INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

CENTRE FOR THE ENVIRONMENT

STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me at the Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati, India under the guidance of Prof. Arun Goyal and Prof. Mohammad Jawed.

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.

March, 2012 *Deepmoni Deka*

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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CERTIFICATE

This is to certify that the work described in this thesis entitled **"Bioethanol production involving cellulase producing *Bacillus subtilis* AS3 using environmentally available thatch grass *(Hyparrhenia rufa)* as weed"** by Ms Deepmoni Deka for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Centre for the Environment, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

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Deepmoni
March, 2012

CONTENTS

Chapter 1

General Introduction

Cellulose is the major polysaccharidic constituent of plant cell wall, comprising *D*-glucopyranose residues linked by β-1,4 glycosidic bonds. Cellobiose is the smallest repetitive unit of cellulose which is ultimately broken down into glucose (Fig 1.1). The individual cellulose chains contain 100 to more than 10,000 glucose units, packed tightly in parallel fashion into microfibrils by extensive inter and intra-chain hydrogen bonding interactions, which confer rigid structural stability (Haigler and Weimer, 1991). The microfibrils aggregate together in varying proportion of crystalline and amorphous forms, regulated by the degree of polymerization, extent of hydrogen bonding and source of cellulose (Wood, 1989; Beguin and Aubert, 1994; Tomme *et al*. 1995; O'Sullivan, 1997; Atalla, 1999; Bayer *et al*. 1998). It is the most abundant renewable energy source which can be potentially used for biofuel production. Cellulolytic microorganisms play an important role in the biosphere by recycling cellulose and converting it into value added products (Wood, 1989).

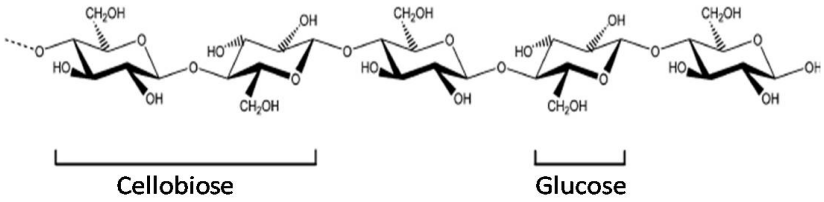

Fig. 1.1 Structure of the cellulose chain with β-1, 4 glycosidic linkages (www.afma.org/f-tutor/cellulose.htm)

1.1 Cellulose degrading enzymes and mechanism of action

1.1.1 Cellulases (EC. 3.2.1)

The structural complexity and rigidity of cellulosic substrates has given rise to remarkable divergence in cellulose degrading enzymes (Fan *et al*. 1980). Microorganisms involved in degrading these complex moieties produce a group of enzymes which are either secreted out or remain membrane bound with different specificities, and act together in synergism. Cellulases are a group of hydrolytic enzymes that hydrolyze insoluble cellulose into cellobiose and ultimately to simple soluble sugars such as glucose and cello-oligosaccharides which afford energy sources for microbial activity (Bhat and Bhat, 1997; Lynd *et al*. 2002; Sukumaran *et al*. 2005). Cellulases are modular enzymes that are composed of independently folding and structurally and functionally discrete units called domains (Henrissat *et al*. 1998; Bayer *et al*. 2004). It is composed of a carbohydrate binding domain (CBD) at the C-terminal joined by a short poly-linker region to the catalytic domain at the N-terminal. Cellulases mostly employ the retention or the inversion mechanism for the hydrolysis of glycosidic bonds (Teeri, 1997; Coutinho and Henrissat, 1999). These catalytic mechanisms involve either the retention or inversion of the configuration of the anomeric carbon, which in both cases is catalyzed by the two

carboxyl groups present at the active site of the enzyme (McCarter and Withers, 1994; Davies and Henrissat, 1995). The complete enzymatic hydrolysis of cellulosic materials is a coordinated action of 3 different enzymes comprising endoglucanase (carboxymethylcellulase, 1,4-β-d-glucan-4-glucanohydrolase;), exocellobiohydrolase (1,4-β-d-glucan glucohydrolase; avicelase), and β-glucosidase (β-d-glucoside glucohydrolase) (Fig 1.2). Endoglucanase produces nicks in the cellulose polymer exposing reducing and non-reducing ends, exoglucanase acts upon these reducing and non-reducing ends of the cellulose chains to liberate cello-oligosaccharides and cellobiose units, and β–glucosidase cleaves the cellobiose to liberate glucose (Bhat and Bhat, 1997). These enzymes can either be free, particularly in aerobic microorganisms, or grouped in a multi-component enzyme complex called as 'cellulosomes' in anaerobic cellulolytic bacteria (Gilbert and Hazelwood, 1993; Bayer *et al*. 1998; Lynd *et al*. 2002). Microbial cellulases are at present the third largest group of industrial enzymes contributing 8% of the global demand. A boost in approximately 100% in the use of cellulase is expected by 2014 if bioethanol from lignocellulosic waste is used as the major additive to the transportation fuel (Bhat, 2000; Singhania *et al*. 2010).

1.1.2 Mechanism of action of cellulase: Induction and regulation

Cellulases are inducible enzymes whose production is controlled by induction and repression mechanisms (Davies and Henrissat, 1995). All cellulolytic microorganisms are known to produce high levels of cellulase enzyme when grown on cellulose as a sole carbon source (Mandels and Reese, 1957). Other substrates such as cellobiose, lactose and sophorose are also known as inducers of cellulase activity (Mandels *et al*. 1962; Lynd *et al*. 2002; Bailey and Tahtiharju, 2003). In

Trichoderma reesei, the expression of cellulase enzyme was found to be induced only in presence of complex substrates like cellulose and its derivatives, but got repressed in presence of easily utilizable substrates such as glucose (Ilmen *et al*. 1997). The mechanism of induction of cellulase is complex because of the insoluble nature of cellulose which makes it unable to enter the microbial cell and trigger the gene. The generally accepted theory of induction of cellulase gene expression by insoluble and polymeric substrate is that, low levels of cellulase activity is constitutively produced by the microorganisms which initiates hydrolysis of cellulose to soluble sugars (Ilmen *et al*. 1997; Bhat and Bhat, 1997). These sugars are then converted into true inducers which enter the cell and either directly or indirectly influence binding protein and promote cellulase gene expression (Bhat and Bhat, 1997). In case of *Trichoderma reesei*, it has been found that the cellobiohydrolase, bound to conidia, hydrolyze the cellulose and release cellobiose and cellobiono-T-1,5- lactone (CBL), which is ultimately taken up by mycelia and promote increased cellulase gene expression and enzyme synthesis (Bhat and Bhat, 1997). Mechanism of action of cellulase in aerobic bacteria is similar to that of fungi but anaerobic bacteria *viz*. *Clostridium* sp. and *Rumionococcus* sp. assemble a multi-component enzyme complex called ‘cellulosomes’ on the cell surface mediating the adherence of bacteria to the substrate and thereby bringing about the degradation (Lamed and Bayer, 1991; Karita *et al*. 1997).

