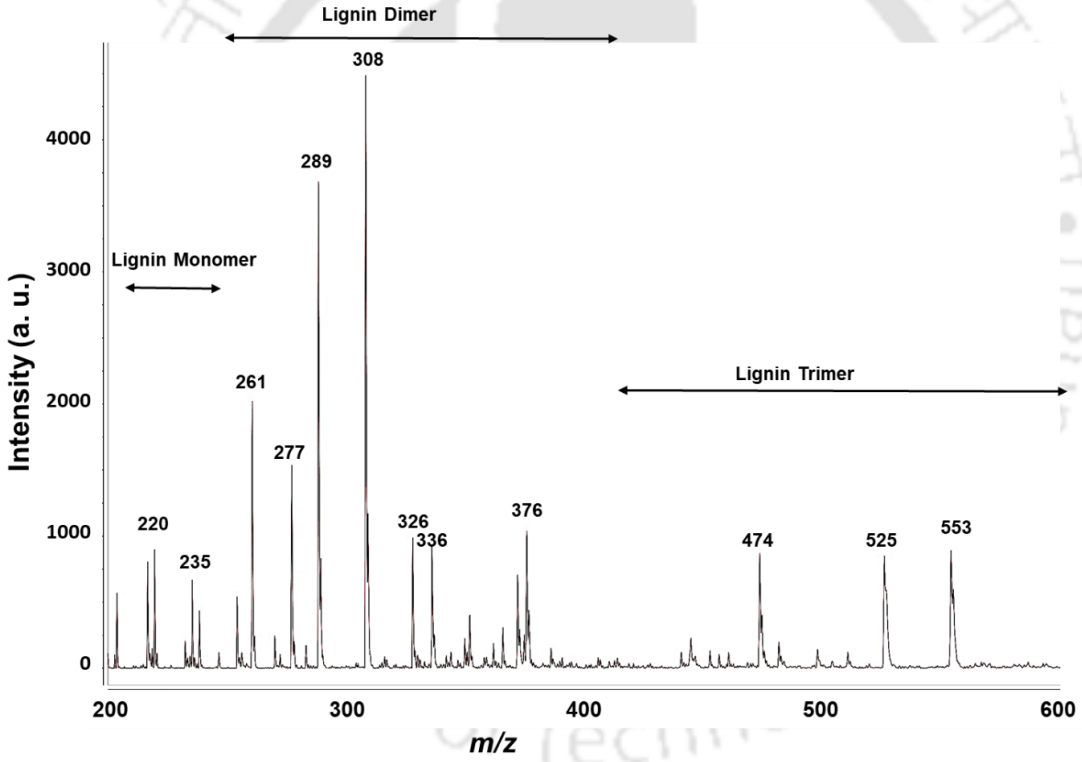

Fig. 4.4. MALDI-TOF MS analysis of apSCTal

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4.3.4.6 Mass balance and economic evaluation of sequential extraction of apSCT xylan and alkali lignin from raw SCT

The economic analysis for sequential extraction of apSCT xylan and alkali lignin from SCT hydrolysate produced during the alkaline pretreatment was carried out to check the feasibility of the process implementation in bioethanol industry (Fig. 4.5).

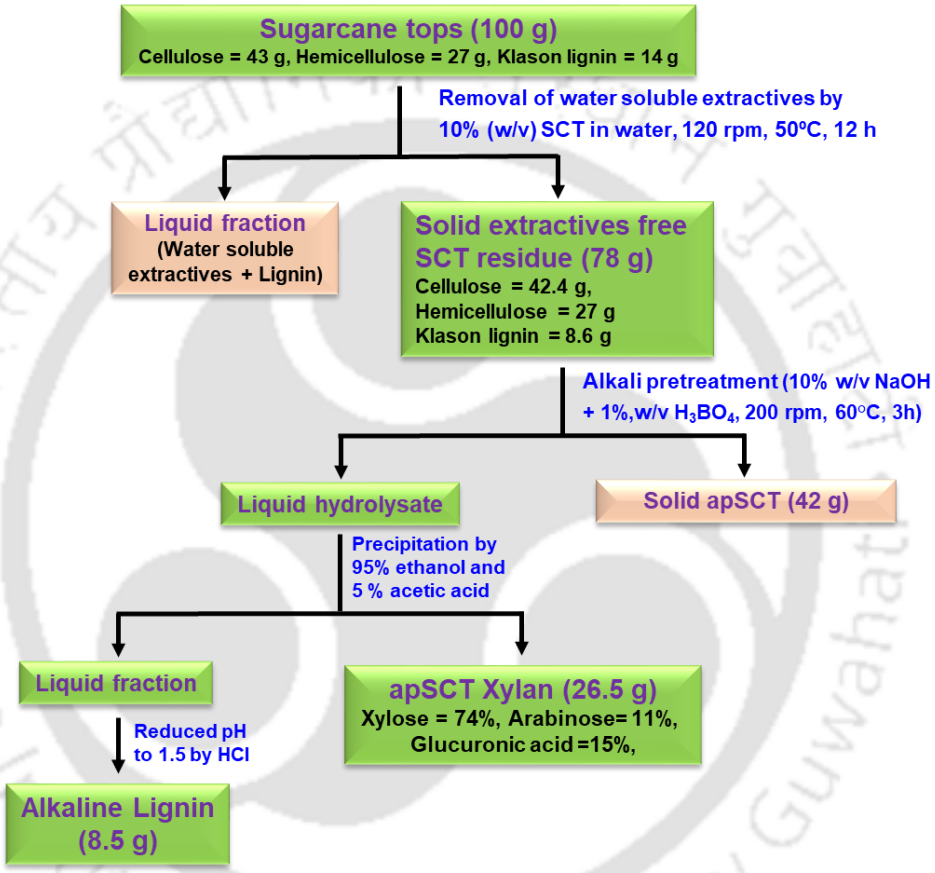

Fig. 4.5. Mass balance of raw SCT to apSCT xylan and alkali lignin extraction

Each product produced in the biorefinery process such as xylan and alkali lignin was evaluated for its value addition. The cost involved in sequential extraction of xylan and lignin from SCT is shown in the Table 4.3.

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Table 4.3. The cost involved in sequential extraction of xylan and lignin from SCT.

Step 1. Removal of water soluble extractives:		
Requirement	**Cost (USD)**	**Required Cost (USD)**
SCT biomass (1 kg)		-
Distilled water (10 L)	0.027/L	0.27
Orbital Shaker 50°C (12 h, 1.7 kW)	0.10/kWh	0.17
Total cost for step 1		**0.44**
Step 2. Alkali pretreatment:		
SCT biomass (780 g)		
Sodium Hydroxide (780 g)	0.94/kg	0.74
Boric Acid (78 g)	5.28/kg	0.412
Distilled water (7.8 L)	0.027/L	0.21
Hot air oven @ 60ºC (3 h, 0.425 kW)	0.10/kWh	0.0425
Total cost for step 2		**1.787**
Step 3. Xylan extraction		
Ethanol (rectified spirit) 11.4 L		This can be reusable
Glacial acetic acid 0.6 L		This can be reusable
Step 4. Lignin precipitation		
HCl to reduce Ph	Negligible	0.1
Total process cost (1+2+3+4)		**~2.33**

According to the market survey, the lowest commercial cost of 25 g of xylan and alkaline lignin is 51 and 26 USD, respectively from Tokyo Chemical Industry, Tokyo, Japan when compared with Megazyme, Wicklow, Ireland and Merck (Sigma-Aldrich), Darstat, Germany. This study also demonstrated that the extracted apSCT xylan and alkali lignin from SCT are of commercial grade and can be utilized as an alternative for commercially available substrate. In this study, only 50% of the commercial cost of xylan and alkali lignin was considered for the economic evaluation. Byproducts produced from the biorefinery process, their commercial and considered cost is shown in the Table 4.4.

Table 4.4. Commercial and considered cost of the byproducts produced from 1 kg of raw SCT.

Byproducts	Yield g/kg raw SCT	Market cost (USD)	Considered cost (USD)	Income (USD)
Xylan	265 g	47/25g	23.5/25g	249.1
Alkali lignin	85 g	25/25 g	12.5/25 g	42.5
Total income				291.6

Market cost of xylan and lignin from TCI, Japan. Assumed considered cost is 50% of commercial cost.

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The total income of the byproducts, apSCT xylan and alkali lignin separated from 1 kg of raw SCT is calculated to be 291.6 USD. As per the economic calculations, about 2.3 USD is required to process 1 kg of raw SCT biomass. The process cost can be further reduced for large scale biorefinery process by purchasing chemicals and reagents in bulk from whole sale market. The overall profit 291.6 USD can be obtained by subtracting the process cost 2.3 USD from the total income 289.3 USD. This study can contribute to the biorefinery process of raw sugarcane tops to bioethanol and other value added products such as xylan and lignin.

Conclusions

The biorefinery approach was demonstrated in this study by sequentially extracting the apSCTx and apSCTal from alkali pretreated SCT hydrolysate. Alkaline pretreatment of SCT reduces the pretreatment processing cost for bioethanol production by recovering 98% of xylan and 61% of alkali lignin from the raw SCT. The composition analysis of of apSCTx by high performance liquid chromatography showed 74% D-xylose, 15% D-glucuronic acid and 11% L-arabinose residues. The analysis of apSCTx by high performance size exclusion chromatography showed molecular size, ~58 kDa. The first derivative of thermogravimetric analysis of apSCTx showed a Td (thermal degradation temperature) at 275ºC. The specific activity of recombinant clostridial endoxylanase (*Ct*Xyn11A) was 1823 ±51 U/mg. Liquid Chromatography-Mass Spectroscopy and Thin-layer chromatography analyses of *Ct*Xyn11A hydrolyzed apSCTx displayed a series of linear xylooligosaccharides ranging between 2-5 degrees of polymerization. The characterization of apSCTal by Field-Emission Scanning Electron Microscopy showed the exposed spherical structures of alkali lignin. Fourier Transform Infrared spectroscopic analysis of apSCTal displayed

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the peaks corresponding to those obtained from commercial alkali lignin. CHNS analysis of apSCTal confirmed the absence of protein impurities. The first derivative of the thermogravimetric analysis of apSCTal showed T_d at 362ºC, similar to the previously reported softwood lignin. The commercial grade purity of the extracted apSCTx and apSCTal was achieved. It was amply demonstrated that xylan can be used as a commercial substrate as well as for prebiotic xylooligosaccharides production. The alkali lignin can be utilized as a potential renewable substrate for aromatic chemicals and energy production.

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Chapter 5

Alkaline pretreatment of sugarcane tops and response surface methodology based recombinant enzymatic saccharification and fermentation for bioethanol production

5.1 Introduction

The energy demand is increasing with populace growth. There is an intensive load on the currently depleting energy resources such as coal and petroleum. The immoderate consumption of these nonrenewable petroleum fuels has caused drastic growth in the level of greenhouse gases (Castillo *et al.*, 2020). At the end of 2050, the energy demand will increase from the existing demand of 13 to 45 billion tons of oil equivalent (Zhang, and Konan, 2010). Therefore, there is an essential need for other sustainable sources of energy and fuels to address the climate change as well as the decreasing oil supply. Bioenergy is harvested from both agricultural or forest residues. The use of renewable biomass resources like agricultural waste has appeared as a reassuring substitute for the synthesis of renewable biofuels like bioethanol, biodiesel and biogas (Thakur *et al.,* 2020). Biodiesel and bioethanol are the two important sustainable energy sources growing as transportation fuels. Preserving the sustainable improvement, the Indian government has made compulsory the blending of 5% bioethanol in gasoline (Singh *et al.,* 2020). Worldwide sugarcane production in 2018

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was about 1.9 billion tonnes. In overall sugarcane production, Brazil contributed about 39% followed by India about 20% (Thakur and Sharma *et al.,* 2021). The stems of sugarcane are mainly used for making the sugarcane juice and further utilized for the production of sugar. Leftover biomass i.e. sugarcane trash/ top (SCT) are usually burned in the field. The SCT contains about 43% of cellulose, 27% of hemicellulose and 19% of lignin, which can be considered as a potential raw material for the bioethanol production (Khaire *et al.,* 2020). To avoid environmental pollution, SCT can have applications in the production of valuable byproducts like xylan, lignin, cellulose, furfural, acetic acid, lactic acid, biobutanol and bioethanol (Canilha *et al.,* 2012; Khaire *et al.,* 2021). A few pretreatment strategies are promising for lignocellulosic biomass for example dilute acid, dilute alkali, ammonia fiber explosion and hydrothermal pretreatment. The reports on pretreatment of SCT biomass by biological pretreatment (Singh *et al.,* 2008), alkali based ammonium fiber explosion pretreatment (Krishnan *et al.,* 2010), acid pretreatment (Sindhu *et al.,* 2011; Srinorakutara *et al.,* 2014), surfactant-assisted ultrasound pretreatment (Sindhu *et al.,* 2013), microwave pretreatment (Maurya *et al.,* 2013), alkali pretreatment (Sindhu *et al.,* 2014, Srinorakutara *et al.,* 2014), formic acid based organosolv pretreatement (Pathak *et al.,* 2020) and crude glycerol assisted pretreatment (Niju *et al.,* 2020) are available. Conventional pretreatment methods involving acids or alkali remove either hemicellulose or lignin from lignocellulosic feedstock (Kucharska *et al.,* 2018). The hot water pretreatment at ≤ 100°C, causes breakdown of carbohydrate-lignin complexes in biomass due to plasticizing action (Vallejos *et al.,* 2017). Alkali pretreatment enhances the enzymatic saccharification of lignocellulosic feedstock as the majority of carbohydrates content is retained while a large fraction of lignin is get separated (Sun *et al.,* 2016). The

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separation of commercial grade cellulose (Khaire *et al.,* 2020) and xylan (Khaire *et al.,* 2021) from delignified SCT biomass is possible by alkaline pretreatment method and this strategy can also lower the pretreatment cost of the process.

The conversion of cellulose to glucose by enzymatic saccharification is a high-cost process and a major bottleneck for the bioethanol industry (Singh *et al.,* 2020). The use of recombinant cellulolytic enzymes can bring the cost effectiveness to the process (Yamada *et al.,* 2013). The rate of enzymatic saccharification reaction and the total reducing sugar yield from feedstock is dependent on the concentration/ ratio of enzyme cocktail (i.e. endoglucanase:cellobiohydrolas:β-glucosidase) used and the use of ezyme concentration relies on the feedstock components and kind of pretreatment process used (Banerjee *et al.,* 2010; Marcos *et al.,* 2013). Accordingly, the selection of cellulase cocktail (type, amount, a ratio of enzymes) is an important factor for the optimum yield of fermentable sugar (Zhou *et al.,* 2009; Kim *et al.,* 2015). A cocktail with the optimum enzyme ratio was speculated based on the rational experiment design and a hypothesis is to study the combining effects of enzymes on the saccharification of lignocellulosic feedstock (Bussamra *et al.,* 2015; Singh *et al.,* 2020).

The aim of this study was to examine the effect of various parameters on the saccharification of alkali pretreated SCT (apSCT) solids and to release maximum reducing sugar by using recombinant cellulolytic enzymes through response surface methodology. The produced glucose from apSCT solids can be further utilized as a substrate for the bioethanol production and exploit this abundantly available feedstock in India.

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5.2 Material and Methods

5.2.1 Materials and sugarcane top collection and biomass processing

Boric acid, sodium chloride, sodium hydroxide was obtained from the Himedia Pvt. Ltd. India. Solvents like acetic acid, hydrochloric acid and butanol were acquired from the Merck India Pvt. Ltd. Sugarcane tops were collected from locality of Guwahati, Assam, India. Collected SCT feedstock was washed by water and cut into 1-2 cm pieces. Then SCT pieces were dried in a hot air oven (Labtech, Mumbai, India) at 60°C for 48 h. After moisture removal, the SCT biomass were milled to 0.85 mm particle size by using a grinder (Philips, HL1606/03) and stored at 25°C.

5.2.2 Removal of water soluble extractives from raw SCT biomass

About 80 g of raw SCT in 800 mL of distilled water was taken in 2 L conical flask and kept at 50°C in a shaker incubator for 12 h to remove the water-soluble extractives without its delignification. The hydrolysate was separated from solid SCT biomass by filtering through cotton cloth and cellulosic solid was dried in a hot air oven at 80°C. Separated hydrolysate was also dried in separate beaker in hot air oven at 80°C. The separate weights of dried hydrolysate and dried solids were taken to calculate the yield of water-soluble extractives content in the raw SCT.

5.2.3 Pretreatment of extractives free sugarcane tops

The extractives free SCT biomass was subjected to alkaline pretreatment (Khaire *et al.,* 2020). 20 g of extractives free SCT was added to 200 mL solution containing 1% (w/v) boric acid and 10% (w/v) sodium hydroxide (pH 13) in a 500 mL reagent bottle and kept on a magnetic stirrer (200 rpm, 60ºC) for 3 h. In alkaline pretreatment, the boric acid acts as a monobasic acid that removes OH groups and enhances the separation of xylan from cellulosic solids (Scott, 1989). After incubation,

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the pretreated slurry was passed through a cotton cloth and separated cellulosic solid was neutralized under tap water and dried at 80ºC for 12h. After drying, the recovered apSCT was weighed and stored at dry place for further experiments. The total cellulose recovered in apSCT was estimated by using standard Technical Association of the Pulp and Paper Industry protocol (1992).

5.2.4 Characterization of alkali pretreated SCT

5.2.4.1 Analysis of monosaccharide composition in apSCT

The composition analysis of apSCT biomass was examined by HPLC (UFLC, Prominence, Shimadzu, Japan). About 5 mg of apSCT biomass was taken in 2 mL Eppendorf tube containing 1 mL of tri-fluoro-acetic acid (2 M, TFA). The reaction mixture was placed in a boiling water bath for 2 h. The hydrolyzed apSCT was centrifuged at 10000g for 5 min. The supernatant was poured into a new 2 mL Eppendorf tube and dried in a hot air oven (at 80ºC, 12h) to evaporate the residual TFA. TFA hydrolyzed apSCT was suspended in 500 µL MilliQ water and then sieved through 0.22 µm pore size syringe (Polyvinylidene fluoride membrane) filter. The TFA hydrolyzed apSCT was analyzed for monosaccharides by HPLC by using refractive index (RI) detector. The 50 mm X 7.8 mm guard column followed by 300 mm X 7.8 mm Phenomenex Rezex ROA (H^+) column were connected to the HPLC system for analysis of TFA hydrolyzed apSCT. H_2SO_4 (0.005N) was used as a mobile phase at a 0.5 mL/min flow rate and 70ºC for 90 min was used for the elution of hydrolyzed apSCT. For the analysis of monosaccharide composition in apSCT, the 1 mg/mL of each standard (glucose, xylose and arabinose) was used.

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5.2.4.2 Functional group analysis of apSCT

The functional groups in both raw (untreated) SCT and apSCT were analyzed by using FT-IR spectroscopy (Perkin Elmer, Spectrum Two, USA) as reported earlier (Khaire *et al.*, 2020). Raw SCT and apSCT were separately mixed with the potassium bromide in a 1:100 (w/w) ratio by using mortar-pestle and under 15-ton hydraulic press, the pellets were prepared. The FT-IR spectra of both the biomass samples were obtained in the range, 4000 to 400 cm^{-1}.

5.2.4.3 Field-Emission Scanning Electron Microscopy (FE-SEM) analysis of raw SCT and apSCT biomass

The surface morphology of raw SCT and apSCT biomass was analyzed by using FE-SEM (Carl Zeiss, Model-Gemini 300, Germany). The carbon tape was fixed on the stab surface before placing the raw SCT or apSCT samples on the carbon tape. Both the samples prepared on the stab was double gold coated in the vacuum chamber to avoid the moisture gain by biomass. The double gold coated samples images were taken at 2 kx magnification by using FE-SEM analyzer for the surface morphology analysis.

5.2.4.4 Crystallinity index analysis of raw SCT and apSCT by X-Ray diffraction

The crystallinity index (*CrI*) of raw SCT and apSCT was measured by XRD (D8 Advance, Bruker, Germany). 1 g of each dried raw SCT and apSCT was analyzed for crystallinity index by scanning over the range of 2θ = 10° - 30° with 0.05° step size. The *CrI* of both raw SCT and apSCT samples was calculated by (Segal *et al.*, 1959).

$$Crystallinity\ Index\ (\%) = \frac{(Ic\ -\ Ia)}{Ic} X\ 100$$

Here, I_c - Intensity at degree, 2Θ = 22° and I_a - Intensity at a degree, 2Θ = 18°.

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5.2.4.5 Elemental analysis of raw SCT and apSCT solids

The elemental analysis of raw SCT and apSCT biomass was analyzed by using CHNS (CHNS: carbon, hydrogen, nitrogen and sulphur) analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Italy). The oxygen content present in raw and apSCT were calculated by using the formula,

$$Oxygen\ \% = 100\% - \sum[Carban(\%) + Hydrogen(\%) + Nitrogen(\%) + Sulphur(\%)]$$

5.2.5 Production and enzyme activity estimation of crude recombinant hydrolytic enzymes

The previously reported recombinant cellulolytic enzymes of endo-β-1,4-glucanase (*Ct*Cel8A) (Jamaldheen *et al.*, 2018) and β-1,4-glucosidase, (*Ht*Bgl) (Sharma *et al.*, 2019) from *Hungateiclostridium thermocellum* were used in this study. Another recombinant enzyme, cellobiohydrolase, *Ct*CBH5A (a gift from Prof. Carlos M.G.A. Fontes, NZYTech Ltd., Lisbon, Portugal) was used in the present study. All the mentioned recombinant enzymes were expressed by using procedure described earlier (Khaire *et al.*, 2020). Enzyme activity of crude *Ct*Cel8A and *Ct*CBH5A enzymes were estimated by determining the released reducing sugar concentration by Nelson (1944) and Somogyi (1945) method and enzyme activity of *Ht*Bgl was estimated by GOD-POD method as reported earlier (Raabo and Terkildsen, 1960).

5.2.6 Optimization of saccharification conditions of apSCT by response surface methodology

The optimization of enzymatic saccharification (in terms of reducing sugar yield) of apSCT was carried out by employing the Design Expert 8.0.7.1 version (Stat-Ease, Inc., Minneapolis, USA). To determine the favorable parameters and their

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combinations for reducing sugar release from apSCT, a second-order Box-Behnken design (BBD) was used.

Table 5.1. Box-Behnken design and the response for apSCT biomass for saccharification by endo-1,4-β-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bg1).

Run Order	Biomass loading (%)	Time (h)	*Ct*Cel8A (U/g of biomass)	*Ct*CBH5A (U/g of biomass)	*Ht*Bg1 (U/g of biomass)	TRS yield (mg/g)
1	3	60	150	200	200	180.51
2	3	36	150	200	300	176.11
3	2	36	150	200	200	240.39
4	2	36	150	100	100	134.50
5	2	36	150	300	300	258.88
6	3	36	150	200	100	238.85
7	2	36	150	200	200	243.25
8	2	36	150	200	200	241.05
9	3	36	150	100	200	138.69
10	2	12	150	100	200	141.66
11	2	12	50	200	200	178.86
12	2	36	50	200	300	191.74
13	2	12	250	200	200	174.57
14	1	60	150	200	200	116.67
15	3	36	150	300	200	182.71
16	3	12	150	200	200	141.99
17	2	60	50	200	200	203.19
18	2	36	250	200	300	267.58
19	2	60	150	200	100	177.43
20	1	36	50	200	200	93.56
21	1	36	150	200	300	112.27
22	2	60	150	100	200	163.12
23	2	36	150	100	300	183.16
24	2	36	50	100	200	119.98
25	3	36	250	200	200	176.11
26	1	36	150	300	200	119.98
27	2	12	150	200	100	163.12
28	2	36	250	100	200	183.82
29	2	36	150	300	100	178.86
30	2	60	150	300	200	233.35
31	2	36	250	200	100	188.88
32	1	36	150	100	200	96.86
33	2	36	150	200	200	250.41
34	1	12	150	200	200	100.16
35	2	12	150	300	200	191.74
36	2	36	50	200	100	148.81
37	2	36	50	300	200	198.90
38	2	60	250	200	200	246.00
39	1	36	250	200	200	118.87
40	2	36	150	200	200	264.17
41	3	36	50	200	200	145.29
42	2	60	150	200	300	248.76
43	1	36	150	200	100	104.57
44	2	12	150	200	300	214.63
45	2	36	150	200	200	247.66
46	2	36	250	300	200	251.84

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Various parameters with different ranges were selected to analyze their effects on the saccharification of apSCT. The temperature 40°C, 150 rpm and citrate-phosphate buffer (50 mM) pH 6.0 were taken as the fixed parameters. The citrate phosphate buffer used in the saccharification process gets absorbed by apSCT at biomass loading above 3.5%. Under this condition, the biomass clumps are formed that hinders the uniform mixing. Therefore, 1-3% of apSCT biomass loading was used in this study. The apSCT biomass loading (1-3%, w/v), time (12-60 h) and recombinant enzyme concentrations (loading of endoglucanase (*Ct*Cel8A) 50-250 U/g and for cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bg1) the loading, 100-300 U/g was taken as variable parameters for the optimization experiment. The selected variables were encoded in the highest, central and lowest level as +1, 0 and -1, respectively. All the experiments were carried out in triplicate sets to check the variability of the results. For estimating the relationship in the experimental and independent responses, the second order polynomial equation was used. The encoded and decoded values for saccharification optimization are showed in Table 5.1. The saccharification of apSCT was done for designed experimental conditions in 2 mL final volume taken in 5 mL tube. The reaction was stopped by keeping the reaction mixture containing test tubes in the water bath (boiling) for 5 min. The resulting hydrolysate was separated by centrifugation at 10000 g for 2 min and reducing sugar released was analyzed by Nelson (1944) and Somogyi (1945) method. The resulting monosaccharide, glucose was also estimated by GOD-POD method. The optimized conditions were validated experimentally for apSCT along with raw SCT for total reducing sugar and glucose yield. Cellulose conversion (%) from apSCT, based on the reducing sugar/ glucose release was calculated by using the formula as reported previously (Abdel-Fattah and Abdel-Naby, 2012).

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$$Cellulose\ conversion\ (\%) = \frac{Reducing\ sugar\ or\ glucose\ release}{Total\ cellulose\ present\ in\ SCT} X\ 100$$

5.2.7 Fermentation of hydrolysate produced from saccharification of apSCT

Hexose fermenting yeast, *Saccharomyces cerevisiae* was revived from glycerol stock at 30°C in MGYP (malt, glucose, yeast extract and peptone) medium as reported earlier (Nedumaran *et al.*, 2020). Sugar and alcohol tolerance of *Saccharomyces cerevisiae* is 25% (v/v) (139 mM) glucose and 10% (171 mM), respectively as reported earlier. About 5% (v/v) of 107 CFU/mL overnight grown *S. cerevisiae* culture inoculum was added to 100 mL of GYE (glucose, yeast extract) medium containing (in g, 0.64 glucose, 0.1 yeast extract, 0.1 KH2PO4, 0.05 MgSO4.7H2O and 0.5 (NH4)2SO4) in distilled water (100 mL) and followed by autoclaving), pH 5.0 in 250 mL Erlenmeyer flask as a standard. In test experiment, 0.64 g equivalent of enzyme saccharified hydrolysate was added instead of glucose. Both the Erlenmeyer flasks were incubated in shaking incubator at 30°C, 150 rpm for 72 h. Ethanol produced by fermentation of saccharified hydrolysate was spectrophotometrically estimated at 578 nm (A_{578}) by using dichromate method (Pilone, 1985). The ethanol yield was calculated by,

$$Ethanol\ yield\ (\frac{g}{g} of\ glucose) = \frac{Ethanol\ produced\ (\frac{g}{L})}{Glucose\ produced\ (\frac{g}{L})}$$

5.2.8 Mass balance of raw SCT biomass to fermentation process

The mass balance of untreated SCT biomass (100 g) for each processing step including removal of water extractives, alkali pretreatement, saccharification process and fermentation process was carried out. The recovery of biomass at each step on dry weight basis was calculated by formula (Guilherme *et al.*, 2017),

$$Biomass\ recovery\ yield\ (\%) = \frac{Recovered\ cellulose\ fiber\ (g)}{Initial\ weight\ of\ raw\ SCT\ (g)} X\ 100$$

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5.3 Results and Discussion

5.3.1 Separation of water soluble extractives from raw SCT biomass

One hundred grams of raw SCT biomass yielded 78 g of solid extractive free SCT residue after the removal of water extractives without delignification. The recovered solid residue contained 42.4 g of cellulose, 27 g of hemicellulose and 8.6 g of Klason lignin as determined by standard TAPPI protocol.

5.3.2 Alkali pretreatment of extractives free SCT biomass

The pretreatment of 78 g of extractives free SCT biomass after alkali pretreatment by 10% (w/v) NaOH and 1% (w/v) boric acid gave 42 g of cellulosic alkali pretreated SCT (apSCT).

5.3.3 Analysis of monosaccharide composition in apSCT biomass

The monosaccharide analysis of apSCT biomass by TFA hydrolysis showed the presence of glucose (87%), xylose (9%), arabinose (1.2%) and glucuronic acid (1.8%).

5.3.4 Surface morphology analysis of raw SCT and apSCT biomass

The FE-SEM images of raw SCT and apSCT biomass are shown in the Fig. 5.1.

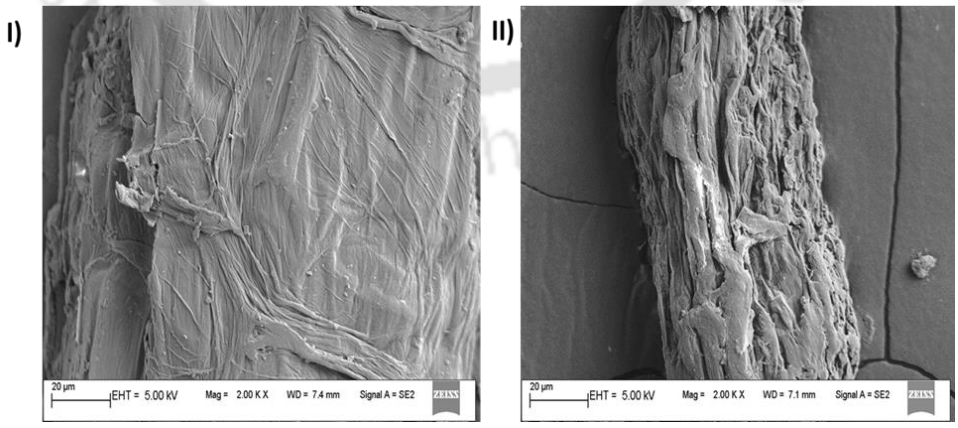

Fig. 5.1. Field-emission scanning electron microscopy analysis images of I) raw SCT biomass and II) apSCT.

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The raw SCT biomass showed smooth and compact structure whereas the apSCT biomass showed the rough and exposed fibrous structure. These exposed fibres in apSCT appear due to hemicellulose removal and more cellulose accumulation (87%), which was also found in previously analysed TFA hydrolysed apSCT.

5.3.5. Crystallinity index analysis of raw SCT and apSCT

The raw SCT and apSCT powder displayed 36% and 47% crystallinity index (*CrI*), respectively (Fig. 5.2).

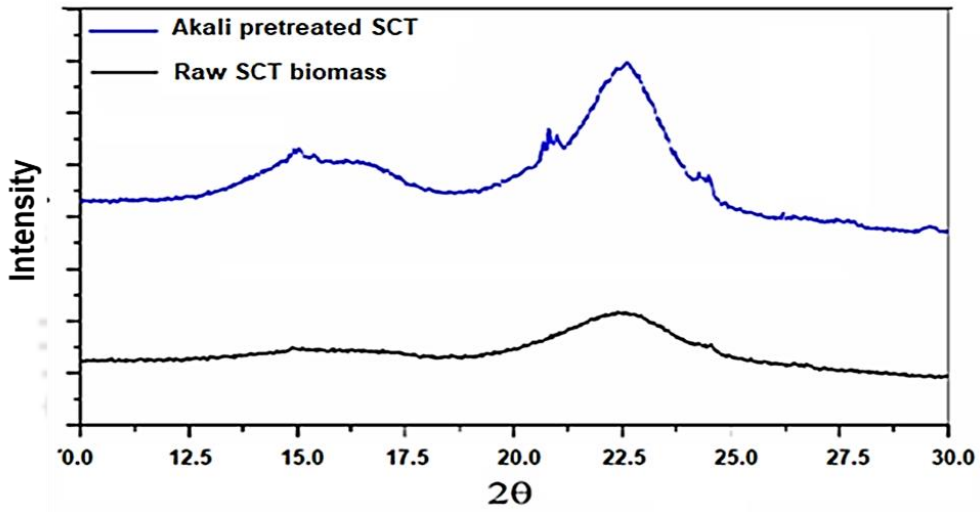

Fig. 5.2. Crystallinity index analysis of raw SCT and apSCT biomass by XRD

The raw SCT and apSCT powder displayed 36% and 47% crystallinity index (*CrI*), respectively. The 11% increase of *CrI* in apSCT as compared with the raw SCT biomass explains more exposure of crystalline cellulose, as also reported earlier (Jamaldheen *et al.*, 2018, Khaire *et al.*, 2020). It was reported that the crystallinity index is directly proportional to the cellulose exposure in apSCT biomass (Kian *et al.*, 2018).

5.3.6 Functional group analysis of raw SCT and apSCT

The FTIR spectra of raw SCT and apSCT biomass are shown in the Fig. 5.3. As per the previous reports, the absorption peaks of cellulose were mainly found between 3660 - 2800 cm^{-1} and 1650 - 800 cm^{-1} wavenumber regions as reported earlier (Khaire

et al., 2020). The peaks in the range of 3660 to 2900 cm^{-1} showed the stretching vibrations between C-H and/or O-H bonds of the apSCT biomass as reported earlier (Rosa *et al.,* 2010). The stretching vibrations of C-H bonds at 2894 cm^{-1} display the overall hydrocarbon constituents present in the raw SCT and apSCT biomass as reported earlier (Jamaldheen *et al.,* 2018).

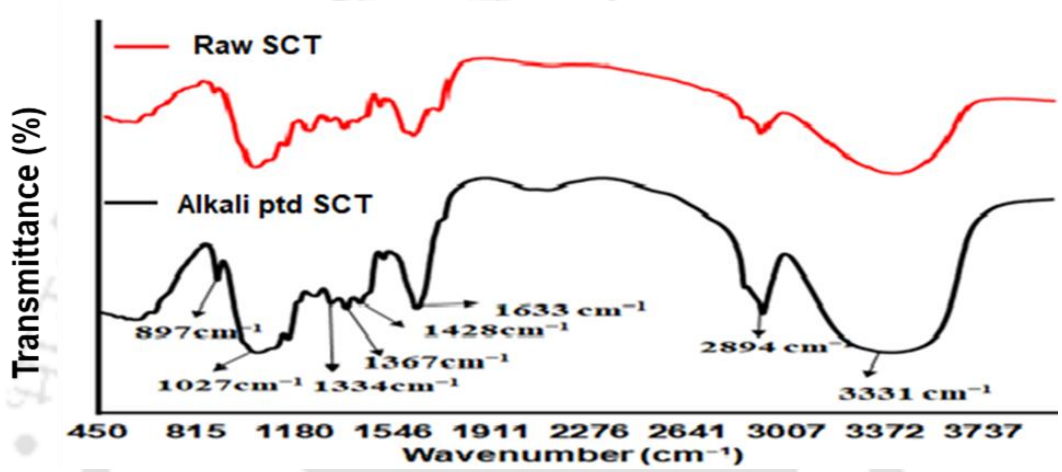

Fig. 5.3. Functional group analysis of raw SCT and apSCT by FT-IR spectroscopy.

A comparatively larger peak at 897 cm^{-1} was observed in apSCT than the raw SCT biomass which is corresponding to the amorphous cellulose as mentioned earlier (Sindhu *et al.,* 2014). A relatively large and prominent peak at 1027 cm^{-1} in apSCT than in raw SCT was observed which is the characteristic peak for C-O vibrations as mentioned in the earlier reports (Krishnan *et al.,* 2010). This result confirmed the more cellulose exposure in apSCT as compared with the raw SCT biomass. Similarly, larger peaks at 3331 cm^{-1} and 3634 cm^{-1} were observed in apSCT than in the raw SCT, corresponding to the intra, and intermolecular O-H bond stretching, respectively, in cellulose, thus supporting the effective exposure of cellulose in apSCT. The appearance of a sharp peak at 1318 cm^{-1} can be ascribed to CH_2 bond vibrations in the apSCT cellulose which were absent in the raw SCT. A similar result was observed in ionic

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liquid pretreated *Asclepias syriaca* seed floss and poplar seed floss (Spiridon *et al.*, 2010). A prominent peak at 1334 cm^{-1} in apSCT displayed the C-C and C-O bond vibrations in cellulose as mentioned in earlier studies (Sindhu et al., 2011). A peak at 1428 cm^{-1} in raw SCT corresponding to the C=C bond stretching present in lignin (phenol ring) as reported earlier was disappeared in the apSCT (Podgorbunskikh *et al.*, 2020). A peak at 1516 cm^{-1}, characteristic of C=C bond present in guaiacyl ring of lignin clearly visible in the spectrum of raw SCT but disappeared in apSCT, as also reported earlier (Sindhu *et al.*, 2014), thus confirming the efficient removal of lignin during alkali pretreatment. This result supported the effective removal of lignin in alkaline pretreatment of SCT. Similarly, a miniature peak at 1633 cm^{-1} observed in raw SCT was disappeared in the apSCT that was associated with the ester bonds in between the lignin and hemicellulose as reported earlier (Gill *et al.*, 2019). These results concluded that the cellulosic fibers are exposed in apSCT and also that the lignin and hemicellulose were effectively removed.

5.3.7 Elemental composition analysis of raw SCT and apSCT

The raw SCT and apSCT were analysed for elemental composition by using CHNS analyser. The CHNS compositions of raw SCT displayed C: 45.2%, H: 7.4%, N: 2.3%, S: 1.1% and O: 44% while CHNS composition of pretreated SCT displayed C: 43.2%, H: 6.29% and O: 50.29%. The absence of sulphur and nitrogen in the apSCT confirmed the removal of protein content and lignin cross linkages as also reported earlier (Karunarathna, *et al.*, 2019; Khaire *et al.*, 2020).

5.3.8 Production of crude recombinant cellulolytic enzymes for the saccharification of apSCT

The protein concentration of expressed crude recombinant cellulolytic enzymes, *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1 enzymes as estimated by Bradford was found to be 9.2

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mg/mL, 6.9 mg/mL and 5.5 mg/mL, respectively. The endoglucanase activity of crude *Ct*Cel8A and cellobiohydrolase activity of crude *Ct*CBH5A as estimated against the CMC-Na was found to be 25.3 U/mL and 4.4 U/mL, respectively. The β-glucosidase activity of crude *Ht*Bg1 enzyme showed 32.3 U/mL against its substrate, cellobiose.

5.3.9 Optimization of enzymatic saccharification of apSCT by response surface methodology

The parameters, biomass loading (%, w/v), recombinant enzymes *i.e.* *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1 loading (U/g) and time (h) were varied under designed experiments as mentioned in the material and method section and the optimization was performed in citrate-phosphate buffer (pH 6, 40°C) for apSCT. The Box-Behnken design was used for optimization of saccharification of apSCT by analysing enzyme loading, time and biomass loading and their response, the TRS yield are shown in the Table 5.1. The second order equation in the terms of actual factors generated by Box-Behnken design for saccharification optimization process is,

Reducing Sugar yield (mg/g of apSCT) =

$$-516.94463 + (371.31766 \times \text{Biomass loading}) + (2.20855 \times \text{Time}) + (0.69811 \times Ct\text{Cel8A}) + (1.19606 \times Ct\text{CBH5A}) + (0.69343 \times Ht\text{Bg1}) + (0.22931 \times \text{Time} \times \text{Biomass loading}) + (0.013759 \times \text{Biomass loading} \times Ct\text{Cel8A}) + (0.052283 \times \text{Biomass loading} \times Ct\text{CBH5A}) - (0.17611 \times \text{Biomass loading} \times Ht\text{Bg1}) + (5.90724E^{-003} \times \text{Time} \times Ct\text{Cel8A}) + (2.09819E^{-003} \times \text{Time} \times Ct\text{CBH5A}) + (2.06379E^{-003} \times \text{Time} \times Ht\text{Bg1}) - (2.72421E^{-004} \times Ct\text{Cel8A} \times Ct\text{CBH5A}) + (8.94310E^{-004} \times Ct\text{Cel8A} \times Ht\text{Bg1}) + (1.60976E^{-003} \times Ct\text{CBH5A} \times Ht\text{Bg1}) - (81.13459 \times \text{Biomass loading}^2) - (0.049342 \times \text{Time}^2) - (2.74026^{E-003} \times Ct\text{Cel8A}^2) - (3.38141E^{-003} \times Ct\text{CBH5A}^2) - (1.63040E^{-003} \times Ht\text{Bg1}^2)$$

The given quadratic model of the response data of apSCT fits well and is shown in the ANOVA Table (Table 5.2). The F-value (18.69) and p-value (<0.0001) of model from ANOVA test displayed that the given model terms are significant and had only a 0.01% chance of noise. P values larger than 0.1000 indicate not significant model terms.

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Table 5.2. ANOVA for quadratic model of apSCT biomass saccharification by endo-1,4-β-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bg1).

Source	SS	Df	Mean^2	F-Valu	p-value	
Quadratic model	1098	20	5491.65	18.69	< 0.0001	Significant
A-Biomass loading	14120.54	1	14120.54	40.00	< 0.0001	
B-Time	4299.90	1	4299.90	14.63	0.0008	
C- *Ct*Cel8A	6697.18	1	6697.18	22.79	< 0.0001	
D-*Ct*CBH5A	12909.21	1	12909.21	43.93	< 0.0001	
E-*Ht*Bg1	8104.45	1	8104.45	27.58	< 0.0001	
AB	121.15	1	121.15	0.41	0.5267	
AC	7.57	1	7.57	0.026	0.8738	
AD	109.34	1	109.34	0.37	0.5474	
AE	202.27	1	202.27	0.69	0.4146	
BC	554.83	1	554.83	1.89	0.1816	
BD	101.43	1	101.43	0.35	0.5621	
BE	98.13	1	98.13	0.33	0.5685	
CD	29.69	1	29.69	0.10	0.7532	
CE	319.92	1	319.92	1.09	0.3067	
DE	246.01	1	246.01	0.84	0.3689	
A^2	60226.00	1	60226.00	204.96	< 0.0001	
B^2	6295.34	1	6295.34	21.42	< 0.0001	
C^2	5827.10	1	5827.10	19.83	0.0002	
D^2	10692.68	1	10692.68	36.39	< 0.0001	
E^2	3846.07	1	3846.07	13.09	0.0013	
Residual	7346.12	25	293.84			
Lack of fit	6950.35	20	347.52	4.39	0.0540	
Pure error	395.77	5	79.15			
Model statistics						
S.D.	17.14		R^2	0.9373		
Mean	181.12		Adj-R^2	0.8872		
C.V. %	9.46		Pred-R^2	0.7579		

In all 5 variable factors studied, the highest F-value of biomass loading implies that this variable is most significant factor for apSCT saccharification and lowest F-value of reaction time indicates least significant variable. As per the models p values, the variables A, B, C, D, E, A^2, B2, C^2, D^2, E^2 are significant. The regression coefficient (R-Square value) of the model was found to be 0.973, moreover the predicted R-Square value of 0.7579 is in reasonable agreement with the Adjusted R-Square value of 0.8872 showed in the Table 2. The correlation of variable factors with each other for reducing

sugar release is shown in the Fig. 5.5. Fig. 5.4I, 5.4II, 5.4III and 5.4IV are showing hyperbolic shaped 3D surface graph of apSCT biomass % vs time, *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1, respectively. The reducing sugar yield (mg/g) increased with the increase in apSCT loading up to 2.14% and then started decline (Fig. 5.4I, 5.4II, 5.4III and 5.4IV). Also, the Fig. 5.4V, 5.4VI, 5.4VII were showing the 3D surface graph of Time vs *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1 enzymes, respectively. Similarly, Fig. 5.4VIII and 5.4IX were showing the hyperbolic 3D graph of *Ct*Cel8A vs *Ct*CBH5A and *Ht*Bg1 enzyme and Fig. 5.4X was for *Ct*CBH5A vs *Ht*Bg1. Similar hyperbolic pattern was observed for the enzymes endo-1,4-β-glucanase (*Ct*Cel8A) (Fig. 5.4II, 5.4V, 5.4VIII and 5.4X), cellobiohydrolase (*Ct*CBH5A) (Fig. 5.4III, 5.4VI, 5.4VIII and 5.4IX) and β-glucosidase (*Ht*Bg1) (Fig. 5.4IV, 5.4VII, 5.4IX and 5.4X). By analyzing the 3-D surface graphs and the ANOVA model, Box-Behnken design predicted the experimental conditions for optimum reducing sugar release from apSCT. These are 2.14% apSCT loading with 213.2 U/g endo-1,4-β-glucanase (*Ct*Cel8A), 272.5 U/g cellobiohydrolase (*Ct*CBH5A) and 299.8 U/g β-glucosidase (*Ht*Bg1) for 49.2 h saccharification time. At above mentioned optimum conditions Box-Behnken design also predicted the total reducing sugar yield of 276.6 mg/g of saccharified apSCT. The experimental total reducing sugar yield upon validation by performing an experiment under predicted optimum conditions, was found to be 265 mg/g (7.95 g/L) of saccharified apSCT. The glucose yield of experimental set up was also estimated by GOD-POD method and it was 214 mg/g of apSCT (6.42 g/L).

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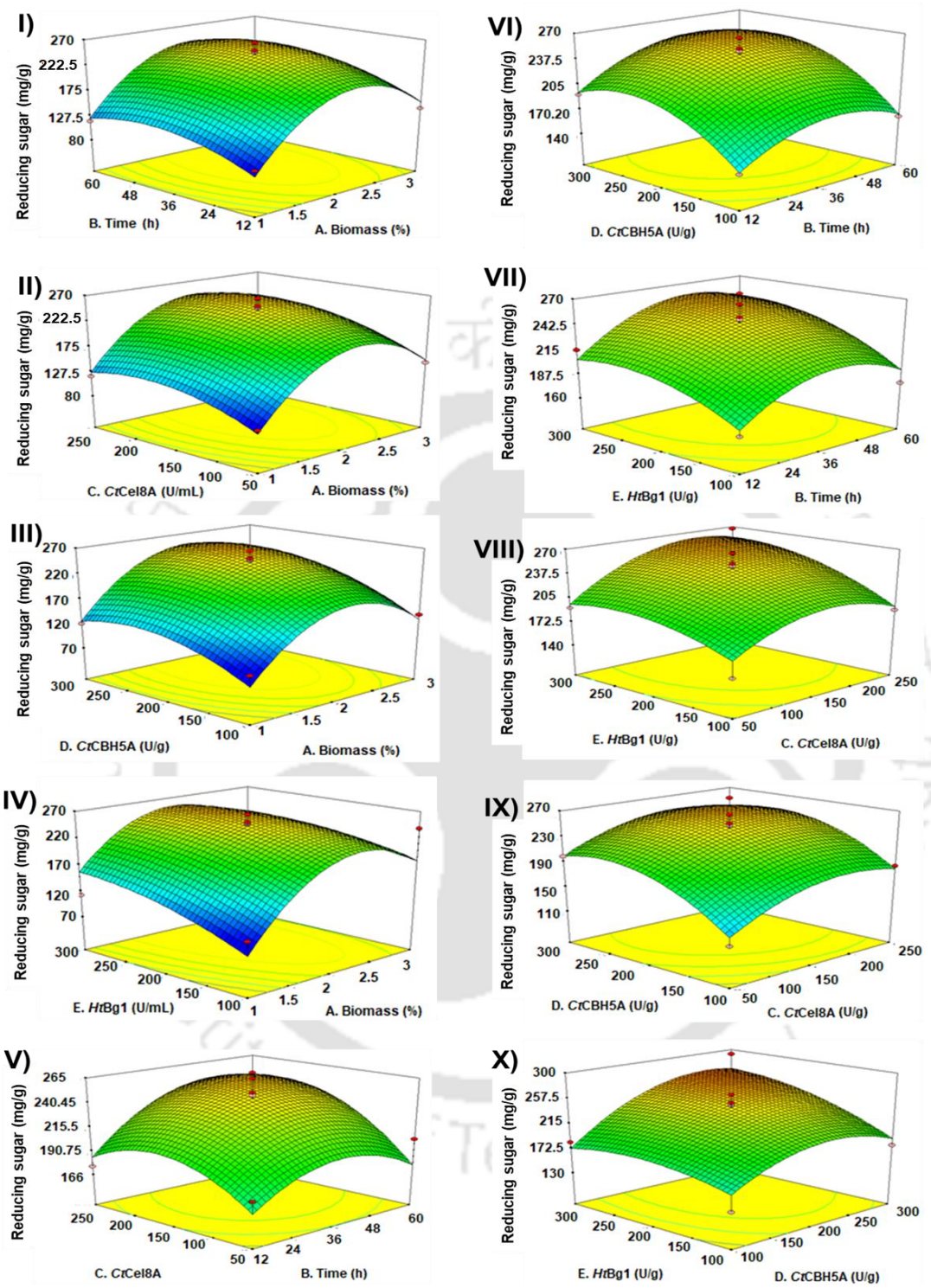

Fig. 5.4. 3-D surface graphs (the interactions between independent variables) of RSM based saccharification optimization of apSCT in terms of reducing sugar released (mg/g). I) Pretreated SCT loading vs Time, II) Pretreated SCT loading vs *Ct*Cel8A, III) Pretreated SCT loading vs *Ct*CBH5A, IV) Pretreated SCT loading vs *Ht*Bg1, V) Time vs *Ct*Cel8A, VI) Time vs *Ct*CBH5A, VII) Time vs *Ht*Bg1, VIII) *Ct*Cel8A vs *Ct*CBH5A, IX) *Ct*Cel8A vs *Ht*Bg1 and *Ct*CBH5A vs *Ht*Bg1.

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The 3% (v/v) sulphuric acid pretreated SCT after saccharification by commercial cellulase (from Zytex India) resulted about 680 mg/g of reducing sugar yield reported earlier (Sindhu *et al.*, 2011). The enzymatic hydrolysis of 3% (w/v) alkali pretreated SCT at 15% (w/w) substrate loading, 50 FPU commercial enzyme loading, 0.125% (w/w) surfactant concentration for 60 h was reported 775 mg/g of reducing sugar yield, which was much higher as compared to our study (Sindhu *et al.*, 2014). These higher yields may due to the species of SCT, type of pretreatment and cellulolytic enzyme used. The conversion of cellulose (%) from apSCT biomass to reducing sugar released and glucose released was found to be 30.4 % and 24.6 %, respectively, which can be further used as a feedstock for the production of bioethanol.

5.3.10 Fermentation of saccharified apSCT biomass

Fermentation of saccharified apSCT biomass carried out by *S. cerevisiae* at 30^{o}C, pH 5 for 72 h, resulted in 1.2 g/L (3.4%) ethanol concentration. Around 0.19 g bioethanol was produced per gram of glucose released during the RSM based optimized saccharification process. The fermentation of optimized alkali hydrogen peroxide followed by acid hydrolysis pretreated SCT gave about 9.9% ethanol concentration in broth (Niju *et al.*, 2020). Dilute acid pretreatment followed by enzymatic saccharification and fermentation of SCT biomass at optimized conditions produced 11.36 g/L of bioethanol using *S. cerevisiae* (Sindhu *et al.*, 2011). The glycerol assisted with transition metal followed by alkali pretreatment of sugarcane trash fermentation by *S. cerevisiae* gave 0.38 g/g of bioethanol yield per gram of pretreated sugarcane trash with 78.9% fermentation efficiency was reported earlier (Raghavi *et al.*, 2016). These higher reported bioethanol yields could be due to the optimized fermentation conditions and depending on the biomass source and pretreatment conditions. The present study

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on bioethanol production by *S. cerevisiae* after saccharification optimization of apSCT resulted in 37.7% of fermentation efficiency, which was lower than those previously reported. However, there is possibility to improve the efficiency of bioethanol production by further optimizing the fermentation parameters such as inoculum percentage, substrate concentration, media composition and other physical parameters. The present pretreatment method used for the processing of the most abundant agricultural waste (SCT) can reduce the processing cost of the biomass and can serve as potential strategy for bioethanol production.

5.3.11 Mass balance of raw SCT to bioethanol fermentation

The mass balance of 100 g raw biomass to bioethanol production is shown in the Fig.5.5. In initial water soluble extractives removal step, 78 g of solid extractives free SCT residue was recovered that contained 42.4 g cellulose, 27 g hemicellulose and 8.8 g Klason lignin. After the alkali pretreatment of extractive free SCT, 42 g of apSCT solid was recovered which contained 87% (w/w) glucose, 9% (w/w) xylose, 1.2% (w/w) arabinose and 1.8% (w/w) glucuronic acid. The RSM based optimization of saccharification of 42 g apSCT gave 265 mg of reducing sugar (or 214 mg of glucose from GOD-POD assay) from 1 g of apSCT, thereby resulting 11.1 g (w/w) reducing sugar (or 9 g, w/w glucose) released from 100 g of raw SCT. The fermentation of released glucose resulted in 19 g of bioethanol production per 100 g of glucose. This is equivalent to 1.7 g of bioethanol per 100 g of raw SCT with 37.7% fermentation efficiency. The laboratory scale economic analysis of SCT biomass to bioethanol production was carried out on the acquired data. The pretreatment cost of 1 kg raw SCT biomass is around 2.30 USD. Under the un-optimized fermentation conditions, comparatively less bioethanol yield, 17 g per kg of raw SCT was obtained.

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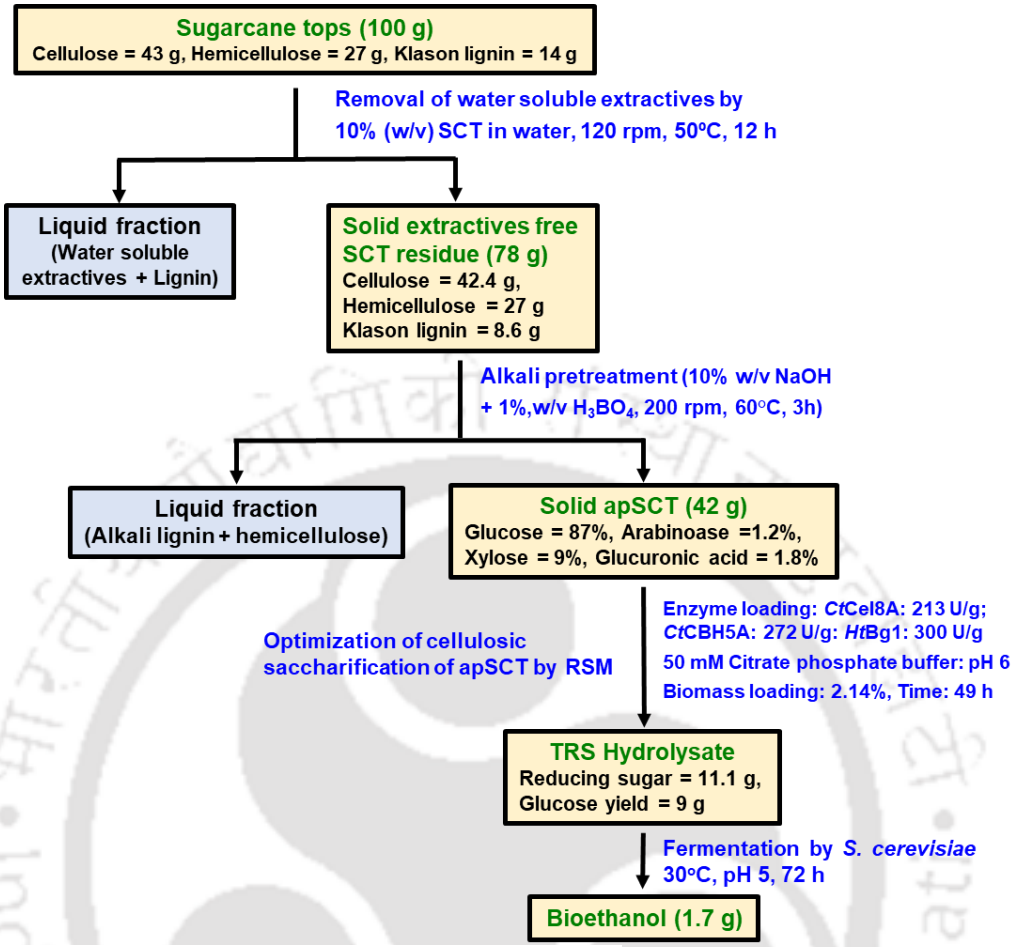

Fig. 5.5. Mass balance for raw SCT biomass to bioethanol fermentation of apSCT biomass.

However, the bioethanol production can be improved by optimizing the fermentation parameters and by enhancing the fermentation efficiency of *S. cerevisiae*. Moreover, the recovery of the by-products from this alkaline pretreatment process such as xylan and alkali lignin can be beneficial under the biorefinery concept. The results from this study may contribute to the development of a SCT based biorefinery.

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Conclusions

Water soluble extractive removal prior to the alkali pretreatment of SCT biomass enhanced the cellulose content in apSCT, which showed advantage over other conventional pretreatment methods. The solid alkali pretreated SCT (apSCT) recovered on Field-emission scanning electron microscopy (FE-SEM) analysis showed exposure of cellulosic fibres as compared with raw SCT. The analyses of apSCT by Fourier Transform Infrared (FT-IR) Spectroscopy, X-ray diffraction (XRD) and High performance liquid chromatography (HPLC) analysis also confirmed the enhanced cellulose content in apSCT. Optimum conditions for response surface methodology based saccharification of apSCT at 40°C, 150 rpm were 2.14% (w/v) apSCT loading in citrate-phosphate buffer (50 mM, pH 6.0), recombinant hydrolytic enzymes (from *Clostridium/Hungateiclostridium thermocellum*) loading for endo-1,4-β-glucanase (*Ct*Cel8A) =213.2 U/g, cellobiohydrolase (*Ct*CBH5A) =272.5 U/g and β-glucosidase (*Ht*Bg1) =299.8 U/g for 49.2 h. Response surface methodology based saccharification optimization of apSCT gave 265 mg/g of TRS (214 mg/g of glucose) yield. This was in close agreement with the predicted TRS (276.6 mg/g) yield. Therefore, SCT can be used as a potential substrate for bioethanol production. Fermentation of released glucose by *S. cerevisiae* yielded 0.19 g (per g of glucose) of bioethanol with 37.7% fermentation efficiency. This can be further improved by statistical optimization of fermentation parameters.

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Chapter 6

Optimization of saccharification and fermentation of alkali pretreated sugarcane tops for higher scale bioethanol production

6.1. Introduction

In recent years, the conversion of agricultural waste to bioethanol has received greater attention owing to the increased costs of petroleum fuels, energy security and environmental pollution (Halder *et al.*, 2019). The agricultural waste is low cost and renewable feedstock that can be utilized for the production of bioethanol which has environmental and economic advantages over fossil-based traditional fuels (Sindhu *et al.*, 2011). Approximately, 200 billion tonnes of agricultural waste is accumulated every year in the agriculture field (Bilal and Iqbal, 2020). Worldwide production of sugarcane in the year 2021 was around 1.9 billion tonnes, of which about 18.4% was contributed by India alone (Thakur and Sharma *et al.*, 2021). The sugarcane tops residues are the most abundant agriculture waste produced in India (Chotirotsukon *et al.*, 2019). Around 7-12 tons of SCT is produced from 1 ha of sugarcane field (Suma and Savitha, 2015). The post harvested green SCT waste can be utilized as an animal fodder. However, the left stubble after it gets dried is burnt in the field which causes the soil and air pollution (De Figueiredo and La Scala Jr, 2011, Pathak *et al.*, 2021). This SCT waste can serve

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as a sustainable, cheap and readily available renewable energy feedstock for the production of bioethanol (Gómez *et al.*, 2014). SCT is composed of 43% cellulose, 27% hemicellulose and 19% lignin (Khaire *et al.*, 2020). Sugarcane stem is utilized to extract the sugarcane juice for sugar and ethanol production. Leftover part of sugarcane crop, the sugarcane tops (SCT) and the sugarcane bagasse comprise the solid waste. The SCT waste can be utilized to produce xylitol, furfural, lactic acid, biobutanol, bioethanol and other value-added products by which environmental pollution can be overcome (Khaire *et al.*, 2021a). The application of SCT for bioethanol production has been demonstrated (Sindhu *et al.*, 2011; Raghavi *et al.*, 2016; Khaire *et al.*, 2021b). Although the bioethanol production from cellulosic waste is a firmly set process, but the use of SCT waste for bioethanol production is not proven as a commercial process. About 250 kg of SCT (Chandel *et al.*, 2012) and 140 kg of bagasse (Dias *et al.*, 2012) is produced from 1 ton of sugarcane crop. Around 50% (w/w) of SCT from this can be collected from field and utilized for the production of bioethanol without harming the nutritional status of the soil (Hassuani *et al.*, 2015). Though the modern techniques are being used for lignocellulosic waste conversion to bioethanol, but the high cost involved in the pretreatment and saccharification processes is major obstacle while taking at industrial level. However, the cost can be reduced by optimizing the saccharification (Sindhu *et al.*, 2014) and fermentation processes (Raghavi *et al.*, 2016; Niju *et al.*, 2020). The pretreatment of agricultural waste for enhancing the cellulose accessibility for enzymatic saccharification is most crucial step (Marulanda *et al.*, 2019). The pretreatment of agricultural waste involving acid or alkali can efficiently remove either hemicellulose or lignin (Kucharska *et al.*, 2018). The alkaline pretreatment has advantages over the acid pretreatment as it can remove maximum lignin fraction by

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retaining the cellulosic fraction (Sun *et al.,* 2016). The use of sodium hydroxide and boric acid demonstrated that it can efficiently separate the commercial grade xylan (Khaire *et al.,* 2021d) and cellulose (Khaire *et al.,* 2021c) from delignified SCT, thereby enhancing the economics of the process. Another major, crucial and cost intensive step in bioethanol production is enzymatic saccharification of pretreated biomass containing cellulose to produce the fermentable monosaccharides (Singh *et al.,* 2020). The complete and efficient utilization of pentose and hexose sugars produced after saccharification of pretreated lignocellulosic biomass can maximize production of bioethanol thereby the profiting industrial process (Krishnan *et al.,* 2000; van Maris *et al.,* 2006). Owing to non-availability of efficient natural fermenting strain, two common approaches such as, the genetically modified strain and co-culture strategy are used to accomplish the fermentation. The use of co-culture showed promising results such as co-culture of *P. stipites with S. cerevisiae* (Taniguchi *et al.,* 1997, Bari *et al.,* 2004), *Z. mobilis* and *P. tannophilus* (Fu *et al.,* 2008) and co-culture of *S. cerevisiae* with a recombinant *E. coli* strain (Laplace *et al.,* 1993). The fermentation using co-culture strategy showed that the pentose sugar is utilized at lower rate due to catabolic repression caused by glucose or oxygen requirement conflict between used fermenting strains (Kordowska-wiater and Targonski, 2002, Peiris, 2009). The current study describes the partial optimization of apSCT biomass saccharification by using commercial cellulase. The present study first time showed new strain combination of consortium of *P. stipites, S. cerevisiae* and *Pachysolen tannophilus* for the production of bioethanol and the effect of various factors on the overall fermentation process. The optimized saccharification and fermentation conditions for apSCT biomass were used for higher scale bioethanol fermentation.

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6.2. Materials and Methods

6.2.1 Materials collection and the processing of SCT biomass

All the required chemicals were purchased from the Himedia Pvt. Ltd. India and solvent/ reagents were procured from Merck India Pvt. Ltd. The commercial cellulase (350 FPU/mL) was procured from Sigma Chem. Co., USA. The SCT biomass was collected from the nearby Guwahati, Assam, India and processed to particle size, 0.85 mm, for further use (Khaire *et al.*, 2021b).

6.2.2 Water-soluble extractives removal from raw SCT

Water-soluble extractives from 1000 g of raw SCT were removed using 10 L distilled water at 50°C for 12 h. Liquid hydrolysate containing water-soluble extractives was separated by using a cotton cloth and the remaining solid was dried at 80°C in a hot air oven for further use. The weight of dried cellulosic solid was measured for biomass recovery calculations.

6.2.3 Alkaline pretreatment of water-soluble extractives free SCT

The water-soluble extractives free SCT (778 g) was pretreated with the 7.78 L alkaline solution containing 1% (w/v) H_3BO_3 and 10% (w/v) NaOH in five Erlenmeyer flasks of 2 L capacity incubated at 200 rpm in an orbital shaker incubator at 60ºC for 3 h as optimized earlier (Khaire et al., 2021b). The acid, H_3BO_3 was added to achieve the enhanced separation of xylan from lignocellulosic SCT as reported earlier (Scott, 1989). The liquid hydrolysate produced after alkaline pretreatment containing xylan and lignin was separated from apSCT solids using a cotton cloth. The separated apSCT was then neutralized by washing with the tap water and dried at 80ºC for 12 h. The dry weight of apSCT was then recorded for the recovery yield calculations and total cellulose

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recovery in apSCT was determined by following the standard Technical Association of the Pulp and Paper Industry (TAPPI) protocol (1992).

6.2.4 Optimization of saccharification conditions for apSCT biomass

The optimization of saccharification of apSCT by using commercial enzyme was carried out by measuring the released total reducing sugar (TRS) as a response. The optimization of saccharification reaction was carried out in 20 mL reaction volume in a 50 mL Erlenmeyer flask. Considering the previous bench-scale experiments, the pH (4.5), temperature (45°C) and shaking (150 rpm) were taken as fixed parameters. The apSCT loading (3 and 5%, w/v), enzyme loading (17.5 or 35 FPU/g $_{\text{of apSCT}}$) and reaction time (24-96 h) were taken as variable parameters. The conditions for each experiment are as follows, Experiment I: 3% (w/v) apSCT and 17.5 FPU/g $_{\text{of apSCT}}$ of commercial enzyme loading, Experiment II: 3% (w/v) apSCT and 35 FPU/g $_{\text{of apSCT}}$ of commercial enzyme loading, Experiment III: 5% (w/v) apSCT and 17.5 FPU/g $_{\text{of apSCT}}$ of commercial enzyme loading and Experiment IV: 5% (w/v) apSCT and 35 FPU/g $_{\text{of apSCT}}$ of commercial enzyme loading. The pH of distilled water was adjusted by using 1 M H_2SO_4. Every experiment was carried out in triplicate. After every 24 h, 500 µL of saccharified hydrolysate was taken for estimation of released TRS estimation by DNS method (Miller, 1959). The cellulose conversion (%) was calculated by considering the TRS released during the saccharification reaction and total cellulose present in apSCT biomass (Abdel-Fattah and Abdel-Naby, 2012).

$$Cellulose\ conversion\ (\%) = \frac{Reducing\ sugar\ produced\ (g)}{Total\ cellulose\ present\ in\ apSCT\ (g)} X\ 100$$

The optimized conditions were used for higher scale saccharification process. Around 40 g of apSCT was taken in 1.2 L distilled water (initial pH adjusted to 4.5) with 35 FPU/g $_{\text{of apSCT}}$ of commercial enzyme in three separate 2 L Erlenmeyer flasks

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and incubated at 45°C, 150 rpm for 48 h. As mentioned earlier, the released TRS in the hydrolysate was determined by the DNS method. The released TRS were further used for bioethanol fermentation.

6.2.5 Optimization of conditions for fermentation of released apSCT sugar

The fermentation reaction was carried out in 20 mL reaction volume in a 50 mL Erlenmeyer flask in a triplet set. The temperature, 35°C and shaking at 100 rpm were taken as the constant factors from previous bench-scale experiments. The optimization of parameters for fermentation of reducing sugar (TRS released from Experiment II: 3% apSCT + 35 FPU/g $_{\text{of apSCT}}$ of cellulase enzyme) was carried out. The TRS loading (10-15 g/L), nitrogen source (3 g/L yeast extract or 1.5 g/L urea), time (24-96 h) and 5% (v/v) fermenting microbes (*S. cerevisiae, P. stipitis* and *P. tannophilus* added individually and as a consortium in the ratio of 1:1:1 from 10^7 CFU/mL overnight grown culture) were used as variable factors. The ethanol released in fermenting hydrolysate was determined by using the dichromate method (Pilone, 1985) and the fermentation efficiency (%) was determined by following the equation (de Farias Silva *et al.*, 2018),

$$Fermentation\ efficiency\ (\%) = \frac{Ethanol\ produced}{0.511\ X\ Sugar\ used}\ X\ 100$$

The optimized fermentation parameters were taken for the higher scale fermentation process in a 5 L bioreactor (BioSpin - 05A, Secunderabad, India). The working volume of around 3 L hydrolysate (from Experiment II: 3% apSCT + 35 FPU/g $_{\text{of apSCT}}$ of cellulase enzyme) containing 15 g/L TRS was subjected to fermentation by adding 5% (v/v) inoculum of consortium of fermenting microbes. The fermentation for bioethanol production was conducted at 35°C and 100 rpm for 24 h. The ethanol released in the hydrolysate was determined by the dichromate method (Pilone, 1985).

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6.2.6 Analytical methods

6.2.6.1 Estimation of reducing sugar by Di-nitro salicylic acid method

The reducing sugar estimation was performed by following the method of (Miller, 1959). One mL of saccharified hydrolysate was taken in a glass test tube followed by addition of 1 mL of DNS reagent to the tube and incubated in boiling water bath for 15 min. After cooling the tube to the room temperature, 3 mL of distilled water was added to the tube. The absorbance at 540 nm (A_{540}) was measured on the UV-Visible spectrophotometer (Varian, Cary 100) and the reducing sugar present in the saccharified hydrolysate was determined by using glucose standard. The DNS standard plot (y=0.0012x and R^2= 0.9881) was prepared by taking 1 mL of different concentrations of glucose (0.1 mg/mL to 1 mg/mL) and blank (1 mL distilled water) in 10 mL test tube.

6.2.6.2 Estimation of ethanol concentration by dichromate method

The cell-free supernatant (0.5 mL) obtained after centrifugation of fermentation broth at 6000*g* at 4°C for 10 min or 0.5 mL distilled water as a blank was taken in 10 mL test tube. The ethanol estimation was carried out by following the method of (Pilone, 1985). 0.5 mL of 1.2 M potassium dichromate solution, 0.5 mL of 50 mM acetate buffer (pH 4.3) and 2.5 mL of 1 N sulfuric acid were added in each tube and mixed well. The reaction was carried out in a boiling water bath for 15 min. The absorbance at 578 nm (A_{578}) was measured on the UV-Visible spectrophotometer (Varian, Cary 100). The ethanol concentration in the fermented cell-free supernatant was determined by using ethanol standard (y=0.4066x and R^2= 0.9943) by taking 0.5 mL of different concentrations (0.6 to 1.9 mg/mL).

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6.2.7 Mass balance and economic evaluation of higher scale saccharification and fermentation process

The higher scale saccharification and fermentation of SCT was initiated from 1 kg of raw SCT. Mass balance was calculated by considering biomass recovery (on a dry basis) at each step in the overall process (Guilherme *et al.*, 2017),

$$\boldsymbol{Biomass\ recovery} = \frac{Recovered\ component\ (g)}{Initial\ weight\ of\ raw\ SCT\ (g)}$$

The economic evaluation of each step was calculated by considering the commercial cost of all components used throughout the process (chemicals, water, electricity and instrumentation). Other costs involved such as manpower, transportation and storage were not considered in economic calculations for the laboratory scale process.

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6.3. Results and Discussion

6.3.1 The higher scale pretreatment of raw SCT

One kilogram of raw SCT gave 778 g of solid SCT biomass after the separation of water-soluble extractives. The water-soluble extractives free SCT contained cellulose- 423 g, hemicellulose- 269 g and Klason lignin- 86 g as determined by the standard TAPPI method. The alkaline pretreatment of 778 g of water-soluble extractives free SCT gave 426 g of cellulosic apSCT solid, used for further study. The monosaccharide composition of apSCT gave glucose (87%, w/w), xylose (9%, w/w), arabinose (1.2%, w/w) and glucuronic acid (1.8%, w/w) residues as reported earlier (Khaire *et al.*, 2021b).

6.3.2 Optimization of bench-scale enzymatic saccharification and its validation at higher scale

The apSCT loading (3 or 5%, w/v), enzyme loading (17.5 FPU/g or 35 FPU/g) and reaction time (24-96 h) were the variable parameters used for the optimization of enzymatic saccharification and the process in 20 mL volume was carried out at pH 4.5, 45°C and 150 rpm and the response, TRS values are listed in Table 6.1.

Table 6.1. Optimization of enzymatic saccharification of apSCT for reducing sugar yield in g/L

		Experiment I	Experiment II	Experiment III	Experiment IV
ApSCT Loading (w/v)		3%	3%	5%	5%
Enzyme Loading (v/w)		5%	10%	5%	10%
Time	0	0	0	0	0
	24	7.67±0.4	13.61±0.2	8.56±0.2	16.6±0.2
	48	9.47±0.3	18.61±0.1	10.1±0.4	19.3±0.1
	72	9.83±0.2	18.21±0.1	9.92±0.2	19.66±0.1
	96	9.79±0.3	17.62±0.4	9.87±0.3	19.63±0.1
Cellulose conversion (%) at 48 h		35.48±0.2	67.16±0.1	20.73±0.2	41.09±0.1
Cellulose conversion (%) (higher scale)			68.7		

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All experiments were conducted at 120 rpm, pH 4.5 and 45°C.

Although the maximum TRS of 19.3 g/L was obtained with Experiment IV, (5%, w/v apSCT with 35 FPU/g $_{\text{of apSCT}}$ of commercial enzyme loading), but the experiment II, (3%, w/v apSCT with 35 FPU/g $_{\text{of apSCT}}$ of commercial enzyme loading) giving TRS of 18.6 g/L was considered for higher scale saccharification and fermentation because of its higher percent of cellulose conversion (Table 1). The cellulose conversion (in %) from apSCT for all four enzymatic saccharification experiment are shown in Fig. 6.1.

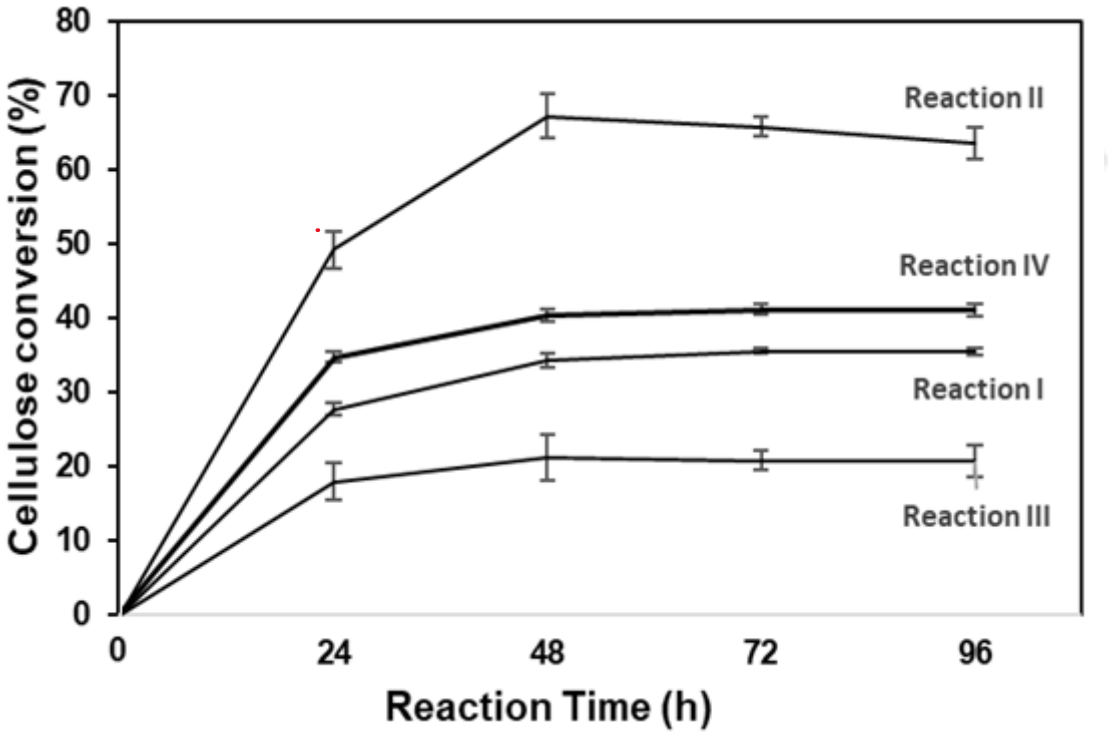

Fig. 6.1. Cellulose conversion (%) during enzymatic saccharification of apSCT under different conditions. Experiment I: 3% (w/v) apSCT and 17.5 FPU/g of apSCT of commercial enzyme loading, Experiment II: 3% (w/v) apSCT and 35 FPU/g of apSCT of commercial enzyme loading, Experiment III: 5% (w/v) apSCT and 17.5 FPU/g of apSCT of commercial enzyme loading and Experiment IV: 5% (w/v) apSCT and 35 FPU/g of apSCT of commercial enzyme loading. All four experiments were carried out at 120 rpm, pH 4.5 and 45°C for different time interval.

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In experiment I, i.e. 3%, w/v apSCT loading with 17.5 FPU/g $_{of\ apSCT}$ of commercial enzyme loading, the maximum 35.48% cellulose conversion was observed at 48 h (Fig. 6.1). The experiment II with 3%, w/v apSCT with 17.5 FPU/g $_{of\ apSCT}$ of commercial enzyme loading gave maximum of 67.2% cellulose conversions at 48 h. Whereas, the experiments III and IV gave lower 20.7% and 41.1% cellulose conversion, respectively. All experiments, showed the enhancement in percent cellulose conversion of apSCT up to 48 h. The enzymatic saccharification of alkali pretreated cellulosic date palm waste reported similar pattern after achieving the 71% cellulose conversion yield (Alrumman, 2016). A previous study, the optimization of saccharification of alkali pretreated SCT by using recombinant cellulolytic enzymes reported 30.4% cellulose conversion (Khaire *et al.*, 2021c). These partially optimized saccharification conditions of experiment II were used for validation at the higher scale. Around 40 g of apSCT taken in 1.2 L distilled water (in triplicate) (initial pH adjusted to 4.5) with 35 FPU/g $_{of\ apSCT}$ of commercial enzyme and incubated at 45°C, 120 rpm for 48 h gave 687 mg/g $_{of\ apSCT}$ of TRS yield which was similar to 672 mg/g obtained for bench-scale saccharification optimization (Table 6.1). The saccharification of 3% (w/v) H_2SO_4 pretreated SCT by commercial enzymes (from Zytex, India) reported similar value, 680 mg/g of TRS yield (Sindhu *et al.,* 2011). The 3% (w/v) alkaline pretreatment of SCT followed by saccharification using commercial cellulase resulted in 775 mg/g TRS yield (Sindhu *et al.,* 2014). The transition metal (assisted by crude glycerol) followed by alkali pretreated SCT reported 780 mg/g of TRS yield in commercial enzymatic saccharification for 48 h (Raghavi *et al.,* 2016). The 7% (v/v) hydrogen peroxide pretreated SCT followed by enzymatic saccharification at 72 h reported 338 mg/g of TRS yield (Dodo *et al.,* 2017). The saccharification of ionic liquid and ammonium

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carbonate pretreated SCT by cellulase (50 FPU/g of pretreated SCT) and xylanase (330 IU/g of pretreated SCT) isolated from fungus *Penicillium chrysogenum* VS4 resulted in 216 mg/g of reducing sugar yield (Sharma *et al.*, 2020). These results are comparable with the obtained results and the slight variation in TRS yield found here may be due to differences in SCT species, pretreatment type and commercial cellulase used. The cellulose conversion (%) of apSCT to reducing sugar was 68.7% (Table 6.1) which was used for bioethanol production and its optimization.

6.3.3 Bench-scale optimization of fermentation of saccharified apSCT and validation at higher scale

The bench-scale optimization of fermentation by using three fermenting microbes under different fermentation conditions was carried. *Saccharomyces cerevisiae* can utilize the hexose sugar, glucose, while *Pichia stipites* can utilize the pentose sugar, xylose and arabinose to produce bioethanol. *P. tannophilus* can efficiently ferment both glucose and xylose. The individual strains of *S. cerevisiae*, *P. stipitis* and *P. tannophilus* and its consortium were grown in different reducing sugar concentrations obtained from saccharification experiment II and different nitrogen sources for 96 h and the released ethanol concentration was measured (Table 6.2). The three individual microbes gave ethanol concentration between 3.8-4.0 g/L at 10 g/L TRS without yeast extract, whereas their mixture at 15 g/L TRS gave maximum 7.58 g/L ethanol with 3 g/L yeast extract. The individual microbe achieved 75 to 79% fermentation efficiency (Table 6.2). The fermenting microbe, *P. tannophilus* showed 79.2%, while *S. cerevisiae* and *P. stipitis* both showed a 75.3% fermentation efficiency at 10 g/L TRS loading. However, the additional TRS loading and nitrogen source loading in fermenting medium showed enhanced fermentation efficiency in the range, 95%-99% (Table 6.2).

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Table 6.2. Optimization of fermentation of saccharified apSCT hydrolysate.

Fermenting Microbes	Reducing sugar conc. (g/L)	Nitrogen Source (g/L)	Ethanol Conc. (g/L)	Fermentation Efficiency (%)
S. cerevisiae	10	-	3.84±0.0	75.28
P. stipites	10	-	3.84±0.0	75.28
P. tannophilus	10	-	4.04±0.0	79.14
Consortium	10	-	3.77±0.1	73.83
Consortium	15	-	7.27±0.0	95.03
Consortium	10	YE: 3.0	4.65±0.1	91.14
Consortium	15	YE: 3.0	7.58±0.1	99.05
Consortium	10	Urea: 1.5	4.67±0.0	91.29
Consortium	15	Urea: 1.5	7.42±0.1	96.99
At Higher scale				
Consortium	15	YE: 3.0	7.43±0.1	96.9

Consortium used at Rratio: S. cerevisiae: P. stipites: P. tannophilus (1:1:1 volume from 10^7CFU/mL culture), YE: Yeast Extract. All experiments were conducted at 100 rpm and 35°C.

The TRS loading and yeast extract were the crucial factors for saccharified apSCT fermentation. The optimized conditions for fermentation of the saccharified TRS from optimized apSCT saccharification giving 99% ethanol (7.58 g/L) were 15 g/L reducing sugar and 3 g/L yeast extract loading using the 5% (v/v) consortium of *S. cerevisiae, P. stipitis, P. tannophilus* in the ratio (1:1:1 after achieving the 1 OD_{600} equivalent microbial growth) at 35°C, 100 rpm for 48 h. The fermentation efficiency can be further increased by optimizing the fermentation at higher glucose concentrations.

The optimized fermentation parameters were used for the higher scale fermentation of the reducing sugar produced from apSCT. In a 5 L bioreactor, around 3000 mL of produced reducing sugar (15 g/L) was fermented by using 5% (v/v) of a consortium of fermenting microbes. About 96.9% fermentation efficiency was achieved within 24 h with 7.43 g/L ethanol, which was similar to 7.58 g/L obtained with optimized bench-scale fermentation. The higher scale fermentation was stopped at 24 h

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in order to reduce the process cost. The bioethanol fermentation of dilute acid pretreated and commercial cellulase saccharified SCT using *S. cerevisiae* reported 11.36 g/L ethanol for 72 h with 50% fermentation efficiency (Sindhu *et al.,* 2011). The enzymatic saccharification of 7% (v/v) H_2O_2 pretreataed SCT followed by fermentation gave 13.7 g/L ethanol yield after 72 h (Dodo *et al.,* 2017). The fermentation of pretreated SCT with transition metal (in crude glycerol) and the alkali followed by saccharification using *S. cerevisiae* reported 0.38 g/g of bioethanol yield per gram of glucose with 78.9% fermentation efficiency (Raghavi *et al.,* 2016). The fermentation of saccharified hydrolysate produced after saccharification of ionic liquid and ammonium carbonate pretreated SCT gave 42.8% fermentation efficiency (Sharma *et al.,* 2020). The results showed that almost the whole of the produced reducing sugar in saccharification of apSCT was utilized by microbial consortium with 96.9% fermentation efficiency, which is significant for large scale bioethanol production.