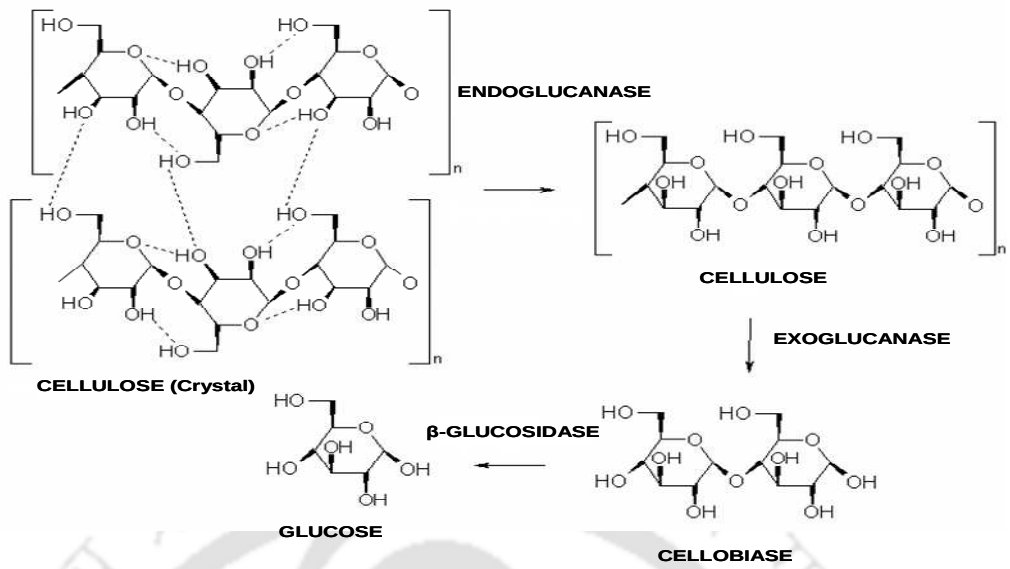

Fig. 1.2 Mechanism of cellulase action on complete hydrolysis of cellulose (www.answers.com/topic/cellulase)

1.2 Screening of microorganisms producing cellulase

Cellulase producing microorganisms have been isolated from a wide variety of sources such as composting heaps, decaying plant material, feces of ruminants, soil and extreme environments like hot-springs and marine habitat (Maki *et al*. 2009; Kuhad *et al*. 2011). Efficient plate screening methods for isolation of cellulolytic microorganisms are available. A simple and reliable method for detecting cellulase activity in microorganisms is by using carboxymethylcellulose-agar medium (Hankin and Anagnostakis, 1977; Teather and Wood, 1982). These methods are based on the formation of complex between cellulose and Congo Red dye (Teather and Wood, 1982). Recently, Kasana *et al*. (2008) found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red gave better results. However, plate-screening methods using dyes are not quantitative or sensitive enough to correlate between enzyme activity and halo size. Use of soluble and insoluble fluorescent labeled polysaccharides such as hydroxyethylcellulose-blue

(HEC- blue) and xylan red to screen bacteria having cellulase and xylanase activities enables higher sensitivity and quantification (Ten *et al*. 2004).

1.3 Cellulase production

Microbial cellulases have recently attained major significance because of their application in converting lignocellulose to bioethanol. Major limitations in their commercial application are low yield, instability and high cost of production (Bhat and Bhat, 1997). To improve the production titers, multifaceted approaches like the use of better bioprocess technologies, employing cheaper and crude raw materials as substrates and bioengineering the microorganisms, have been adopted (Lynd *et al*. 2002; Sukumaran *et al*. 2005). While majority of the suggested processes described are batch process, a few protocols like fed-batch (Wen *et al*. 2005) and continuous mode (Schafner and Toledo, 1992; Ju and Afolabi, 1999; Bailey and Tahtiharju, 2003) with improved enzyme production have also been reported.

The array of microorganisms involved in cellulase production includes protozoa, white rot and soft rot fungi (Tanaka *et al*. 2009; Shrestha *et al*. 2009; Lo *et al*. 2010), anaerobic fungi (Ljungdahl, 2008; Dashtban *et al*. 2009) and aerobic and anaerobic bacteria (Gilkes *et al*. 1991; Thirumale *et al*. 2001; Kumar *et al*. 2004; Wang *et al*. 2009). Most of the cellulases exploited commercially are from filamentous fungi such as *Trichoderma, Aspergillus, Penicillium, Fusarium, Humicola* and *Phanerochaete* which produce enzymes of high activity at moderate temperature (Javed *et al*. 2007; Ahamed and Vermette, 2008; Mathew *et al*. 2008; Bak *et al*. 2009; Lo *et al*. 2010, de Siqueira *et al*. 2010). The prime hindrance in the use of commercially available fungal enzymes is their slow growth and sensitivity to high temperature and pH (Chowdary *et al*. 2001). Additionally, absence of prominent

β-glucosidase activity causes in-efficient saccharification process (Schulein, 1988). Bacteria are now being widely explored because of their rapid growth rate, expression of multi-enzyme complexes, stability at extremes of temperature and pH, lesser feedback inhibition and capability to withstand variety of environmental stress (Gilkes, 1991; Gilbert and Hazlewood, 1993; Bhat, 2000; Kotchoni *et al*. 2003; Maki *et al*. 2009). These bacteria are aerobic, anaerobic, mesophilic, thermophilic, alkaliphilic, acidophilic and even halophilic. These strains not only have the capability to survive the harsh conditions during the bioconversion processes but produce enzymes that are stable under extreme conditions which ensure increased enzymatic hydrolysis, fermentation and product recovery (Maki *et al*. 2009). There are several reports on production of cellulases from bacteria such as *Bacillus*, *Clostridium*, *Cellulomonas*, *Ruminococcus* and *Streptomyces* spp. (Robson and Chambliss, 1989; Thirumale *et al*. 2001; Rajoka, 2004; Bayer *et al*. 2006; Nascimento *et al*. 2009; Rastogi *et al*. 2009). The cellulosome of the bacterium *Clostridium thermocellum*, in addition to having 50-fold higher specific activity than *Trichoderma* sp. against crystalline cellulose also displayed highest rate of cellulose utilization (Demain *et al*. 2005; Fontes and Gilbert, 2010).