6.3.4 Mass balance and economic evaluation of higher scale saccharification and fermentation process

The mass balance of higher scale fermentation of 1000 g SCT to bioethanol production was calculated and shown in Fig. 6.2. From the initial 1000 g SCT, 778 g of solid extractive-free SCT was recovered after the water-soluble extractives removal pretreatment. After the alkaline pretreatment step, around 426 g of cellulosic apSCT solid was recovered. The saccharification of apSCT using commercial enzyme resulted in a 68.7% conversion yield which was 287.9 g of reducing sugar. Fermentation of released reducing sugar gave 143 g of ethanol production, equivalent to the 96.9% fermentation efficiency of *S. cerevisiae, P. stipitis, P. tannophilus* consortium. These conditions can be considered for the utilization of sugarcane waste for industrial-level bioethanol production. After the alkaline pretreatment step, around 426 g of cellulosic

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apSCT solid was recovered. The saccharification of apSCT using commercial enzyme resulted in a 68.7% conversion yield which was 287.9 g of reducing sugar.

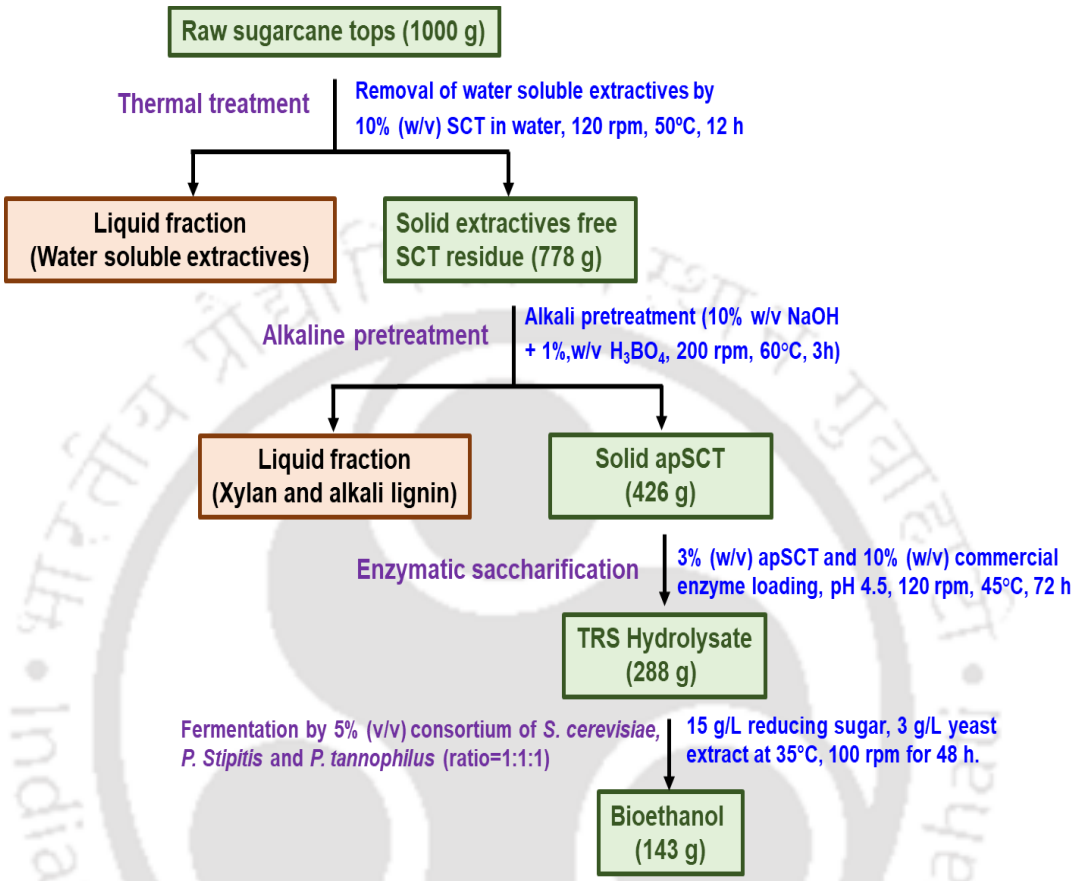

Fig. 6.2. Mass balance for bioconversion process of raw SCT to bioethanol.

Fermentation of released reducing sugar gave 143 g of ethanol production, equivalent to the 96.9% fermentation efficiency of *S. cerevisiae, P. stipitis, P. tannophilus* consortium. These conditions can be considered for the utilization of sugarcane waste for industrial-level bioethanol production. The economic evaluation of higher scale saccharification and fermentation from SCT was carried out to check the feasibility of its implementation in bioethanol industry. The cost involved in conversion of SCT biomass to bioethanol and value added products is shown in the Table 6.3. In this study, 143 g (0.18 L) of bioethanol was produced from 1000 g of raw SCT. As per the calculations about around 5.6 kg of raw SCT is required to produce 1 L of

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bioethanol. As per the economic calculations, 114 USD is required to process 1 kg of raw SCT biomass. The required cost for 1 L bioethanol production is approximately, 632.8 USD.

Table 6.3. The cost involved in conversion of SCT biomass to bioethanol and value added product.

Step 1. Removal of water soluble extractives:		
Requirement	**Cost (USD)**	**Required Cost(USD)**
SCT biomass (1 kg)	-	-
Distilled water (10 L)	0.027/L	0.27
Orbital shaker 50°C (12 h, 1.7 kW)	0.10/kWh	0.17
Step 2. Alkali pretreatment:		
SCT biomass 780 g		
Sodium hydroxide (780 g)	0.94/kg	0.74
Boric acid (78 g)	5.28/kg	0.412
Distilled water (7.8 L)	0.027/L	0.21
Hot air oven @ 60ºC (3 h, 0.425 kW)	0.10/kWh	0.0425
Step 3. Saccharification:		
Hot air oven (72h; 3.6 kW x 20 =72 kW)	0.10/kWh	7.2
Water (125.700 L)	0.027/L	3.39
Enzyme (42.6 mL)	-	100
Saccharification cost	-	110.59
Step 4. Fermentation:		
Fermenter 24 h; (1.2 kW X 20= 24 kW)	0.10/kWh	2.4
Ethanol recovery (Distillation) (1.2 kW X 10= 1.2 kW)	0.10/kWh	1.2
Total Cost		114.19
Total cost for overall process (1+2+3+4)		**114.00**

The bench scale bioethanol production from *Miscanthus* after considering the other byproduct cost was 1.31 USD per liter of bioethanol (Kang *et al.,* 2019). This huge cost can be compensated by the total income (289.3 USD/kg of raw SCT) produced from commercial grade xylan and lignin (as described in the chapter 4) extracted from raw SCT. The process costs can be further reduced for large scale biorefinery process by purchasing chemicals and reagents in bulk from whole sale market. Use of electricity in kWh per kg of biomass will also be reduced for large scale biorefinery process. The

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results and data obtained from this study can contribute to the industrial level bioethanol biorefinery plant.

Conclusions

In this investigation, the partial optimization of saccharification and fermentation of apSCT was carried out for the higher (3 L) scale bioethanol production. The saccharification of apSCT carried out in 20 mL volume gave optimized conditions, 3%, w/v apSCT, 10%, v/w_{apSCT} commercial enzyme and 48 h, at pH-4.5 and 45°C giving 67.2%,w/w cellulose conversion with 18.6 g/L total reducing sugars (TRS). These optimized conditions used for higher scale saccharification, at 3.6 L taking 120 g apSCT resulted 68.7% (w/w_{apSCT}**)** cellulose conversion with 18.8 g/L TRS. The enzymatic saccharification of 1000 g of apSCT resulted 687 g of reducing sugar which is significant for the consideration for the fermentation process. Fermentation of above saccharified hydrolysate containing TRS (15 g/L) supplemented with 3 g/L yeast extract and 5% (v/v) consortium of fermenting microbes (*Saccharomyces cerevisiae, Pichia stipitis* and *Pachysolen tannophilus,* 1:1:1 with each 10^7 CFU/mL) carried out for 48 h at initial pH 5, 100 rpm and 35°C in 20 mL gave 7.5 g/L of bioethanol which is better compared with individual fermenting microbes. The bioethanol production from the produced reducing sugar after saccharification of apSCT at bioreactor level yielded 7.43 g/L of ethanol within 24 h with 96.9% fermentation efficiency. This can be further optimized and improved at the industrial level fermentation process.

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Future Prospects

This thesis has presented the potential of sugarcane top as a feedstock for bioethanol biorefinery. Being a most abundant agrowaste, SCT can serve as the feedstock for bioethanol, xylan and lignin biorefinery. The future scope of this work are as follows:

1. Xylan produced from delignification followed by alkaline pretreatment (Chapter 3) and water extractives removal prior to alkaline pretreatment (Chapter 4) can be characterized for their industrial applications such as food packaging, bioplastics and in paper making.
2. Xylooligosacharides produced by the enzymatic hydrolysis of xylan extracted from delignification followed by alkaline pretreatment (Chapter 3) and water extractives removal prior to alkaline pretreatment (Chapter 4) with endo-1,4-β-xylanase (*Ct*Xyn11A) can have potential as a prebiotic in nourishing products.
3. The lignin extracted from water extractives removal prior to alkaline pretreatment (Chapter 4) can be further studied for its potential as a renewable substrate for aromatic chemicals and energy production.
4. The pretreatment, saccharification and fermentation of alkali pretreated SCT can also be exploited for large and industrial scale biorefinery process.
5. The economic evaluation of the biorefinery process using advanced tools and software can also be exploited.

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Journal publications

From thesis:

1. **Kaustubh Chandrakant Khaire,** Vijayanand Suryakant, Moholkar, Puneet Pathak, Nishi Kant Bhardwaj and Arun Goyal* (2022)**.** Optimization of saccharification and fermentation of alkali pretreated sugarcane tops for higher scale bioethanol production using microbial consortium. Available at SSRN: https://ssrn.com/abstract=4209086 or http://dx.doi.org/10.2139/ssrn.4209086
2. **Kaustubh Chandrakant Khaire,** Vijayanand Suryakant Moholkar and Arun Goyal* (2022) A biorefinery approach for sequential extraction of commercial grade xylan and alkali lignin from alkali pretreated sugarcane tops hydrolysate. Industrial Crops and Products, 187, 115545. **(IF-6.5)**
3. **Kaustubh Chandrakant Khaire,** Vijayanand Suryakant Moholkar and Arun Goyal* (2021) Alkaline pretreatment and response surface methodology based recombinant enzymatic saccharification and fermentation of sugarcane tops. Bioresource Technology, 341, 125837. **(IF-11.9)**
4. **Kaustubh Chandrakant Khaire,** Kedar Sharma, Abhijeet Thakur, Vijayanand Suryakant Moholkar, and Arun Goyal* (2021) Extraction, characterization of xylan from sugarcane leaf tops and its application. Journal of Biosciences and Bioengineering, 131(6), 647-654. **(IF-3.2)**
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6. **Kaustubh Chandrakant Khaire,** Vijayanand Suryakant Moholkar and Arun Goyal* (2021). Separation and characterization of cellulose from sugarcane tops and its saccharification by recombinant cellulolytic enzymes. *Preparative Biochemistry & Biotechnology*, *51*(8), 811-820. **(IF-3.1)**

Others

7. Abhijeet Thakur†, Aakash Sharma†, **Kaustubh Chandrakant Khaire,** Vijayanand Suryakant, Moholkar, Puneet Pathak, Nishi Kant Bhardwaj and Arun Goyal* (2021) Efficient saccharification of sugarcane hemicellulose by recombinant hemicellulases. ACS omega, 6(17), 11772-11782. **(IF-4.1)** †Equal contribution.
8. S Selveshwari, **Kaustubh Chandrakant Khaire,** Aditee Kadam and Sutirth Dey* (2022). The effect of migration and variation on populations of *Escherichia coli* adapting to complex fluctuating environments. bioRxiV. doi: https://doi.org/10.1101/2022.03.22.485294

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Book chapters

1. **Kaustubh Chandrakant Khaire,** Sanjeet Mehariya and Bikash Kumar* (2022) Valorization of Municipal Wastes in Circular Economy. In Waste Management in the Circular Economy" to be released by Springer Nature, Switzerland **(Accepted).**
2. **Kaustubh C. Khaire,** Premeshworii D. Maibam, Abhijeet Thakur and Arun Goyal* (2022) Biomedical and Pharmaceutical Applications of Xylan and Its Derivatives. In: Brienzo M. (eds) Hemicellulose Biorefinery: A Sustainable Solution for Value Addition to Bio-Based Products and Bioenergy, Clean Energy Production Technologies. 447-465. Springer, Singapore. https://doi.org/10.1007/978-981-16-3682-0_14
3. Abhijeet Thakur, Kedar Sharma, **Kaustubh C. Khaire,** Vijayanand Suryakant Moholkar and Arun Goyal* (2020) Enzymes: Key role in conversion of waste to biofuel. Ed Sonali Mohapatra, In Microbial Fermentation and Enzyme Technology, CRC Press. https://doi.org/10.1201/9780429061257-16
4. **Kaustubh C. Khaire,** Seema Patel, Parmeshwar V. Gavande, Vijayanand S. Moholkar and Arun Goyal* (2019) Chapter 7 "Extremophilic biofilms: Exploring the prospects". Ed Rathinam and Sani; Introduction to Biofilm Engineering, 141-157, ACS Symposium Series; American Chemical Society: Washington, DC.

Invited Talk

1. Invited to **deliver a lecture** in XVIII BRSI Convention and the International Conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals, and Health (BREEECH 2021), December 1-4, 2021, at CSIR-Indian Institute of Petroleum, Dehradun, Uttarakhand, India.
2. Invited as a **Resource Person** to deliver a Special lecture (online) on lignocellulosic agricultural waste to bioethanol and other value-added products, organized by Department of Biotechnology, Shivchhatrapati college, November 27, 2021, at Shivchhatrapati college, Aurangabad, Maharashtra, India.

Awards and Honour

3. **Received Best poster award** in International Conference on Biotechnology for Sustainable Agriculture, Environment and Health (BSAEH-2021) for poster entitled "Alkaline pretreatment and RSM based recombinant enzymatic saccharification of sugarcane tops for production of bioethanol", April 4-8, 2021, MNIT, Jaipur, Rajasthan, India.

Conferences

From thesis

1. **Kaustubh Chandrakant Khaire*,** Puneet Pathak, Vijayanand Suryakant Moholkar, Nishi Kant Bhardwaj and Arun Goyal (2022) Optimization of saccharification and fermentation of alkali pretreated sugarcane tops for higher scale bioethanol production using microbial consortium. 62nd Annual Conference "Microbes and Society: Current Trends and Future Prospects (MSCTFP-2022)", September 21-23, 2022, University of Mysore, Karnataka, India **(Oral Presentation).**
2. **Kaustubh Chandrakant Khaire*,** Puneet Pathak, Vijayanand Suryakant Moholkar, Nishi Kant Bhardwaj and Arun Goyal (2022) Optimization of saccharification and fermentation of alkali pretreated sugarcane tops for higher scale bioethanol production using microbial consortium. North East Research conclave, Sustainable Science and Technology, Assam Biotech Conclave 2022, May 20-22, 2022, Indian Institute of Technology Guwahati, Assam, India. **(Poster)**
3. **Kaustubh Chandrakant Khaire*,** Vijayanand Suryakant Moholkar and Arun Goyal (2022) A biorefinery approach of sequential extraction of xylan and alkali lignin from alkali pretreated sugarcane tops hydrolysate. Research and Industrial Conclave 2022, An Amalgamation of Academia, Industry & Start-ups. January 21-23, 2022, Indian Institute of Technology Guwahati, Assam, India. **(Oral Presentation)**
4. **Kaustubh Chandrakant Khaire*,** Vijayanand Suryakant Moholkar and Arun Goyal (2021) Bioconversion of sugarcane tops to bioethanol and other value-added products: A biorefinery approach. International Conference on advances in chemistry and biology of carbohydrates (CARBO XXXV), December 4-5, 2021, Forest research institute Dehradun Uttarakhand, India. **(Poster)**
5. **Kaustubh Chandrakant Khaire*,** Vijayanand Suryakant Moholkar and Arun Goyal (2021) Alkaline pretreatment and RSM based recombinant enzymatic saccharification of sugarcane tops for production of bioethanol. International Conference on Biotechnology for Sustainable Agriculture, Environment and Health (BSAEH-2021), April 4-8, 2021, MNIT, Jaipur, Rajasthan, India. **(Received best Poster award)**
6. **Kaustubh Chandrakant Khaire*,** Kedar Sharma, Abhijeet Thakur, Vijayanand Suryakant Moholkar and Arun Goyal (2020) Extraction and separation of xylan and cellulose from sugarcane tops and their characterization. Research Conclave, March 19-22, 2020, IIT Guwahati, Assam. (**(Poster)**

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7. **Kaustubh Chandrakant Khaire*,** Vijayanand Suryakant Moholkar and Arun Goyal (2020) Separation of cellulosic part from sugarcane tops/trash and optimization of enzymatic saccharification using recombinant hydrolytic enzymes. International conference on Innovative Research in Applied Sciences, Microbiology, Agriculture, Ecology, Humanities and Business Management for Sustainable Development, March 17-18, 2020, University of North Bengal, West Bengal, India. **(Poster)**
8. **Kaustubh Khaire*,** Kakali Borah, Arun Goyal and V. S. Moholkar, (2018) Pretreatment optimization of sugarcane leaf tops for recovery of holocellulose. Third International conference on Sustainable Energy and Environmental Challenges 2018 (SEEC 2018) December 19-22, IIT Roorkee, Uttarakhand, India. **(Oral Presentation)**
9. **Kaustubh Chandrakant Khaire*,** Kedar Sharma, Arun Goyal and Vijayanand Suryakant Moholkar (2019) Extraction, characterization of xylan from sugarcane tops. IV International Conference on Sustainable Energy & Environmental Challenges (IV SEEC) 27th to 29th November 2019, CSIR-National Environmental Engineering Research Institute, Nagpur, Maharashtra, India **(Oral presentation)**
10. **Kaustubh Chandrakant Khaire***, Arun Goyal and Vijayanand Suryakant Moholkar (2019) Potential use of cellulose recovered from xylan extracted sugarcane leaf tops for bioethanol production. International Conference on New Horizons in Biotechnology, November 20-24, 2019; Trivandrum, Kerala, India. **(Poster)**

Others

11. Avinash Anand*, **Kaustubh Chandrakant Khaire** and Vijayanand Suryakant Moholkar (2022) Utilization of food waste for the production of biohydrogen by using response surface methodology based enzymatic saccharification optimization. International Conference on Biotechnology for Sustainable Bioresources and Bioeconomy (BSBB-2022), December 7-11, 2022, Indian Institute of Technology, Guwahati, India. **(Poster)**
12. Aakash Sharma*, **Kaustubh Chandrakant Khaire,** Abhijeet Thakur and Arun Goyal (2020) Efficient utilization of hemicellulosic fraction of sugarcane leaf tops for the production of bioethanol by recombinant hemicellulases. Research Conclave, March 19-22, 2020, IIT Guwahati, Assam. **(Poster)**
13. Akshita Kanwar*, Parmeshwar V. Gavande, **Kaustubh Chandrakant Khaire,** Arun Dhillon and Arun Goyal (2022) Construction, cloning, expression, purification and biochemical characterization of a chimeric cellulose enzyme system, *At*Bg1-L1-*At*Cel8A from a thermophilic bacterium *Acetivibrio thermocellus* ATCC 27405 for the production of lignocellulosic bioethanol. North East Research conclave, Sustainable Science and Technology, Assam Biotech Conclave 2022, May 20-22, 2022, Indian Institute of Technology Guwahati, Assam, India**. (Poster)**
14. Kedar Sharma*, Sudhir Morla, **Kaustubh Chandrakant Khaire**, Abhijeet Thakur, Vijayanand Suryakant Moholkar, Sachin Kumar and Arun Goyal

(2019) Extraction, characterization of xylan from neem sawdust and its application in xylanase mediated production of anticancer xylooligosaccharides. International Carbohydrate Conference on Emerging Frontiers in Carbohydrate Chemistry and Glycobiology, Dec. 5-7, 2019, University of Lucknow, UP, India. **(Poster)**

15. Kedar Sharma*, **Kaustubh Chandrakant Khaire,** Abhijeet Thakur, Vijayanand Suryakant Moholkar and Arun Goyal (2019) Acacia xylan as a potential commercial xylan and its application in production of xylooligosaccharides. International Conference on New Horizons in Biotechnology, November 20-24, 2019, Trivandrum, Kerala, India. **(Poster)**
16. Kedar Sharma*, Sudhir Morla, **Kaustubh Chandrakant Khaire,** Abhijeet Thakur, Vijayanand Suryakant Moholkar, Sachin Kumar and Arun Goyal (2019) Xylanase mediated production of xylooligosaccharides from neem sawdust xylan and its anticancer potential. International Conference on Nutraceuticals and Chronic Diseases (INCD 2019), September s23-25, 2019 Indian Institute of Technology Guwahati, Assam, India. **(Poster)**
17. Kedar Sharma*, Abhijeet Thakur, **Kaustubh Khaire** and Arun Goyal (2018) Molecular characterization of halo and organic solvent stable xylanase from *Pseudopedobacter saltans* and its application in xylooligosaccharides production from Kans grass biomass. International Conference on Biotechnological Research and Innovation for Sustainable Development, 15th BRSI convention. CSIR- Indian Institute of Chemical Technology (CSIR-IICT), Nov. 22-25, 2018, Hyderabad, India. **(Poster)**

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VITAE

The author was born on October 1, 1993 in Pune (Maharashtra). He passed the Secondary Examination (10th Class) in 2009 and Higher Secondary Examination conducted (12th Class) in 2011 under the Maharashtra board of school education. He completed B.Sc. (Biotechnology) from MGM's Institute of Biosciences and Technology, Dr. Babasaheb Ambedkar Marathwada University in June 2014 and M.Sc. (Biotechnology) from School of Biotechnology, Devi Ahilya Vishwavidhyalaya Indore in June 2016. He worked as a Junior Research Fellow at IISER, Pune for 1 year (August, 2016-June, 17).

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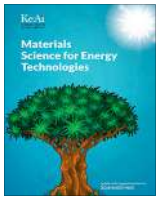

Bioconversion of sugarcane tops to bioethanol and other value added products: An overview

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ABSTRACT

The increasing use of non-renewable resources and its continuous depletion has become a major concern nowadays. The production of renewable fuels from agricultural lignocellulose waste is sustainable and economically feasible. Sugarcane top is one of the most abundant lignocellulosic biomasses in India. More conveniently, it is burned in the field being in large quantity and the high-cost involved in its collection and transportation. The pretreatment process is one of the cost intensive steps in the bioethanol production, owing to the manpower and expensive chemicals requirement to separate cellulose, hemicellulose and lignin from lignocellulosic biomass. In recent times, the advent of novel recombinant carbohydrate-active enzymes from different microbial sources by genetic manipulation has led to great advances in saccharification of pretreated biomass leading to higher production of bioethanol. The multienzyme complexes called cellulosomes are capable of hydrolyzing cellulose and hemicellulose. This study is aimed at production of xylan, bioethanol and other value-added products from sugarcane tops. Isolation, separation and characterization of xylan, cellulose and lignin from sugarcane tops can be most efficient industry oriented approach due to their xylo-oligosaccharides or alternative substrate, bioethanol and oil based petro-chemical applications. The saccharified hydrolysate can be used for bioethanol production by fermenting with *Saccharomyces cerevisiae*. Xylooligosaccharides can also be produced by enzymatic hydrolysis of xylan extracted from agro-waste and have applications in controlling blood sugar level, boosting immunity, improving intestinal function and reducing fatigue. Xylan can be utilized for the production of xylo-oligosaccharides, food coating, packaging films and other pharmaceutical applications. Lignin is mainly used as a rubber intensifier, polyolefin and rubber packing. It is also used in making cement, composite materials, unsaturated polyester and vinyl ester as filler and co-monomer.

Contents

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1. Introduction

The Indian economy depends on agriculture. Over half of the Indian populace legitimately relies upon agriculture for their business. Various crops are grown in a year in India attributable to the assorted variety in the atmosphere. Significantly, maize, sorghum, sugarcane, rice, wheat and other crops are grown in India [17]. The consumable portion of the plant is utilized to draw energy for the metabolic working of living beings, while the non-consumable part is known as agro-waste. Every year around, 200 billion tons of lignocellulosic wastes from agriculture are created worldwide [58] while, 200 million tons of lignocellulosic wastes are consistently produced in India [141]. The huge amount of agricultural waste produced each year is neither adding any financial advantages to the farmer nor it is useful for nature. The development of any nation, for the most part, depends on three columns, i.e., advancement at the economic, environmental and social levels. The financial improvement of any nation whether it is developing or developed relies intensely on the usage of non-renewable or fossil fuels [33]. These resources perform an important role in making lifestyle simple at different levels running from transportation, in-home appliances, in different industrial procedures for the fulfillment of our day to day needs. As a result of the restricted accessibility with unsustainable utilization of fossil fuels, the research shift from a linear economy (fossil-based) to a circular economy (bio-economy/ bio-based) is expanding [33]. The transformation of renewable resources into economically important products like animal feed, human food, bioenergy/biofuel and bio-based industrial products production by biotechnological mediation is named as bioeconomy [33]. Farming practices are the principal component of bioeconomy. The requirement for food and energy supply is expanding with the increase in population and it implies an incredible pressure on agribusiness cultivating and its processing industries [121].

Organic waste biomass produced from crops and food processing industries is named as lignocellulosic waste. Around $1.3x10^{10}$ metric tons of lignocellulosic waste is produced every year and its management is turning out to be a thriving issue [87]. In the last few years, the burning of lignocellulosic biomass by farmers to clean the cultivating land has contributed immensely towards global climate change, air and water pollution. The lignocellulosic waste biomass is rich in carbon and other important crucial supplements and consequently, it can be utilized for the production of bio-based products and bioenergy [87]. To satisfy the huge energy demand, the most ideal route is to use lignocellulosic wastes for energy production. The energy demands are rapidly expanding. In 2010, the energy demand was equivalent to the 13 billion tons of oil and it should rise to 45 billion tons of oil by 2050 [171]. The utilization of nonrenewable energy sources, for example, coal, oil and petroleum gas to meet the energy demand will cause exhaustion of their reserves. This additionally also prompts to increase in fuel cost with the expanding demand. To achieve energy necessity, renewable sources are focused such as atomic energy, bioenergy, hydro energy, solar energy and wind energy. Bioenergy contributes the most elevated share of 14% (18% considered as a100%) out of 18% of the total sustainable energy supply [14]. Bioenergy is produced either from agricultural residues or the forest residues. The lignocellulosic waste collected from the agricultural field can be converted into the source of energy e.g. biodiesel, biohydrogen, bioethanol, biobutanol and methane, etc. In most of the countries, biofuels are produced as a sustainable alternative for reducing the consumption of rapidly depleting conventional fuels and harmful gas emissions [19].

The world is searching for an option of conventional fuels and sustainable fuels such as biofuels are getting consideration from the most recent two decades. Biofuels production includes three stages: pretreatment, saccharification and fermentation. The pretreatment delignifies the agricultural waste biomass and subsequently makes it available for enzymatic hydrolysis and in this manner as an outcomes C5 and C6 mono sugars are released. The released mono sugars are changed over to biofuels with the assistance of fermenting microorganisms. Contingent upon the feedstock utilized for biofuel production, biofuels are arranged in the following generations: first generation, second generation, third generation and fourth generation biofuels. The first generation biofuels rely upon animal fats, edible oil seeds and crops. First generation biofuel production procedure is a basic, financially less costly (no requirement of pretreatment) and ecofriendly. Major disadvantage of biofuel production from edible sources is that, this competes with the food meant for human consumption. From cottonseed oil, 77% biodiesel alongwith 20% methanol production was accomplished by pretreatment with 0.5% (w/v) sodium hydroxide [101]. In the second-generation biofuels nonedible lignocellulosic biomass, for example, grain straw, sugarcane bagasse and forest residual wastes are utilized. Lignocellulosic biomass is waste produced during the crop harvesting process and can serve as an alternative promising source for biofuels production [14]. Bioethanol has advantages over other biofuels such as high heat

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of vaporization, high octane number (108), low boiling point and similar energy content [137]. Up to 85% (v/v) bioethanol blend in gasoline can be utilized in vehicles without alteration of the existing engine [137]. The use of petroleum as well as greenhouse gas emission can be significantly reduced by the bioethanol blending in gasoline. The main issue with the bioethanol is the high cost associated with its pretreatment strategies. Other significant cost requirements are the expense on collection and transportation of feedstock, chemicals, enzymes, detoxification process and ethanol recovery [137]. The enzymatic hydrolysis is one of the significant steps towards reducing the production cost [153]. The third-generation biofuel is centered around the usage of algal biomass for biodiesel production. Algae develops at a higher growth rate and has no food crop rivalry and can also utilize wastewater and seawater. The serious issues with the third generation biofuel are higher energy prerequisites for algal development, lower lipid content and more noteworthy odd is the contamination [142]. In fourth-generation biofuel feedstock utilized are green algae and different organisms with high growth rate, high lipid content and more CO_2 catching capacity. The obstacle in fourth-generation biofuel is a greater expense for the basic research.

2. Types of waste

Waste is a wide term that involves anything useless. The accumulation of waste causes damage to the land, pollution and several health issues. Waste can be arranged on the accompanying premise:

I The state of waste

a) Gaseous waste [85]
b) Liquid waste [29]
c) Solid waste [29]

II Waste source

a) Agricultural waste [125]
b) Commercial waste [8]
c) Industrial waste [27]
d) Municipal waste [10]

III The physical, chemical and biological properties

a) Biodegradable waste [130]
b) Non-biodegradable waste [105]

To conquer the energy shortage and to dispose of the waste aggregation, methodology for the conversion of waste into energy was recommended [79]. The agricultural waste is biodegradable, solid and lignocellulosic. The plant cell wall is made up of carbohydrates, lignin and proteins. Carbohydrates are made up of cellulose and hemicellulose. Cellulose is the most abundant polysaccharide made up of glucose and hemicellulose is heterogeneous having various side chains and its backbone is mainly made up of arabinose, xylose, glucose, mannose, etc. Other than cellulose and hemicellulose, pectins are also present in the cell wall.

3. Lignocellulosic waste

Lignocellulosic waste is referred to as the plant dry matter or biomass. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin molecules [62]. The principle polysaccharides of primary cell walls are cellulose, hemicellulose and pectin. The hemicelluloses interact with celluloses to form a hemicellulose network with the cellulose micro-fibrils. The pectin contains nearly 30% of dry weight in the primary cell wall [28]. The secondary cell wall has more cellulose as compared to the primary cell wall but it lacks pectin and because of that, the plants secondary cell wall is much harder. The basic component of the secondary cell wall is hemicellulose [28]. The lignin is situated in the external cell wall of ligocellulosic biomass. The structure of lignocellulosic biomass is shown in Fig. 1.

Generally, cellulose is situated inside a lignin shell, while the hemicellulose with an irregular and undefined structure is situated within the cellulose and between the lignin and cellulose. The hydrogen bonding is present between the linin and cellulose, as well as the hemicellulose and cellulose. Moreover, covalent linkages, predominantly ether bonds, have been proposed to be present between lignin and cellulose [69]. Therefore, the lignin extracted from natural lignocellulose always contains some carbohydrate impurities [69]. The cell walls of plants mainly consist of hemicelluloses, cellulose and lignin in a ratio of 3:4:3 [28]. The chemical structure of cellulose, hemicellulose and lignin is shown in Fig. 2. This ratio varies from source to source, for example, softwood, hardwood and grasses. Besides these molecules, lignocellulose also contains a small number of proteins and pectin. The elemental content of lignocellulosic material is around 50% Carbon, 44% Oxygen, 6% Hydrogen, and 0.05–0.4% Nitrogen [28]. These lignocellulosic structural components have a great ability to prevent water loss from a plant leaves. The intercellular layer in the plant is mainly made up of pectic polymers, which are amorphous and have strong plasticity and hydrophobicity [168]. Pectin structure

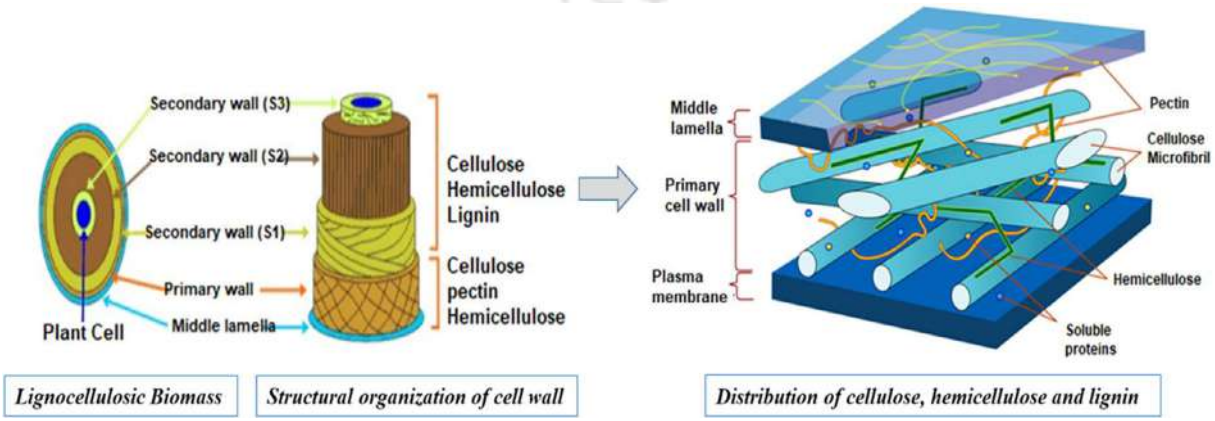

Fig. 1. Structure of lignocellulosic biomass [72].

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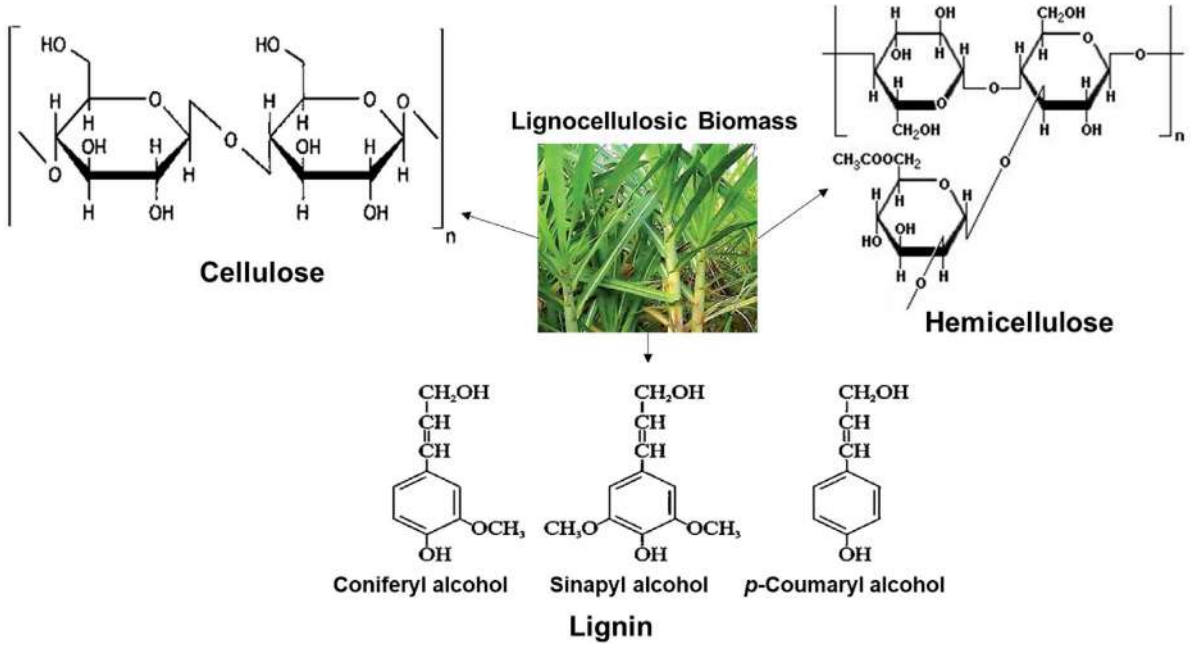

Fig. 2. Chemical structure of lignin, cellulose and hemicellulose.

can easily break by acidic or enzymatic treatment leading to the separation of plant cells [30].

3.1. Cellulose

One of the principal structural components of the plant cell wall is cellulose. Cellulose accounts for about 15–30% dry weight of the total primary cell wall. Cellulose is a high molecular weight polysaccharide made up of repeating units of glucose monomers. Cellulose is strong, crystalline and hydrolysis resistant and hemicelluloses have an amorphous and random structure with little strength as compared to the cellulose [11]. The cellulose microfibrils form a parallel structure of the cell wall with a diameter of about 10–25 nm and the elementary fibril diameter is approximately, 2 and 4 nm. This systematic arrangement of cellulose fibers in micro-fibrils is called a micelle [28]. Cellulose is an organic compound $(C_6H_{10}O_5)_n$ is not soluble in dilute acid/alkali solutions and water at room temperatures. Cellulose establishes 30–50% of the total lignocellulosic biomass with a worldwide production evaluated to be 120–140 billion tons per year [166]. Cellulose is principally present in plant cell walls. The composition of cellulose is reliant on the type of plant, species and age [86]. The primary cell walls of all angiosperms and gymnosperms are dominantly made up of cellulosic polysaccharide. The cottonseed hair contains 91% of cellulose, which speaks to its pure structure in contrast with wood cellulose. The cellulose is a polymer consisting of a straight chain of several thousand β-1 → 4 linkage of D-glucose units [81]. Every repeating β-D-glucopyranose unit is turned 180° to its next β-D-glucopyranose unit [158], which bring about the arrangement of the cellulose chain. These cellulose chains further aggregate or crosslink by framing intra- and intermolecular van der Waals and hydrogen bonding to create the cellulose microfibrils. The normal level of polymerization in the primary cell wall is approximately 6000 units and up to 14,000 units of glucose in the secondary cell walls [28]. Cellulose microfibrils are profoundly crystalline and hydrophobic, which contributes significantly to the recalcitrance of biomass [63]. The cellulose is a principle structural component of plants, bacteria, algae, other biomasses and the oomycetes and some bacterial species can secrete it to produce biofilms [28].

3.2. Hemicellulose

Hemicellulose are also called as polyose [84]. It is made up of several heteropolysaccharides such as arabinoxylans, which are present along with the cellulose in all plant cell walls. Hemicellulose polymers include arabinoxylan, xylan, glucomannan, glucuronoxylan and xyloglucan. These polymers contain various types of sugar monomers besides glucose. Other monosaccharide sugars in hemicelluloses include arabinose, mannose, xylose, galactose and rhamnose. In hemicellulose, a high amount of the D-sugars (C5) and sometimes small amounts of L-sugars (C5) are present. In most cases, the abundant monomeric sugar present in hemicellulose polymer is xylose although mannose can be the most abundant sugar present in softwoods. The modified form i.e. acidified sugars such as glucuronic acid and galacturonic acids are also be found in the hemicelluloses besides the regular sugar residues [28].

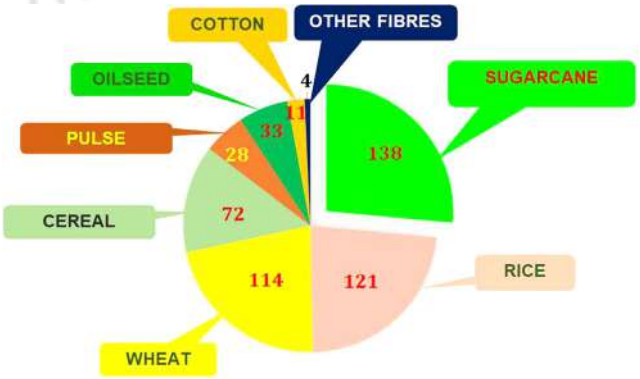

Fig. 3. Annual agriculture waste production in India (in Million Tons) (Report on State of Indian Agriculture 2015–16).

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Hemicelluloses are easily hydrolyzed by dilute acid or base as well as the number of hemicellulose enzymes. Around 15–35% of total lignocellulosic biomass is made up of hemicellulose [86]. Hemicelluloses are branched, single chain and amorphous heteropolysaccharide in nature. Hemicelluloses are composed of monomeric sugars, for example, pentose (arabinose and xylose), hexoses (glucose and mannose), uronic acids and acetylated sugars with degree of polymerization of 500–3000 monomeric units [41]. These D-pyranol sugars, for example, xylose, mannose and glucose are connected through β-1,4-glycosidic linkage at O4 position [102]. The side chains of hemicelluloses are small in length, which makes hemicellulose non-crystalline in nature [37]. The different functional groups present in branches and their number vary depending on the type of the biomass [86]. Hemicelluloses are subdivided into four primary categories relying upon the 1 → 4 linked monosaccharides present in the main chain of polysaccharides, which are arabinans, xylans, mannans and xyloglucans [131]. The most abundant hemicellulose is the xylan and its composition fluctuates among the species [86]. The most significant role of hemicellulose is to add the strengthening of the plant cell wall by associating with the lignin and cellulose molecules [131].

3.3. Lignin

Lignin is a class of complex organic polymers present in the tissues of vascular plants and some algae that forms the important structural materials for their support. Lignin plays a significant role in the plant cell wall formation (bark and wood) that leads to the rigidity of plants. Chemically, lignins are cross-linked phenolic polymers [65]. Lignin is an amorphous, highly branched and heterogeneous aromatic polymer that lacks primary structure. Lignin is made up of three fundamental phenylpropane monomers as repetitive units viz. *p*-coumaryl alcohol, sinapyl alcohol and coniferyl alcohol, which are commonly named as "monolignols." The softwood lignin is mostly made up of coniferyl alcohol, the hardwood lignin is made up of sinapyl alcohols and coniferyl alcohol, while the lignin present in grasses contains all the three previously mentioned monolignols [46]. Lignin is present in the secondary cell wall of plants and contributes to the transportation of water and nutrients to the xylem in vascular plants by offering mechanical support to the cells. Its recalcitrance nature grants the resistance against plant pathogens [12]. The composition of lignin differs from species to species. Lignin contains 63.4% Carbon, 30% Oxygen, 5.9% Hydrogen, 0.7% ash. A lignin molecule is unusual because of its heterogeneity and lack of definition to its primary structures. The lignin is fundamentally utilized as a fuel, yet it very well may be artificially altered to be utilized as a chelating agent [55] for the expulsion of heavy metals [38], or as a precursor for adhesives [13], activated carbon [50] and surfactants [35] like value-added product synthesis. Worldwide, the yearly commercial production of lignin polymer is around 1.1 million metric tons. It is used in a broad range of applications where the lignin purity is not required [28]. Lignin is covered with the bundle cells, for example, sclerenchyma cells and wood fibers. It increases the rigidity of the cell wall. Lignin content in the plant cell wall varies from woody plants (27–32%) to herbaceous plants (14–25%) [28]. The protective tissue of plants is also made up of wax, suberin, cutin and other polymers. The epidermis cell present on the plant surfaces is made up of cutin. The cork cells in plants secondary protecting tissue contain a suberin and cutin and sometimes it is covered with wax [4].