Bacillus spp. are most sought after as they produce diverse range of cellulases that are stable under extreme conditions (Singh *et al*. 2001; Singh *et al*. 2004; Ariffin *et al*. 2006; Hirasawa *et al*. 2006; Lee *et al*. 2008; Rastogi *et al*. 2009; Rastogi *et al*. 2010; Trivedi *et al*. 2011; Annamalai *et al*. 2011). Among *Bacillus* spp. *B. subtilis* continues to be a dominant workhorse due to its capacity to secrete large quantities of extracellular cellulolytic enzymes (Aa *et al*. 1994; Mawadza *et al*. 1996; Schallmey *et al*. 2004; Li *et al*. 2008; Kim *et al*. 2009; Yin *et al*. 2010; Deka *et al*. 2011). Horikoshi *et al*. (1984) first identified a *Bacillus* strain secreting alkaline cellulase.

Bacilli have been reported to secrete alkaline cellulases which have huge potential for industrial applications (Hirasawa *et al*. 2006). *Bacillus subtilis* is an ubiquitous bacterium isolated from various sources such as soil, feces of ruminants, compost, hot spring and marine habitat (Mayendea *et al*. 2006; Rastogi *et al*. 2009; Kim *et al*. 2009; Rastogi *et al*. 2010; Yin *et al*. 2010; Deka *et al*. 2011). The bacterium produces an endospore that enables it to endure extreme conditions of heat and desiccation (Claus and Berkeley, 1986). Since it is non-pathogenic, it is safe for free handling. It is therefore, used as model organism for genetic manipulation. The genus *Bacillus* consists of a diverse group of organisms as evidenced by the wide range of DNA base ratios of approximately 32-69 mol% (G + C) content (Claus and Berkeley, 1986).

The growth dependent cellulase production is influenced by a number of process conditions like temperature, pH, aeration, medium constituents and process modes like batch, fed batch, semi-continuous and continuous (Domingues *et al*. 2000; Bailey and Tahtiharju, 2003; Wen *et al*. 2005; Jatinder *et al*. 2006; Immanuel *et al*. 2006; Ahamed and Vermette, 2008; Lo *et al*. 2010). Every organism stands apart in its requirement of process conditions and nutritional factors for maximum enzyme production. Therefore, each of them has to be considered separately and the growth requirements have to be optimized accordingly.

The fermentation temperature ranging from 30-40°C supported optimum growth and cellulase production by Bacilli (Singh *et al*. 2004; Rastogi *et al*. 2009). Temperature above and below the optimum level inhibits the cellulase activity probably due to inhibition of multi-enzyme complex system of the cell (Sohail *et al*. 2009). Optimum cellulase production from *Bacillus subtilis* subsp. *subtilis* A-53 was found to be at 30°C and pH 6.8 (Kim *et al*. 2009). *Bacillus amyloliquefaciens* DL-3

and *Bacillus subtilis* YJ1 performed better at 37°C at neutral pH (Lee *et al*. 2008; Yin *et al*. 2010). These reports differed from the finding of Ray *et al*. (2007) who recorded maximum yield at 40°C by *Bacillus subtilis* and *Bacillus circulans*. Similarly, Immanuel *et al*. (2006) also reported maximum cellulase production by *Cellulomonas, Bacillus* and *Micrococcus* sp. at 40°C and neutral pH. Singh *et al*. (2004) also recorded similar results at 40°C and pH 9.5. Mawadza *et al*. (2000) reported 70°C and 80° C as optimum temperature for growth of thermophilic *Bacillus* sp. CH43 and *Bacillus* sp. HR 68.

Surrounding medium pH is an important factor affecting cellulase activity. Most of the *Bacillus* spp. grows at pH in the range of 7.0-7.5. Ariffin *et al*. (2006) reported a combination of 37°C and pH 7.0 as optimum for *Bacillus pumilus* EB3. Similarly Immanuel *et al*. (2006) and Rastogi *et al*. (2009) reported pH 7.0 as optimum for cellulase production by *Bacillus* sp. Ray *et al*. (2007) reported pH 7.0-7.5 as optimum for growth of *Bacillus subtilis* and *Bacillus circulans*. *Bacillus* spp. are also active at alkaline pH (Kim *et al*. 2005; Hirasawa *et al*. 2006). Singh *et al*. (2001) observed that pH 9.0 favoured cellulase production and the growth and activity drastically reduced when pH dropped below 6.0. Kim *et al*. (2005) reported growth at pH 10.0 to be ideal for maximum cellulase production by *Bacillus* sp. HSH-810. Acharya and Chaudhury (2011) and Hirasawa *et al*. (2006) reported pH higher than 9.0 as optimum for cellulase production. This differed from the findings of Otajevwo and Aluyi (2010) who recorded 35°C and pH 5.0 as optimal for cellulase activity of *Bacillus subtilis* and *Bacillus circulans*.

Some *Bacillus* spp. are salt tolerant and alkaliphilic in nature. Trivedi *et al*. (2011) reported halostable cellulase producing *Bacillus flexus* which could retain 70% of enzyme activity at salt concentration of 15%. The enzyme activity of

alkaliphilic and halo tolerant *Bacillus agaradhaerens* was found to be induced in presence of NaCl (Hirasawa *et al*. 2006). Similarly Singh *et al*. (2004) reported 1.2 fold rise in cellulase activity in presence of 50mM NaCl at pH 8.0 in alkaliphilic *Bacillus sphaericus* JS1.

Agitation is one of the important culture parameters that maintains homogenous conditions, disperses dissolved oxygen into smaller bubbles thereby increasing the interfacial area and oxygen mass transfer rate for enhancing both substrate utilization and microbial activity (Singh *et al*. 2000). However, agitation speed above the optimum value did not improve the enzyme activity, which may be attributed to increased shear stress on the cells leading to reduced enzyme production (Purkarthofer *et al*. 1993). Bin and Hussaini (2006) noticed 2 fold increase in cellulase activity under shaking condition as compared to static condition in *Bacillus amyloliquefaciens* UMAS 1002. Agitation speed in the range of 120-200 rpm was found optimum for cellulase production (Mawadza *et al*. 2000; Singh *et al*. 2004; Liang *et al*. 2009; Annamalai *et al*. 2011). Rastogi *et al*. (2009) and Trivedi *et al*. (2011) reported agitation speed of 120 rpm as optimum for cellulase production by *Bacillus* spp.

Cellulases are inducible enzymes and different carbon sources play important role in affecting the enzymatic levels (Lee and Koo, 2001; Kumar *et al*. 2008). Carboxymethylcellulose (CMC) has been reported to be good cellulase inducer for different cellulolytic microbes (Domingues *et al*. 2000; Narasimha *et al*. 2006; Niranjane *et al*. 2007; Ahamed and Vermette, 2008; Annamalai *et al*. 2011). Presence of 1% CMC increased the rate of cellulase synthesis in *Bacillus* spp. (Singh *et al*. 2001; Arriffin *et al*. 2008; Kim *et al*. 2009). Similarly, Li *et al*. (2008) reported maximum cellulase activity from *Bacillus* sp., grown in medium supplemented with

1 % CMC. Other substrates such as cellobiose, lactose and sophorose are also known as inducers of cellulase activity (Mandels *et al*. 1962; Lynd *et al*. 2002; Bailey and Tahtiharju, 2003). Cellobiose and glucose have been reported to be good inducers for many cellulolytic microbes (Mandels *et al*. 1962; Rodriguez *et al*. 1996). *Brevibacillus* sp. JXL showed much higher cellulase activity when cellobiose or glucose were used as co-substrates (Liang *et al*. 2009). Lactose has also been found to enhance cellulase production by 3-4 times in *Microbacterium* sp. (Sadhu *et al*. 2011).

Among different sources of nitrogen, organic nitrogen served as a better source than the inorganic nitrogen for cellulase production (Singh *et al*. 2001; Ray *et al*. 2007; Arriffin *et al*. 2008; Jo *et al*. 2008; Abou-Taleb *et al*. 2009). Peptone was more effective than urea and ammonium nitrate for cellulase activity in *Bacillus amyloliquefaciens* DL-3 (Jo *et al*. 2008). Annamalai *et al*. (2011) found peptone to be a good source for enhancing cellulase production from *Bacillus licheniformis*. Yeast extract scored over peptone and urea for cellulase production (Arriffin *et al*. 2008; Li *et al*. 2008). Arriffin *et al*. (2008) reported 2 fold increase in cellulase production in presence of 0.2% yeast extract by *Bacillus pumilus* EB3. At 0.7% yeast extract, cellulase production was optimum in *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C2 (Abou-Taleb *et al*. 2009). Singh *et al*. (2001) reported enhanced production of cellulase by *Bacillus* sp. when it was grown in presence of tryptone. On the other hand, Rajoka (2004) found $NaNO_3$, KNO_3 and NH_4NO_3 as best nitrogen source for cellulase production from *Cellulomonas flavigena*. A 2 fold enhancement in avicelase production was reported from *Geobacillus* sp. after optimization of culture conditions using factorial design with addition of yeast extract and ammonium sulfate (Abdel-Fattah *et al*. 2007).

1.4 Optimization strategies for cellulase production

Optimization is an important step used in bioprocesses to determine the most suitable culture conditions and concentration of medium components which produce the best possible response (Weuster-Botz, 2000). Traditionally, optimization in bioprocesses is carried out by monitoring the effect of one variable at a time (OVAT) on an experimental response (Immanuel *et al*. 2006; Ray *et al*. 2007; Ariffin *et al*. 2008; Shanmughapriya *et al*. 2010). This method however, does not bring out the interaction effect of various variables on enzyme production. The conventional methods for multifactor experimental designs are time consuming and incapable of detecting the true optima (Rajoka 2004; Wang and Lu, 2005; Sohail *et al*. 2009; Acharya and Chaudhary, 2011). In order to overcome this problem, the optimization of bioprocesses is carried out by using multivariate statistic techniques (Latifian *et al*. 2007; Alam *et al*. 2008; Jahani *et al*. 2008; Nagar *et al*. 2010; Deka *et al*. 2011). Response surface methodology (RSM) is the most relevant empirical modeling technique among the statistical techniques used in analytical optimization. It is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which describes the behaviour of a data set with the objective of making statistical predictions (Box and Hunter, 1975; Salimi and Esfahani, 2009). The first step to optimize a process is to identify the factors which have shown significant effect on the process. Two level fractional factorial design developed by Plackett and Burman, (1946) has been extensively used to screen significant factors for process optimization (Ghanem *et al*. 2000; Hao *et al*. 2006; Youssef and Berekaa, 2009; Geetha and Gunasekaran, 2010; Deka *et al*. 2011). It is based on the first order polynomial model. To identify an optima with the selected variables, experimental designs for quadratic response surfaces have been used, such

as three level factorial, Box-Behnken and central composite design (Wen *et al*. 2005; Jatinder *et al*. 2006; Maeda *et al*. 2010). Among these, central composite design was employed for cellulase production (Techapun *et al*. 2002; Singh *et al*. 2009). The central composite design presented by Box and Wilson (1951) is based on the second order polynomial model. Abdel–Fattah *et al*. (2007) reported 2 fold increase in avicelase activity from *Geobacillus* sp. after optimization of medium components using Plackett-Burman and Box-Behnken designs. Statistics based experimental designs were applied to optimize the fermentation medium for the production of cellulase by *Bacillus subtilis* AS3. Seven different medium components were screened for their significance on enzyme production using Plackett-Burman factorial design. A 2^2 full factorial central composite design was used to establish the relationship between enzyme production and three medium factors namely CMC, yeast extract and peptone. The optimal levels of medium components determined were CMC (1.8%), peptone (0.8%) and yeast extract (0.479%). The maximum enzyme activity predicted by the model was 0.49 U/mL which was in good agreement with the experimental value 0.43 U/mL showing 6-fold increase as compared to unoptimized medium (Deka *et al*. 2011). Several commercial software packages like Design Expert (STAT-Ease, Inc., USA) and Minitab (Minitab, Inc., USA) are available for designing experiments using Plackett–Burman, central composite and Box-Behnken designs and their analyses.