4. Selection of feedstock for second generation bioethanol production

Unabated exploitation of non-renewable energy resources and its continuous depletion is become a biggest concern for now a day. The production of renewable fuels from agricultural waste is sustainable and economically achievable. Worldwide, approximately 1.9 billion tonnes of sugarcane is produced [Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), USA [51]]. In tropical regions, Sugarcane (*Saccharum officinarum)* is one of the majorly cultivated crops [56]. Annual agro wastes produce in India is shown in Fig. 3.

Sugarcane tops (SCT) is one of the most abundant agricultural waste produced in India [34]. More than 199 million metric tons of sugarcane was reported to be produced in different regions of India in 2009 [148]. The sugarcane plant is showed in Fig. 4A and the dried leaves and tops (Fig. 4B) are often called as sugarcane top/trash (SCT).

SCT are produced during harvesting of sugarcane stem and are suitable as raw material for second generation bioethanol productions because of their high availability. A few waste management systems such as recycling, landfills, pyrolysis are used in disposal of the agrowaste. Some of these techniques can cause soil and air pollution in surrounding environment. Even though fertility of soil is mainly depending on the chemical and physical property of environment but it also affected by human management practise. One of these practices demonstrated by farmers is burning the SCT after the sugarcane harvest. Around 7–12 tons of SCT can be produced from 1 ha of the sugarcane field [149]. Owing to the huge production quantity and associated high-cost for collection and trans-

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Fig. 4. A) Sugarcane plant and **B)** Dried sugarcane leaf top (SCT).

Table 1
The composition analyses of SCT (%, w/w, dry weight basis).

Cellulose	Hemicellulose	Lignin	Ash	Extractives	Others	Reference
36.1	28.3	26.2	2.1	5.3	–	[99]
34.4	18.4	24.0	11.7	11.5	–	[114]
36.1	26.9	26.2	2.1	5.3	–	[123]
33.6	28.9	31.8	5.7	–	–	[39]
33.3	27.4	26.1	2.6	–	10.6	[90]
33.5	27.1	25.8	2.5	–	–	[36]
40.5	30.8	22.8	2.1	2.5	–	[54]
38.1	25.4	21.1	8.2	–	7.2	[34]
40.4	33.2	17.4	6.4	–	2.8	[16]

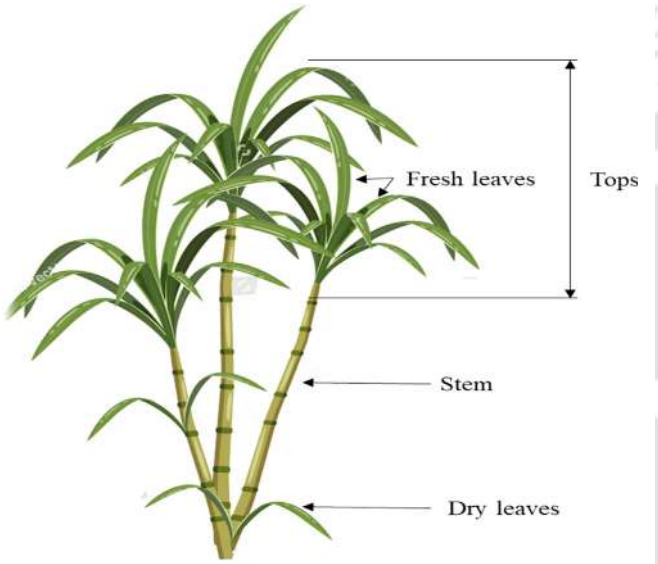

Fig. 5. Morphology of the sugarcane plant.

portation, mostly trash is burnt in the field. In any case, when SCT is burnt, a large portion of the nutrients and organic matter are lost, prompting the soil pollution [96]. Farmers ordinarily burn the waste, to reduce work, but it reduces the germination efficiency of the soil [149]. Moreover, most of the nutrients and the lignocellulosic matter is lost while burning the tops, leading to the pollution of the environment [42].

SCT is a cheap and potential renewable raw substrate for bioethanol production [54]. From 1 ton of sugarcane plant, about 140 Kg of bagasse [44] and 250 Kg of SCT [26] were produced. Approximately, 50% of SCT can be removed from the agricultural field and used for bioethanol production without compromising the nutritional status of soil [64]. Up to 66% (w/w) of SCT could be used in the bioethanol industries if collected as dry leaves [52]. This post-harvest SCT can serve as a low cost and readily available substrate for bioethanol production [148]. The use of SCT for ethanol production is favored because its production process can be annexed to the sugar/ethanol units already in place for lower investment, infrastructure, logistics and energy supply [106]. In such scenario, more ethanol can be produced by also utilizing SCT from the same amount of sugarcane, without compromising the cultivation aera. The yield of ethanol is 6,000 L/hectare of the sugarcane crop [106]. It was estimated that the ethanol production could reach 10,000 L/hectare, if only half of the SB generated is harnessed for the production of biofuel [106]. In India, 7.3 billion litres of ethanol is needed and this is growing annually at the rate of 6.6%. If the ethanol cannot be produced domestically, the whole purpose of achieving energy security is defeated [147]. Depending upon taxonomical interpretation sugarcane is any of 6 to 37 species of tall enduring grasses of the genus Saccharum (family: Poaceae, Class: Andropogoneae). The Sugarcane plant is a local warm calm atmosphere, basically found in tropical regions like Asia Pacific, Africa, India and Brazil [22]. The composition analyses of SCT determined by different studies is shown in Table 1.

4.1. Structure of sugarcane plant

The sugarcane plant is formed by the straw (tops) and stem. The morphology of the sugarcane plant is shown in Fig. 5. Sugarcane stem is separated before the processing of sugarcane to extract a juice which is finally utilized for sugar or ethanol production. Sugarcane bagasse (SB) is the waste of sugarcane stem after the collection of juice. SCT is formed by dry or fresh leaves and tops accessible prior to collecting. Fresh leaves are yellow and green in shading and tops are the piece of sugarcane plant in between the terminal stalk node and the top end. Dry leaves are brownish [104]. The sugarcane leaves are used as a fuel (combustion), crude feedstock for pyrolysis and

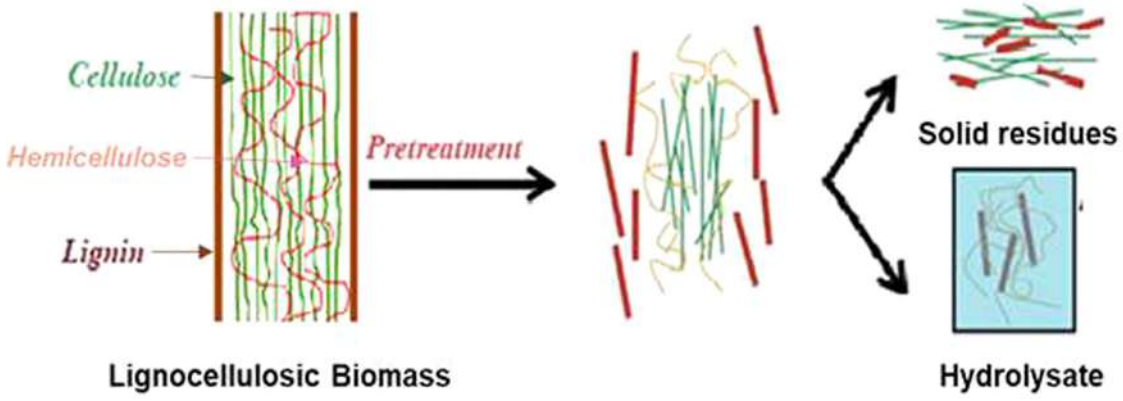

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Fig. 6. Schematic presentation of pretreatment of lignocellulosic biomass process.

Table 2
Different types of pretreatment of lignocellulosic biomass and their advantages and disadvantages.

Pretreatment	Process	Advantage	Disadvantage	Reference
Thermo-mechanical	Grinding, milling and shearing	Reduce particle size and increase the surface/ volume ratio	High capital cost	[15]
Auto Hydrolysis	Supercritical CO_2 explosion	Enhances the yield	Mixture of monosaccharides	[162]
Acid pretreatment	Dilute/conc. acid (H_2SO_4, HCl)	Enhance sugar production, dissolve hemicelluloses and lignin	Causes the sugar degradation, toxic and hazardous	[15,34]
Alkali pretreatment	NaOH, NH_3, alkaline H_2O_2	Increase surface area, reduce crystallinity, breakdown and separation of carbohydrate and lignin structure	Saccharification and fermentation processes, deacetylation of the structural units	[49,133]
Physical pretreatment	Steam-explosion, microwave heating	No toxic by-products	High energy Consumption	[31,59]
Biological pretreatment	Brown, white and soft rot-fungi	Low energy requirement and mild operation conditions	Rate of this process very low, Require more time	[92]

gasification (methane to methanol biosynthesis). The tops are mainly used for animal feed, anaerobic methane fermentation or direct energy utilization (after drying) [104].

Characterization of SCT, SB and stem, can be done on the basis of following perceptions. The sucrose collection is more prominent at the bottom of the sugarcane stem and the cellulose content and reducing sugars is prevalent in the upper part of stem and tops. The length of the plants stem relies upon the assortment of the sugarcane plant and the cultural care given. So a grown-up stem can from under 2–4 m in size, influencing the length and quantity of internodes. The shading relies upon the chlorophyll content and anthocyanins present in the plant. The moisture content in sugarcane biomass varies from part to part, such as 13.5% (leaves) to 82.3% (SCT) [22]. All composition present is experimentally same in carbon (~45%), oxygen (~43%), hydrogen (~6%), sulfur (~0.1%) and nitrogen (0.5–1%) [104].

5. Pretreatments

Pretreatment of lignocellulosic biomass mainly involve the efficient separation of every component of complex feedstock enhancing their accessibility. A combination of pretreatments is beneficial as compared to conventional single pretreatment methods as this reduces the operational steps and overall cost of the bioethanol production process [76]. The lignin present in plant cell wall negatively impacts the pretreatment process [119]. Delignification of biomass can enhance the access of cellulosic and hemicellulosic components for the enzymatic saccharification. For the delignification of lignocellulosic waste, pretreatment is necessary. The process of pretreatment of lignocellulosic biomass is shown as schematic presentation in Fig. 6.

The task of hydrolysing the lignocellulose to monosaccharides is still a technical challenge, because of the indigestibility of cellulose structure. Different types of pretreatment and their advantages and disadvantages are listed in Table 2. Because of the structural characteristics of lignocellulosic biomass the pretreatment becomes an essential step for enhancing the cellulose accessibility for enzymes to release of fermentable sugars in the hydrolysis step and reducing the enzyme usage [92]. Biological, physical, chemical and physicochemical pretreatment strategies for lignocellulosic biomass are utilized before the enzymatic hydrolysis [132]. The pretreatment strategies can be utilized independently or in combination for upgrading the enzyme efficiency in the saccharification process

The pretreatment strategies can be utilized independently or in combination to enhance the enzyme efficiency in saccharification process [74]. Pretreatment methods such as liquid hot water, wet oxidation, alkali, steam explosion, dilute acid hydrolysis and ammonia fiber expansion, etc. are used to pretreat the agriculture waste [132]. Combined pretreatment methods are considered more advantageous over single pretreatment as it overcomes the challenges encountered in single pretreatments, such as inefficient monosaccharides production, the formation of inhibitors at high concentration and extreme pretreatment conditions. Therefore, the combined pretreatments result in higher productivity of bioethanol. Alkaline pretreatment of the biomass combined with dilute acid, Dilute acid pretreatment combined with steam explosion and microwave assisted alkali pretreatements are effective for the removal of lignin and hemicellulose [89]. A rice straw pretreated with 2% (v/v) H_2SO_4 at 165 °C for 2 min followed by a steam explosion at 180 °C for 20 min gave higher xylose yield [31]. The alkali pretreatment followed by microwave irradiation can enhance the chemical reaction rate leading to an explosive effect among the biomass particles. The microwave-based heating in the presence of alkali was employed instead of the conventional heating for the pretreatment of switch grass [83]. Microwave assisted alkali pretreated rice straw showed the effective removal of lignin and hemicellulose [172].

The hydrolysate produced during pretreatment process contains hemicellulose, lignin and other plant extractive. This hydrolysate can be used for the production of other value added products such as furfural, hydroxy methyl furfural (HMF), acetic acid and lignin. Lignin separated from the hydrolysate after the enzymatic hydrolysis from Iogen process can be used for the production of electricity [161]. Xylan extraction before the use of cellulosic portion for bioethanol production is an approach to increase the value of hemicellulose components. Extracted xylan can be utilized in papermaking, pharmaceutical and food industries [146]. The removal of hemicellulosic components also enhances the enzymatic hydrolysis of cellulose [146]. This biorefinery concept may add value to the hemicellulose portion and also can provide the hemicellulose free solids which could be suitable for bioethanol fermentation. It can also improve the economic value of the sugarcane tops.

6. Extraction of xylan

Hemicellulose is the second most abundant biopolymer produced by green plants. Xylan is predominant hemicellulose found in secondary cell walls of SB. Xylan is made up of xylose backbone with a different degree of substitutions (such as arabinose and glucuronic acids), which directly affects the structural and functional characteristics [102]. Based on the method of extraction and source of plant/tissue used, the structure and composition of extracted xylan will vary [113]. The backbone of xylan consists of β-1 → 4-linked xylose residues, which is often substituted with methylated α-D-*gluco*-pyranosyl uronic acid and α-L-*arabino*-furanosyl, ferulic

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acid, *p*-coumaric acid as the side chains [109]. Apart from these substitutions, heteroxylan also contains D-galactose, D-xylose, L-rhamnose, D-glucose, acetyl groups and different dimeric and trimeric side chains [97]. Extracted xylans are suitable for biopolymer applications however some lignin fractions are also solubilized with xylan [71]. Another simpler traditional procedure of xylan extraction is based on alkaline pretreatment followed by ethanol precipitation of xylan, but it gives a lower xylan yield [146]. The extraction of xylan from different biomass and recovery yield was listed in Table 3.

Xylan extracted from SB by the alkaline method showed low purity of isolated xylan and detected 16–18% of residual lignin [32]. However, the alkaline method for xylan extraction from SB showed a higher yield in de-lignified biomass [32]. Modifications in traditional alkaline hemicellulose extraction methods by including de-lignification with $NaClO_2$ followed by xylan extraction provides a high yield of the polymeric xylan [67]. The xylan extraction before the enzymatic saccharification of cellulosic components can add value to the hemicellulose part and can provide the cellulosic solids which can be readily hydrolyzed and further used for bioethanol production. Hemicellulose separation from lignocellulosic biomass can also enhance the hydrolysis by cellulolytic enzymes [95]. This concept can add value to the hemicellulose part and can also provide hemicellulose-free solids (cellulosic part) which can be suitable feedstock for bioethanol production [77].

7. Saccharification of lignocellulosic biomass

The plentiful accessibility of cellulose in raw biomass makes it alluring for producing numerous industrially significant products. Unfortunately, a great part of the cellulosic waste is frequently discarded by burning [20]. However, it is viewed as a global phenomenon. With the assistance of the cellulolytic framework, cellulose can be hydrolyzed to glucose which is a multi-utility substrate, and is lot less expensive [61]. Polysaccharides are broken down gradually by the microbes. Microorganism secretes the collection of enzymes that degrade the polysaccharides to individual monosaccharides [111]. The task of hydrolyzing lignocellulose to monomeric sugars is still a technical challenge, because of the hard digestibility of cellulose structure [22]. The lignocellulosic biomass can be hydrolyzed by cellulase produced from both fungal and bacterial species. The fungal species e.g. Aspergillus niger, Trichoderma reesei, Sporotrichum pulverulentum, Penicillium funiculosum and Myrothecium verrucaria etc. can produce higher activity cellulase [126]. From these fungal species, the Trichoderma has the potential to offer high enzyme yield so is mostly used for cellulase enzyme production [144]. The enzymes like β-glucosidase (EC3.2.1.2), endoglucanase (E.C3.2.1.4) and exoglucanase (EC3.2.1.74) secreted from T. reesei act synergistically in the cellulose to glucose conversion process [57]. The bacterial species belonging to the genus Bacillus, Clostridium, Cellulomonas, Ruminococcus, Acetovibrio, *Streptomyces*, Bacteroides, Microbispora, Thermomonospora and Erwinia produce cellulase when grow on cellulose as a carbon source [82]. The cellulase enzyme synthesized by bacterial species is not much efficient to degrade a cellulose crystallinity as compared with the fungal enzymes. The enzymes produced from microbes are preferred compared to the enzymes from fungal sources due to the several advantages such as easy to handle, fast growth, cost-effective, consistency in production and genetically easy for the modification. The gene encoding the cellulase enzyme can be expressed in the *E. coli* to take advantage of a fast-growing model organism for enhancement of the enzyme productivity (Singh *et al.*, 2013). The enzyme expression is induced by the addition of IPTG (Isopropyl β-D-1- thiogalactopyranoside) [7]. In the saccharification process, the activity of the hydrolytic enzymes is measured by the amount of enzyme requires to produce 1 μmole of reducing sugar (D-glucose) per min under optimum condition. Immobilization of enzyme over polystyrene nanosphere mimics the bacterial cellulosome consisting of organized cellulases with improved catalytic efficiency as compared with free cellulases [9]. The saccharification of sugarcane biomass and its yield from various reports is listed in Table 4. The same concept applied over superparamagnetic nanoparticles increases the stability and enzyme

Table 3
Xylan extraction from various agricultural wastes.

Raw material	Extraction method	Yield	Reference
Wheat straw	OrganoSolv	41% of the total hemicellulose	[151]
Corn cob	5% (w/v) NaOH, 60 °C, ultrasonication, 1 h	36.1% of total xylan	[70]
Corn cob	Classical extraction, 5% (w/v) NaOH, 1 h	31.5% of total xylan	[70]
Corn cob	Ultrasonication, 10 min cycle, 1 h, 25 °C	52.7% of the total hemicellulose	[47]
Wheat straw	Ultrasonication, 0.5 M KOH, 30 min	25.5% of total dried biomass	[152]
Sugarcane bagasse	Ultrasonication, 10% (w/v) NaOH, 15 h	97.3% of total hemicellulose	[120]
Wheat straw	Steam explosion	80% of the total hemicellulose	[68]
Cotton stalks	24% (w/v) KOH + 1% (w/v) $NaHBH_4$, 3 h	0.4 g/2g of cotton stalk	[1]
Wheat straw	0.5 M NaOH, 185 °C, 30 min	49.3% of total xylan	[122]
Wheat straw	24% (w/v) KOH + 1% (w/v) $NaHBH_4$, 3 h	20.6% of total dried raw biomass	[2]
Maize stem	8% (w/v) NaOH, 50 °C, 3 h	21% of total dried biomass	[155]
Rye Bran	1 M NaOH, 95 °C, 45 min	8.6% of total xylan	[128]
Corn stalks	10% (w/v) NaOH, 20 °C	54% of the original xylan	[48]
Grape stalks	Hot water, 180 °C, 30 min	8.09% of total dried biomass	[6]
Rye Bran	Water extraction, 95 °C, 45 min	3.2% of total xylan	[128]
Sweet sorghum stem	2.5% (w/v) KOH, 75 °C, 3 h	76% of the total hemicellulose	[154]
Pear pomace	4 M NaOH at 80 °C for 18 h	14% of total dried biomass	[117]
Finger millet	1 M BaOH + 1 M KOH, 25 °C, 18 h	6–10% of dried raw biomass	[115]
Rapeseed straw	0.5 M NaOH, 105 °C, 20 min	25% of total dried biomass	[156]
Sugarcane Bagasse	Hot water, 170 °C, 1 h	33% of total hemicellulose	[170]
Sugarcane bagasse	Hot water	69% of the total hemicellulose	[162]
Sugarcane bagasse	Twin screw extruder	54% of total hemicellulose	[164]
Rice bran	Water extraction, 25 °C, 2 h	73.5% of total xylan	[110]
Finger millet seed coat	Water extraction, 25 °C, 2 h	79.7% of total xylan	[110]
Acacia sawdust	10% NaOH + 1% H_3BO_4, 60 °C, 3 h	23.5% of dried biomass	[134]
Neem sawdust	10% NaOH + 1% H_3BO_4, 60 °C, 3 h	22.5% of total biomass	[135]

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Table 4
Total sugar yield from the sugarcane waste by cellulolytic enzymes produced from various sources.

Sugarcane waste	Source of Enzyme	Total Sugar Yield in mg/g dry substrate	Time (h)	References
SB	Commercial cellulase	400	72	[129]
SB	*Trichoderma sp.*	457	72	[91]
ST	*Trichoderma reesei*	331	48	[173]
SB	*Trichoderma reesei*	365	48	[133]
SCT	Commercial cellulase	789	60	[139]
SCT	Commercial cellulase	669	60	[140]
SCT	*Trichoderma reesei*	329	48	[133]
ST	*Trichoderma reesei*	340	48	[133]
ST	cellulysin® cellulose	380	–	[45]

SB = Sugarcane bagasse; ST = Sugarcane trash; SCT = Sugarcane tops

activity [78]. It also helps in the magnetic recovery of enzymes for reuse [78].

8. Enzymes involved in saccharification

The plant biomass gets delignified after pretreatment and in this manner it gets available for enzymatic saccharification. For bioethanol production, the significant components in pretreated biomass are cellulose and hemicellulose, can be hydrolyzed by cellulases and hemicellulases respectively. Pectinases are likewise supplemented to decrease the cellulase and hemicellulase loading in the saccharification of plant biomass [169]. A few, microorganisms produce these enzymes in an abundant amount. To enhance the production of these enzymes, the genes responsible for these proteins are cloned in a suitable vector and overexpressed [162,25,165]. The technologies such as site-directed mutagenesis [49] and protein engineering are additionally used to improve the catalytic efficiency of enzymes [23]. Change of amino acid phenylalanine to alanine (flexible residue) by the side-directed mutagenesis adjacent to the active site of endoglucanase (*Ct*GH5) resulting 2-fold enhancement of endoglucanase activity. Chimera of β-glucosidase (*Ct*GH1) and endoglucanase (*Ct*GH5-F194A) enzyme was showed enhance in both endoglucanase and β-glucosidase activities [103].

8.1. Cellulases

The cellulases breakdown cellulose to glucose. Cellulases are the most requested enzymes/catalysts for their applications in biofilms and other industries. Cellulases are utilized in the feed [145], paper [60], cotton [53] and the food industries [94]. Plant biomass contains 40–50% of cellulose, consequently can be utilized for saccharification by cellulases for the production of biofuels. Cellulases are of three kinds i) endoglucanase; ii) exoglucanase (cellobiohydrolase) and iii) β-glucosidase dependent on their substrate specificities.

8.1.1. Endoglucanase

Endoglucanase (EC 3.2.1.4) arbitrarily acts against amorphous cellulase and produces cello-oligosaccharides of the fluctuating degree of polymerization (DP). Endoglucanases are available in glycoside hydrolase (GH) families 5, 8, 9, 26, 44, 48, 74 and 124 (http://www.cazy.org/). These oligosaccharides work as probiotics and have useful food applications [116]. For biofuel production, monosaccharides are required by the fermenting microorganisms [119]. Endoglucanase isn't sufficient to produce glucose from cellulose and needs other types of cellulase enzymes for the complete hydrolysis of cellulose.

8.1.2. Exoglucanases

Exoglucanases (EC 3.2.1.91 and EC 3.2.1.176) is also called cellobiohydrolase, which acts against amorphous cellulose, crystalline cellulose and oligosaccharides. The exoglucanase acts on the reducing end are called as a cellobiohydrolase-I (EC 3.2.1.176) and are available in GH family 7 and 48. The cellobiohydrolase-II (EC 3.2.1.91) severs at the non-reducing end of cellulose and is found in GH families 5, 9 and 48. The major product released by both enzymes is cellobiose, which is further broken down into two molecules of glucose.

8.1.3. β-Glucosidase

β-Glucosidase (EC 3.2.1.21) enzyme mainly acts on cellobiose and separates glucose from non-reducing end. The β-Glucosidases are belongs to the GH families 1, 2, 3, 5, 9, 30 and 39. β-Glucosidases cannot hydrolyze cellulose. Therefore, all the three enzymes endoglucanase, cellobiohydrolase and β-glucosidase are essential for the complete hydrolysis of lignocellulosic biomass.

8.2. Hemicellulases

In contrast to cellulose, hemicellulose is heterogeneous in structure and comprises 20–40% of plant biomass and is connected with cellulose, gelatin and lignin [160]. Hemicelluloses have both C5 (arabinose, xylose) and C6 (glucose, galactose, mannose) sugars in the xylan backbone with different side chains. Xylan, glucuronoxylan, arabinoxylan, xyloglucan, galactan, arabinogalactan, mannan, glucomannan, galactomannan and arabinan are the major hemicellulose present in plant biomass [93]. Because of the multifaceted nature in the structure of hemicellulose, a variety of catalytic enzymes are required for its complete hydrolysis.

8.2.1. Xylanolytic enzymes

Xylan is the most plenteous hemicellulose that contains xylose in the backbone. Endoxylanase (3.2.1.8) cleaves xylan main chain and produces xylo-oligosaccharides. In carbohydrate-active enzymes database (CAZy) endoxylanase are reported from GH families 3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62, 98 and 141 [88]. Endoxylanase alone cannot release xylose from xylan and other auxiliary hemicellulases are essentially required for the complete hydrolysis of xylan to produce xylose. β-Xylosidase (EC 3.2.1.37) breaks down the xylan and xylobiose from the non-reducing end to release xylose. β-Xylosidase is necessary along with endoxylanase for the complete hydrolysis of xylan [163]. β-Xylosidases are available in GH family 1, 3, 5, 30, 39, 43, 51, 52, 54, 116 and 120. Xylan backbone is substituted with 4-O-methyl glucuronic acid, arabinose, acetate and ferulic or *p*-coumaric acid as the side chains. These side chains meddle in the xylan hydrolysis. Side chain

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expelling enzymes, for example, ferulic acid esterase/*p*-coumaric acid esterase (EC 3.1.1.73), acetyl xylan esterase (EC 3.2.1.72), arabinofuranosidase (EC 3.2.1.55) and glucuronidase (EC 3.2.1.139) release the side chain and boost the xylan hydrolysis [5].

8.2.2. Mannan hydrolytic enzymes

β-Mannanase (EC 3.2.1.78) hydrolyzes the principle chain of glucomannan, galactomannan and galactoglucomannan in an endolytic way are produce monno-oligosaccharides. β-Mannanases are available in GH families 5, 9, 26, 44, 113 and 134. Manno-oligosaccharide is hydrolyzed to mannose from non-reducing terminal by *exo*-β-mannosidase enzyme. Exo-β-mannosidases belong to the GH families 1, 2 and 5.

8.3. Pectinolytic enzymes

The polysaccharide structure of pectin is extremely complex is composed of about 17 different monosaccharides and more than 20 linkages. Due to this, pectin degrading enzymes are also classified based on their site of action in the pectin polymer [18]. Pectin degrading enzymes are available in carbohydrate esterase (CE), glycoside hydrolase (GH) and polysaccharide lyase (PL) classes. The pectin degrading enzymes, for example, gelatin acetylesterase (EC 3.1.1.6), gelatin methylesterase (EC 3.1.1.11), polygalacturonase (3.2.1.15), rhamnogalacturonan I endohydrolase (EC 3.2.1.171), unsaturated rahmnogalactouronyl hydrolase (EC 3.2.1.172), rhamnogalacturonan I galactohydrolase (EC 3.2.1.173), rhamnogalacturonan I rhamnohydrolase (EC 3.2.1.174), exopolygalacturonase (EC 3.2.1.67), pectate lyase (EC 4.2.2.2), exopectate lyase (EC 4.2.2.9), gelatin lyase (EC 4.2.2.10), and rhamnogalacturonan lyase (EC 4.2.2.23). These enzymes are found in CE (1,3,4,8 and 16) family, GH (4, 28, 78, 105 and 126) family and PL (1, 2, 3, 4, 9, 10, 11 and 26) family [88]. The cellulases, hemicellulases and pectinases act explicitly and breakdown the polysaccharide into monosaccharides are listed in Table 5.

9. Xylo-oligosaccharides and their applications

The xylo-oligosaccharides (XOS) are produced by the breaking down the glycosidic bonds present in the main chain of xylan polysaccharides. This can be achieved by subjecting the xylan to higher temperature, chemical reagents or biological catalysts.

9.1. XOS production by chemical and physical treatments

The β-1,4 glycosidic bond in xylan can be broken down by acids. To avoid the direct hydrolysis of xylan into monosaccharides, the acid concentration used is low. At higher acid concentration, monomers formed can further undergo hydrolysis to give furfural/hydroxy-methyl-furfural. [22,66].

9.2. XOS production by enzymatic hydrolysis

The xylan has to be isolated from lignocellulosic material by using a suitable pretreatment method before the XOS can be recovered by enzymatic hydrolysis. The characteristics of extracted xylan depend on the type of biomass and pretreatment used [21]. The extracted xylan can be hydrolyzed by endo β-1,4-xylanase having no β-xylosidase enzyme activity to produce XOS without the monomers. The xylanases with varying substrate specificities can produce various hydrolysis products and therefore, controlling the degree of polymerization of XOS can be difficult [3]. The specificity of the substrate varies in endoxylanases from GH10 and GH11 families. The GH10 family xylanases are less specific for xylan and can produce oligosaccharides with substituents at a non-reducing end of XOS. The true xylanase, GH11 family, are specific for xylan and cleave the unbranched region of xylan and can produce XOS with a higher degree of polymerization than the GH10 family xylanase [98].

9.3. Applications of XOS

The XOS can be used as a partial substitute for other sugars in the food industries. They also promote beneficial bacterial growth due to their prebiotic property. The food quality and consumer health can be improved by using these prebiotic XOS [73]. The quantity of monomeric units as well as the degree of polymerization of XOS depend on the source of extracted xylan. Xylan may also contain the side chains of acetyl and arabinofuranosyl residues. These xylans will lead to the synthesis of branched XOS [43]. Uronic acid, as a branch in XOS, is known for its anti-allergic and antioxidant properties [73]. The reduction of protein degradation in muscles for the production of energy is another biological function of XOS [43]. XOS also have applications in colon cancer prevention [24], as a substitute in antibiotics, in agricultural and pharmaceutical industries [100].

Table 5
Enzymes for saccharification of plant biomass.

Enzymes	EC number	Substrate	Product
Endoglucanase	3.2.1.4	Cellulose	Gluco-oligosaccharides
Endoxylanase	3.2.1.8	Xylan	Xylo-oligomers
Polygalacturonase	3.2.1.15	Polygalacturonan	Digalacturonate
β-Glucosidase	3.2.1.21	Cellobiose	Glucose
β-Galactosidase	3.2.1.23	β-Galactoside	D-galactose
Exo-β-1,4-mannosidase	3.2.1.25	Manno-oligosaccharides	Mannose
β-Xylosidase	3.2.1.37	Xylo-oligosaccharides	Xylose
α-Arabinofuranosidase	3.2.1.55	Arabinoxylan	Arabinose
Exopolygalacturonase	3.2.1.67	Polygalacturonan	Galacturonic acid
Ferulic acid esterase	3.1.1.73	Xylan	Ferulic acid
ρ-Coumaric acid esterase	3.1.1.-	Xylan	ρ-Coumaric acid
Acetyl xylan esterase	3.1.1.72	Xylan	Acetate
β-Mannanase	3.2.1.78	Mannan	Mannooligosaccharides
Endogalactanase	3.2.1.89	Galactan	Galactooligosaccharides
Cellobiohydrolase	3.2.1.91	Cellulose	Cellobiose
α-Glucuronidase	3.2.1.139	Glucuronoxylan	Glucuronic acid
Pectate lyase	4.2.2.2	Polygalactosyluronic acid	Pectin oligosaccharides
Exopectate lyase	4.2.2.9	Polygalactosyluronic acid	Digalacturonate
Pectin lyase	4.2.2.10	Methylated pectin	Oligosaccharides

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10. Microbial fermentation

The nature of the fermenting microbe and the characteristics of an enzyme are the deciding factors for choosing the mode of fermentation for bioethanol production. Different modes of fermentation used in the production of bioethanol have been described in the following sub-sections. The fermentation processes are classified as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP).

10.1. Separate hydrolysis and fermentation

In separate hydrolysis and fermentation (SHF) process, the hydrolysis of pretreated biomass and the fermentation of monosaccharides to ethanol operate in two separate units. SSF can withstand high solid loading by intermittent feeding (Koppram. and Olsson 2014). This separation of the enzymatic hydrolysis process from fermentation allows enzymes to operate at a higher temperature (45–50 °C) and fermentation at a slightly lower temperature (30–37 °C) for better performance of individual process [124]. The SHF fermentation of hydrolyzate obtained from saccharification of pretreated SCT gave 11.4 g/L ethanol yield [138]. The 1.5% (w/v) sulfuric acid pretreated SCT gave 4.71 g/L or 8% (w/w) of ethanol yield after SHF by using S. cerevisiae for 24 h [75].

10.2. Simultaneous saccharification and fermentation

The simultaneous saccharification and fermentation (SSF) process combines the hydrolysis and fermentation in a single operational unit. The comparison of SHF and SSF modes with different lignocellulosic feedstocks like corn stover and wheat straw showed that SSF contributes to higher ethanol productivity and yield than SHF [107]. Moreover, no inhibitory compound was formed during SSF process. The SSF has the advantage of immediately utilizing the monosaccharides released during saccharification for ethanol fermentation [80]. The major drawback of this process is the difference in operating conditions since the saccharification and fermentation operate at different optimal parameters. SSF requires compatible saccharification and fermentation conditions, with a similar pH, temperature and optimum substrate concentration. SSF process also produces the value-added products such as furfural, glycerol and furans, etc. which reduces the production costs in the industry [40]. The use of single container for the saccharification and fermentation process is one of the major advantages of the SSF, which reduces both saccharification and fermentation process time and capital cost. Another noticeable advantage of SSF is the lower formation of inhibitory components from hydrolysis process, which improves the overall performance of the process [92].

10.3. Simultaneous saccharification and Co-Fermentation

Simultaneous Saccharification and Co-Fermentation (SSCF) is a type of fermentation where pentose in the hydrolysate is also fermented by using pentose fermenting microorganism along with the glucose fermentation. In co-fermentation process, pentose utilizing yeasts like *Pichia fermentans* and *Pichia stipites* are co-cultured with *S. cerevisiae* for glucose fermentation. *P. stipitis* is capable of performing both aerobic and oxygen limiting fermentations and has the natural ability to ferment xylose directly into ethanol [167]. The ability of *P. stipitis* to produce ethanol from xylose is advantageous as it can combat the inability of *Saccharomyces cerevisiae* to ferment pentose [167]. In the overall fermentation process by *P. stipitis*, 3 mol of xylose are converted to 5 mol of ethanol with a theoretical yield of 0.51 g ethanol/g xylose [167]. The SSCF system comprising enzyme cocktail of cellulase (GH5) and hemicellulase (GH43) with co-fermentation of *Saccharomyces cerevisiae* and *Candida shehatae* gave an ethanol titre of 23 g/L which was 2-fold higher than single enzyme (GH5, cellulase) with single culture (*Saccharomyces cerevisiae*) system employing 5% (w/v) pretreated wild grass. Since, the saccharification and fermentation have different operating conditions, the selection of suitable fermenting microbes for SSF is a challenging task [40].

10.4. Consolidated bioprocessing

Consolidated Bioprocessing (CBP) involves the use of a single microbial community for all three subsequent bioconversion process, viz. i) production of cellulolytic and xylanolytic enzyme ii) enzymatic saccharification of delignified biomass and iii) fermentation of monosaccharides to ethanol. Therefore, this mode of fermentation has the advantage of low capital and operational costs over other modes of fermentation. However, the crucial part in this mode of fermentation is the need for compatibility in the operating parameters of enzyme production, saccharification and fermentation. The native bacterial strains, *S. cerevisiae* and *Trichoderma* sp. have been currently in use for CBP. The disadvantage of the use of genetically engineered strain is the low yield of ethanol [150]. *Clostridium thermocellum* can produce proficient enzymes for lignocellulose hydrolysis and can do fermentation too in a CBP [108]. Around 99% of crystalline cellulose hydrolysis was accomplished by CBP approach [143]. The pretreatment used for sugarcane biomass and ethanol yield obtained are listed in Table 6.

The high ethanol yield is due to the SSF, was found when addition of various nutrients and K. fragilis is used, were able to ferment at higher temperature is closer to the cellulase optimal temperature [80]. Besides *S. cerevisiae* and K. fragilis, other microorganisms such as *Escherichia coli* and *Zymomonas mobilis* are also being selected through metabolic engineering for the production of bioethanol [127]. The current trends of bioethanol production by using *S. cerevisiae* can be achieved from the different

Table 6
Pretreatment for sugarcane biomass and ethanol yield.

Sugarcane biomass	Pretreatment	FermentationTime (h)	EthanolYield	Reference
SB	H_3PO_4 + Steam explosion	12	19 g/L	[59]
SCT	6% Glycerol + 5% NaOH + 1% $FeCl_3$	72	31.9 g/L	[118]
SS	1.5%(w/w) H_2SO_4	8	74.7%	[112]
SCT	Acid pretreatment	8	65.5%	[112]
SB	Acid pretreatment	8	54.7%	[112]
SCT	1.6 M H_2SO_4	24	6.9 g/L	[45]
SCT	1.6 M H_2SO_4	72	11.7 g/L	[45]
SCT	Laccase enzyme pretreatement	24	27.2 g/L	[136]
SCT	Acid hydrolysis	72	13.7 g/L	[45]

SB = Sugarcane bagasse; SS = Sugarcane Straw; SCT = Sugarcane tops

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perspectives such as inhibitors and substrates reduction in biomass hydrolysates, co-culturing with different microorganisms, growth variables and various immobilization techniques [159].

11. Conclusions

The details of various types of pretreatment methods used for sugarcane top processing for production of bioethanol and other value added products such as xylan, lignin, furfural have been reported here. This also reports the methods for reducing the processing cost for the bioethanol production. Sugarcane tops are low cost and one of the most abundant source of biomasses for bioethanol production. SCT contains about 40–45% cellulose, 25–30% hemicellulose and 15–19% lignin. Isolation, separation and characterization of xylan, cellulose and lignin from SCT can be the most efficient industry-oriented approach owing to their applications in oligosaccharides, bioethanol and oil-based petrochemical production. Xylan can be also utilized for the production of food coating, packaging films and other pharmaceutical applications. Useful and economically adhesive properties of lignin can be improved through the addition of aldehyde, phenol or by lignin modification itself. The cost-effective separation method for xylan and lignin extraction on a large scale from SCT has still remained a matter of further research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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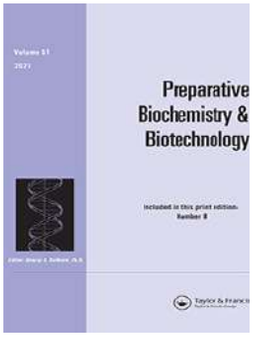

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Separation and characterization of cellulose from sugarcane tops and its saccharification by recombinant cellulolytic enzymes

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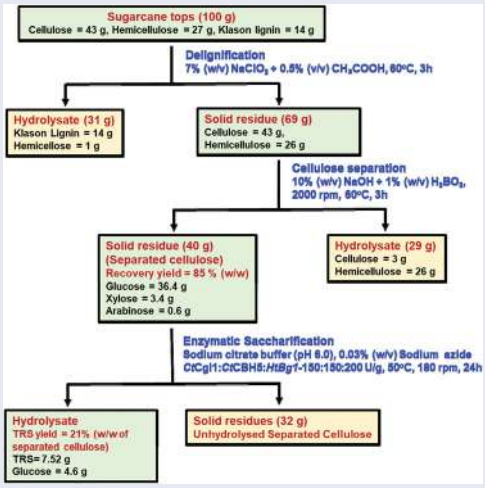
ABSTRACT

In the present study, the cellulose from sugarcane tops (SCT) was separated and characterized for its purity. Approximately, 85% (w/w) of total cellulose present in raw SCT was recovered by using alkaline method. The monosaccharide analysis of SCT cellulose by HPLC showed 91% D-glucose, 7.5% D-xylose and 1.5% D-arabinose residues. Surface morphology study of dried cellulosic fibers by FESEM exhibited the fibrous structure. The FTIR analysis of separated cellulose displayed the peaks corresponding to the peaks obtained from commercial cellulose, confirming its purity. The crystallinity index (*CrI*) of separated cellulose increased to 49% after delignification and xylan extraction from 36% of raw SCT. The typical TGA curve of separated SCT cellulose showed decomposition and mass reduction at 327 °C resulting in single decomposition peak in TGA analysis, confirming its purity. CHNS analysis supported the purity of separated cellulose by confirming absence of nitrogen and sulfur. The separated cellulose was hydrolyzed by recombinant endo-β-1,4-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) from *Clostridium themocellum* and β-1,4-glucosidase (*Ht*Bgl) from *Hungateiclostridium thermocellum* at pH 5.8, 50 °C for 24 h, resulting in the production of 188 mg/g of total reducing sugar (TRS). The separated cellulose from SCT can be utilized as an alternative substrate for commercialization and for bioethanol production.

KEYWORDS

Clostridium thermocellum; crystallinity index; FTIR; separated cellulose; sugarcane tops

GRAPHICAL ABSTRACT



HIGHLIGHTS

- 85% of total cellulose recovery yield was obtained from raw sugarcane top (SCT)
- SCT cellulose contains 91%, D-glucose, 7.5% D-xylose and 1.5% D-arabinose residues
- Crystallinity index (*CrI*) of separated cellulose increased from 36% to 49% of raw SCT
- TGA curve of separated SCT cellulose showed the single decomposition peak at 327 °C
- Separated cellulose hydrolyzed by recombinant enzymes gave 188 mg/g TRS yield

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Introduction

Unabated exploitation of nonrenewable energy resources and their depletion has become a major concern today. The production of renewable fuels from agricultural waste is sustainable and economically feasible. In the tropical regions, *Saccharum officinarum* or Sugarcane is one of the major cultivated agricultural crops.[1] Worldwide approximately, 1.91 billion tonnes of sugarcane is produced [Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), USA, 2018]. Sugarcane trash/tops (SCT) is one of the most abundant agricultural waste produced in India.[2] More than 199 million metric tons of sugarcane were reported to be produced in different regions of India.[3] Owing to the huge quantity of production of SCT and the high-cost involved in its collection and transportation, mostly the trash is burnt in the field.