1.5 Purification of cellulase

Various methods such as precipitation by ammonium sulphate and solvent extraction by acetone and ethanol, ultra filtration and chromatography have been used for enzyme purification from *Bacillus* sp. In general, for extracellular enzymes after separating the culture from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by means of ultrafiltration, salting out by ammonium sulfate or solvent extraction methods using acetone and ethanol (Mawadza *et al*. 2000; Huang and Monk, 2004; Singh *et al*. 2004). After concentration, further purification is achieved by various chromatographic procedures such as ion-exchange and hydrophobic interaction chromatography (CM-cellulose, DEAE-cellulose, phenyl-sepharose or DEAE-Sepharose and HiTrap Q, affinity chromatography) (Apiraksakorn *et al*. 2008) or gel filtration chromatography *viz*., sephadex, sephacryl, DEAE-sephadex (Singh *et al*. 2001; Singh *et al*. 2004; Kim *et al*. 2005; Bischoff *et al*. 2006; Kotchoni *et al*. 2006; Li *et al*. 2006; Kim *et al*. 2009; Yin *et al*. 2010; Trivedi *et al* 2011).

1.5.1 Precipitation techniques

Protein precipitates are aggregates of protein molecules large enough to be visible and collected by centrifugation. The distribution of hydrophilic and hydrophobic residues at the surface of a protein determines its solubility properties. The solubility of a protein is the result of polar interaction with the aqueous solvent, ionic interaction with salts and repulsive electrostatic interaction between like charged molecules. Salting out with ammonium sulfate is a convenient, non-denaturing way to concentrate a protein. It is achieved by dehydration of protein molecule in the microenvironment. In solution, a large number of water molecules

are bound to the sulfate ion which reduces the amount of water available to interact with the protein molecules. At a particular concentration of ammonium sulfate $(NH_4)_2SO4$, an insufficient quantity of unbound water will be available to keep a given protein species in solution, resulting in the precipitation of that protein. Material precipitating prior to 25% NH_4SO_4 saturation is generally particulate, pre-aggregated or very high molecular weight protein (Rosenberg, 1996). Many proteins can also be precipitated by the addition of water miscible organic solvents such as ethanol and acetone which displaces water from the protein surfaces resulting in decrease in solvation layer around the protein. Precipitation occurs more readily when the pH of the solution is close to the iso-electric point (pI) of the target protein. Also, a larger protein will precipitate at lower concentrations of organic solvent than a smaller protein with similar properties. Most proteins precipitate in the range of 20-50% (v/v) acetone.

1.5.2 Chromatography

Cellulase has been purified using columns containing hydroxyapatite, DEAE-cellulose, DEAE-sephadex, phenyl-sepharose, HiTrap Q, sephadex and sephacryl. Mawadza *et al*. (2000) reported purification of cellulase from *Bacillus* sp. by acetone precipitation and then subjecting it to hydrophobic interaction chromatography on phenyl-sepharose. Bischoff *et al*. (2006) reported purification by phenyl-sepharose chromatography and desalting by ultrafiltration and finally using ion exchange chromatography. Lee *et al*. (2008) and Kim *et al*. (2009) reported three steps of purification such as ammonium sulphate precipitation, HiTrap Q and two cycles of ion exchange chromatography with Mono Q HR column.

1.6 Characterization of cellulase

1.6.1 Optimum pH and stability of cellulase

Acidic and basic amino acids located on the surface of enzymes determine the net charge, charge distribution and reactivity of the catalytically active groups. State of ionization of amino acids varies with pH of the environment. The state of ionization near active site determines the overall activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990). Alteration in the state of ionization of amino acids in a protein leads to distortion of ionic bonds resulting in enzyme becoming inactive.

Most of the alkaline cellulases have optimum pH between 9.0-11.0 (Christakopoulos *et al*. 1999; Singh *et al*. 2004; Shanmughapriya *et al*. 2010). An optimum pH of 9.0 was reported for cellulase from *Bacillus* sp. KSM-N252 (Ito, 1997), *Bacillus* sp. VG1 (Singh *et al*. 2001) and *Bacillus* sp. HSH-10 (Kim *et al*. 2005). Trivedi *et al*. (2011) reported optimum cellulase activity from *Bacillus flexus* at pH 10.0 which remained stable up to pH 12.0. Annamalai *et al*. (2011) obtained optimum cellulase activity at pH 9.0 from *Bacillus licheniformis* which also remained stable up to pH 12.0 even after 1 h incubation at 80°C. Conversely, an optimum pH of 5.0 was reported for cellulases of *Bacillus* sp. CH43 and *Bacillus* sp. HR68 (Mawadza *et al*. 2000), *Bacillus* sp. AC-1 (Li *et al*. 2006) and *Bacillus* sp. DUSELR13 (Rastogi *et al*. 2010).

1.6.2 Optimum temperature and thermal stability of cellulase

The thermal stability of enzymes largely depends on its primary structure. A high content of hydrophobic amino acids provides a compact structure, whereby the enzyme does not easily get denatured with change in the external environment. In addition, disulfide bridges and other bonds in the protein result in high resistance to heat inactivation and chemical denaturation. Similarly, presence of divalent cations and polysaccharides also stabilize the molecule (Linderstrom-Lang and Schellman, 1959). Cellulases from *Bacillus* spp. have been reported to be stable showing optimum activity over temperature range of 40-80°C (Endo *et al*. 2001; Singh *et al*. 2001; Singh *et al*. 2004; Lee *et al*. 2008). An optimum temperature of 70°C was reported for cellulase of *Bacillus* sp. AC-1 and *Bacillus* sp. CH43 and retained 80% stability after incubation at 50°C for 60 min (Li *et al*. 2006; Mawadza *et al*. 2000). Rastogi *et al*. (2010) reported maximum cellulase activity at 75°C from *Bacillus* sp. retaining 75% of its initial activity after incubation at 70°C for 60 min. Trivedi *et al*. (2011) reported optimum cellulase activity from *Bacillus flexus* at 45°C. An optimum temperature of 60°C was reported from cellulase of *Bacillus subtilis* YJ1 and *Bacillus licheniformis* and retained stability even after 1 h incubation at 50°C (Yin *et al*. 2010; Annamalai *et al*. 2011). Kim *et al*. (2005) reported optimum cellulase activity from *Bacillus* sp. HSH-810 at 50°C and showed 60% of the maximum activity after incubation at 60°C for 1 h. A salt-activated endoglucanase from alkaliphilic *Bacillus agaradhaerens* JAM-KU023 showed increased optimal thermostability at 50-60°C in presence of 0.2M NaCl and pH range from 7.0-9.4 (Hirasawa *et al*. 2006). A novel thermophilic, bacterium *Brevibacillus* sp. JXL retained 50% activity after 1 h at 100°C (Liang *et al*. 2009).

1.6.3 Substrate specificity

Cellulases in general have broad substrate specificity and are active against a number of synthetic substrates (Aono *et al*. 1995; EI-Helow and EI-Ahawany, 1999; Mawadza *et al*. 2000; Singh *et al*. 2001; Yang *et al*. 2008). But, the cellulases from *Bacillus* spp. appeared to be more active against CMC (Li *et al*. 2006). Kim *et al*. (2005) and Yin *et al*. (2010) reported maximum cellulase activity against CMC from *Bacillus* sp. HSH-810 and *Bacillus subtilis* YS1 respectively, and negligible activity against avicel, cotton fibre, filter paper, xylan and p-nitrophenylglucopyranoside (p-NPG). Singh *et al*. (2004) reported maximum activity with CMC and lichenan from *Bacillus sphaericus*. Some alkaline cellulases from *Bacillus* spp. show significantly high activity towards specific substrates such as β-glucan (Mawadza *et al*. 2000) and lichenan (Singh *et al*. 2001; Ozaki and Ito, 1991; Hakamada *et al*. 2002). Mawadza *et al*. (2000) and Lee *et al*. (2008) reported significant activity with avicel and filter paper from *Bacillus* sp. HR and *Bacillus amyloliquefaciens* apart from CMC. Similarly, high level activity towards avicel, xylan and filter paper apart from CMC was reported from *Bacillus circulans* (Kim, 1995). Deka *et al*. (2011) reported a 2.8 and 3 fold rise in enzyme activity from *Bacillus subtilis* AS3 using lichenan and β-glucan as substrate as compared to CMC optimized medium. *Brevibacillus* sp. JXL found to produce cellulases active against a broad spectrum of substrates such as crystalline cellulose, CMC, xylan, cellobiose, glucose and xylose (Liang *et al*. 2009). Some of the biochemical properties of cellulases from *Bacillus* spp. are listed in Table 1.1.

Table 1.1 Biochemical properties of cellulases from *Bacillus* spp.

Strains	Optimum temp (°C)	Optimum pH	Thermo stability (°C)	pH stability	Mol wt (kDa)	Ref
Bacillus sp. VG1	65	9-10	60	7-10	85	Singh *et al.* (2001)
Bacillus sphaericus JS1	60	7.0	60	8.0	42	Singh *et al.* (2004)
Bacillus sp. HSH-810	50	9.0	50	8.0	80	Kim *et al.* (2005)
Bacillus amyoliquefaciens DL-3	50	7.0	50	4 - 9	53	Lee *et al.* (2008)
Salinivibrio sp. NTU-05	35	7.5	10-40	6.5-8.5	29	Wang *et al.* (2009)
Bacillus subtilis subsp. *subtilis* A-53	50	6.5	50	6.5	56	Kim *et al.* (2009)
Bacillus licheniformis AU01	50	9-10	70-80	11-12	33	Annamalai *et al.* (2011)
Bacillus subtilis AS3	45	9.2	20-50	------	30	In this study

1.7 Applications of cellulases

Cellulases have a wide range of applications in textile, laundry, pulp and paper, fruit juice extraction and animal feed industries (Bhat, 2000; Maki *et al.* 2009). In addition, cellulases have been implicated in saccharification of lignocellulosic biomass to fermentable sugars which can be used for the production of bioethanol, lactic acid, single cell protein and other industrially important chemicals (Sanchez and Cardona, 2005).

1.7.1 Cellulases in pulp and paper industry

In the pulp and paper industry cellulases are used to decrease the viscosity of the processed material during the pulping process (bio-mechanical pulping). This saves energy during refining and modifies fibre properties which improves drainage of recycled fibers and increases operational efficiency of paper mills (Leatham *et al.* 1990; Pere *et al.* 1995). Cellulases have also been used to release ink from fiber surfaces found in used paper material by partial hydrolysis (bio-de-inking) for reducing environmental pollution (Buchert *et al.* 1998). In addition, it is used to

improve sheet-strength property of the end-product and characterization of fibers by selective solubilization of pulp carbohydrates (Buchert *et al*. 1996).

1.7.2 Cellulases in textile and detergent industries

In textile industry, cellulases are extensively used in bio-stoning of denim as a sustainable alternative to stone-washing, which gives faded, worn and aged appearance, through careful removal of excess dye from the fabric. They are also efficient in softening the textile without causing fiber damage and ensuring maximum production of good quality garments. Short exposure to cellulases results in increased functional life of washing machines, which undergo less wear and tear (Galante *et al*. 1998). Cellulases are extensively used in the production of environment friendly washing powders. They improve the detergent performance as they restore the softness and brightness of cotton fabric by selectively removing small and fuzzy fibrils from the surface (Uhlig, 1998). The same phenomenon occurs during cellulase action in bio-polishing of fabric leading to color brightness, uniformity and smooth and glossy appearance (Cortez *et al*. 2001).

1.7.3 Cellulases in beer and wine industry

In beer brewing, cellulases are used to improve the brewing process from poor quality barely. Their function is to avoid gel formation that may cause poor filtration, slow run-off time and low extract yield (Oksanen *et al*. 1985). In wine making, they are employed to obtain better skin degradation, improved color extraction, easier must clarification, better extraction and improved quality and stability of the end product (Caldini *et al*. 1994). Cellulases are also used for

extraction and clarification of fruit and vegetable juices and production of nectars and purees (Uhlig, 1998).

1.7.4 Cellulases in animal feed

Cellulases are widely used as additives to monogastric and ruminant feed. Their role is to (i) eliminate anti-nutritional factors present in grains and vegetables, (ii) degrade certain cereal compounds to improve nutritional value of feed, (iii) supplement the digestive enzymes of animals when those are inadequate specially in the post-weaning period, (iv) improve feed conversion rate, and (v) enable the utilization of less expensive feed components (Fontes *et al*. 2004).