Sugarcane top is the field residue remaining after the harvesting of sugarcane stalk, while sugarcane bagasse is the fibrous residue left after milling of sugarcane stalk. Chemical composition analysis of SCT showed 40% cellulose, 33% hemicellulose and 17% lignin content, making it an attractive feedstock for bioethanol production.[4] SCT can serve as the cheap and potential renewable raw substrate for biofuels.[3] From 1 ton of sugarcane plant, about 140 kg of bagasse[5] and 250 kg of SCT[6] are produced. Approximately, 50% of SCT can be removed from the agricultural field and used for the bioethanol production without compromising the nutritional status of the soil.[7] Up to 66% (w/w) of SCT could be used in the bioethanol industries if collected as dry leaves. This post-harvest SCT is low cost and readily available substrate for bioethanol production.[3] The SCT can also be used as a raw material for pyrolysis to gas, char and oil production,[8] gasification,[9] methanol synthesis and anaerobic methane[10] and bioethanol fermentation.[3] SCT is also used as a ruminant feed (fresh or dried) for cow and buffalo.[11] Hemicellulose separation before the hydrolysis of cellulosic part of feedstock is the new approach to increase the value of biomass. This concept can add value to the hemicellulose part and also can provide hemicellulose-free solids (cellulosic part) which can be suitable feedstock for bioethanol production. Separated hemicellulose can be used in papermaking, pharmaceutical and food industries.[12] The use of both cellulosic and hemicellulosic fractions can also increase the economic value of agricultural waste. In this study, the cellulose was separated from SCT and investigated for its purity. There is no report on the separation and characterization of cellulose from sugarcane trash. This approach of separation of cellulose and xylan from SCT may increase the commercial value of both cellulose and hemicellulose components for their use in respective industries. The technique involving the recombinant cellulosic enzymes in saccharification of cellulose separated from SCT is reproducible and is cost effective as compared with the saccharification by commercial enzymes. The structural properties of separated cellulose demonstrated that it can be used as commercial substrate as well as an alternate substrate for bioethanol production. This study focuses on the structure analysis of cellulose separated from SCT and its enzymatic hydrolysis by recombinant cellulolytic enzymes.

Material and methods

Material and feedstock collection and its processing

All chemicals (Sodium chlorite, sodium hydroxide, boric acid, etc.) were bought from Himedia, India. Ethanol, acetic acid and Thin Layer Chromatography (TLC) plate were procured from Merck India Pvt. Ltd. The SCT was collected from field nearby Guwahati, Assam, India. The feedstock was washed under tap water and chopped into one to two-inch pieces. The chopped biomass was rinsed with deionized water and dried in the oven at 60–70 °C for 24 h. Dried biomass was ground by grinder (Philips, HL1606/03) and passed through a standard test sieve of 0.85 mm pore size.

Composition analysis of raw SCT

One gram of powdered SCT was dried in hot air oven at 105 °C for 24 h. Then biomass was weighed before and after drying for the determination of moisture content. 0.5 g of raw SCT biomass was used for holocellulose and hemicellulose estimation and 0.1 g of raw SCT was taken for acid soluble lignin (ASL)/Klason lignin estimation.[13] Holocellulose content was estimated by Browning method.[14] Hemicellulose and Klason lignin were estimated by the standard TAPPI protocols.[15]

Delignification of the sugarcane trash

About 50 g of moisture-free SCT powder was suspended in 500 mL distilled water in a 1000 mL reagent bottle. This was followed by addition of sodium chlorite (20 g) and glacial acetic acid (2.5 mL), mixed thoroughly and heated in water bath at 60 °C for 3 h. The hydrolysate was then removed by filtration through Whatman no. 1 filter paper and biomass was neutralized by washing with distilled water.[16] The de-lignified SCT was dried at 80 °C for 24 h and used further for other experiments.

Separation of cellulose from de-lignified sugarcane trash

The removal of hemicellulose is required to achieve pure cellulose from SCT. The cellulose was recovered from SCT by following the alkaline separation method as reported earlier.[17] 20 g of de-lignified SCT was mixed with 200 mL of a mixture containing 10 (%, w/v) of sodium hydroxide and 1 (%, w/v) of boric acid. The higher concentration of sodium hydroxide to remove the hemicellulose has been also reported previously.[18] The suspension was stirred at 2000 rpm, 60 °C for 3 h and filtered through cheese cloth. The separated solid residues as cellulosic fibers were neutralized by washing under tap water and final washing with distilled water. The separated cellulosic fibers were dried at 80 °C for 12 h and used for further studiess. The dried

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weight of separated cellulose was noted for determining the cellulose yield. Cellulose yield was calculated by,

$$\text{Cellulose yield } (\%) = \frac{\text{Dried weight of cellulose fiber}}{\text{Total cellulose present in raw biomass}} \times 100$$

Here, the total cellulose present in raw SCT biomass was calculated by the difference between total holocellulose estimated by Browning[14] method and total hemicellulose estimated by standard TAPPI[15] protocols.

Monosaccharide analysis of cellulose separated from SCT

The monosaccharide composition analysis of cellulose separated from SCT was performed by treating 5 mg separated cellulose with 1 mL of 2 M Tri-fluro-acetic acid (TFA) in a 1.5 mL Eppendorf tube and by heating in a boiling water bath for 3 h. The hydrolyzed cellulose was kept in hot air oven at 80 °C for 12 h to remove the remaining residual TFA. The dried TFA hydrolyzed cellulose sample was mixed in 500 µL degassed Milli-Q water and then filtered through a syringe filter using by PVDF membrane (Pore size: 0.22 µm). The filtered sample was transferred to the HPLC vials (Axiva Sichem Biotech, India) and analyzed for monosaccharides present by High Performance Liquid Chromatography (HPLC) (UFLC, Prominence, Shimadzu, Japan) using a RI detector. 10 µL of TFA treated SCT cellulose was loaded and passed through the guard column (50 mm × 7.8 mm), followed by passing through the connected monosaccharide separation column phenomenex Rezex ROA (H+) (300 mm × 7.8 mm). The elution was performed by 0.005 N H_2SO_4 solution at 70 °C at 0.5 mL/min flow rate for 90 min run time per sample. 1 mg/mL each of glucose, xylose, arabinose or glucuronic acid was used as standard for composition determination.

Field-emission scanning electron microscopy (FE-SEM) analysis of raw SCT and separated cellulose

The surface morphology of raw SCT and separated cellulose were analyzed by FE-SEM (Carl Zeiss, Model-Gemini 300, Germany). The carbon tape was fixed on the stab surface before placing the raw SCT and separated cellulose samples. Both the samples prepared on the stab was double gold coated and kept in the vacuum chamber to remove the moisture from the samples. Then sample image was taken by FE-SEM (Carl Zeiss, Model-Gemini 300, Germany) at 2000X magnification.

Crystallinity study of raw SCT and separated cellulose by X-ray diffraction

XRD analysis was done to compare the crystallinity indices of raw SCT and separated cellulose. The dried raw SCT and separated cellulose from SCT (1 g of each) was assessed for their respective crystallinity indices (*CrI*) by X-ray diffractometer (D8 Advance, Bruker, Germany). Each sample was scanned over a range of $2\theta = 10°–30°$ with a step size of 0.05°. The *CrI* of SCT samples was calculated by using following formula.[19]

$$CrI\ (\%) = ((I_{crystalline} - I_{amorphous}))/I_{crystalline} \times 100$$

where $I_{crystalline}$ is the intensity at degree, $2\Theta = 22°$ and $I_{amorphous}$ is intensity at a degree, $2\Theta = 18°$.[13]

Fourier transform infrared (FT-IR) analysis of separated cellulose

The functional groups present in separated cellulose were determined by the FT-IR analysis. The SCT cellulose and cellulose (HiMedia Laboratories, India) samples were mixed with KBr in the ratio 1:100 (w/w) and ground by using mortar and pestle. The pellet was prepared from ground powder with the help of 15 tonne hydraulic press. The prepared pellet was scanned on FT-IR spectrophotometer (Perkin Elmer, Spectrum Two, USA) between wavenumber range, 4000–400 cm^{-1}.[20]

Thermal analysis of cellulose separated from SCT

The thermo-gravimetric and differential thermal properties of cellulose separated from sugarcane trash were analyzed by thermal analyzer (Netzsch, Model: STA449F3A00). For TGA analysis, 6 mg of SCT cellulose was taken in alumina crucible and heated at a rate of 10 °C min^{-1} from 30 to 500 °C temperature. The nitrogen gas was used at a flow rate of 60 mL/min. The weight loss and heating rate of TGA analyzer were continuously recorded throughout the experiment.

Elemental composition analysis of separated SCT cellulose

Ultimate analysis of biomass gives the information regarding the composition of Carbon, Hydrogen, Nitrogen and Sulfur (CHNS) of separated cellulose fibers. Carbon, nitrogen, sulfur and hydrogen present in cellulosic fibers were determined by the CHNS analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Italy), whereas oxygen was determined by calculating the difference in the percentage.

Production of recombinant hydrolytic enzymes for hydrolysis of SCT samples

The gene encoding endo-β-1,4-glucanase (*Ct*Cel8A) of family 8 glycoside hydrolase[13] and β-1,4-glucosidase, (*Ht*Bgl) of family 1 glycoside hydrolase cloned and reported earlier[13,21] were used in the present study. The plasmid containing gene encoding cellobiohydrolase, *Ct*CBH5A of family 5 glycoside hydrolase cloned from *Clostridium thermocellum* (renamed *Hungateiclostridium thermocellum*) was a gift from Prof. Carlos M.G.A. Fontes, NZYTech Ltd., Lisbon, Portugal. The plasmids containing the genes encoding endo-β-1,4-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-1,4-glucosidase (*Ht*Bgl) from *Hungateiclostridium thermocellum* were transformed in *Escherichia coli* BL21 (DE3) cells and grown as reported earlier.[13] In 5 mL of Luria-Bertani (LB) medium, 50 µg/mL of kanamycin were

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added 25 ml culture tubes and 50 µL of each from the glycerol stock was inoculated separately and incubated at 37 °C, 180 rpm for 12 h. The revived *E. coli* cells expressing *Ct*Cel8A, *Ct*CBH5A or *Ht*Bg1 enzymes were inoculated at 1% (v/v) inoculum in 400 mL LB medium in 1 L Erlenmeyer flasks containing kanamycin (final conc. 50 µg/mL) and incubated at 37 °C, 180 rpm for 2–3 h till the OD at 600 nm reached 0.4. Then 1 mM IPTG was added to each culture flask to induce the respective enzyme production and incubated at 24 °C, 180 rpm for 18 h. The cell cultures were centrifuged at 8000 g, 4 °C for 10 min. The cell pellet was re-suspended in 15 mL lysis buffer (50 mM sodium phosphate, pH 7.5) and the cell free extract obtained was subjected to sonication for 30 min (5 s on/10 s off pulse, 33% amplitude, Sonics, Vibra cell). The sonicated cells were centrifuged at 16,000 g, 4 °C for 1 h. The N-terminal hexa-His tag containing *Ct*Cel8A, *Ct*CBH5A and *Ht*Bgl enzymes were purified by 5 mL Sepharose column (Hi-Trap Chelating, GE Healthcare). The purified *Ct*Cel8A, *Ct*CBH5A and *Ht*Bgl enzymes were extensively dialyzed against 50 mM sodium phosphate buffer (pH 7.5). The concentration of enzymes was determined by Bradford method by taking absorbance at 595 nm (A_{595}) on a UV spectrophotometer (Varian, Cary 100). Bovine serum albumin (BSA) was used as a standard.

Enzyme activity assay of recombinant hydrolytic enzymes against SCT

The endo-β-1,4-glucanase (*Ct*Cel8A) enzyme activity was determined by incubating 5 µL (0.1 mg/ml) of enzyme with 1% (w/v) carboxymethyl cellulose (CMC) (Sigma, USA) in 50 mM citrate phosphate buffer (pH 5.8) in a 0.1 mL reaction mixture at 70 °C for 1 min. The enzyme activity was calculated by determining the reducing sugar release by the method reported earlier.[22,23] The total amount of reducing sugar released was calculated using standard plot of D-Glucose. The 1 unit (U) of enzyme activity was defined as the amount of *Ct*Cel8A enzyme required in mg to produce 1 µmole of D-glucose per min under optimum condition.

The cellobiohydrolase (*Ct*CBH5A) enzyme activity was determined by taking 5 µL (0.1 mg/ml) of enzyme with 1% (w/v) carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 6) in a 0.1 mL reaction mixture and incubating at 60 °C for 2 min. The enzyme activity was calculated by determining the released reducing sugar concentration by method of Nelson[22] and Somogyi.[23] The amount of reducing sugar released was calculated by using the standard plot of D-Glucose. The 1 unit (U) of enzyme activity was defined as the amount of *Ct*CBH5A enzyme required in mg to produce 1 µmole of D-glucose per min under optimum condition.

The β-1,4-glucosidase (*Ht*Bgl) enzyme activity was determined by using cellobiose as substrate. The enzymatic hydrolysis was performed in 1 mL reaction mixture containing 1% (w/v) cellobiose in 50 mM citrate phosphate buffer (pH 6.5), 5 µL of the enzyme (0.1 mg/mL) by incubating at 65 °C for 5 min. The enzyme activity was calculated by estimating the release of glucose by taking absorbance at 505 nm (A_{505}) on a UV–Visible spectrophotometer (Varian, Cary 100 Bio). One unit of enzyme activity for cellobiose was defined as the amount of *Ht*Bgl enzyme in mg was required to release 1 µmole of glucose per min.[24]

Enzymatic saccharification of SCT samples

The enzymatic hydrolysis of raw SCT and separated cellulose was carried out by taking 1% (w/v) biomass in 10 mL 50 mM citrate phosphate buffer, pH 6 in 15 mL centrifuge tube in triplicate sets. The recombinant enzymes, endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bgl) in a ratio 150:150:200 U/g were added to each of the reaction tube. Sodium azide 0.03% (w/v) was added to each reaction mixture containing tube to prevent the contamination. The reaction mixture was incubated at 50 °C in a shaker incubator at 180 rpm for 24 h. The reaction was stopped by boiling the reaction mixture for 5 min in boiling water bath and then centrifuged at 13,000 g, 25 °C for 10 min, to collect the supernatant containing released sugar. Total reducing sugar (TRS) released was calculated by the method of Nelson[22] and Somogyi.[23] The supernatant was used for the TLC analysis. The standard glucose and cellobiose samples were also loaded along with the hydrolyzed samples of raw SCT and SCT cellulose in separate lanes on TLC plate and dried for 5 min. The plate was kept in the TLC chamber within the mobile phase having methanol, acetic acid and water in a ratio 2:1:1. The migrated hydrolyzed products were analyzed by immersing the plate in a solution containing 0.5% (w/v) α-naphthol in methanol and sulfuric acid (95:5) solution.[25]

Results and discussion

Analysis of structure composition of raw SCT

The moisture content of raw SCT was found to be 4.3% (w/w). Raw SCT contained 70.1% (w/w) holocellulose, 27.2% (w/w) hemicellulose and 13.4% (w/w) acid soluble lignin/Klason lignin. The total amount of cellulose present in raw SCT was estimated by calculating the difference between holocellulose (70.1%, w/w) and hemicellulose contents (27.2%, w/w) determined by Browning[14] and TAPPI[15] methods, which was found to be 43% (w/w).

Delignification of raw SCT

The raw SCT after delignification by sodium chlorite method gave 34.5 g of delignified SCT, resulting in 69% (w/w) yield of delignified SCT. The delignification was confirmed by standard TAPPI protocol.

Separation of cellulose from delignified SCT

Twenty grams of delignified SCT gave 11.6 g of cellulose, resulting in an overall 40% (w/w) yield based on raw SCT. The recovery yield of cellulose was 85% (w/w) of total cellulose. The cellulose recovered from SCT is shown in the Figure 1.

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Monosaccharide analysis of separated SCT cellulose

The monosaccharide composition analysis of separated cellulose by HPLC displayed the presence of 91% of D-glucose, 7.5% of D-xylose and 1.5% of D-arabinose residues (Figure 2).

Surface morphology analysis of raw SCT and cellulose separated from SCT

The FESEM electromicrographs of the cellulose separated from SCT (Figure 3B) showed clear fibrous structures as compared to the raw SCT (Figure 3A) as also reported earlier.[26] This fibrous structure of separated cellulose could due to prior delignification and xylan removal leading to the exposure of cellulose fibers appearing as fibrous bundles, which are absent in raw SCT.

Figure 1. Digital image showing the cellulose separated from sugarcane top (SCT).

X-Ray diffraction analysis of raw SCT and cellulose separated from SCT

The powdered raw SCT and cellulose separated from SCT displayed 36% and 49% crystallinity index (*CrI*), respectively. These results explained that the increase in crystallinity index (Figure 4) of separated cellulose was due to the prior delignification and xylan removal that resulted in the exposure of the crystalline cellulose, as also reported earlier.[27] The crystallinity index of commercial cellulose II reported was 61%.[28] This difference between the crystallinity index of cellulose II and cellulose separated from SCT may be due to the difference in their purity.

Functional group analysis of cellulose separated from SCT

The FTIR spectra of cellulose separated from SCT and commercial cellulose are shown in Figure 5. The absorption peaks for cellulose were observed in two regions at wavenumbers ranges (3660–2800 cm^{-1} and 1650–800 cm^{-1}). The stretching vibrations of C–H and O–H bonds of the polysaccharide showed peaks in the range, 3660–2900 cm^{-1}.

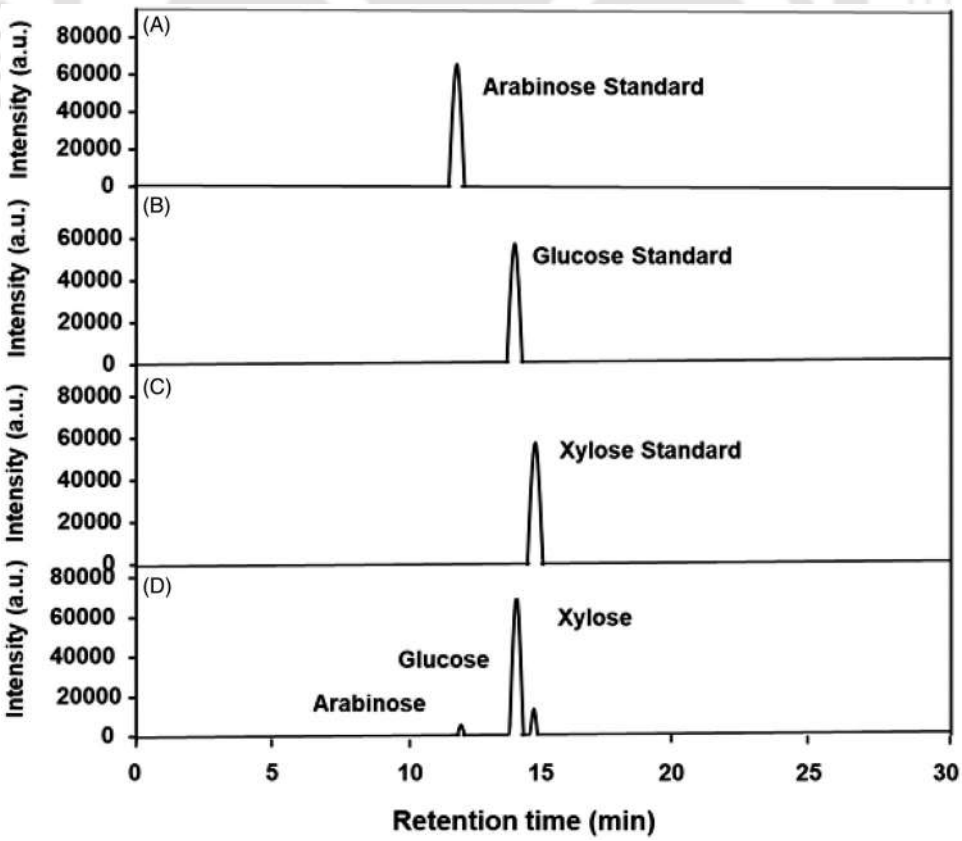

Figure 2. The composition analysis of SCT cellulose by high performance liquid chromatography (HPLC). (A) Arabinose standard (B) Glucose standard, (C) Xylose standard for HPLC and (D) HPLC analysis of TFA treated SCT cellulose.

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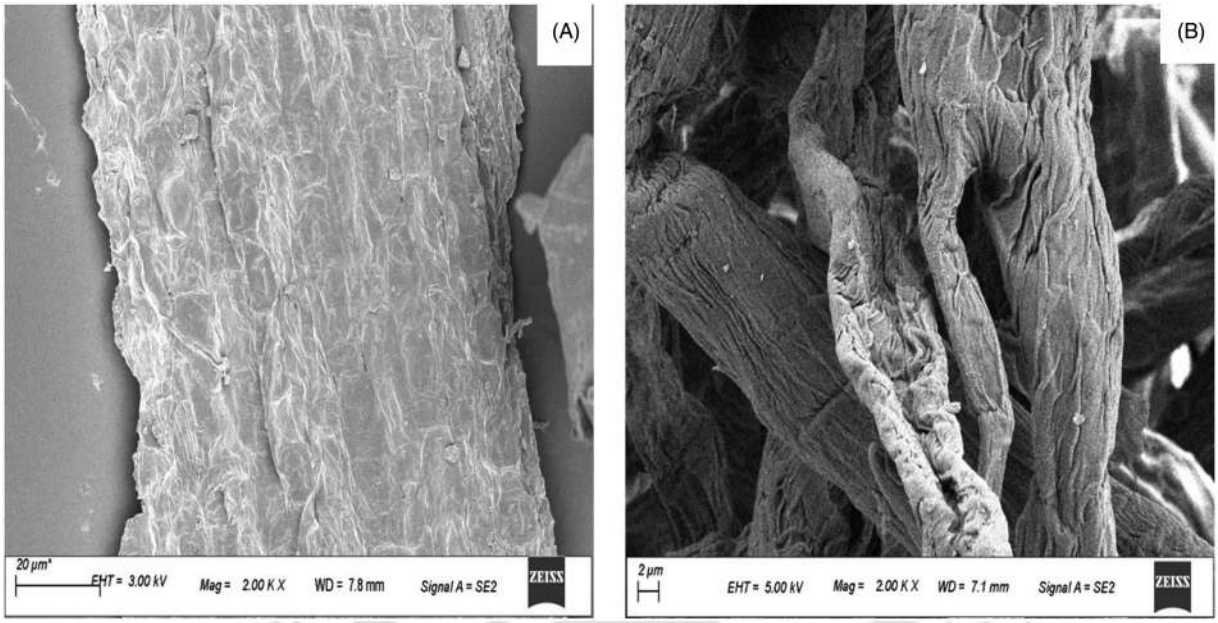

Figure 3. FE-SEM image of raw biomass (A) and separated cellulose (B) from SCT.

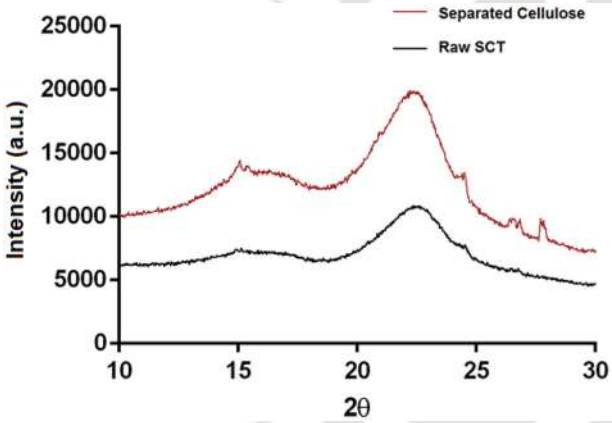

Figure 4. XRD analysis of raw SCT and separated cellulose from SCT.

However, the stretching vibration of O–H group in cellulose showed a broad peak at 3331 cm^{-1} as also reported previously.[29,30] The intra- and intermolecular vibrations[31] and C–H stretching vibration present in overall hydrocarbon constituents is attributed at 2894 cm^{-1}.[13] Bands assigned particularly for cellulose were found in the region, 1630 to 900 cm^{-1}.[30] A peak at 1633 cm^{-1} represented the absorbed water vibration in separated cellulose. The bending and stretching vibrations of $-CH_2$ and –CH, –OH and C–O bonds in separated cellulose were observed at 1428, 1367, 1334, 1027 and 896 cm^{-1} as also reported earlier.[32] The crystalline structure of cellulose is associated with the peaks at around 1430–1420 cm^{-1}, while amorphous region in cellulose is associated with the band at 897 cm^{-1} as also mentioned earlier.[30] The CH and CH_2 bond vibrations in cellulose showed the peaks at 1428 cm^{-1} and 1337 cm^{-1}, respectively.[33] All above mentioned peaks in FTIR spectrum observed in the separated cellulose were also found in the commercial cellulose confirmed the purity of cellulose separated from SCT.

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TGA analysis of cellulose separated from SCT

TGA analysis of cellulose separated from SCT was used to estimate the amount of remaining inorganic impurities in the separated cellulose fibers (Figure 6). Thermal analysis of separated cellulose showed weight loss between 80 °C and 150 °C, which are associated with the dehydration process. The decomposition process of separated cellulose initiated at 180 °C. This decomposition process ended at 400 °C and is strictly related to the thermal decomposition of cellulose (organic carbon) in the oxidizing atmosphere as also mentioned earlier.[34] The typical TGA curve for separated SCT cellulose showed 65% mass reduction and DTG decomposition at 327 °C. The microcrystalline cellulose from Sigma Aldrich, USA showed similar DTG decomposition curve at 322 °C and 70% mass reduction in TGA as also reported earlier.[35] After 400 °C, the decomposition of residual biomass leads to the formation of CO, CO_2, H_2O and char as also reported earlier.[36] Breakage of the glycoside linkage after 400°, reduces the degree of polymerization within the cellulosic fibers, leads to the formation of organic compounds such as alkenes and other hydrocarbon derivatives as reported earlier.[37] In the present study, the single decomposition peak obtained in DTG analyses confirmed the SCT cellulose is pure and there are no other impurities (xylan or proteins) in it.

Elemental composition analysis of cellulose separated from SCT

The air dried cellulose separated from SCT was used to determine the elemental composition by CHNS analyzer. The element composition analysis of powdered cellulose displayed that the carbon, hydrogen and oxygen contents are 41.45%, 4.4% and 45.85%, respectively. The absence of nitrogen and sulfur in the extracted cellulose showed that the cellulose is pure and is devoid of the protein[38] and lignin cross linkages.[39]

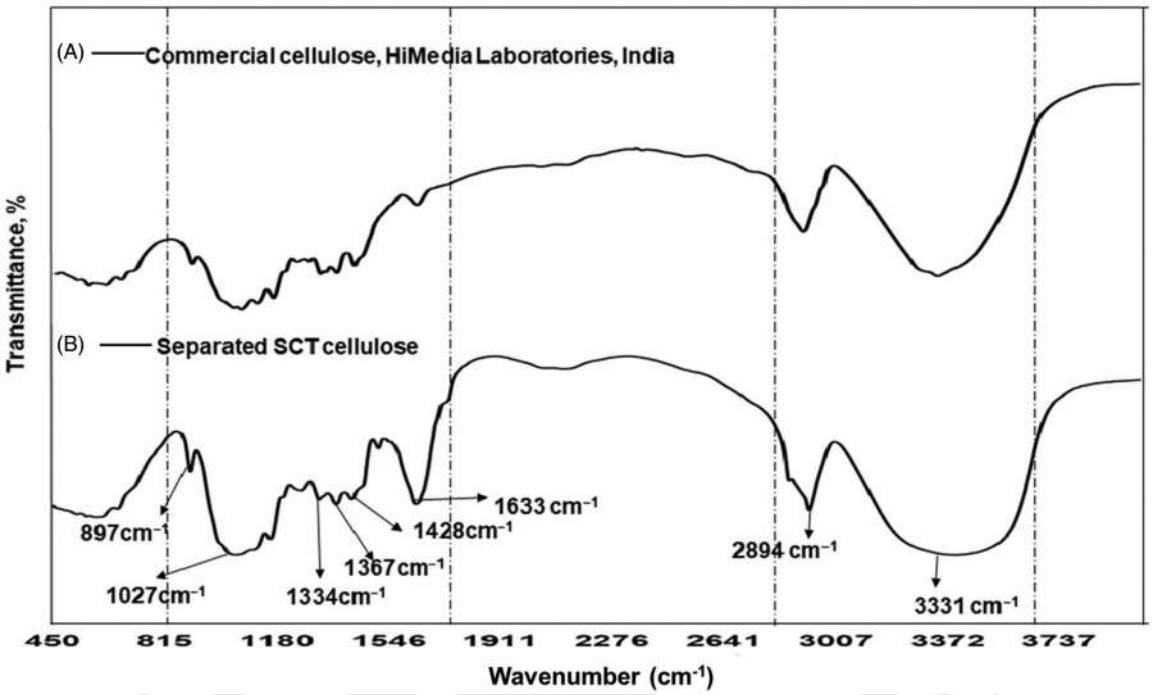

Figure 5. FT-IR analysis of (A) commercial cellulose (HiMedia laboratories, India) and (B) separated cellulose from SCT.

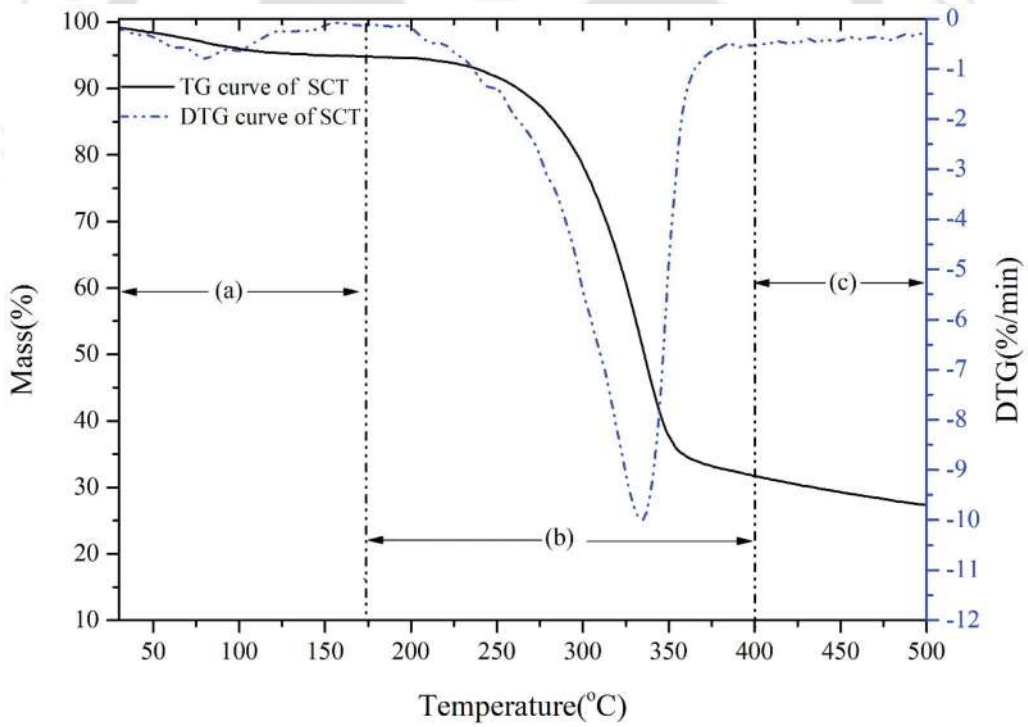

Figure 6. TGA and DTG analysis of SCT cellulose. Region (a) showed the dehydration of water present in the separated cellulose from SCT. Region (b) indicated the thermal degradation/decomposition of separated cellulose into organic compounds and region (c) denoted the decomposition of residual biomass.

Production of recombinant cellulolytic enzymes for saccharification

The protein concentration of purified endo-β-1,4-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-1,4-glucosidase (*Ht*Bgl) enzymes was 4.2 mg/mL, 2.9 mg/mL and 2.5 mg/mL, respectively. The purified *Ct*Cel8A and *Ct*CBH5A enzymes showed endoglucanase activity, 108 U/mg and cellobiohydrolase activity, 30 U/mg, respectively, against CMC-Na. The purified *Ht*Bgl showed β-glucosidase activity, 58 U/mg against cellobiose.

Enzymatic saccharification of raw SCT and cellulose separated from SCT

The enzymatic hydrolysis of raw SCT by using endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and

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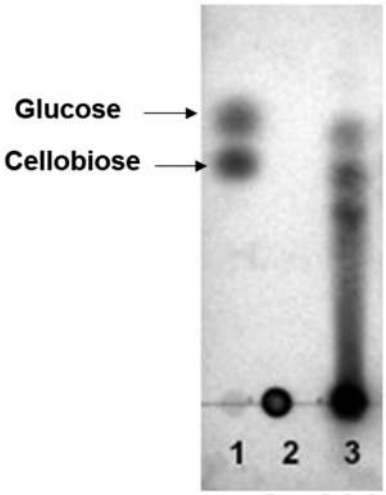

Figure 7. TLC analysis of hydrolyzed products of raw SCT and SCT cellulose by recombinant endoglucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and *β*-glucosidase (*Ht*Bgl) enzyme from *Clostridium thermocellum* at 50 °C for 24 h. Lane 1, Standards (Glucose and Cellobiose), Lane 2, Unhydrolysed product of raw SCT, Lane 3, Hydrolyzed products of separated SCT cellulose.

β-glucosidase (*Ht*Bgl) gave the TRS yield, 2.83 mg/g of raw SCT, whereas, of separated cellulose yielded TRS, 188 mg/g of separated cellulose. The GOD-POD analysis of enzyme hydrolyzed separated cellulose yielded glucose, 115 mg/g of separated cellulose. The TRS (188 mg/g of separated cellulose) released from 1 g of separated cellulose gave 21% of glucose yield (showed by HPLC analysis) from total 91% glucose present in separated cellulose. The TLC analysis of enzymatic hydrolyzed raw SCT showed no hydrolyzed product in hydrolysate. However, the SCT cellulose clearly showed cello-oligosaccharides of various degrees of polymerization in the hydrolysate (Figure 7). As seen in the FESEM image discussed above, prior delignification and xylan removal of raw SCT increases the accessibility of separated cellulose by disrupting the interlinkages between crystalline and amorphous cellulose that cause steric hindrance and exposure of cellulosic fibers toward the cellulolytic enzymes. However, much higher glucose yield was reported for other biomasses by using commercial enzymes.[40] The lower TRS yield obtained here could be attributed to the difference in biomass, the type of the pretreatment and also the enzymes used. The saccharification of SCT cellulose by recombinant enzymes *Ct*Cel8A, *Ct*CBH5A and *Ht*Bgl can be further enhanced by optimizing the process parameters.

Conclusion

The agro-waste, SCT was explored for recovery of potentially a commercial cellulose. The separated cellulose was found to contain 70% (w/w) holocellulose content, which is a significant fraction for production of monosaccharide that can be used in bioethanol production. The monosaccharide analysis of SCT cellulose after TFA hydrolysis by HPLC displayed the presence of 91% D-glucose, 7.5% D-xylose and 1.5% D-arabinose residues. The 85% of recovery yield was obtained from the total cellulose present in SCT biomass. The exposed cellulose fibers observed by FESEM analysis and the enhanced crystallinity by XRD analysis supported the purity of separated cellulose. The presence of crystalline and amorphous cellulose was confirmed by FTIR spectrum. TGA of SCT cellulose gave single decomposition temperature at 327 °C and CHNS analysis confirmed absence of impurities. The enzymatic saccharification of 1 g cellulose separated from SCT by recombinant endo-β-1,4-glucanase (*Ct*Cel8A), cellobiohydrolase (*Ct*CBH5A) and β-1,4-glucosidase (*Ht*Bg1) yielded 188 mg/g (TRS) of separated cellulose of reducing sugar and 115 mg/g of glucose per gram of separated cellulose, respectively. This resulted the 21% of reducing sugar conversion from saccharification of separated SCT cellulose. The enzymatic hydrolysis using these enzymes did not show efficient saccharification yield, which might be due to the increase in crystallinity of SCT cellulose. However, there is a scope of enhancing the yield by optimization of enzyme hydrolysis parameters. The above results showed the purity of separated cellulose and this process can help in improving the bio-refinery concept for the bioethanol production and the pure separated cellulose can be also used for other commercial applications.

Disclosure statement

The authors do not have any potential conflict of interest.

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Extraction and characterization of xylan from sugarcane tops as a potential commercial substrate

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Xylan is the major hemicellulose present in sugarcane stem secondary cell walls. Xylan is composed of xylose backbone with a high degree of substitutions, which affects its properties. In the present study, the xylan from sugarcane tops (SCT) was extracted and characterized. Compositional analysis of xylan extracted from SCT (SCTx) displayed the presence of 74% of D-xylose residues, 16% of D-glucuronic acid residues and 10% of L-arabinose. High performance size exclusion chromatographic analysis of SCTx displayed a single peak corresponding to a molecular mass of ~57 kDa. The Fourier transform infrared spectroscopic analysis of SCTx displayed the peaks corresponding to those obtained from commercial xylan. FESEM analysis of SCTx showed the granular and porous surface structure. Differential thermogravimetric analysis (DTG) of SCTx displayed two thermal degradation temperatures (T_d) of 228°C, due to breakdown of the side chains of glucuronic acid and arabinose and 275°C, due to breakdown of xylan back bone. The presence of arabinose and glucuronic acid as a side chains was confirmed by the DTG and thermogravimetric analysis. The CHNS analysis of SCTx showed the presence of only carbon and hydrogen supporting its purity. The recombinant xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* displayed a specific activity of 1394 ± 51 U/mg with SCTx, which was higher than those with commercial xylans. The thin layer chromatography and electrospray ionization mass spectroscopy analyses of *Ct*Xyn11A hydrolysed SCTx contained a series of linear xylo-oligosaccharides ranging from degree of polymerization 2-6 and no substituted xylo-oligosaccharides because of the endolytic activity of enzyme. The extracted xylan from SCT can be used as an alternative commercial substrate and for oligo-saccharide production.

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[**Key words:** Sugarcane leaf tops; Xylan; Xylanase; Xylo-oligosaccharides; Thermal degradation temperature]

Sugarcane (*Saccharum officinarum*) is one of the major cultivated crops in tropical regions. Every one ton of processed sugarcane produces 140 kg of bagasse (1) and 250 kg (dry mass) of sugarcane trash/sugarcane tops (SCT) residues (2). By means of the quantity, sugarcane was the world's largest crop produced in 2016 and was 1.9 billion tons as reported by Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), USA. The worldwide annual production of SCT is 279 million metric tons that could be used for cellulosic ethanol production (2). Around 50% of SCT can be removed from the field without affecting the nutritional properties of soil (3). The dry leaves can be collected from agricultural field up to 66% and used as a feedstock in biofuel industries (2). These post harvested residues are abundant, readily available and are cheap source of lignocellulosic biomass (4). SCT contains three main components *viz.* cellulose, hemicellulose and lignin. SCT is the field residue remaining after the harvesting of sugarcane stalk, while sugarcane bagasse is the fibrous residue left after milling of sugarcane stalk. The SCT contains 43% cellulose, 27% hemicellulose and 17% lignin (5) while SB contains 34% cellulose, 24% hemicellulose and 25% lignin (6). Cellulose is a straight chain polymer of glucose units having β-1,4-glycosidic linkages. Hemicelluloses are a group of complex plant polysaccharides that are biosynthesized in large quantities in most trees and terrestrial plants. Hemicellulose is the second most abundant biopolymer produced by plants. Xylan is the major hemicellulose found in secondary cell walls of sugarcane bagasse. Xylan is made up of xylose backbone with a high degree of substitutions of arabinose and glucuronic acids, which directly affect its structure and functional characteristics (7). Based on the method of extraction and source of plant/tissue used, the structure and composition of extracted xylan varies (8). The backbone of xylan consists of β-1,4-linked xylose residues, which is often substituted with methylated α-D-glucopyranosyl uronic acid, α-L-arabinofuranosyl, ferulic acid and *p*-coumaric acid in the side chain (9). Xylans also contain D-galactose, D-xylose, L-rhamnose, D-glucose, acetyl groups and different dimeric and trimeric side chains (10).

A chosen extraction method for xylan extraction compromises among extraction yield, selectivity and degree of polymerization (DP) (11). The extracted xylans can be used for biotechnological applications, however, some lignin fractions are also associated (12).