1.7.5 Cellulases in lignocellulose to bioethanol process

Lignocellulosic biomass forms the most abundant renewable resource but their use is limited due to lack of cost effective technologies. Cellulases have potential applications in saccharification of lignocellulosic residues to fermentable sugars which in turn can be used for production of bioethanol, lactic acid, single cell protein and other industrially important chemicals (Cherry and Fidantsef, 2003, Emtiazi *et al*. 2003, Tanaka *et al*. 2006 and Sanchez and Cardona, 2005). The strategy currently employed for microbial cellulases mediated conversion of lignocellulosic biomass to ethanol is a four step process involving (i) pretreatment of the lignocellulosic biomass residues to remove lignin and hemicellulose fraction, (ii) enzymatic hydrolysis of the cellulosic residue to generate fermentable sugars (e.g. hexose and pentose), (iii) use of a fermentative microorganism to produce alcohol from the hydrolyzed cellulosic material and finally (iv) the product purification and recovery (Kovacs *et al*. 2009; Li *et al*. 2009; Saha *et al*. 2011). However, the

commercial exploitation of lignocellulosic fermentation largely depends upon the efficient hydrolysis of lignocellulosic substrates. Efficient enzymatic hydrolysis with maximum sugar yields is the key factor for successful conversion of lignocellulosic biomass to ethanol. In addition to the recalcitrant nature of the substrate, other factors that limit cellulase efficiency during the hydrolysis are end product inhibition, thermal deactivation of the native protein, nonspecific binding to lignin (Yang and Wyman, 2004) and irreversible adsorption of enzymes to the heterogeneous substrate (Taniguchi *et al*. 2005). To improve hydrolytic efficiency, it is necessary to optimize cellulase production and develop an efficient cellulase based catalysis system (Sukumaran *et al*. 2005). Screening of autochthonous *Bacillus* spp. and genetic manipulation will enable developing 'super strains' producing more efficient cellulases (Baker *et al*. 2005).

1.8 Bioethanol production by simultaneous saccharification and fermentation

Use of bioethanol as an alternative fuel derived by lignocellulosic fermentation process will significantly reduce the consumption of crude oil and eventually the net carbon dioxide emission. Popular raw materials such as corn and sugarcane currently used for bioethanol production are less preferred owing to their primary importance as a source of food (Zaldivar *et al*. 2001). Utilization of cheaper substrates such as crop residues and farm waste would make bio-ethanol production more cost-effective. These lignocellulosic residues which contain an abundance of fermentable carbohydrates in the form of hexose and pentose polymers, have a huge potential as an alternative, renewable source for production of bioethanol and other value-added products (Wyman, 2007; Fujii *et al*. 2009).

Among all processes for bioethanol production from lignocellulosic biomass, simultaneous saccharification and fermentation (SSF) appears as a promising one. It is a one step process involving combination of enzymatic hydrolysis of cellulose with simultaneous fermentation of its main product (glucose) to ethanol in the same bioreactor, thereby reducing the infrastructure cost (Bollok *et al*. 2000; Ballesteros *et al*. 2004). SSF process first described by Takagi *et al*. (1977), scores over separate hydrolysis and fermentation in offering higher saccharification rate and ethanol yield by eliminating end product inhibition and decreased risk of contamination due to presence of ethanol. Further, it requires low enzyme loading and utilizes enzymes instead of chemicals to depolymerize structural carbohydrates like cellulose and hemi-cellulose (Olofsson *et al*. 2008; Santos *et al*. 2010; Saha *et al*. 2011). A major challenge in improving the SSF process is matching the temperature conditions required for optimum performance of the enzyme and the fermenting microorganism (Bollok *et al*. 2000). The optimum temperature for cellulase enzymes is higher than the maximum limit for most yeasts used for industrial ethanol production (Ballesteros *et al*. 2004; Kiran Sree *et al*. 2000). *Saccharomyces cerevisiae* provides a better choice for production of ethanol, due to its inherent ability to utilize various substrates, high ethanol tolerance and ability to withstand a range of metabolic inhibitions (Casey and Ingledew, 1986). While, both *Saccharomyces cerevisiae* and *Zymomonas mobilis* are known to utilize hexoses predominantly, the key enzymes namely alcohol dehydrogenase and pyruvate decarboxylase were reported to be best expressed in *Zymomonas mobilis* (Sprenger, 1996). *Zymomonas mobilis* ferments sugars by Entner–Doudoroff pathway which generates lower ATP per catabolized glucose in contrast to the classical Embden–Meyerhoff–Parnas pathway (Olofsson *et al*. 2008). As a result, lower biomass yield with higher ethanol concentration are

obtained as compared to *Saccharomyces cerevisiae*. As a consequence, in recent years research is focused on gram-negative facultative anaerobic bacterium, *Zymomonas mobilis*, as promising alternative ethanol producer because of its high sugar uptake and ethanol production, high ethanol tolerance and amenability to genetic manipulation (Santos *et al*. 2010). Ability to use hemicellulose component in biomass feedstock is vital for successful conversion of lignocellulose to bioethanol. *Saccharomyces cerevisiae* and *Zymomonas mobilis*, lack ability to ferment hemicellulose derived pentose (C5) sugars. The organisms commonly used to ferment pentose (C5) sugars are *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae* (Olofsson *et al*. 2008). *Candida shehatae* is a pentose fermenting yeast which has ability to ferment pentose sugars to ethanol by using pentose phosphate pathway. The key enzymes involved are xylose reductase (XR) and xylitol dehydrogenase (XDH). But, these microbes need microaerophilic conditions and are sensitive to inhibitors, high concentration of ethanol and low pH resulting in low ethanol yield (Hahn-Hagerdal *et al*. 1991; Chandrakant and Bisaria, 1998).

1.9 Pretreatment of lignocellulosic biomass

The major challenge to effective cellulose utilization for ethanol production is the degree of crystallinity of the lignocellulosic biomass. The lignocellulosic complex is made up of a matrix of cellulose and lignin moieties bound by hemicellulose chains. Pretreatment breaks down the matrix and makes the substrate accessible for enzymatic hydrolysis (Lee *et al*. 1994; Mosier *et al*. 2005). The basic purpose of pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity and enzymatic hydrolysis (Lynd *et al*. 2002; Kumar *et al*. 2009). Several pretreatment techniques like physical, physico-chemical

and chemical processes have been used to accelerate hydrolysis of lignocellulosic biomass (Sun and Cheng, 2002; Hendricks and Zeeman, 2009).

1.9.1 Physical pretreatment

Lignocellulosic biomass is comminuted by a combination of chipping, grinding and milling to reduce particle sizes thereby increasing the available surface area for enzymatic attack (Miller *et al*. 1976). The drawback of the process is high energy requirement. Pyrolysis is another method where lignocellulosic biomass is pretreated at temperature higher than 300°C. This results in rapid decomposition of cellulose to gaseous products and residual char (Shafizadeh and Bradbury, 1979).