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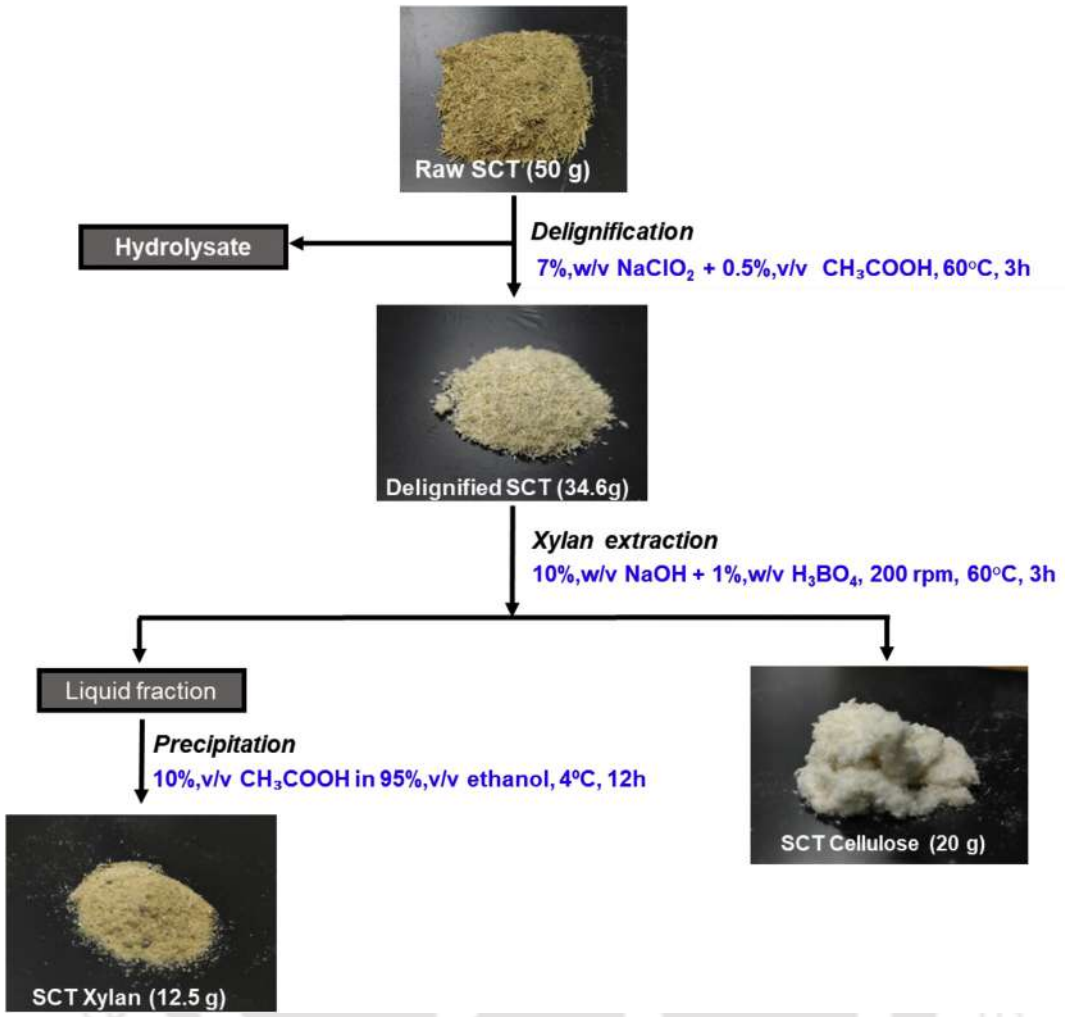

FIG. 1. Scheme showing the xylan extraction process from sugarcane tops.

Another simple traditional procedure of xylan extraction is by alkaline method followed by ethanol precipitation of xylan, however, the yield is generally low (13). However, the alkaline method used for xylan extraction from delignified sugarcane bagasse showed a higher yield though the xylan extracted was of low purity and displayed 16–18% residual lignin (14). The modification in traditional alkaline hemicellulose extraction methods by including de-lignification with sodium chlorite followed by the extraction gave high xylan yield (15). The xylan can be recovered from pretreated biomass, before the enzymatic saccharification of the cellulosic component, and used for other value added products thus leaving the cellulosic solids for bioethanol production. It has been reported that the hemicellulose removal can also enhance the cellulolytic hydrolysis (16). The xylan extraction from SCT is not much explored. Therefore, the present study on the extraction and characterization of xylan from SCT was undertaken. The extracted and purified xylan was structurally and functionally characterized and demonstrated as a commercial xylan for laboratory and industrial purposes. The extracted xylan was used to produce xylo-oligosaccharides by hydrolysis by using recombinant clostridial xylanase. Xylo-oligosaccharides have prebiotic potentials and are used in numerous nourishment products.

MATERIALS AND METHODS

Material and feedstock collection and its processing SCT (residue remaining after the harvesting of sugarcane stalk) was collected from Machkhowa, Guwahati, Assam, India. Sodium chlorite, sodium hydroxide, boric acid and other chemicals were purchased from Himedia Pvt. Ltd. (Mumbai, India). Reagents (ethanol and acetic acid) and thin layer chromatography (TLC) plate were purchased from Merck India Pvt. Ltd. (Mumbai, India). Beechwood xylan, oat spelt xylan and 4-*O*-methyl D-glucurono D-xylan from Sigma Chemical Corporation (St. Louis, MO, USA) and xylan from Carbosynth, (Compton, UK) were purchased. The feedstock was washed before and after chopping with tap water and rinsed with distilled water. Chopped SCT were dried in the oven at 60°C for 24 h and then ground to powder using a mixer grinder (HL1606/03, Philips, Amsterdam, Netherlands) and passed through a 0.85 mm sieve.

Delignification of SCT SCT was dried at 80°C for 24 h to remove the moisture content. Delignification was done in 1 L reagent bottle (Borosil, Ahmedabad, India) by adding 500 mL of distilled water and 50 g of air-dried SCT powder. Approximately, 35 g of sodium chlorite ($NaClO_2$) was added and mixed well followed by addition of 2.5 mL glacial acetic acid. The bottle was closed and incubated at 60°C in a water bath in a fume hood for 3 h. After incubation, the hydrolysate was removed by filtering using Whatman no. 1 filter paper under tap water till de-lignified SCT was neutralized. De-lignified SCT was dried at 80°C for 24 h (17). The delignification of SCT was confirmed by standard TAPPI protocol.

Extraction of xylan from SCT The extraction of xylan from SCT was carried out by alkaline xylan extraction method as shown in Fig. 1. The de-lignified SCT biomass recovered from previous step, i.e., 34.6 g was mixed with 346 mL solution containing 10% (w/v) sodium hydroxide (NaOH) and 1% (w/v) boric acid (H_3BO_4) and stirred at 200 rpm on a magnetic stirrer at 60°C for 3 h. The suspension was filtered through cotton cloth and xylan was precipitated with 3 volumes of ice-cold ethanol (95%, v/v) containing 10% (v/v) acetic acid and incubated at 4°C for 12 h. The impurities were removed by washing with 95% (v/v) ethanol and suspension was centrifuged at 10,000 *g* for 10 min to recover all the precipitated xylan. The extracted xylan (SCTx) was collected and lyophilized by lyophilizer (Labconco, Kansas City, MO, USA) and stored at 25°C for further experiments. The total xylan yield was determined by considering the amount of

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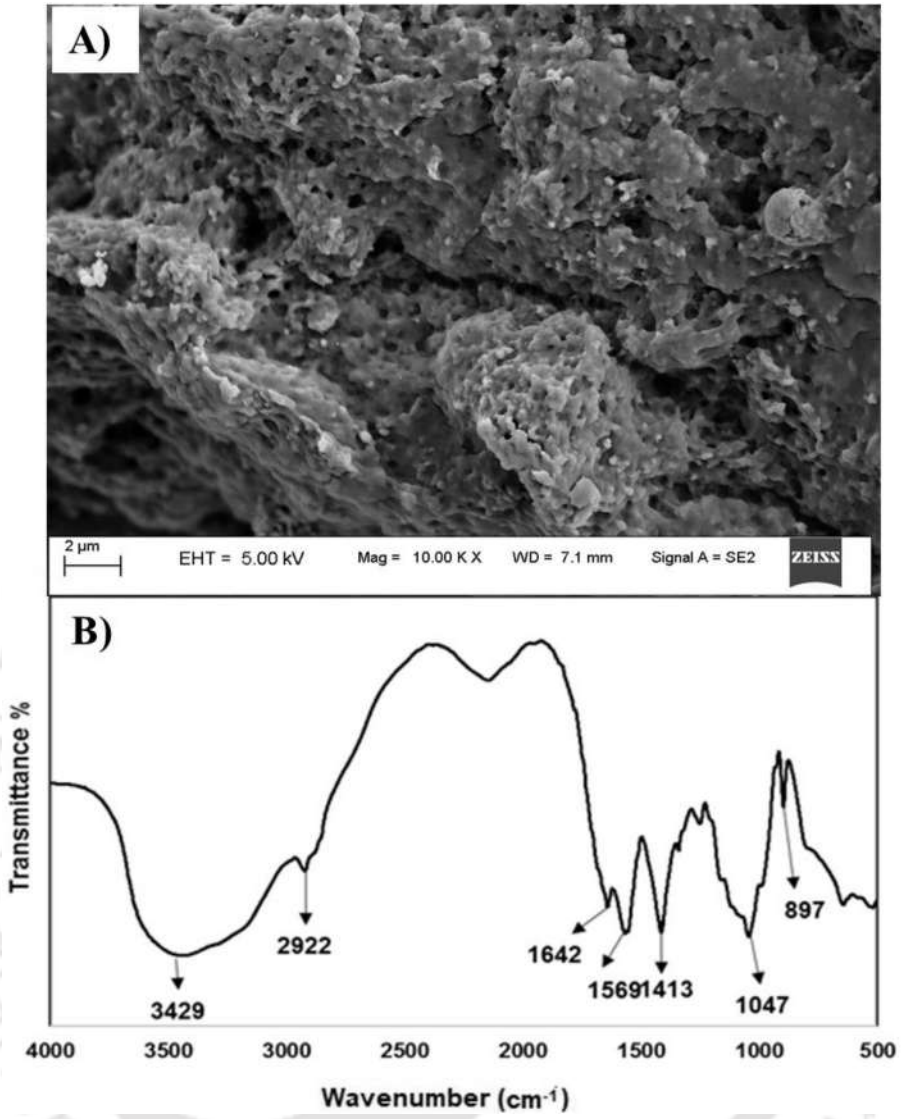

FIG. 2. (A) Field emission scanning electron micrograph (FE-SEM) analysis of extracted xylan. (B) Fourier transform infrared (FT-IR) spectroscopic analysis of SCTx.

hemicellulose in raw SCT biomass and extracted SCTx sample. The xylan yield was calculated as follows:

$$\text{Xylan yield } (\%) = \frac{\text{Dried weight of extracted SCTx}}{\text{Total hemicellulose present in raw SCT}} \times 100 \qquad (1)$$

Field-emission scanning electron microscopy analysis of SCTx The surface morphology of SCTx was analysed by using Field-emission scanning electron microscopy (FE-SEM, Carl Zeiss, Model-Gemini 300, Carl Zeiss, Jena, Germany). The SCTx sample was prepared on carbon tape and placed over the stub surface. A double gold coating of the sample was performed on a prepared stub and kept in the vacuum chamber before imaging by FE-SEM.

Fourier transform infrared spectroscopic analysis of SCTx Fourier transform infrared (FT-IR) spectroscopic analysis for SCTx was performed to determine the functional groups present. Extracted SCTx and potassium bromide (KBr) were mixed in ratio 1:100 (w/w) and ground with the help of mortar and pestle and a pellet was prepared under 15-ton hydraulic press. The sample was scanned on an FT-IR spectrophotometer (Spectrum Two, PerkinElmer, Waltham, MA, USA) and spectrum was recorded with in the wavenumber range, 4000–400 cm^{-1}.

Elemental analysis and specific optical rotation of SCTx Carbon, nitrogen, sulphur and hydrogen present in SCTx were determined by CHNS analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Pavia, Italy), whereas the oxygen was determined by using the following formula:

$$\text{Oxygen}(\%) = 100(\%) - (\text{Sum of present carbon, hydrogen and nitrogen}) \qquad (2)$$

The extracted xylan from SCT and commercial xylans, xylan (Carbosynth) and beechwood xylan (Sigma Chemical Corporation) at 0.5% (w/v) concentration were separately added in 2 mL of 50 mM NaOH and dissolved by warming at 60°C. The leftover insoluble xylo-polysaccharide was separated by centrifuging the xylan sample at 13,000 *g* for 15 min. The clear solution was transferred to the polariscope tube. The specific optical rotation of extracted xylan from SCT and commercial xylans were recorded by the digital polarimeter (MCP150, Anton Paar, Graz, Austria) at 25°C maintained by the peltier temperature controller.

Analysis of average molecular mass of SCTx The SCTx was analysed for average molecular mass by high performance size exclusion chromatography (HP-SEC) by using Phenomenex Polysep-GFC-P6000 column connected with a guard column (Phenomenex Polysep-GFC-P) of HPLC system (Shimadzu, Kyoto, Japan) equipped with RI detector. 10 µL of SCTx (1 mg/mL) and dextran standards (1 mg/mL concentration of 10 kDa, 20 kDa, 40 kDa, 70 kDa, 100 kDa, 200 kDa, 270 kDa and 500 kDa) were loaded sequentially and eluted by 100 mM $NaNO_3$ (mobile phase) at a 0.5 mL/min flow rate. With the help of dextran standards, the molecular mass of SCTx was calculated.

Thermal degradation of SCTx The changes in physical properties of SCTx were measured by observing the change in temperature, by the thermogravimetric analysis (TGA). TGA and differential thermogravimetric analysis (DTG) of SCTx were performed by using a thermal analyzer (model STA449F3A00, Netzsch, Selb, Germany). For TGA and DTG, 6 mg of SCTx was taken in an alumina crucible and heated from ambient temperature to 500°C at a rate of 10°C min^{-1} and the nitrogen gas was purged at a flow rate of 60 mL/min.

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Monosaccharide composition analysis of SCTx The carbohydrate composition of SCTx was determined by treating 5 mg with 1 mL of 2 M tri-fluoro-acetic acid (TFA) for complete hydrolysis. The hydrolyzed xylan was kept at 80°C in hot air oven for overnight to remove residual TFA. The dried sample was dissolved in 500 µL degassed Milli-Q water and filtered through a syringe filter using PVDF membrane (pore size: 0.22 µm). The 10 µL TFA treated SCTx sample was then transferred to 500 µL HPLC vials and analyzed for the different sugars with the help of RI detector by high performance liquid chromatography (HPLC) (UFLC, Prominence, Shimadzu). TFA treated SCTx was loaded and passed through the guard column (50 mm × 7.8 mm) followed by the connected Phenomenex Rezex ROA (H+) column (300 mm × 7.8 mm). The samples were eluted by using 0.005 N H_2SO_4 as a mobile phase at 70°C and at a 0.5 mL/min flow rate for 90 min run time. The standards, xylose, arabinose and glucuronic acid at 1 mg/mL of each, were used for composition determination.

Enzyme activity analysis of xylanase against SCTx and other xylans The enzyme, endo-β-1,4-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* was expressed, purified and its enzyme activity was determined by following the method as reported earlier (18). The enzyme, 5 µL (5 µg/mL) was added to 0.5% (w/v) SCTx, beechwood xylan, birchwood xylan, oat spelt xylan and 4-*O*-methyl D-glucorono D-xylan or xylan (from Carbosynth) in 50 mM sodium phosphate buffer (pH 7.5) in 100 µL reaction mixture and incubated at 65°C for 1 min. The enzyme activity was calculated by estimating the reducing sugar released, by Nelson–Somogyi method using D-xylose as standard. One unit (U) of enzyme activity was defined as the amount of *Ct*Xyn11A required to produce 1 µmole of reducing sugar (D-xylose) per min under optimum conditions.

Xylo-oligosaccharides production from SCTx by xylanase SCTx (1%, w/v) was dissolved in 990 µL of 50 mM sodium phosphate buffer (pH 7.5) and 10 µL (5 µg/mL) of *Ct*Xyn11A was added in 2.0 mL microcentrifuge tube and the reaction mixture was incubated in a water bath at 50°C for 1 h. After that, 3 volumes of 95% (v/v) ethanol was added to it and the mixture was centrifuged at 10,000 *g* for 10 min to separate the residual unhydrolyzed polysaccharide. The supernatant was separated and kept at 80°C for 12 h to evaporate the ethanol. The yield of concentrated xylo-oligosaccharides released from SCTx was determined by using the phenol–sulfuric acid method (19). The qualitative analysis and identification of xylo-oligosaccharides released from SCTx was performed by TLC by using a mobile phase of chloroform:acetic acid:water (6:7:1). The TLC plate was stained with staining solution of (0.5%, w/v) α-naphthol in methanol and sulphuric acid (95:5) solution. Xylo-oligosaccharides with DP 2–5 and alduronic acid mixture was used as standard for identifying the hydrolyzed products. The produced xylo-oligosaccharides species present in xylo-oligosaccharide mixture was detected by electrospray ionization mass spectroscopy (ESI-MS) analysis as reported earlier (20).

RESULTS AND DISCUSSION

Delignification of SCT and extraction of xylan from SCT The raw SCT after delignification by sodium chlorite method gave 34.6 g of delignified SCT, resulting in 69.2% (w/w) yield of delignified SCT. The delignified SCT colour was white as shown in Fig. 1. The delignification was confirmed by standard TAPPI protocol. This delignified SCT (34.6 g) was used for xylan preparation by alkaline extraction and subsequent precipitation that gave 12.5 g of xylan (SCTx) after lyophilization, resulting in an overall 25% (w/w) yield based on raw SCT (Fig. 1). The xylan extracted from SB by using alkali method gave about 19.6% (w/w) yield (21). The xylan extracted from acacia by using similar extraction process gave 23.5% (w/w) yield (20). The xylan extraction from bleached corn stover by DMSO mediated extraction process gave a lower yield of 8.7% (w/w) (18). The lower yields of about 12.3% (w/w) from bambara and 13.6% (w/w) from cowpea were reported by alkali mediated xylan extraction method (22).

FE-SEM analysis of SCTx The surface morphology of dried SCTx on analysis by FE-SEM showed rough and highly porous surface with irregular granules (Fig. 2A). The previous reports on xylan extracted from rice bran and finger millet (23), from pineapple peel waste (24) and from acacia saw dust (21) also showed the irregular and rough surface morphology.

FT-IR analysis of SCTx The FT-IR spectrum of SCTx (Fig. 2B) showed a discrete peak at 3429 cm^{-1} for stretching vibrations of the hydroxyl (OH) group, which was similar to the peak for OH stretching as reported earlier (25). The peak at 2922 cm^{-1}

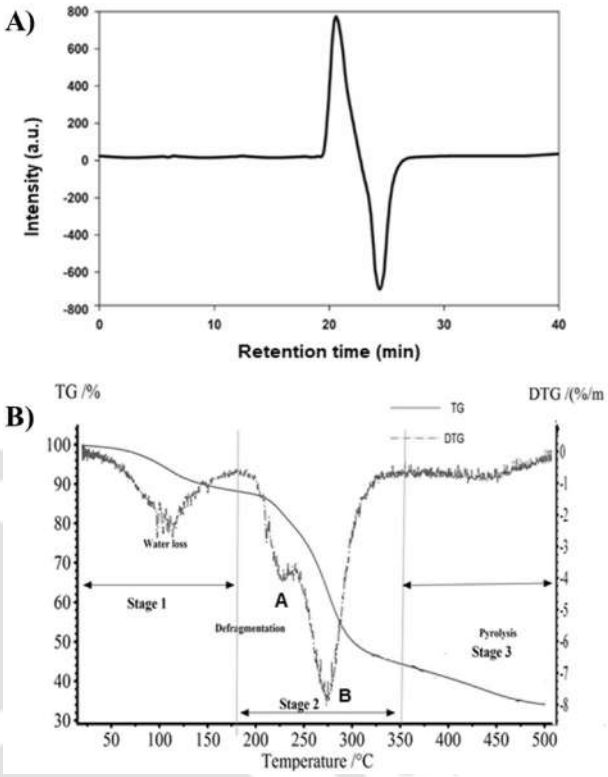

FIG. 3. (A) HP-SEC profile of xylan extracted from SCT and (B) thermogravimetric analysis (TGA) showing the decomposition of SCTx (from 160°C to 350°C) and differential thermogravimetric analysis (DTG) of SCTx displaying the breakdown of arabinose and glucuronic acid side chains at 228°C (stage A) and thermal degradation temperature (T_d) of xylose back bone at 275°C (stage B).

revealed symmetric CH stretching in extracted SCTx, which was similar to the peak previously reported (26). The symmetric and asymmetric stretching modes of the carboxyl group (-COOH) of glucuronic acid in SCTx were found at 1569 cm^{-1} and 1413 cm^{-1}, respectively as also reported earlier (27,28). A small peak at 1642 cm^{-1} in SCTx denoted the presence of little amount of water, as also earlier reported (29,30). The β-glycosidic linkage in SCTx showed a small absorption peak at 897 cm^{-1} that is considered as the anomeric region as mentioned earlier (30,31). The stretching vibration of the OH side group and the C–O–C glycosidic group overlap in the region, 1200–1000 cm^{-1} in the FT-IR spectrum. The β-1,4 backbone of extracted xylan represented by the peak at 1047 cm^{-1} as reported earlier (32).

Elemental composition analysis and specific optical rotation of SCTx The elemental composition analysis of SCTx showed 35.2% carbon, 4.6% hydrogen and approximately, 60.2%, oxygen content. The absence of nitrogen and sulphur in the extracted xylan showed the purity of xylan that is devoid of the protein and lignin content as also mentioned earlier (33). The specific optical rotation estimated for extracted xylan from SCT was −72°. The commercial xylans, the xylan from Carbosynth and beechwood xylan (Sigma Chemical Corporation) showed optical rotation of −70.36° and −68.40°, respectively. Similar results, of 72.65° of specific optical rotation was reported for the xylan extracted from Acacia (21). The similar optical rotation of extracted xylan to the commercial xylan confirmed that the SCTx is composed of β-linked D-xylose monosaccharides, α-linked D-glucuronic acid and L-arabinose residues.

Molecular mass determination of SCTx The HP-SEC analysis of SCTx showed a single peak that corresponded to the molecular

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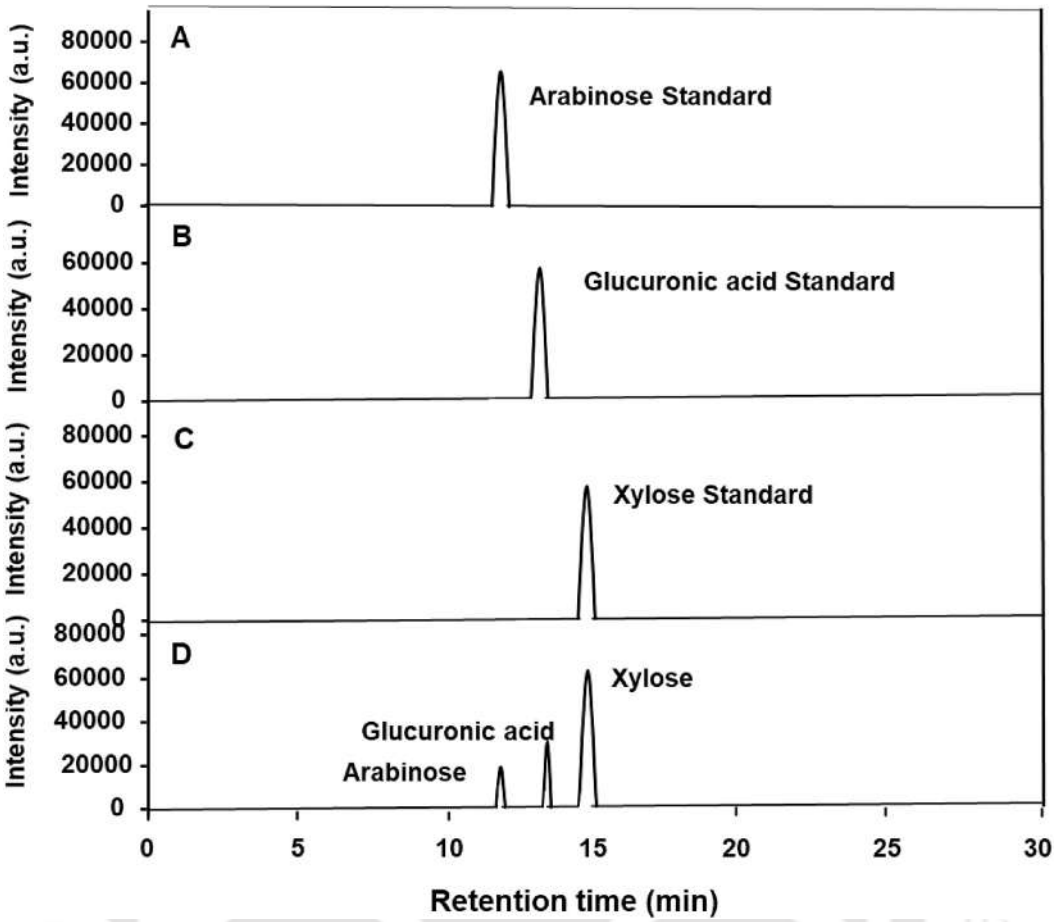

FIG. 4. (A) High performance liquid chromatography (HPLC) analysis of TFA treated SCTx, (B) arabinose standard, (C) glucuronic acid standard and (D) xylose standard for HPLC.

mass of ~57 kDa (Fig. 3A). The molecular mass of SCTx was similar to 56.6 kDa of glucuronoxylan isolated from Eucalyptus (34) and was higher than 49 kDa of xylan from seeds of *Cassia obtusifolia* (35). The molecular mass of xylan extracted from acacia using the similar extraction method was ~70 kDa (20). However, much lower molecular mass of extracted xylan from beechwood (20.84 kDa) (36) and from waste fibers (15 kDa) (30) has been also reported earlier.

DTG and TGA of extracted SCTx The thermal stability and thermal decomposition analyses of SCTx were studied by DTG and TGA, respectively. The maximum thermal degradation (T_d) temperature is designated as DTG_{max} and can be used for analyzing the sample stability. The reaction occurring at 100°C (Fig. 3B, stage 1) are endothermic and mainly attributing to the removal of moisture content, when SCTx is heated. The DTG profile of SCTx showed two peaks, one at 228°C (Fig. 3B, stage 2A) and second at 275°C (Fig. 3B, stage 2B), indicating that there are side chains present in SCTx. The DTG curve represented the breakdown of arabinose and glucuronic acid side chains at 228°C and xylose back bone at 275°C and these peaks corresponded to the T_d or DTG_{max} (Fig. 3). The two-step degradation for beechwood xylan was reported in which, the first step was associated with the breakage of the side chain units and the second step was associated with the depolymerisation of xylan backbone (37). The SCTx showed decomposition or weight loss in the temperature range, 200–350°C. The xylan from rice bran, finger millet seed coat and beech wood showed the weight losses at the temperature ranges, 132–334°C, 220–380°C and 247–340°C, respectively (23). The birchwood xylan (commercial) and wheat straw xylan also showed the weight losses between 220°C and 350°C, similar to SCTx (38).

The TGA analysis of SCTx displayed that it was stable up to 158°C and above this temperature, the thermal degradation started. The decomposition of SCTx occurred in three stages. The first stage of decomposition was between 70°C and 160°C and the weight loss was 8%, which denotes the moisture or water loss in SCTx as also reported earlier for commercial xylan (39) and hemicellulose from banana rachis (40). In the second stage, significance weight loss (52%) was observed between 160°C and 400°C due to the decomposition and fragmentation of SCTx into smaller molecules such as CH_3COOH, CH_4, CO, CO_2, and HCOOH as also reported earlier (40,41). The third stage of decomposition represented the pyrolysis of residual biomass that started from 400°C. In this process, the remaining residual xylan was pyrolyzed to ash.

Monosaccharide analysis of SCTx The monosaccharide composition analysis of SCTx by HPLC displayed the retention time

TABLE 1. Comparison of xylanase (*Ct*Xyn11A) activity against SCTx and commercial xylans.

Xylan	Specific activity (U/mg)
Xylan extracted from SCT (SCTx)	1394 ± 51
Xylan[a]	1220 ± 42
Beechwood xylan[b]	1218 ± 13
Birchwood xylan[b]	894 ± 58
Oat spelt xylan[b]	751 ± 43
4-*O*-Methyl D-glucorono D-xylan[b]	332 ± 92

[a] Carbosynth.
[b] Sigma Chemical Corporation.

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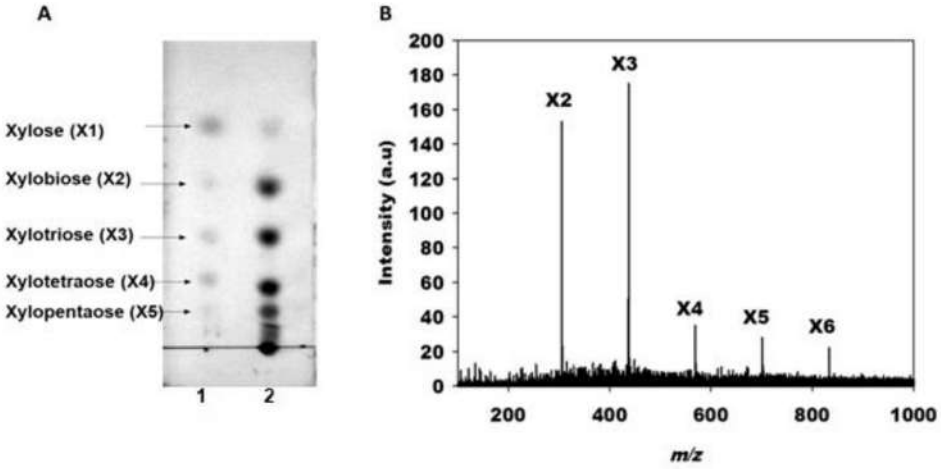

FIG. 5. (A) TLC analysis of hydrolyzed products of SCTx by endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum*. Lane 1, standards (xylose, DP2 to DP5); lane 2, *Ct*Xyn11A hydrolysed SCTx products. (B) ESI-MS analysis of endo-1,4-β-xylanase (*Ct*Xyn11A) hydrolysed products of SCTx.

of xylose, D-glucuronic acid and L-arabinose sugar similar to those with the standards (Fig. 4). The presence of 74% (w/w) D-xylose residues, 16% (w/w) of D-glucuronic acid residues and 10% (w/w) of L-arabinose content was determined by using the standards. The presence of higher percentage of xylose in SCTx shows that the backbone is composed of D-xylose monomers; arabinose and glucuronic acid are present as side chains. The major side-chain component in sugarcane bagasse is L-arabinofuranose. In sugarcane bagasse, alkali (40%, w/v NaOH) mediated extracted xylan, the arabinose/xylose and D-glucuronic acid/xylose ratios reported were 0.12 and 0.09, respectively (13). The arabinose/xylose ratio in xylan extracted from SCT by 10% NaOH was 0.14, similar to the previously reported xylan extracted from sugarcane bagasse (SBx). However, the D-glucuronic acid/xylose ratio found was higher (i.e., 0.22) in SCTx than reported for SBx. These ratios suggested that the low NaOH load (10%, w/v) in xylan extraction method protected the side chains in SCTx whereas, the high NaOH load (40%, w/v) removed the side chain groups from SBx, especially the D-glucuronic acid (13).

Enzyme activity analysis of SCTx by xylanase The enzyme activity of xylanase (*Ct*Xyn11A) from *C. thermocellum* against the xylans from different sources is listed in Table 1. The specific activity of *Ct*Xyn11A against SCTx (molecular mass: 57,000) was 1394 ± 51 U/mg, which was higher than the studied commercial xylans (beechwood xylan, oat spelt xylan and 4-*O*-methyl D-glucurono D-xylan purchased from Sigma Chemical Corporation and xylan from Carbosynth). The xylanase enzyme activity depends on the xylan's structure and its solubility. Birchwood xylan and beechwood xylan contain similar percentage (87.7%) xylose and lower content of glucose, arabinose and galactose. However, the relative content of hexuronic acid is different (10.2% in birchwood xylan and 7% in beechwood xylan) and therefore differs in relative solubilities. Similarly, oat spelt xylan also contains mixture of xylose (84%) and arabinose, glucose and galactose and also very less soluble (42). Owing to the higher water solubility, beechwood, xylan (Carbosynth) and SCTx showed the high enzyme activity compared to other xylans (Table 1). 4-*O*-Methyl D-glucurono D-xylan (MGX) has high degree of substitution (70–80%) as compared to other xylans (43). Xylanase (*Ct*Xyn11A) showed less activity against MGX, which may due to non-accessibility of the main chain of the substrate owing to the presence of high degree of side chain substitutions. These results amply demonstrated that the alkali extracted SCTx can serve as a commercial substrate.

Identification and production of xylanase mediated xylo-oligosaccharides The total xylo-oligosaccharide yield from 10 mL hydrolysis of 1% (w/v) SCTx by 50 μL of *Ct*Xyn11A (5 μg/mL) was 41.2 ± 1.7 mg as estimated by phenol sulfuric acid method (44). The TLC analysis of mixture of xylo-oligosaccharides produced by enzymatic hydrolysis of SCTx showed the presence of series of xylo-oligosaccharides of DP ranging from DP2 to DP5 (Fig. 5A). The hydrolysis of 4-*O*-methyl glucuronoxylan by recombinant *Ct*XynGH30 (45) and *Ps*Xyn10A (46) released the identical types of xylo-oligosaccharides. The TLC analysis of hydrolysed products also showed higher range of unresolved xylo-oligosaccharides. Therefore, to obtain the precise information about presence of any substitution and DP, the xylo-oligosaccharides mixture was further analysed by mass spectrometry. The ESI-MS analysis of *Ct*Xyn11A hydrolysed xylo-oligosaccharides mixture revealed that the mixture contains a series of linear xylo-oligosaccharides ranging from DP2 to DP6 and no substituted xylo-oligosaccharides (Fig. 5B). The mixture includes xylobiose (*m*/*z* 305.01), xylotriose (*m*/*z* 437.0), xylotetraose (*m*/*z* 569.0), xylopentaose (*m*/*z* 701.0) and xylohexaose (*m*/*z* 833.0). The molecular mass of acidic and neutral xylo-oligosaccharides matches well with the earlier reports (47). The ESI-MS analysis of xylo-oligosaccharides produced by alkali hydrolysis of extracted xylan from Acacia saw dust displayed the production of series of xylo-oligosaccharides (DP2–DP8) (20). Acetyl glucuronoxylan hydrolysed by acetylxylan esterase resulted in a mixture containing linear xylo-oligosaccharides from DP2 to DP6 (47). The utilization of enzyme assisted production of xylo-oligosaccharides in various applications such as antioxidative agent, as antiproliferative agent in healthcare (48) and as food supplement (49) has been extensively made. Therefore, the extracted xylan apart from its role as a substitute of commercial substrate, can also be used for diverse biotechnological applications.

Thus the higher xylanase activity achieved on SCTx than on commercial xylans established that it can be taken for commercial production and that the xylo-oligosaccharides produced from it can be used in various health-care and food applications.

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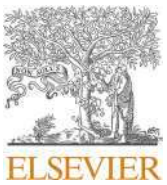

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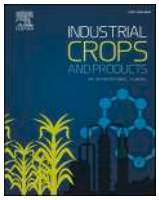

A biorefinery approach for sequential extraction of commercial grade xylan and alkali lignin from alkali pretreated sugarcane tops hydrolysate

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ABSTRACT

Sequential extraction of xylan and lignin from alkali pretreated sugarcane tops (apSCT) hydrolysate was carried out. The xylan extracted from apSCT hydrolysate (apSCTx) was 26.5 g and the alkali lignin (apSCTal) was 8.5 g from 100 g of raw SCT. The composition analysis of apSCTx by high performance liquid chromatography showed 74% D-xylose, 15% D-glucuronic acid and 11% L-arabinose residues. The analysis of apSCTx by high performance size exclusion chromatography showed molecular size, ~58 kDa. The first derivative of thermogravimetric analysis of apSCTx showed a Td (thermal degradation temperature) at 275°C. The elemental (CHNS) analysis of apSCTx displayed the presence of only carbon and hydrogen. The absence of nitrogen and sulfur in the extracted apSCTx displayed that the xylan is pure and without the protein and lignin. The specific activity of recombinant clostridial endoxylanase (*Ct*Xyn11A) was 1823 ± 51 U/mg. Liquid Chromatography-Mass Spectroscopy and Thin-layer chromatography analyses of *Ct*Xyn11A hydrolyzed apSCTx displayed a series of linear xylooligosaccharides ranging between 2 and 5 degrees of polymerization. apSCTx can serve as a potential commercial substrate and also for xylooligosaccharides production. The characterization of apSCTal by Field-Emission Scanning Electron Microscopy showed the exposed spherical structures of alkali lignin. Fourier Transform Infrared spectroscopic analysis of apSCTal displayed the peaks corresponding to those obtained from commercial alkali lignin. CHNS analysis of apSCTal confirmed the absence of protein impurities. The first derivative of the thermogravimetric analysis of apSCTal showed T_d at 362°C, similar to the previously reported softwood lignin. The extracted apSCTal can be used as feedstock for the production of aromatic chemicals. The biorefinery approach of sequential extraction of apSCTx and apSCTal from alkali pretreated SCT hydrolysate waste can enhance the economics of the extraction process.

1. Introduction

The major part of Indian population relies on the agriculture and agri-based industries for their living. Barley, maize, rice, sugarcane and wheat are the major crops in India. Yearly around 200 billion tonnes of sugarcane tops (SCT) is produced in India (Khaire et al., 2021a). This leftover residual biomass is usually burnt in agricultural land which leads to environmental pollution. The composition of SCT from different locations shows cellulose (40–45%), hemicellulose (25–30%) and lignin (15–19%) (Khaire et al., 2020). SCT can be utilized as a substrate for the production of various value-added products such as acetic acid, biobutanol, bioethanol, cellulose, furfural, lactic acid, lignin and xylan (Canilha et al., 2012; Khaire et al., 2021a).

The hemicellulose is made up of a mixture of heteropolysaccharides such as glucomanan, galactomannan, xyloglucan, glucuronoxylan and xylan. The xylan separation before enzymatic hydrolysis of biomass can enhance the enzymatic digestibility of cellulose in biomass (Meng and Ragauskas, 2014; Huang et al., 2022). The characteristic structure of xylan is dependent on the plant source and the extraction method used (Petzold-Welcke et al., 2014). The main chain of xylan is made up of xylose residues via β-1,4-glycosidic linkages with other side chain residues such as arabinose, glucose, galactose, acetyl groups, rhamnose as well as dimeric and trimeric side chains (Moine et al., 2007). Traditionally, alkaline treatment followed by ethanol precipitation method is

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used for the extraction of xylan from various plant sources. However, this method generally shows low xylan yield. The optimization of extraction process can enhance the xylan yield (Banerjee et al., 2019). Commercial grade xylan was extracted from sugarcane tops by alkaline treatment method (Khaire et al., 2021c). Xylan is extensively used in industrial applications such as food packaging, bio-catalyst in pollutant removal (Ananpattarachai and Kajitvichyanukul, 2016), Bioplastics (Brodin et al., 2017), as additives in paper making (Sharma et al., 2020a) and as drug carriers in pharmaceutical applications (Khaire et al., 2022). The xylooligosacharides produced from xylan extracted from neem saw dust can have potential as a prebiotic in nourishing products (Sharma et al., 2020c).

The most abundant aromatic biopolymer found on the Earth is lignin accounting for almost 30% organic carbon (Kajaste, 2014). More than 70 million tons of lignin is produced annually which receives considerable attention as a bio-based precursor (Molinari et al., 2013). Lignin is mainly recovered from sulfate and Kraft pulping processes in paper and pulp industry (Stewart, 2008). However, only 2% of the produced lignin is utilized for value-added products whereas the rest is burnt as low-energy value fuels. Lignin is highly branched, irregular three-dimensional and amorphous phenolic polymers which provides a cover for structural support and protection against biological and chemical attacks (Poovaiah et al., 2014; Lu et al., 2017). The chemical structure of lignin is extremely complex and it is made up of three basic phenylpropanoid monomers, syringyl (S), guaiacyl (G) and p-hydrophenyl (H) units (Shigeto et al., 2017). The lignin composition and content varies in different lignocellulosic biomasses because of radical coupling reactions of monomers forming a phenolic, cross-linked and racemic polymer using its biosynthesis (Shigeto et al., 2017). It also varies with the source of tissue and the age of the lignocellulosic biomass (Healey et al., 2016). The percentage of lignin in lignocellulosic biomass varies from 10% to 40%, depending on the species, variety, maturity and developmental growth conditions (Shigeto et al., 2017). The typical lignin content in softwoods, hardwoods and grasses are 24–33%, 19–28% and 15–25%, respectively. The different composition and structure of lignin are dependent on the functional group (carbonyl, carboxyl, hydroxyl and methyl etc.) linkage with aliphatic or aromatic moieties in different proportions (Kai et al., 2016). Lignin contains a number of functional groups like aliphatic, phenolic, hydroxyl and the carbonyl groups, enabling it to undergo several modifications. Lignin can be utilized as complexing, binding, dispersing and emulsion-stabilizing agent for several applications (Liao et al., 2020). Lignin can also be used for the sustainable production of aromatic chemicals such as ferulic acid, vanillin and phenol (Zhang et al., 2022). Lignin also has some novel applications such as packaging material, 3D-printing materials, flame retardants, surfactants, biocompatible and slow releasing fertilizers, lignin-epoxy resins and biobased polyurethanes (Bertella and Luterbacher, 2020).

The hemicellulose and lignin separation during the pretreatment process and their utilization as a substrate by other industries for value addition is the ultimate goal of a biorefinery.

The extraction of xylan and lignin from sugarcane tops is not yet explored. The alkali pretreated SCT hydrolysate is waste produced from the alkaline pretreatment of SCT biomass for bioethanol production (Khaire et al., 2021b). The current study is based on the sequential extraction and characterization of xylan and alkali lignin from the alkali pretreated SCT hydrolysate. Therefore, the utilization of the waste SCT hydrolysate for production of commercial grade xylan and alkali lignin will enhance the economics of a biorefinery process.

2. Materials and methods

2.1. Materials and biomass processing

The sugarcane tops biomass was collected from the nearby vicinity of Guwahati, India. After collection, the SCT biomass was washed under tap water and then cut into pieces (1–2 cm). Moisture was removed from the pieces by drying at 60 °C for 48 h in the hot air oven (Labtech, Mumbai, India). Then the dried SCT pieces were milled to powder (particle size: 0.85 mm) with the help of mixer-grinder (Philips, HL1606/03) and stored at 25 °C for future experiments. All reagent or analytical grade chemicals and reagents were procured from Himedia Pvt. Ltd. and Merck India Pvt. Ltd., respectively.

2.2. Pretreatment of SCT biomass

The water soluble extractives were removed from 100 g of raw SCT (in 1000 mL of distilled water) by incubating at 50 °C for 12 h in a shaker incubator. Then the solid SCT biomass was separated from the hydrolysate by filtering through a muslin cloth and dried it at 80 °C in hot air oven (Khaire et al., 2021c). About 76 g of extractive free SCT biomass was further pretreated with 760 mL alkaline solution containing 10% (w/v) sodium hydroxide and 1% (w/v) boric acid in 1 L reagent bottle on a magnetic stirrer at 60ºC, 200 rpm for 3 h. The slurry was filtered by passing through muslin cloth and the 600 mL of alkali pretreated SCT (apSCT) hydrolysate was collected in a new reagent bottle for sequential extraction of xylan and alkali lignin. The separated solid biomass residue was previously studied for statistical optimization of enzymatic saccharification and bioethanol production (Khaire et al., 2021b).

2.3. Sequential extraction of xylan and alkali lignin from alkali pretreated SCT hydrolysate

The xylan from apSCT (apSCTx) was precipitated in step one by adding 3 volumes (1800 mL) of solution containing ice-cold rectified spirit (ethanol: 95%, v/v) and glacial acetic acid (10%, v/v) to 1 volume (600 mL) of apSCT hydrolysate. The reagent bottle was kept for incubation at 4ºC for 12 h. The suspension was then centrifuged at 4ºC and 10,000 g for 10 min to collect all the precipitated apSCTx. Used ethanol was recovered by partial distillation process before lignin precipitation. In the second step, the alkali lignin (apSCTal) from the remaining supernatant was recovered by precipitating by lowering its pH to 1.5 by adding hydrochloric acid. The precipitated apSCTal was recovered by centrifugation at 10,000 g for 10 min. The sequentially extracted apSCTx and alkali lignin were stored at 25ºC after lyophilization (Lyophilizer: Labconco, Kansas City, MO, USA). Total apSCTx and apSCTal yield from SCT biomass were determined by considering the hemicellulose and lignin present in raw SCT and in extracted samples. The apSCTx yield was calculated by,

$$apSCTx\ \ yield\ \ (\%) = \frac{Dried\ \ weight\ \ of\ \ apSCTx}{Total\ \ hemicellulose\ \ present\ \ in\ \ raw\ \ SCT} X\ \ 100$$

The apSCTal yield was calculated by,

$$apSCTal\ \ \ (\%) = \frac{Dried\ \ \ weight\ \ \ of\ \ \ apSCTal}{Total\ \ \ lignin\ \ \ present\ \ \ in\ \ \ raw\ \ \ SCT} X\ \ \ 100$$

2.4. Composition analysis of apSCTx

The Tri-fluro-acetic acid (TFA) hydrolyzed apSCTx sample was prepared as per the previously mentioned method (Khaire et al., 2021c). The prepared apSCTx sample was solubilized in degassed water (Milli-Q: 500 µL) and passed through the 0.22 µm PVDF membrane by using a syringe filter to HPLC vials (Axiva Sichem Biotech, India). The 10 µL sample was then analyzed for the different monosaccharides by using High-Performance Liquid Chromatography (HPLC) (UFLC, Prominence, Shimadzu, Japan) and RI detector. The arabinose, glucuronic acid and xylose were taken as standard monosaccharides.

2.5. Analysis of average molecular mass of extracted apSCTx

The apSCTx was analyzed for the average molecular mass by using

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high-performance size exclusion chromatography method as described earlier (Sharma et al., 2020b). The dextran standards markers (10, 20, 40, 70, 100, 200, 270 and 500 kDa) and apSCTx (1 mg/mL) were analyzed by using 100 mM $NaNO_3$ solution as a mobile phase at a flow rate 0.5 mL/min. The molecular weight of apSCTx was calculated with the help of dextran standards.

2.6. Ultimate elemental composition analysis of apSCTx and apSCTal

The CHNS analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Pavia, Italy) was used for the analysis of carbon, hydrogen, nitrogen and sulphur content present in apSCTx and apSCTal whereas the present oxygen content was calculated by using the formula,

$$Oxygen(\%) = 100(\%) - (\text{Sum of present carbon, hydrogen and nitrogen})$$

2.7. Specific optical rotation of apSCTx

The apSCTx sample was prepared by adding 0.5% (w/v) apSCTx in 2 mL sodium hydroxide (50 mM) solution and dissolved by warming at 60ºC. The insoluble apSCTx was removed from sample by centrifugation at 13,000 g for 15 min. The clear supernatant was collected in the clean polariscope tube. The specific optical rotation of apSCTx was recorded at 25 °C (Peltier temperature controller) by using the digital polarimeter (MCP150, Anton Paar, Graz, Austria).

2.8. Functional group and the surface morphology analysis of apSCTx and apSCTal

The functional group present in apSCTx and apSCTal were examined by using the Fourier Transform Infrared (FTIR) spectrophotometer (Perkin Elmer, Spectrum Two, USA) by scanning at 4 cm^{-1} resolutions for wavenumber ranges from 450 to 4000 cm^{-1} (Khaire et al., 2021c). The morphological structure imaging of apSCTx was studied by using the Field-Emission Scanning Electron Microscopy (FESEM) (Carl Zeiss, Model-Gemini 300, Germany) (Sharma et al., 2020c).

2.9. Thermal degradation analysis of apSCTx and apSCTal

Thermogravimetric (TG) and differential thermogravimetric (DTG) analyses were carried out for studying the changes in physical properties of apSCTx and apSCTal with increase in the temperature by using a thermal analyzer (Netzsch, Model: STA449F3A00). The sample was prepared as mentioned earlier and heated at a rate of 10ºC min^{-1} from 30 to 500ºC in a nitrogen environment (Khaire et al., 2021c).

2.10. Enzyme activity analysis of xylanase (CtGH11A)

Endo-β-1,4-xylanase (*Ct*GH11A) from *Clostridium thermocellum* was expressed in *E. coli* BL21(DE3) cells and purified as reported earlier (Jamaldheen et al., 2019). The enzyme activity of *Ct*GH11A was estimated by taking 0.5% (w/v) substrate (apSCTx or SCTx: xylan extracted from delignified SCT) with 5 µL (5 µg/mL) of enzyme in 95 µL sodium-phosphate buffer (50 mM, pH 7.5) in a 2 mL Eppendorf tube and incubated at 65 °C for 1 min (Jamaldheen et al., 2019). The enzyme activity of *Ct*GH11A was estimated by the released reducing sugar that was measured by following the Nelson (1944) and Somogyi (1945) method by using D-xylose as a standard.

2.11. Xylanase mediated xylooligosaccharides production

ApSCTx (0.1 g) was solubilized in 10 mL of sodium phosphate buffer (50 mM, pH 7.5) and partially digested with 10 µL of recombinant xylanase (*Ct*GH11A, stock conc. 5 µg/mL) at 50ºC for 1 h. The residual unhydrolyzed xylan was precipitated by adding 3 volumes of absolute ethanol followed by centrifugation at 10,000 g for 10 min. The separated supernatant was dried at 80ºC for 12 h and the concentration of xylooligosaccharides was estimated by following the phenol-sulfuric acid method (Khaire et al., 2021c). The qualitative identification of xylooligosaccharides produced from apSCT was determined by TLC analysis by using mixture of standard xylooligosaccharides of degree of polymerization (DP) 2–5 (xylobiose, xylotriose, xylotetraose and xylopentaose). The degree of polymerization of produced xylooligosaccharides was also confirmed by LC-MS analysis following the method reported earlier (Sharma et al., 2020b).

2.12. UV spectroscopic analysis of apSCTal

The content of alkali-soluble lignin, the purity and the components of the apSCTal was studied by UV spectroscopy (Thermo Fisher Scientific, MA USA) in the range 200–400 nm and compared (Lu et al., 2017).

2.13. Matrix-assisted laser desorption ionization coupled with time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of apSCTal

The MALDI-TOF-MS spectrometer (Bruker Daltonic, MA, USA) was used to record the high-resolution mass spectra of apSCTal at linear positive mode with a pulsed nitrogen laser (Ionization at 337 nm, the pulse rate: 10 Hz, acceleration voltage: ± 20 kV). A 5.2 kV lens was used to focus the laser light and a total of 300 shots were provided for apSCTal sample.

2.13.1. Sample preparation

Lignin sample at 1 mg/mL was prepared in water:acetonitrile: ethanol (10:50:40, v/v). The matrix, 2,5 dihydroxy benzoic acid was prepared at 1 mg/mL in a mixture of water: acetone (10:90 v/v). 1 µL of apSCTal sample was mixed with 10 µL of matrix solution and then 1 µL of the mixed sample was spotted on MALDI target and dried before the analysis. The spectrum was recorded in positive ionization mode for the lignin sample.

2.14. Mass balance and economic evaluation of sequential extraction of apSCT xylan and alkali lignin from raw SCT

The mass balance of sequential extraction of apSCT xylan and alkali lignin from 100 g of raw SCT for was carried out on the basis of the dry weight recovery. Economic evaluation of xylan and lignin extraction was carried out for 1 kg raw SCT biomass. The economic evaluation of each step was calculated by considering the commercial cost of all components used throughout the process (chemicals, water, electricity and instrumentation). Other costs involved such as manpower, transportation and storage were not considered in economic calculations for the laboratory scale process. The overall profit was calculated by.

$$\text{Overall profit} = Total\ income - Process\ cost$$

3. Results and discussion

3.1. Sequential extraction of xylan (apSCTx) from alkali pretreated SCT hydrolysate

One hundred gram of raw SCT contained 43 g of cellulose, 27.2 g of hemicellulose and 14 g Klason lignin. Alkaline pretreatment of 100 g of raw SCT biomass gave 42 g solid biomass (87% of glucose, 9% of xylose, 1.2% arabinose and 1.8% of glucuronic acid residues as reported earlier (Khaire et al., 2021b). This pretreatment resulted in production of around 600 mL of alkali pretreated SCT hydrolysate which was used for sequential extraction of xylan and lignin. Around 26.5 g (26.5%, w/w) of apSCTx was recovered from 600 mL alkali pretreated SCT hydrolysate which was 98.2% (w/w) of total xylan (27%, w/w) present in the raw SCT biomass. The xylan extracted from SCT biomass after delignification process using similar extraction process reported 25% (w/w) xylan yield from raw SCT (Khaire et al., 2021c). The xylan extracted from sugarcane

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Table 1
Xylan extraction methods and yield from different waste biomass.

Biomass	Xylan Extraction Method	Extraction Yield	Reference
Sugarcane bagasse	40%, w/v NaOH, 60 °C, 2 h	53% of total xylan	Sporck et al. (2017)
Sugarcane tops	Delignification, (18%, w/v NaOH + 0.26 M $NaBH_4$), 18 h, room temperature + 24%, w/v KOH	83% of total xylan	Flórez-Pardo et al. (2019)
Pineapple peel	10%, w/v NaOH, 45 °C, 16 h	87.6% of total xylan	Banerjee et al. (2019)
Acacia sawdust	Delignification, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60 °C, 3 h	23.5% of dry biomass	Sharma et al. (2020b)
Neem sawdust	Delignification, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60 °C, 3 h	22.5% of dry biomass	Sharma et al. (2020c)
Sugarcane tops	Delignification, 10%, w/v NaOH + 1%,w/v H_3BO_4, 60 °C, 3 h	92.6% of total xylan	Khaire et al. (2021c)
Sugarcane bagasse	0.53%, w/v NaClO, 52°C, 75 min	72.7% of total xylan	Gupta et al. (2022)
Sugarcane tops	Extraction, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60 °C, 3 h	98.2% of total xylan	This study

bagasse by following the alkali extraction method yielded 19.6%, w/w (de Figueiredo et al., 2017). The delignification of acacia sawdust followed by xylan extraction by the alkali extraction process resulted 23.5% (w/w) yield (Sharma et al., 2020b). A comparative account of xylan extraction methods and yield from different waste biomass is given in Table 1.

3.2. Characterization of apSCTx

3.2.1. Monosaccharide analysis of apSCTx

ApSCTx was analysed for its monosaccharide composition after TFA hydrolysis by HPLC. The HPLC analysis showed 74% (w/w) xylose, 15% (w/w) Glucuronic acid and 11% (w/w) arabinose residues in apSCTx. Due to the xylan backbone, the higher xylose percentage was found in the apSCTx and glucuronic acid and arabinose are found as side chains.

3.2.2. Molecular mass estimation of apSCTx

In HPSEC analysis of apSCTx, the molecular mass was found to be ~58 kDa, which was similar to the previously reported xylan extracted from SCT biomass (~57 kDa) (Khaire et al., 2021c). This was also similar to 56.6 kDa of glucuronoxylan extracted from Eucalyptus (Biely et al., 2013). The higher molecular mass of xylan (~70 kDa) was reported for acacia xylan extracted by using a similar extraction method (Sharma et al., 2020b).

3.2.3. Elemental composition analysis and specific optical rotation of apSCTx

The air-dried apSCTx was used to determine the elemental composition by CHNS analyzer. The element composition analysis of apSCTx displayed the carbon, hydrogen and oxygen contents, 38.81%, 6.42% and 54.77%, respectively. The absence of nitrogen and sulfur in the extracted apSCTx displayed that the xylan is pure and without the protein and lignin cross-linkages as also reported earlier (Khaire et al., 2020).

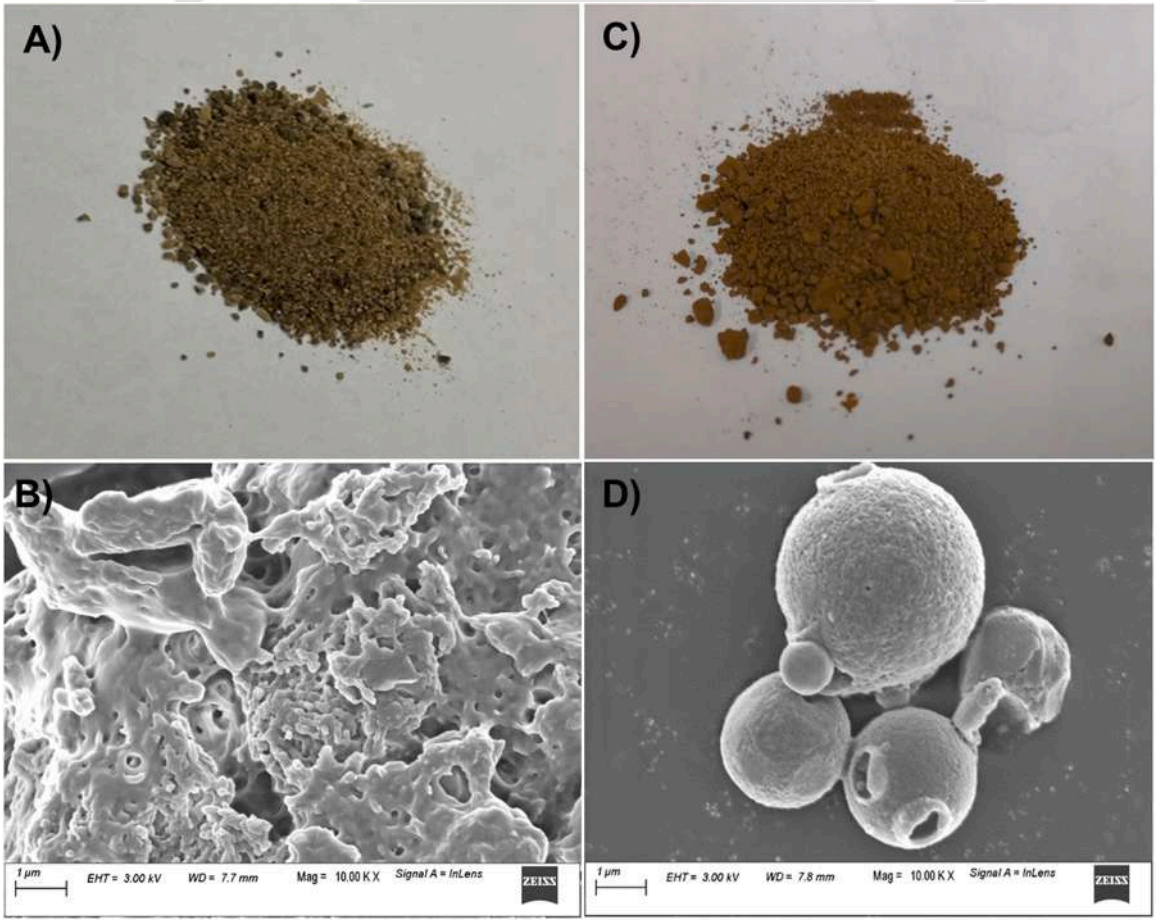

Fig. 1. A) SCT xylan separated from alkali pretreated SCT hydrolysate, B) Field emission scanning electron micrograph (FE-SEM) of apSCTx, C) Alkali SCT lignin separated from alkali pretreated SCT hydrolysate and D) Field emission scanning electron micrograph (FE-SEM) of apSCTal.

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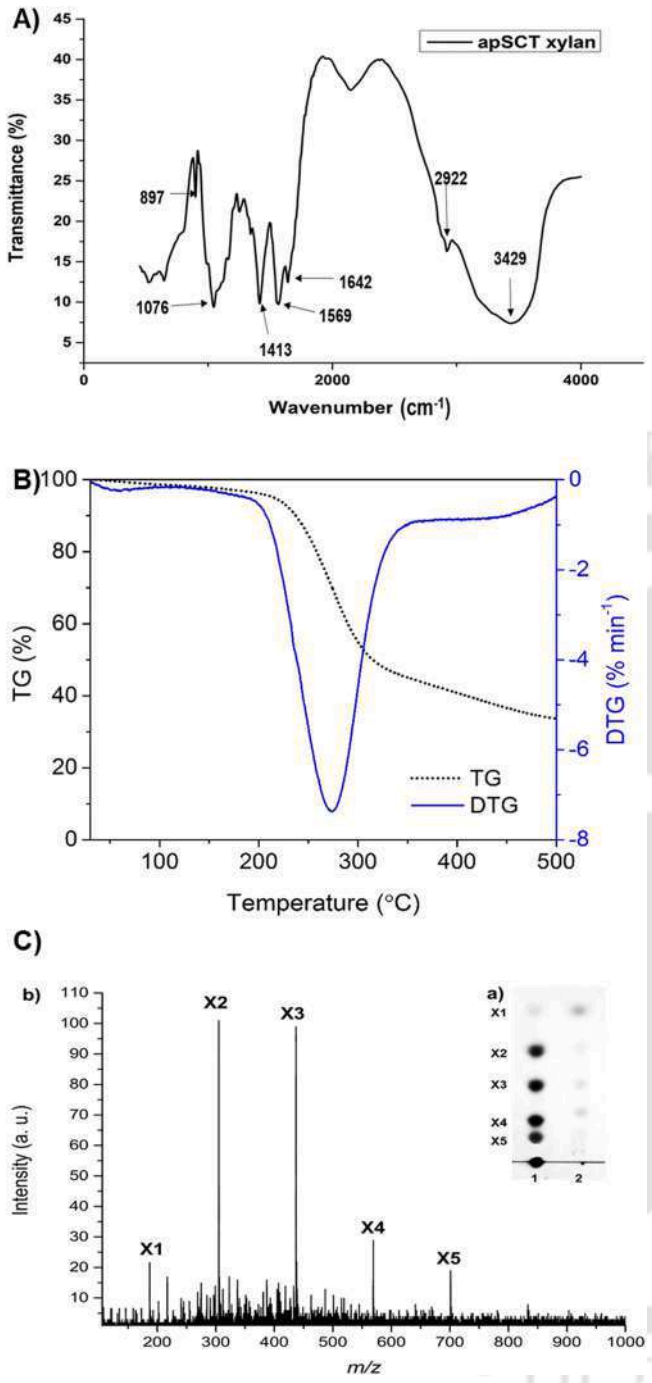

Fig. 2. A) Fourier Transform Infrared (FT-IR) spectroscopic analysis of apSCTx, B) Thermogravimetric analysis (TGA) and Derivative Thermogravimetric Analysis (DTG) of apSCTx displaying single thermal degradation temperature (T_d) of 275ºC and C) Endo β(1,4) xylanase (*Ct*Xyn11A) mediated oligosaccharides a) Thin-layer chromatography based analysis of. endo β(1,4) xylanase (*Ct*Xyn11A) hydrolyzed products of apSCTx: Lane 1, *Ct*Xyn11A hydrolyzed apSCTx products, Lane 2, Standards (xylose, DP2 to DP5) and b) ESI-MS analysis of endo β(1,4) xylanase (*Ct*Xyn11A) hydrolyzed products of apSCTx.

The specific optical rotation (SOR) determined for apSCTx was − 70°, similar to the SOR (−72°) reported previously for xylan extracted from delignified SCT biomass (Khaire et al., 2021c). The commercial xylans, beechwood xylan (from Sigma Chem. Co., USA) and xylan (from Carbosynth, UK) showed SOR of − 68.40° and − 70.36°, respectively as reported earlier (Khaire et al., 2021c). The similar value of SOR of apSCTx to the commercial xylans confirmed that the apSCTx is also composed of L-arabinose, α-linked D-glucuronic acid and β-linked D-xylose monosaccharide residues.

3.2.4. *Functional group analysis of apSCTx*

The FTIR spectrum of apSCTx (Fig. 2A) displayed a discrete peak at 3429 cm^{-1}, denoting the hydroxyl (OH) groups stretching vibrations which are similar to the peak obtained for OH group stretching in xylan extracted from roots of *Cudrania tricuspidata* (Shi et al., 2014). The 2922 cm^{-1} peak demonstrated the symmetric CH stretching in xylan extracted from sugarcane bagasse as also reported earlier (Luo et al., 2015). The asymmetric and symmetric stretching mode of the carboxyl group (-COOH) of glucuronic acid was reported at 1413 cm^{-1} and 1569 cm^{-1}, respectively as studied previously (Corradini et al., 2018). Water absorption in apSCTx was displayed at 1642 cm^{-1} as reported earlier (Zhang et al., 2016). A small absorption peak of β-glycosidic linkage at 897 cm^{-1} was observed in the anomeric region (Kacuráková et al., 2000; Zhang et al., 2016). The stretching vibration of the C-O-C glycosidic group and OH side groups overlap the ring vibrations in a region between 1200 and 1000 cm^{-1} as reported earlier (Khaire et al., 2021c). The extracted xylan β-1,4 backbone demonstrated a peak of 1047 cm^{-1}, as also reported earlier (Kacurakova et al., 2000). This characteristic functional groups similar to previously reported commercial xylans confirms the purity of extracted apSCTx.

3.2.5. *FESEM analysis of apSCTx*

The highly porous irregular granular and rough surface morphology was observed in the FESEM image of apSCTx extracted from apSCT hydrolysate (Fig. 1B). A similar morphological structure was also reported previously for xylan extracted from finger millet and rice bran xylan (Palaniappan et al., 2017), pineapple peel waste (Banerjee et al., 2019), acacia sawdust (Sharma et al., 2020b), neem sawdust (Sharma et al., 2020c) and sugarcane tops (Khaire et al., 2021c).

3.2.6. *DTG and TGA analysis of apSCTx*

The thermal decomposition and stability of apSCTx were studied by TG and DTG analyses, respectively (Fig. 2B). The TG analysis of apSCTx showed that xylan was stable up to 215 °C and there after the thermal degradation started. The decomposition of apSCTx occurred in 3 steps. In the first step, the decomposition between 70ºC to 200ºC showed the 6% weight loss that denotes the water loss in apSCTx as also mentioned earlier for banana rachis xylan (Deumaga et al., 2020) and commercial xylan (Zhang et al., 2016). The decomposition between 200ºC and 400ºC temperature in the second step showed 54% weight loss due to fragmentation of apSCTx into CO, CO_2, CH_4, CH_3COOH and HCOOH as also mentioned in previous reports (Deumaga et al., 2020; Khaire et al., 2021c). In the final step of apSCTx decomposition, pyrolysis of residual xylan to ash occurred above 400ºC.

The DTG_{max} is the maximum thermal degradation (Td) temperature of apSCTx and was analyzed by DTG (Fig. 2B). The overall thermal degradation gave a single peak at 275 °C, which is similar to the depolymerization of xylan extracted from delignified SCT as reported earlier (Khaire et al., 2021c). This endothermic reaction occurred due to the weight loss or decomposition of apSCTx between 230 °C and 350 °C which is similar to the xylan from rice bran (132–334 °C), from finger millet seed coat (220–380ºC), from beechwood (247–340ºC) (Palaniappan et al., 2017), from birchwood and wheat straw (220–350ºC) (Ruzene et al., 2007).

3.2.7. *Enzyme activity analysis of apSCTx by xylanase*

The specific activity of endoxylanase *Ct*Xyn11A against apSCTx (Molecular mass: 58000) was 1823 ± 51 U/mg, which was similar to 1876 ± 73 U/mg from the xylan extracted from delignified SCT and also the commercial xylans as reported earlier (Khaire et al., 2021c). Owing to the higher enzyme activity, apSCTx can be considered as a commercial substrate.

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Table 2
Lignin extraction methods and yield from sugarcane waste.

Biomass	Lignin Extraction Method	Extraction Yield	Reference
Sugarcane bagasse	0.5 M NaOH, Autoclaving at 120 °C, 1 h	7.5% of total dry biomass	Costa et al. (2017)
Sugarcane bagasse	95%, w/w acetic acid + 0.1%, v/v HCl, 187 °C, 40 min	55% of Klason lignin	Pinheiro et al. (2017)
Sugarcane bagasse	Ionic liquid 1-ethyl-3-methyl imidazolium acetate, 140 °C, 120 min	90% of total lignin	Saha et al. (2018)
Sugarcane bagasse	7.0%, w/v NaOH, 135 °C, 48 min	79% of total lignin	Ratanasumarn and Chitprasert (2020)
Sugarcane bagasse	Acid catalyst (H_2SO_4, 0.5%, w/w) at 150 °C, 30 min	10% of total dry biomass	Kauldhar et al. (2021)
Sugarcane tops	Extractive removal, 10%, w/v NaOH + 1%, w/v H_3BO_4, 60 °C, 3 h	61% of total alkali lignin	This study

3.2.8. *Xylanase mediated production of xylooligosaccharides*

The total xylooligosaccharide produced from 1% (w/v) apSCTx in 10 mL reaction mixture after hydrolysis by 50 μL of endoxylanase enzyme (*Ct*Xyn11A: 5 μg/mL) was found to be 27.6 ± 2.5 mg by phenol sulfuric acid method. The TLC analysis of apSCTx hydrolysed products showed the presence of xylooligosaccharides of degree of polymerization (DP), from 2 to 5 (Figure 2Cb). The identical type of oligosaccharides was produced in xylan extracted from delignified SCT (Khaire et al., 2021c). The produced oligosaccharides were further analyzed by LCMS for the confirmation of the degree of polymerization. The ESI-MS analysis of endoxylanase treated apSCT hydrolysate sample showed a series of linear chain xylooligosaccharides of DP from 2 to 5 with no substitution of other residues. The xylooligosaccharides produced from apSCTx sample included xylobiose, xylotriose, xylotetraose and xylopentaose with *m/z*: 305.0, 437.0, 569.0 and 701.0, respectively (Figure 2Ca). The ESI-MS analysis of endoxylanase mediated xylooligosaccharides produced from acacia xylan (Sharma et al., 2020b) and SCT xylan (Khaire et al., 2021c) displayed a similar series of xylooligosaccharides (DP2-DP8 and DP2-DP6, respectively). These enzymatically produced xylooligosaccharides can be utilized in a variety of applications such as antiproliferative agents, as an antioxidative agent in healthcare (Aachary et al., 2015) and as prebiotics (Sharma et al., 2020c). Apart from a substitute from the commercial substrate, apSCTx can be also utilized for various biotechnological applications (Kumar et al., 2021).

3.3. *Sequential extraction of apSCTal from alkali pretreated SCT hydrolysate*

After the xylan (apSCTx) extraction (by precipitation) from 600 mL apSCT hydrolysate, the remaining liquid fraction was further used for apSCTal extraction by precipitation using HCl at pH 1.5. Around 8.5 g of apSCTal was recovered (Fig. 1C) from 500 mL of liquid fraction (from 100 g initial biomass) resulting in an overall 60.7% (w/w) yield based on total alkali lignin present in the raw SCT biomass (14%, w/w). The lignin extracted by dioxane and hydrochloric acid after cyclohexane-ethanol (1:1, v/v) pretreatment gave 10% (w/w) recovery yield from raw sugarcane bagasse (Hoareau et al., 2004). The lignin extraction from sugarcane bagasse by alkaline pulping with sodium hydroxide solution after hot water pretreatment gave 13% (w/w) yield (Wahba et al., 2015). Table 2 shows a comparative analysis of different lignin extraction methods and yield from sugarcane waste.

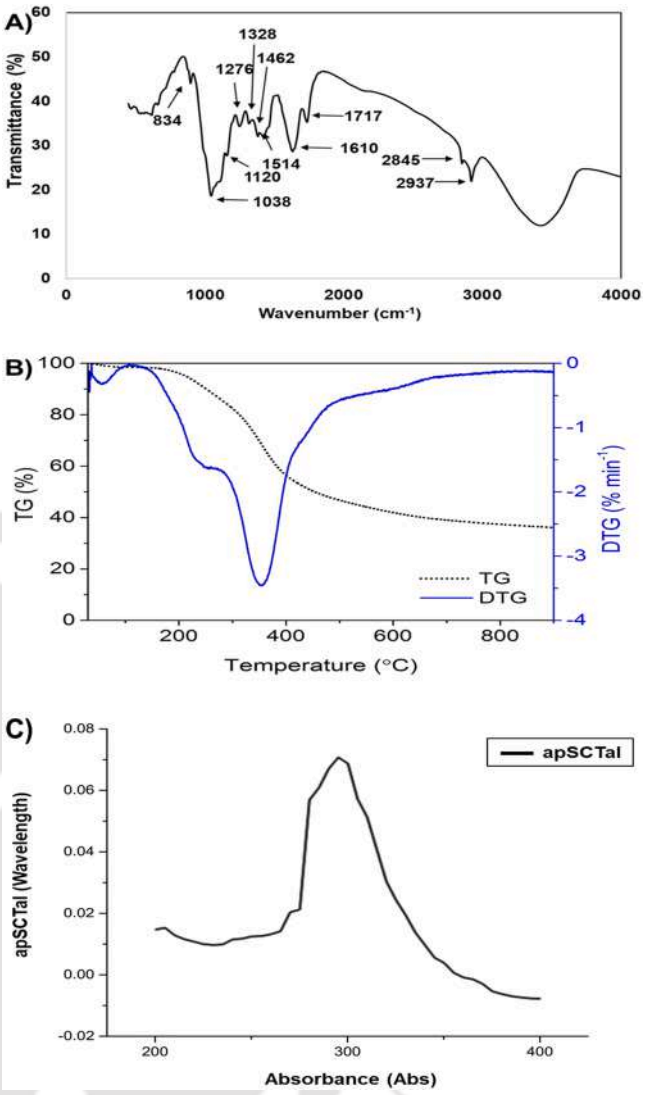

Fig. 3. A) Fourier Transform Infrared (FT-IR) spectroscopic analysis of apSCTal, B) Thermogravimetric analysis (TGA) and Derivative Thermogravimetric Analysis (DTG) of apSCTal displaying thermal degradation temperature (T_d) of 358°C and C) Qualitative spectrometric analysis of apSCTal.

3.4. *Characterization of apSCTal*

3.4.1. *Elemental compositional analysis of apSCTal*

The air-dried apSCTal was used for determining its elemental composition by CHNS analyzer. The element composition analysis of powdered alkali lignin displayed that the carbon and hydrogen contents to be 40.52% and 5.58% respectively. The absence of sulfur and nitrogen in the separated alkali lignin from SCT showed that the lignin is pure (without lignin cross-linkages) and is devoid of the protein (Khaire et al., 2020).

3.4.2. *Surface morphology and functional group analysis of apSCTal*

The electron micrograph of apSCTal by FESEM showed spherical structures (Fig. 1D). These spherical structures may have formed due to the surface tension during the acid precipitation of apSCTal (HCl) as also reported earlier for Kraft lignin extracted from oil palm (H_2SO_4) (Ibrahim et al., 2011). This similar structural morphology of apSCTal can confirms the presence of lignin in the apSCT hydrolysate.

FTIR spectrum of apSCTal (Fig. 3A) revealed that the functional groups present are similar to those previously reported lignin (Ibrahim et al., 2011). A predominant absorption peak was found at 3404 cm^{-1}

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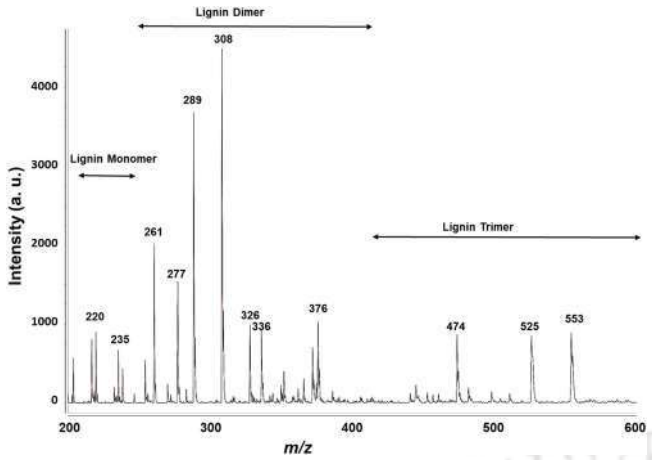

Fig. 4. MALDI-TOF MS analysis of apSCTal.

denoting the aliphatic and aromatic –OH bands stretching vibration present in the extracted apSCTal (Mousavioun et al., 2012). The strong absorption peak in alkali lignin at 2937 cm^{-1} indicated the stretching of C–H band vibration in -CH2, -CH3O, and -CH3 groups (Liu et al., 2014). The C-H stretching in -OCH3 group of apSCTal was represented by 2845 cm^{-1}. C═O stretching of unconjugated ketone, carbonyl and ester groups were seen at 1717 cm^{-1} as mentioned in earlier reports (Sun et al., 2013; Liu et al., 2014). The C═C stretching of the aromatic skeleton of apSCTal was represented by a peak at 1610 cm^{-1} (Nan et al., 2020). A 1514 cm^{-1} absorption peak found in FTIR spectra of apSCTal represents the C–C stretching in the aromatic skeleton of lignin as mentioned previously (Sun et al., 2013). A C–O stretching in apSCTal denotes the peak at 1328 cm^{-1}, which represents the syringyl group (Prado et al., 2016). A plane deformation of Ar–CH group in syringyl was represented by a peak at 1120 cm^{-1}. An ether (C–O-C) was confirmed by the presence of a peak at 1038 cm^{-1} (Liu et al., 2014). A dominant peak at 834 cm^{-1} denoting the C-H stretching out of plane symmetry in apSCTal as also reported earlier (Liu et al., 2014). The characteristic functional groups similar to previously reported alkali lignin confirms the purity of apSCTal.

3.4.3. TG and DTG analysis of apSCTal

The TG curve of apSCTal (Fig. 3B) showed the first decomposition curve between 70ºC to 100ºC with 6% weight loss representing the loss of water absorbed by lignin which was similar to the previous reports on oil plam showing 8% weight loss between 70ºC to 100ºC (Ibrahim et al., 2011). During the second step of decomposition between 200ºC and 500ºC, almost 68.75% of weight loss was observed due to the fragmentation of alkaline SCT lignin as also mentioned previously (Liu et al., 2014). In the final step, the decomposition of residual lignin occurred after 500ºC. The first derivative of TG (DTG) of apSCTal was analyzed for DTG_{max} (maximum thermal degradation (Td) temperature). The DTG curve showed 2 decomposition peaks, a small peak at 275 °C for xylan as contaminant (Khaire et al., 2021c) and a clear dominant peak at 358 °C (Fig. 3B) representing apSCTal, as reported earlier (Ibrahim et al., 2011).

3.4.4. UV spectrophotometric analysis of recovered apSCTal

The UV scan of apSCTal showed the a symmetrical syringyl unit in lignin at 295 nm (Fig. 3C). The phenolic hydroxyl groups of alkali lignin in neutral solution at absorption maxima at 295 nm was also reported earlier (Lu et al., 2017). No other absorption peak in UV spectrum of apSCTal also supported the purity of the extracted lignin.

Table 3
Commercial and considered cost of the byproducts produced from 1 kg of raw SCT.

Byproducts	Yield g/kg raw SCT	Market cost (USD)	Considered cost (USD)	Income (USD)
Xylan	265 g	47/25 g	23.5/25 g	249.1
Alkali lignin	85 g	25/25 g	12.5/25 g	42.5
Total income				291.6

Market cost of xylan and lignin from TCI, Japan. Assumed considered cost is 50% of commercial cost.

3.4.5. Matrix-assisted laser desorption ionization (MALDI) coupled with time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of apSCTal

A series of alkaline SCT lignin (apSCTal) oligomers were characterized by MALDI TOF (Fig. 4). The most abundant oligomers were found between the mass range of *m/z* 200–555 Da with maximum number of residues at *m/z* = 308 Da with highest covered area of 2959 square unit, which is similar to the previously reported MALDI TOF for switchgrass lignin (Richel et al., 2012). Fig. 4 is tentatively showing the molecular structure of different cations. MALDI-TOF MS analysis of apSCTal showed the series of the degree of polymerization and the modified monolignol subunits bellow *m/z* = 250 Da. These specific MS peaks recorded with variable intensities represented the syringyl (S), guaiacyl (G) and p-hydrophenyl (H) units derived from sinapyl, coniferyl, and p-coumaryl alcoholic precursors. The *m/z* ranges from 250 to 400 Da denotes the specific linkages between any two phenylpropane subunits or their precursors as mentioned previously for Switchgrass lignin (Richel et al., 2012). However, the autohydrolysis of lignin during extraction process makes it difficult to analyze the absolute molar mass of lignin as also reported earlier (Fernández-Rodríguez et al., 2019). The most abundant ions over a mass range of *m/z* 308 Da with 243 S/N ratios displayed in Fig. 4 represent the dimeric linkages between lignin monomers. The signals at *m/z* 474, 525 and 553 displayed the tri-oligomers of S, G or H subunits formed by α→5 and β→O→4 linkages with linear and regular subunit structures. This confirmed that the produced apSCTal is composed of sinapyl, coniferyl, and *p*-coumaryl alcoholic precursors which can be further used for the production of aromatic chemicals. The separated lignin can have applications in biomedical fields as nanofibril composite for antimicrobial property (Huang et al., 2020), bioactive substances for anti-inflammatory or antioxidative property (Wang et al., 2021) and therapeutic agents for degenerative orthopaedic diseases (Pei et al., 2022).

3.5. Mass balance and economic evaluation of sequential extraction of apSCT xylan and alkali lignin from raw SCT

The economic analysis for sequential extraction of apSCT xylan and alkali lignin from SCT hydrolysate produced during the alkaline pretreatment was carried out to check the feasibility of the process implementation in bioethanol industry (Fig. 1). Each product produced in the biorefinery process such as xylan and alkaline lignin was evaluated for its value addition. According to the market survey, the lowest commercial cost of 25 g of xylan and alkaline lignin is 51 and 26 USD, respectively from Tokyo Chemical Industry, Tokyo, Japan when compared with Megazyme, Wicklow, Ireland and Merck (Sigma-Aldrich), Darstat, Germany. This study also demonstrated that the extracted apSCT xylan and alkali lignin from SCT are of commercial grade and can be utilized as an alternative for commercially available substrate. In this study, only 50% of the commercial cost of xylan and alkali lignin was considered for the economic evaluation. Byproducts produced from the biorefinery process, their commercial and considered cost is shown in the Table 3. The total income of the byproducts, apSCT xylan and alkali lignin separated from 1 kg of raw SCT is calculated to be 454.9 USD. As per the economic calculations, about 2.3 USD is required to process 1 kg of raw SCT biomass. The process cost can be further

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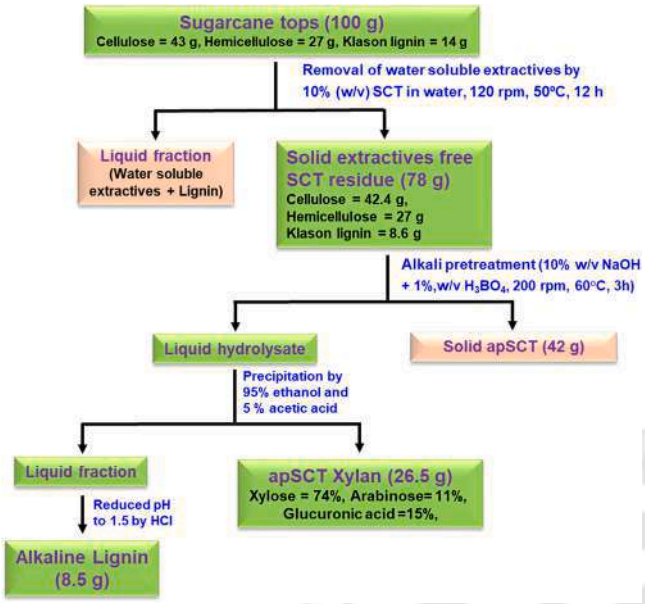

Fig. 5. Mass balance of raw SCT to apSCT xylan and alkali lignin extraction.

reduced for large scale biorefinery process by purchasing chemicals and reagents in bulk from whole sale market. The overall profit 291.6 USD can be obtained by subtracting the process cost 2.3 USD from the total income 289.3 USD. This study can contribute to the biorefinery process of raw sugarcane tops to bioethanol and other value added products such as xylan and lignin. Fig. 5.

4. Conclusions

The biorefinery approach was demonstrated in this study by sequentially extracting the apSCTx and apSCTal from alkali pretreated SCT hydrolysate. Alkaline pretreatment of SCT reduces the pretreatment processing cost for bioethanol production by recovering 98% of xylan and 61% of alkali lignin from the raw SCT. The commercial grade purity of the extracted apSCTx and apSCTal was achieved. It was amply demonstrated that xylan can be used as a commercial substrate as well as for prebiotic xylooligosaccharides production. The alkali lignin can be utilized as a potential renewable substrate for aromatic chemicals and energy production.

CRediT authorship contribution statement

Kaustubh Chandrakant Khaire: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Vijayanand Suryakant Moholkar:** Supervision, Visualization, Investigation. **Prof. Arun Goyal:** Supervision, Resources, Writing- Reviewing and Editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.115545.

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Alkaline pretreatment and response surface methodology based recombinant enzymatic saccharification and fermentation of sugarcane tops

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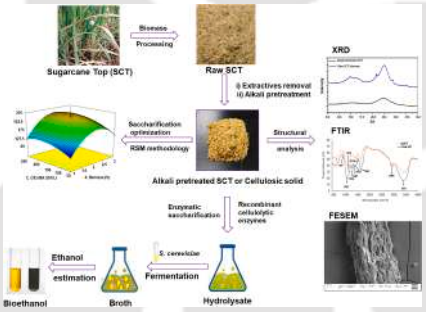
HIGHLIGHTS

- Alkali process can efficiently separate cellulose, xylan and alkali lignin from SCT.
- At optimized saccharification condition for apSCT yielded 265 mg/g reducing sugar.
- Glucose yield at optimized conditions was found to be 214 mg/g of apSCT.
- Fermentation process produced about 0.19 g of bioethanol per g of glucose.
- Fermentation efficiency of S. *cerevisiae* was found to be 37.7%

GRAPHICAL ABSTRACT



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ABSTRACT

In present study, the water-soluble extractives removal prior to alkali pretreatment of sugarcane tops (SCT) was carried out. The solid alkali pretreated SCT (apSCT) recovered on Field-emission scanning electron microscopy (FE-SEM) analysis showed exposure of cellulosic fibres as compared with raw SCT. The analyses of apSCT by Fourier Transform Infrared (FT-IR) Spectroscopy, X-ray diffraction (XRD) and High performance liquid chromatography (HPLC) analysis also confirmed the enhanced cellulose content in apSCT. Optimum conditions for response surface methodology based saccharification of apSCT at 40 °C, 150 rpm were 2.14% (w/v) apSCT loading in citrate–phosphate buffer (50 mM, pH 6.0), recombinant hydrolytic enzymes (from *Clostridium/Hungateiclostridium thermocellum*) loading for *endo*-1,4-β-glucanase (CtCel8A) = 213.2 U/g, cellobiohydrolase (CtCBH5A) = 272.5 U/g and β-glucosidase (*Ht*Bg1) = 299.8 U/g for 49.2 h. Under optimized saccharification conditions, the total reducing sugar yield was 265 mg/g (glucose 214 mg/g) of apSCT. Fermentation of produced glucose by S. *cerevisiae* gave 0.19 g/g glucose of bioethanol.

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1. Introduction

The energy demand is increasing with populace growth. There is an intensive load on the currently depleting energy resources such as coal and petroleum. The immoderate consumption of these nonrenewable petroleum fuels has caused drastic growth in the level of greenhouse gases (Castillo et al., 2020). At the end of 2050, the energy demand will increase from the existing demand of 13 to 45 billion tons of oil equivalent (Zhang, and Konan, 2010). Therefore, there is an essential need for other sustainable sources of energy and fuels to address the climate change as well as the decreasing oil supply. Bioenergy is harvested from both agricultural or forest residues. The use of renewable biomass resources like agricultural waste has appeared as a reassuring substitute for the synthesis of renewable biofuels like bioethanol, biodiesel and biogas (Thakur et al., 2020). Biodiesel and bioethanol are the two important sustainable energy sources growing as transportation fuels. Preserving the sustainable improvement, the Indian government has made compulsory the blending of 5% bioethanol in gasoline (Singh et al., 2020). Worldwide sugarcane production in 2018 was about 1.9 billion tonnes. In overall sugarcane production, Brazil contributed about 39% followed by India about 20% (Thakur and Sharma et al., 2021). The stems of sugarcane are mainly used for making the sugarcane juice and further utilized for the production of sugar. Leftover biomass i.e. sugarcane trash/ top (SCT) are usually burned in the field. The SCT contains about 43% of cellulose, 27% of hemicellulose and 19% of lignin, which can be considered as a potential raw material for the bioethanol production (Khaire et al., 2020). To avoid environmental pollution, SCT can have applications in the production of valuable byproducts like xylan, lignin, cellulose, furfural, acetic acid, lactic acid, biobutanol and bioethanol (Canilha et al., 2012, Khaire et al., 2021a). A few pretreatment strategies are promising for lignocellulosic biomass for example dilute acid, dilute alkali, ammonia fiber explosion and hydrothermal pretreatment. The reports on pretreatment of SCT biomass by biological pretreatment (Singh et al., 2008), alkali based ammonium fiber explosion pretreatment (Krishnan et al., 2010), acid pretreatment (Sindhu et al., 2011, Srinorakutara et al., 2014), surfactant-assisted ultrasound pretreatment (Sindhu et al., 2013), microwave pretreatment (Maurya et al., 2013), alkali pretreatment (Sindhu et al., 2014, Srinorakutara et al., 2014), formic acid based organosolv pretreatement (Pathak et al., 2020) and crude glycerol assisted pretreatment (Niju et al., 2020b) are available. Conventional pretreatment methods involving acids or alkali remove either hemicellulose or lignin from lignocellulosic feedstock (Kucharska et al., 2018). The hot water pretreatment at ≤ 100 °C, causes breakdown of carbohydrate-lignin complexes in biomass due to plasticizing action (Vallejos et al., 2017). Alkali pretreatment enhances the enzymatic saccharification of lignocellulosic feedstock as the majority of carbohydrates content is retained while a large fraction of lignin is get separated (Sun et al., 2016). The separation of commercial grade cellulose (Khaire et al., 2020) and xylan (Khaire et al., 2021b) from delignified SCT biomass is possible by alkaline pretreatment method and this strategy can also lower the pretreatment cost of the process.

The conversion of cellulose to glucose by enzymatic saccharification is a high-cost process and a major bottleneck for the bioethanol industry (Singh et al., 2020). The use of recombinant cellulolytic enzymes can bring the cost effectiveness to the process (Yamada et al., 2013). The rate of enzymatic saccharification reaction and the total reducing sugar yield from feedstock is dependent on the concentration/ ratio of enzyme cocktail (i.e. endoglucanase:cellobiohydrolas:β-glucosidase) used and the use of enzyme concentration relies on the feedstock components and kind of pretreatment process used (Banerjee et al., 2010; Marcos et al., 2013). Accordingly, the selection of cellulase cocktail (type, amount, a ratio of enzymes) is an important factor for the optimum yield of fermentable sugar (Zhou et al., 2009; Kim et al., 2015). A cocktail with the optimum enzyme ratio was speculated based on the rational experiment design and a hypothesis is to study the combining effects of enzymes on the saccharification of lignocellulosic feedstock (Bussamra et al., 2015; Singh et al., 2020).

The aim of this study was to examine the effect of various parameters on the saccharification of alkali pretreated SCT (apSCT) solids and to release maximum reducing sugar by using recombinant cellulolytic enzymes through response surface methodology. The produced glucose from apSCT solids can be further utilized as a substrate for the bioethanol production and exploit this abundantly available feedstock in India.

2. Materials and methods

2.1. Materials and sugarcane top collection and biomass processing

Boric acid, sodium chloride, sodium hydroxide was obtained from the Himedia Pvt. Ltd. India. Solvents like acetic acid, hydrochloric acid and butanol were acquired from the Merck India Pvt. Ltd. Sugarcane tops were collected from the locality of Guwahati, Assam, India. A collected SCT feedstock was washed by water and cut into 1–2 cm pieces. Then SCT pieces were dried in a hot air oven (Labtech, Mumbai, India) at 60 °C for 48 h. After moisture removal, the SCT biomass were milled to 0.85 mm particle size by using a grinder (Philips, HL1606/03) and stored at 25 °C.

2.2. Removal of water soluble extractives from raw SCT biomass

About 80 g of raw SCT in 800 mL of distilled water was taken in 2 L conical flask and kept at 50 °C in a shaker incubator for 12 h to remove the water-soluble extractives. The hydrolysate was separated from solid SCT biomass by filtering through cotton cloth and cellulosic solid was dried in a hot air oven at 80 °C. Separated hydrolysate was also dried in separate beaker in hot air oven at 80 °C. The separate weights of dried hydrolysate and dried solids were taken to calculate the yield of water-soluble extractives content in the raw SCT.

2.3. Pretreatment of extractives free sugarcane tops

The extractives free SCT biomass was subjected to alkaline pretreatment (Khaire et al., 2020). 20 g of extractives free SCT was added to 200 mL solution containing 1% (w/v) boric acid and 10% (w/v) sodium hydroxide (pH 13) in a 500 mL reagent bottle and kept on a magnetic stirrer (200 rpm, 60 °C) for 3 h. In alkaline pretreatment, the boric acid acts as a monobasic acid that removes OH groups and enhances the separation of xylan from cellulosic solids (Scott, 1989). After incubation, the pretreated slurry was passed through a cotton cloth and separated cellulosic solid was neutralized under tap water and dried at 80 °C for 12 h. After drying, the recovered apSCT was weighed and stored at dry place for further experiments. The total cellulose recovered in apSCT was estimated by using standard Technical Association of the Pulp and Paper Industry (1992).

2.4. Characterization of alkali pretreated SCT

2.4.1. Analysis of monosaccharide composition in apSCT

The composition analysis of apSCT biomass was examined by HPLC (UFLC, Prominence, Shimadzu, Japan). About 5 mg of apSCT biomass was taken in 2 mL Eppendorf tube containing 1 mL of Tri-fluoro-acetic acid (2 M, TFA). The reaction mixture was placed in a boiling water bath for 2 h. The hydrolyzed apSCT was centrifuged at 10000g for 5 min. The supernatant was poured into a new 2 mL Eppendorf tube and dried in a hot air oven (at 80 °C, 12 h) to evaporate the residual TFA. TFA hydrolyzed apSCT was suspended in 500 μL MilliQ water and then sieved through 0.22 μm pore size syringe (Polyvinylidene fluoride membrane) filter. The TFA hydrolyzed apSCT was analyzed for monosaccharides by HPLC by using refractive index (RI) detector. The 50 mm X 7.8 mm guard column followed by 300 mm X 7.8 mm Phenomenex Rezex ROA (H^+) column were connected to the HPLC system for analysis

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Table 1
Box-Behnken design and the response for apSCT biomass for saccharification by *endo*-1,4-β-glucanase (CtCel8A), cellobiohydrolase (CtCBH5A) and β-glucosidase (*Ht*Bg1).

Run Order	Biomass loading (%)	Time (h)	*Ct*Cel8A (U/g of biomass)	*Ct*CBH5A (U/g of biomass)	*Ht*Bg1 (U/g of biomass)	Reducing sugar yield (mg/g)
1	3	60	150	200	200	180.51
2	3	36	150	200	300	176.11
3	2	36	150	200	200	240.39
4	2	36	150	100	100	134.50
5	2	36	150	300	300	258.88
6	3	36	150	200	100	238.85
7	2	36	150	200	200	243.25
8	2	36	150	200	200	241.05
9	3	36	150	100	200	138.69
10	2	12	150	100	200	141.66
11	2	12	50	200	200	178.86
12	2	36	50	200	300	191.74
13	2	12	250	200	200	174.57
14	1	60	150	200	200	116.67
15	3	36	150	300	200	182.71
16	3	12	150	200	200	141.99
17	2	60	50	200	200	203.19
18	2	36	250	200	300	267.58
19	2	60	150	200	100	177.43
20	1	36	50	200	200	93.56
21	1	36	150	200	300	112.27
22	2	60	150	100	200	163.12
23	2	36	150	100	300	183.16
24	2	36	50	100	200	119.98
25	3	36	250	200	200	176.11
26	1	36	150	300	200	119.98
27	2	12	150	200	100	163.12
28	2	36	250	100	200	183.82
29	2	36	150	300	100	178.86
30	2	60	150	300	200	233.35
31	2	36	250	200	100	188.88
32	1	36	150	100	200	96.86
33	2	36	150	200	200	250.41
34	1	12	150	200	200	100.16
35	2	12	150	300	200	191.74
36	2	36	50	200	100	148.81
37	2	36	50	300	200	198.90
38	2	60	250	200	200	246.00
39	1	36	250	200	200	118.87
40	2	36	150	200	200	264.17
41	3	36	50	200	200	145.29
42	2	60	150	200	300	248.76
43	1	36	150	200	100	104.57
44	2	12	150	200	300	214.63
45	2	36	150	200	200	247.66
46	2	36	250	300	200	251.84

of TFA hydrolyzed apSCT. H_2SO_4 (0.005 N) was used as a mobile phase at a 0.5 mL/min flow rate and 70 °C for 90 min was used for the elution of hydrolyzed apSCT. For the analysis of monosaccharide composition in apSCT, the 1 mg/mL of each standard (glucose, xylose and arabinose) was used.

2.4.2. Functional group analysis of apSCT

The functional groups in both raw (untreated) SCT and apSCT were analyzed by using FT-IR spectroscopy (Perkin Elmer, Spectrum Two, USA) as reported earlier (Khaire et al., 2020). Raw SCT and apSCT were separately mixed with the potassium bromide in a 1:100 (w/w) ratio by using mortar-pestle and under 15-ton hydraulic press, the pellets were prepared. The FT-IR spectra of both the biomass samples were obtained in the range, 4000 to 400 cm^{-1}.

2.4.3. Field-Emission scanning electron microscopy (FE-SEM) analysis of raw SCT and apSCT biomass

The surface morphology of raw SCT and apSCT biomass was analyzed by using FE-SEM (Carl Zeiss, Model-Gemini 300, Germany). The carbon tape was fixed on the stab surface before placing the raw SCT or apSCT samples on the carbon tape. Both the samples prepared on the stab was double gold coated in the vacuum chamber to avoid the moisture gain by biomass. The double gold coated samples images were taken at 2 kx magnification by using FE-SEM analyzer for the surface morphology analysis.

2.4.4. Crystallinity index analysis of raw SCT and apSCT by X-Ray diffraction

The crystallinity index (*CrI*) of raw SCT and apSCT was measured by XRD (D8 Advance, Bruker, Germany). 1 g of each dried raw SCT and apSCT was analyzed for crystallinity index by scanning over the range of $2\theta = 10^\circ - 30^\circ$ with 0.05° step size. The *CrI* of both raw SCT and apSCT samples was calculated by (Segal et al., 1959).

$$CrystallinityIndex(\%) = \frac{(Ic - Ia)}{Ic} X100$$

Here, I_c - Intensity at degree, $2\Theta = 22^\circ$ and I_a - Intensity at a degree, $2\Theta = 18^\circ$.

2.4.5. Elemental analysis of raw SCT and apSCT solids

The elemental analysis of raw SCT and apSCT biomass was analyzed by using CHNS (CHNS: carbon, hydrogen, nitrogen and sulphur) analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Italy). The oxygen content present in raw and apSCT were calculated by using the

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formula,

$$Oxygen\% = 100\% - \sum [Carban(\%) + Hydrogen(\%) + Nitrogen(\%) + Sulphur(\%)]$$

2.5. Production and enzyme activity estimation of crude recombinant hydrolytic enzymes

The previously reported recombinant cellulolytic enzymes of *endo*-β-1,4-glucanase (*Ct*Cel8A) (Jamaldheen et al., 2018) and β-1,4-glucosidase, (*Ht*Bgl) (Sharma et al., 2019) from *Hungateiclostridium thermocellum* were used in this study. Another recombinant enzyme cellobiohydrolase, *Ct*CBH5A (a gift from Prof. Carlos M.G.A. Fontes, NZYTech Ltd., Lisbon, Portugal) was used in the present study. All mentioned recombinant enzymes were expressed by using procedure described earlier (Khaire et al., 2020). Enzyme activity of crude *Ct*Cel8A and *Ct*CBH5A enzymes were estimated by determining the released reducing sugar concentration by Nelson (1944) and Somogyi (1945) method and enzyme activity of *Ht*Bgl was estimated by GOD-POD method as reported earlier (Raabo and Terkildsen, 1960).

2.6. Optimization of saccharification conditions of apSCT by response surface methodology

The optimization of enzymatic saccharification (in terms of reducing sugar yield) of apSCT was carried out by employing the Design Expert 8.0.7.1 version (Stat-Ease, Inc., Minneapolis, USA). To determine the favorable parameters and their combinations for reducing sugar release from apSCT, a second-order Box-Behnken design (BBD) was used. Various parameters with different ranges were selected to analyze their effects on the saccharification of apSCT. The temperature, 40 °C, 150 rpm and citrate–phosphate buffer (50 mM), pH 6.0 were taken as the fixed parameters. The citrate phosphate buffer used in the saccharification process gets absorbed by apSCT at biomass loading above 3.5%. Under this condition, the biomass clumps are formed that hinders the uniform mixing. Therefore, 1–3% of apSCT biomass loading was used in this study. The apSCT biomass loading (1–3%, w/v), time (12–60 h) and recombinant enzyme concentrations (loading of endoglucanase (*Ct*Cel8A) 50–250 U/g and for cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ht*Bg1) the loading, 100–300 U/g was taken as variable parameters for the optimization experiment. The selected variables were encoded in the highest, central and lowest level as + 1, 0 and −1, respectively. All experiments were carried out in triplicate sets to check the variability of the results. For estimating the relationship in the experimental and independent responses, the second order polynomial equation was used. The encoded and decoded values for saccharification optimization are showed in Table 1. The saccharification of apSCT was done for designed experimental conditions in 2 mL final volume taken in 5 mL tube. The reaction was stopped by keeping the reaction mixture containing test tubes in the water bath (boiling) for 5 min. The resulting hydrolysate was separated by centrifugation at 10,000 g for 2 min and reducing sugar released was analyzed by Nelson (1944) and Somogyi (1945) method. The resulting monosaccharide, glucose was also estimated by GOD-POD method. The optimized conditions were validated experimentally for apSCT along with raw SCT for total reducing sugar and glucose yield. TLC analysis of raw SCT and apSCT was performed by following the previously reported procedure (Khaire et al., 2020). Cellulose conversion (%) from apSCT, based on the reducing sugar/ glucose release was calculated by using the formula as reported previously (Abdel-Fattah and Abdel-Naby, 2012).

$$Celluloseconversion(\%) = \frac{ReducingsugarorGlucoserelease}{TotalcellulosepresentinSCT} X100$$

2.7. Fermentation of hydrolysate produced from saccharification of apSCT

Hexose fermenting yeast, *Saccharomyces cerevisiae* was revived from glycerol stock at 30 °C in MGYP (malt, glucose, yeast extract and peptone) medium as reported earlier (Nedumaran et al., 2020). 5% (v/v) of *S. cerevisiae* culture inoculum was added to 100 mL of GYE (glucose, yeast extract) medium containing (in g, 1.0 glucose, 0.1 yeast extract, 0.1 KH_2PO_4, 0.05 $MgSO_4{\cdot}7H_2O$ and 0.5 $(NH_4)_2SO_4$) in distilled water (100 mL) and followed by autoclaving), pH 5.0 in 250 mL Erlenmeyer flask as a standard. In test experiment, 1 g equivalent of enzyme saccharified hydrolysate was added instead of glucose. Both the Erlenmeyer flasks were incubated in shaking incubator at 30 °C, 150 rpm for 72 h. Ethanol produced by fermentation of saccharified hydrolysate was spectrophotometrically estimated at 578 nm (A_{578}) by using dichromate method (Pilone, 1985). The ethanol yield was calculated by,

$$Ethanolyield(\frac{g}{g}ofglucose) = \frac{Ethanolproduced(\frac{g}{L})}{Glucoseproduced(\frac{g}{L})}$$

2.8. Mass balance of raw SCT biomass to fermentation process

The mass balance of untreated SCT biomass (100 g) for each processing step including removal of water extratcives, alkali pretreatement, saccharification process and fermentation process was carried out. The recovery of biomass at each step on dry weight basis was calculated by formula (Guilherme et al., 2017),

$$Biomassrecoveryyield(\%) = \frac{Recoveredcellulosefiber(g)}{InitialweightofrawSCT(g)} X100$$

3. Results and discussion

3.1. Separation of water soluble extractives from raw SCT biomass

One hundred grams of raw SCT biomass yielded 78 g of solid extractive free SCT residue after the removal of water extractives. The recovered solid residue contained 42.4 g of cellulose, 27 g of hemicellulose and 8.6 g of Klason lignin as determined by standard TAPPI protocol.

3.2. Alkali pretreatment of extractives free SCT biomass

The pretreatment of 78 g of extractives free SCT biomass after alkali pretreatment by 10% (w/v) NaOH and 1% (w/v) boric acid gave 42 g of cellulosic alkali pretreated SCT (apSCT).

3.3. Analysis of monosaccharide composition in apSCT biomass

The monosaccharide analysis of apSCT biomass by TFA hydrolysis showed the presence of glucose (87%), xylose (9%), arabinose (1.2%) and glucuronic acid (1.8%).

3.4. Surface morphology analysis of raw SCT and apSCT biomass

The FE-SEM images of raw SCT and apSCT biomass are shown in the Fig. 2A. The raw SCT biomass showed smooth and compact structure whereas the apSCT biomass showed the rough and exposed fibrous structure. These exposed fibres in apSCT appear due to hemicellulose removal and more cellulose accumulation (87%), which was also found in previously analyzed TFA hydrolyzed apSCT.

3.5. Crystallinity index analysis of raw SCT and apSCT

The raw SCT and apSCT powder displayed 36% and 47% crystallinity index (*CrI*), respectively. The 11% increase of *CrI* in apSCT as compared

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Table 2
ANOVA for quadratic model of apSCT biomass saccharification by *endo*-1,4-β-glucanase (CtCel8A), cellobiohydrolase (CtCBH5A) and β-glucosidase (*Ht*Bg1).

Source	SS	Df	Mean square	F-Value	p-value	
Quadratic model	1098	20	5491.65	18.69	< 0.0001	Significant
A-Biomass loading	14120.54	1	14120.54	40.00	< 0.0001	
B-Time	4299.90	1	4299.90	14.63	0.0008	
C- *Ct*Cel8A	6697.18	1	6697.18	22.79	< 0.0001	
D-*Ct*CBH5A	12909.21	1	12909.21	43.93	< 0.0001	
E-*Ht*Bg1	8104.45	1	8104.45	27.58	< 0.0001	
AB	121.15	1	121.15	0.41	0.5267	
AC	7.57	1	7.57	0.026	0.8738	
AD	109.34	1	109.34	0.37	0.5474	
AE	202.27	1	202.27	0.69	0.4146	
BC	554.83	1	554.83	1.89	0.1816	
BD	101.43	1	101.43	0.35	0.5621	
BE	98.13	1	98.13	0.33	0.5685	
CD	29.69	1	29.69	0.10	0.7532	
CE	319.92	1	319.92	1.09	0.3067	
DE	246.01	1	246.01	0.84	0.3689	
A^2	60226.00	1	60226.00	204.96	< 0.0001	
B^2	6295.34	1	6295.34	21.42	< 0.0001	
C^2	5827.10	1	5827.10	19.83	0.0002	
D^2	10692.68	1	10692.68	36.39	< 0.0001	
E^2	3846.07	1	3846.07	13.09	0.0013	
Residual	7346.12	25	293.84			
Lack of fit	6950.35	20	347.52	4.39	0.0540	
Pure error	395.77	5	79.15			
Model statistics						
S.D.	17.14		R^2	0.9373		
Mean	181.12		Adj-R^2	0.8872		
C.V. %	9.46		Pred-R^2	0.7579		

with the raw SCT biomass explains more exposure of crystalline cellulose, as also reported earlier (Jamaldheen et al., 2018, Khaire et al., 2020). It was reported that the crystallinity index is directly proportional to the cellulose exposure in apSCT biomass (Kian et al., 2018).

3.6. Functional group analysis of raw SCT and apSCT

As per the previous reports, the absorption peaks of cellulose were mainly found between 3660 and 2800 cm^{-1} and 1650–800 cm^{-1} wavenumber regions as reported earlier (Khaire et al., 2020). The peaks in the range of 3660 to 2900 cm^{-1} showed the stretching vibrations between C–H and/or O–H bonds of the apSCT biomass as reported earlier (Rosa et al., 2010). The stretching vibrations of C–H bonds at 2894 cm^{-1} display the overall hydrocarbon constituents present in the raw SCT and apSCT biomass as reported earlier (Jamaldheen et al., 2018). A comparatively larger peak at 897 cm^{-1} was observed in apSCT than the raw SCT biomass which is corresponding to the amorphous cellulose as mentioned earlier (Sindhu et al., 2014). A relatively large and prominent peak at 1027 cm^{-1} in apSCT than in raw SCT was observed which is the characteristic peak for C-O vibrations as mentioned in the earlier reports (Krishnan et al., 2010). This result confirmed the more cellulose exposure in apSCT as compared with the raw SCT biomass. Similarly, larger peaks at 3331 cm^{-1} and 3634 cm^{-1} were observed in apSCT than in the raw SCT, corresponding to the intra, and intermolecular O–H bond stretching, respectively, in cellulose, thus supporting the effective exposure of cellulose in apSCT. The appearance of a sharp peak at 1318 cm^{-1} can be ascribed to CH_2 bond vibrations in the apSCT cellulose which were absent in the raw SCT. A similar result was observed in ionic liquid pretreated *Asclepias syriaca* seed floss and poplar seed floss (Spiridon et al., 2011). A prominent peak at 1334 cm^{-1} in apSCT displayed the C–C and C-O bond vibrations in cellulose as mentioned in earlier studies (Sindhu et al., 2011). A peak at 1428 cm^{-1} in raw SCT corresponding to the C = C bond stretching present in lignin (phenol ring) as reported earlier was disappeared in the apSCT (Podgorbunskikh et al., 2020). A peak at 1516 cm^{-1}, characteristic of C = C bond present in guaiacyl ring of lignin clearly visible in the spectrum of raw SCT but disappeared in apSCT, as also reported earlier (Sindhu et al., 2014), thus confirming the efficient removal of lignin during alkali pretreatment. This result supported the effective removal of lignin in alkaline pretreatment of SCT. Similarly, a miniature peak at 1633 cm^{-1} observed in raw SCT was disappeared in the apSCT that was associated with the ester bonds in between the lignin and hemicellulose as reported earlier (Gil et al., 2019). These results concluded that the cellulosic fibers are exposed in apSCT and also that the lignin and hemicellulose were effectively removed.

3.7. Elemental composition analysis of raw SCT and apSCT

The raw SCT and apSCT were analyzed for elemental composition by using CHNS analyzer. The CHNS compositions of raw SCT displayed C: 45.2%, H: 7.4%, N: 2.3%, S: 1.1% and O: 44% while CHNS composition of pretreated SCT displayed C: 43.2%, H: 6.29% and O: 50.29%. The absence of sulphur and nitrogen in the apSCT confirmed the removal of protein content and lignin cross linkages as also reported earlier (Karunarathna, et al., 2019; Khaire et al., 2020).

3.8. Production of crude recombinant cellulolytic enzymes for the saccharification of apSCT

The protein concentration of expressed crude recombinant cellulolytic enzymes, *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1 enzymes as estimated by Bradford was found to be 9.2 mg/mL, 6.9 mg/mL and 5.5 mg/mL, respectively. The endoglucanase activity of crude *Ct*Cel8A and cellobiohydrolase activity of crude *Ct*CBH5A as estimated against the CMC-Na was found to be 25.3 U/mL and 4.4 U/mL, respectively. The β-glucosidase activity of crude *Ht*Bg1 enzyme showed 32.3 U/mL against its substrate, cellobiose.

3.9. Optimization of enzymatic saccharification of apSCT by response surface methodology

The parameters, biomass loading (%, w/v), recombinant enzymes i.e. *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1 loading (U/g) and time (h) were varied under designed experiments as mentioned in the material and method

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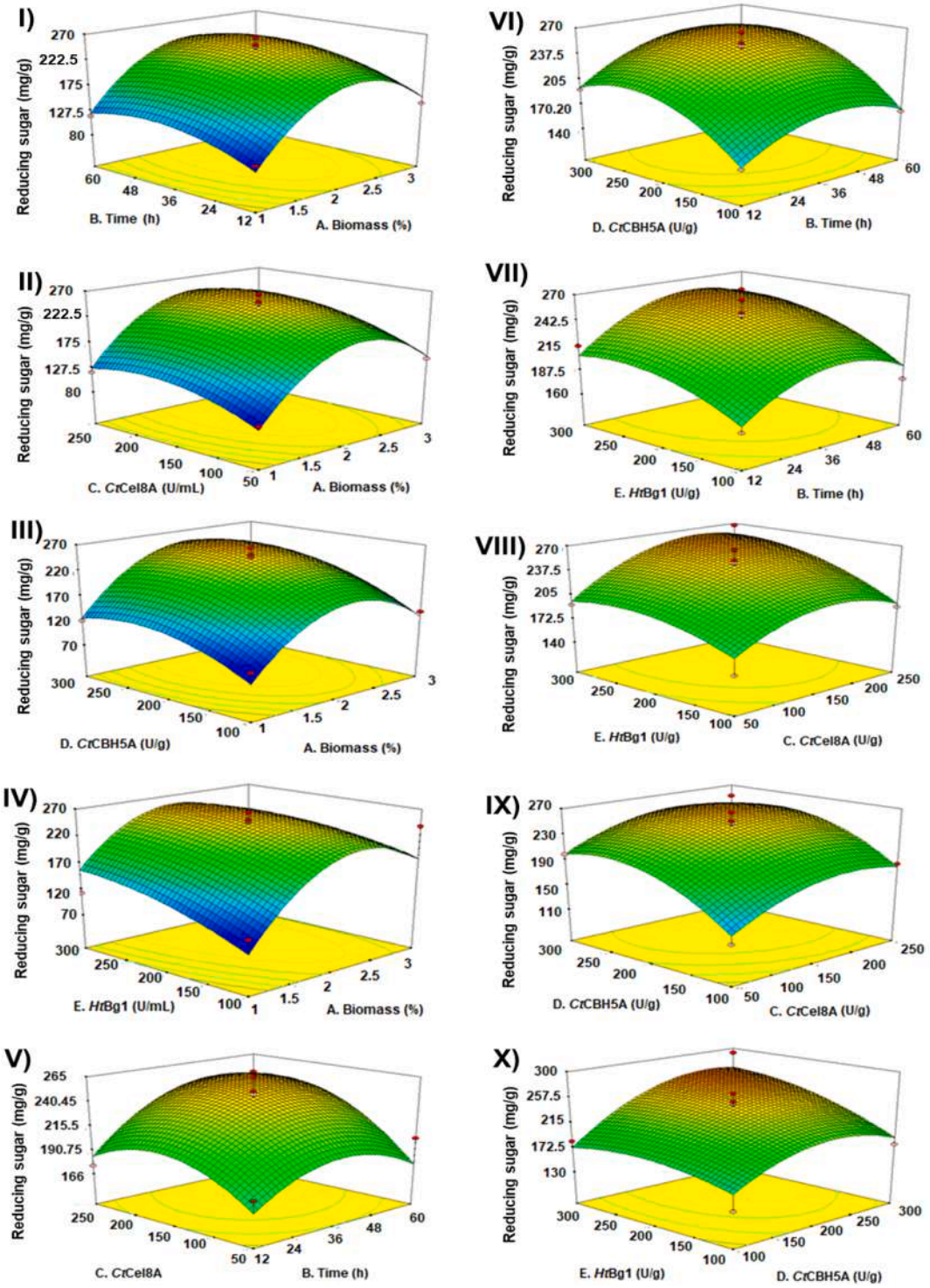

Fig. 1. 3-D surface graphs (the interactions between independent variables) of RSM based saccharification optimization of apSCT in terms of reducing sugar released (mg/g). I) Pretreated SCT loading vs Time, II) Pretreated SCT loading vs *Ct*Cel8A, III) Pretreated SCT loading vs *Ct*CBH5A, IV) Pretreated SCT loading vs *Ht*Bg1, V) Time vs *Ct*Cel8A, VI) Time vs *Ct*CBH5A, VII) Time vs *Ht*Bg1, VIII) *Ct*Cel8A vs *Ct*CBH5A, IX) *Ct*Cel8A vs *Ht*Bg1 and *Ct*CBH5A vs *Ht*Bg1.

section and the optimization was performed in citrate–phosphate buffer (pH 6, 40 °C) for apSCT. The Box-Behnken design was used for optimization of saccharification of apSCT by analyzing enzyme loading, time and biomass loading and their response, the TRS yield are shown in the Table 1. The second order equation in the terms of actual factors generated by Box-Behnken design for saccharification optimization process is,

Reducing Sugar yield (mg/g of apSCT)

$$= -516.94463 + (371.31766 \text{ X Biomass loading}) + (2.20855 \text{ X Time}) + (0.69811 \text{ X } Ct\text{Cel8A}) + (1.19606 \text{ X } Ct\text{CBH5A}) + (0.69343 \text{ X } Ht\text{Bg1}) + (0.22931 \text{ X Time X Biomass loading}) + (0.013759 \text{ X Biomass loading X } Ct\text{Cel8A}) + (0.052283 \text{ X Biomass loading X } Ct\text{CBH5A}) - (0.17611 \text{ X Biomass loading X } Ht\text{Bg1}) + (4.90724E^{-003} \text{ X Time X } Ct\text{Cel8A}) + (2.09819E^{-003} \text{ X Time X } Ct\text{CBH5A}) + (2.06379E^{-003} \text{ X Time X } Ht\text{Bg1}) - (2.72421E^{-004} \text{ X } Ct\text{Cel8A X } Ct\text{CBH5A}) + (8.94310E^{-004} \text{ X } Ct\text{Cel8A X } Ht\text{Bg1}) + (1.60976E^{-003} \text{ X } Ct\text{CBH5A X } Ht\text{Bg1}) - (81.13459 \text{ X}$$

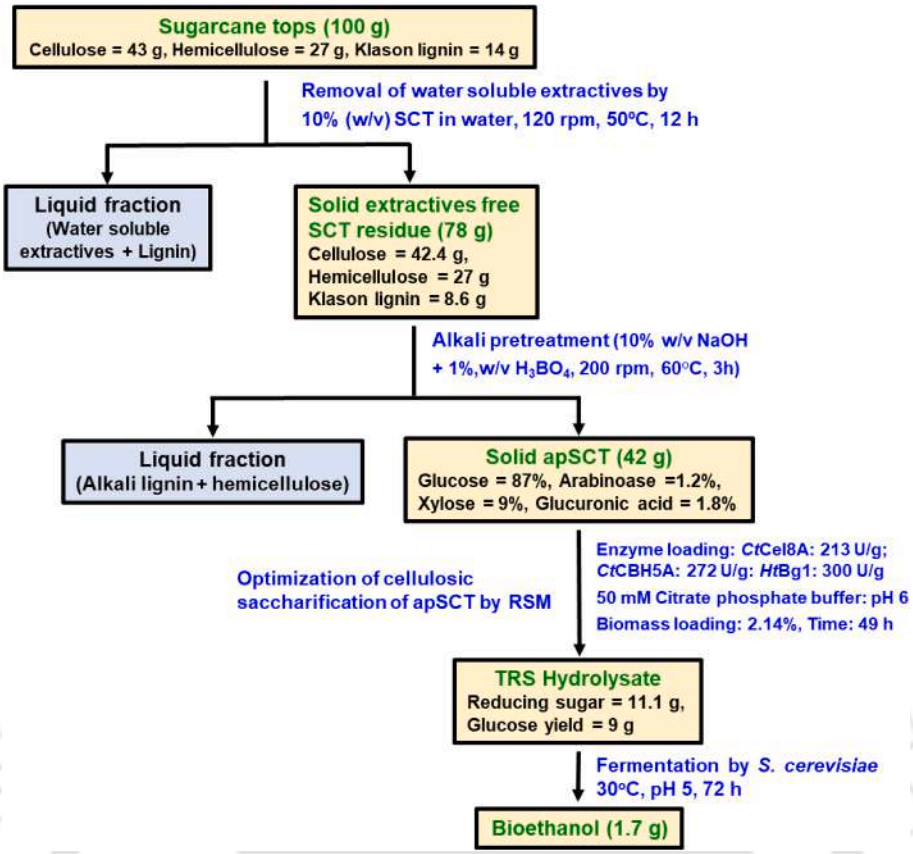

Fig. 2. Mass balance for raw SCT biomass to bioethanol fermentation of apSCT biomass.

Biomass loading^2) – (0.049342 X Time^2) – (2.74026^{E-003} X *Ct*Cel8A^2) – (3.38141E^{-003} X *Ct*CBH5A^2) – (1.63040E^{-003} X *Ht*Bg1^2)

The given quadratic model of the response data of apSCT fits well and is shown in the ANOVA Table (Table 2). The F-value (18.69) and p-value (<0.0001) of model from ANOVA test displayed that the given model terms are significant and had only a 0.01% chance of noise. P values larger than 0.1000 indicate not significant model terms. In all 5 variable factors studied, the highest F-value of biomass loading implies that this variable is most significant factor for apSCT saccharification and lowest F-value of reaction time indicates least significant variable. As per the models p values, the variables A, B, C, D, E, A^2, B2, C^2, D^2, E^2 are significant. The regression coefficient (R-Square value) of the model was found to be 0.973, moreover the predicted R-Square value of 0.7579 is in reasonable agreement with the Adjusted R-Square value of 0.8872 showed in the Table 2. The correlation of variable factors with each other for reducing sugar release is shown in the Fig. 1. Fig. 1I, 1II, 1III and 1IV are showing hyperbolic shaped 3D surface graph of apSCT biomass % vs time, *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1, respectively. The reducing sugar yield (mg/g) increased with the increase in apSCT loading up to 2.14% and then started decline (Fig. 1I, 1II, 1III and 1IV). Also, the Fig. 1V, 1VI, 1VII were showing the 3D surface graph of Time vs *Ct*Cel8A, *Ct*CBH5A and *Ht*Bg1 enzymes, respectively. Similarly, Fig. 1VIII and 1IX were showing the hyperbolic 3D graph of *Ct*Cel8A vs *Ct*CBH5A and *Ht*Bg1 enzyme and Figure X was for *Ct*CBH5A vs *Ht*Bg1. The similar hyperbolic pattern was observed for the enzymes *endo*-1,4-β-glucanase (CtCel8A) (Fig. 1II, 1 V, 1VIII and 1X), cellobiohydrolase (CtCBH5A) (Fig. 1III, 1VI, 1VIII and 1IX) and β-glucosidase (*Ht*Bg1) (Fig. 1IV, 1VII, 1IX and 1X). By analyzing the 3-D surface graphs and the ANOVA model, Box-Behnken design predicted the experimental conditions for optimum reducing sugar release from apSCT. These are 2.14% apSCT loading with 213.2 U/g *endo*-1,4-β-glucanase (CtCel8A), 272.5 U/g cellobiohydrolase (CtCBH5A) and 299.8 U/g β-glucosidase (*Ht*Bg1) for 49.2 h saccharification time. At above mentioned optimum conditions Box-Behnken design also predicted the total reducing sugar yield of 276.6 mg/g of saccharified apSCT. The experimental total reducing sugar yield upon validation by performing an experiment under predicted optimum conditions, was found to be 265 mg/g of saccharified apSCT. The glucose yield of experimental set up was also estimated by GOD-POD method and it was 214 mg/g of apSCT. The predominant spot of glucose can be seen in the TLC plate which supported the successful saccharification of apSCT biomass. The 3% (v/v) sulphuric acid pretreated SCT after saccharification by commercial cellulase (from Zytex India) resulted about 680 mg/g of reducing sugar yield reported earlier (Sindhu et al., 2011). The enzymatic hydrolysis of 3% (w/v) alkali pretreated SCT at 15% (w/w) substrate loading, 50 FPU commercial enzyme loading, 0.125% (w/w) surfactant concentration for 60 h was reported 775 mg/g of reducing sugar yield, which was much higher as compared to our study (Sindhu et al., 2014). These higher yields may due to the species of SCT, type of pretreatment and cellulolytic enzyme used. The conversion of cellulose (%) from apSCT biomass to reducing sugar released and glucose released was found to be 30.4 % and 24.6 %, respectively, which can be further used as a feedstock for the production of bioethanol.

3.10. Fermentation of saccharified apSCT biomass

Fermentation of saccharified apSCT biomass carried out by *S. cerevisiae* at 30 °C, pH 5 for 72 h, resulted in 1.02 g/L (3.4%) ethanol concentration. Around 0.19 g bioethanol was produced per gram of glucose released during the RSM based optimized saccharification process. The fermentation of optimized alkali hydrogen peroxide followed by acid hydrolysis pretreated SCT gave about 9.9% ethanol concentration in broth (Niju et al., 2020a). The dilute acid pretreatment followed by enzymatic saccharification and fermentation of SCT biomass at

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optimized conditions produced 11.36 g/L of bioethanol using *S. cerevisiae* (Sindhu et al., 2011). The glycerol assisted with transition metal followed by alkali pretreatment of sugarcane trash fermentation by *S. cerevisiae* gave 0.38 g/g of bioethanol yield per gram of pretreated sugarcane trash with 78.9% fermentation efficiency was reported earlier (Raghavi et al., 2016). These higher reported bioethanol yields could be due to the optimized fermentation conditions and depending on the biomass source and pretreatment conditions. The present study on bioethanol production by *S. cerevisiae* after saccharification optimization of apSCT resulted in 37.7% of fermentation efficiency, which was lower than those previously reported. However, there is possibility to improve the efficiency of bioethanol production by further optimizing the fermentation parameters such as inoculum percentage, substrate concentration, media composition and other physical parameters. The present pretreatment method used for the processing of the most abundant agricultural waste (SCT) can reduce the processing cost of the biomass and can serve as potential strategy for bioethanol production.

3.11. Mass balance of raw SCT to bioethanol fermentation

The mass balance of 100 g raw biomass to bioethanol production is shown in the Fig. 2. In initial water soluble extractives removal step, 78 g of solid extractives free SCT residue was recovered that contained 42.4 g cellulose, 27 g hemicellulose and 8.8 g Klason lignin. After the alkali pretreatment of extractive free SCT, 42 g of apSCT solid was recovered which contained 87% (w/w) glucose, 9% (w/w) xylose, 1.2% (w/w) arabinose and 1.8% (w/w) glucuronic acid. The RSM based optimization of saccharification of 42 g apSCT gave 265 mg of reducing sugar (or 214 mg of glucose from GOD-POD assay) from 1 g of apSCT, thereby resulting 11.1 g (w/w) reducing sugar (or 9 g, w/w glucose) released from 100 g of raw SCT. The fermentation of released glucose resulted in 19 g of bioethanol production per 100 g of glucose. This is equivalent to 1.7 g of bioethanol per 100 g of raw SCT with 37.7% fermentation efficiency. The laboratory scale economic analysis of SCT biomass to bioethanol production was carried out on the acquired data. The pretreatment cost of 1 kg raw SCT biomass is around 2.30 USD. Under the un-optimized fermentation conditions, comparatively less bioethanol yield, 17 g per kg of raw SCT was obtained. However, the bioethanol production can be improved by optimizing the fermentation parameters and by enhancing the fermentation efficiency of *S. cerevisiae*. Moreover, the recovery of the by-products from this alkaline pretreatment process such as xylan and alkali lignin can be beneficial under the biorefinery concept. The results from this study may contribute to the development of a SCT based biorefinery.

4. Conclusions

Water soluble extractive removal prior to the alkali pretreatment of SCT biomass enhanced the cellulose content in apSCT, which showed the advantage over other conventional pretreatment methods. Response surface methodology based saccharification optimization of apSCT gave 265 mg/g of TRS (214 mg/g of glucose) yield. This was in close agreement with the predicted TRS (276.6 mg/g) yield. Therefore, SCT can be used as a potential substrate for bioethanol production. Fermentation of released glucose by *S. cerevisiae* yielded 0.19 g (per g of glucose) of bioethanol with 37.7% fermentation efficiency. This can be further improved by statistical optimization of fermentation parameters.

CRediT authorship contribution statement

Kaustubh Chandrakant Khaire: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Vijayanand Suryakant Moholkar:** Supervision, Visualization, Investigation. **Arun Goyal:** Supervision, Resources, Writing – review & editing, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.125837.

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