1.9.2 Physico-chemical pretreatment

Physico–chemical pretreatments are more effective than physical methods. Steam explosion (autohydrolysis) is the most cost-effective and commonly used physico–chemical method (McMillan, 1994). In this method, chipped and grounded biomass is rapidly heated with high pressure steam for a short duration followed by suddenly releasing the pressure making the material undergo an explosive decompression (Grous *et al*. 1986). The duration of exposure to temperature in the range of 160–230°C during steam explosion depends upon the type of substrate. The efficiency of steam explosion is regulated by factors like temperature, residence time, particle size and moisture content (Duff and Murray, 1996). Steam explosion breaks the cellulose-hemicellulose-lignin complex and removes the hemicellulose while increasing the surface area. The drawback of the process is formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis and fermentation during downstream process. Thus, the pretreated biomass need to be

washed to remove the inhibitory material alongwith removing water soluble hemicellulose decreasing the overall saccharification yields (Sun and Cheng, 2002). This method has been recognized as the most cost-effective method for hardwood (poplar, oak, birch, maple) and agricultural residues, but less effective for softwood (pine, cedar) (Clarke and Mackie, 1987).

Ammonia fibre explosion (AFEX) is another method where lignocellulosic biomass are exposed to liquid ammonia at high temperature and pressure (Garlock *et al*. 2011). The advantage of AFEX pretreatment is that it concurrently removes lignin and hemicellulose while decrystallizing cellulose. It is more effective for herbaceous and agricultural residues than for hardwood and softwood (McMillan, 1994). Sun and Cheng (2002) reported 90% hydrolysis of cellulose and hemicellulose with AFEX pretreatment of bermuda grass and bagasse. AFEX pretreatment has the advantage of minimizing formation of cellulase inhibitors for the downstream biological processes. This is due to high temperature and pH maintained during the process resulting in higher saccharification yields. However, the method is cost intensive because of the use and recovery of ammonia (Mosier *et al*. 2005).

Carbon dioxide (CO_2) explosion is also used for pretreatment of lignocellulosic materials which uses the same principle of AFEX and steam explosion (Zheng *et al*. 1998). The CO_2 explosion results in the formation of carbonic acid which increases the hydrolysis rate but the saccharification yields are relatively low compared to steam or ammonia explosion pretreatment (Sun and Cheng, 2002).

1.9.3 Chemical pretreatment

Chemical pretreatments employ chemical agents like ozone, acids, alkalis, peroxide and organic solvents. Inorganic acids such as H_2SO_4 and HCl have also been used for lignocellulosic biomass pretreatment. Concentrated acids are powerful agents for cellulose hydrolysis, but they are toxic, corrosive and need to be recovered after hydrolysis to make the process economical and environmentally feasible (McMillan, 1994). Dilute acid pretreatment is favourable as high reaction rates were achieved improving significantly the cellulose hydrolysis (Schell *et al*. 2003). However, the process cost has been found to be usually higher than steam explosion or AFEX (Sun and Cheng, 2002). Moreover, pH has to be neutralized during the downstream processes.

Some bases can also be used for pretreatment of lignocellulosic materials. Pretreatment with dilute NaOH causes swelling, leading to an increase in internal surface area, decrease of polymerization degree and crystallinity, separation of structural linkages between lignin and carbohydrates and disruption of the lignin structure (Mosier *et al*. 2005). The effectiveness of this method depends on the lignin content of the biomass (Sun and Cheng, 2002). In general, utilization of sodium hydroxide or solvents such as ethanol or methanol (organosolv process) has been found to cause dissolution of lignin, but their high costs make these methods uneconomical for large scale operation (Lynd *et al*. 2002).

Ozone can also be used to degrade lignin and hemicellulose in many lignocellulosic materials. Ozonolysis effectively removes lignin, does not produce any toxic residues and the reaction is carried out at ambient conditions (Vidal and Molinier, 1988). However, the process is not cost effective as it requires large amount of ozone.

Phosphoric acid-acetone pretreatment is a recently introduced method (Li *et al*. 2009). It requires a temperature of 50°C at normal atmospheric pressure alongwith a combination of a nonvolatile cellulose solvent (phosphoric acid) and a volatile organic solvent (acetone). Among other methods it was found to be advantageous as it removed almost all the hemicellulose and partially lignin content, recycled both the solvents and obtained high sugar yield. This pretreatment process is an effective method for perennial plants as well as hard wood (Kim and Mazza, 2008) and produces 'clean' cellulose which facilitates enzymatic hydrolysis and yeast fermentation.

1.10 Objectives of the present study

Cellulases have been studied extensively because of their ever increasing applications in textile, laundry, pulp and paper, fruit juice extraction, animal feed industry, waste management, pollution control, medical & pharmaceutical industry, protoplast production and genetic engineering. In addition, they find use in saccharification of lignocellulosic wastes to fermentable sugars which can be used for production of bioethanol, lactic acid, single cell protein and other industrially important chemicals. Impending depletion of non-renewable energy sources and search for clean energy have generated interest in microbial cellulases involved in lignocellulose conversion to bioethanol. Commercial exploitation of cellulases has remained restricted because of their low yield, instability and high cost of production. It is therefore, necessary to look for microbes producing more cellulases with higher stability, activity and versatile applicability. Bacteria are now being widely explored for cellulase production because of their rapid growth, expression of multi-enzyme complexes, stability at extremes of temperature and pH, lesser feedback inhibition and ability to withstand variety of environmental stress. They produce cellulases that are stable under extreme conditions, during bioconversion processes, which accelerate enzymatic hydrolysis, fermentation and product recovery.

The aim of the current study was to isolate and characterize fast growing and efficient cellulase producing bacterial strains. The colonies were selected based on the zone of clearance observed on CMC agar medium after Congo red staining. Colonies showing largest CMC hydrolyzing halo were picked up and tested for enzyme activity. Gram staining, catalase and urease tests were carried out for the selected strains. The morphology of the isolates were studied using scanning electron microscope (SEM). 16S rRNA sequencing based molecular identification of the

isolates was carried out and taxonomic position was traced by constructing a phylogenetic tree.

Growth parameters of the organism like incubation time, inoculum concentration and substrate concentration requirement were studied. Optimization of medium composition and levels of pH, temperature and agitation supporting maximum yield of cellulase were ascertained using strategies like one variable at a time technique (OVAT), response surface methodology (RSM) and multi-response analysis. Purification of cellulase was carried out by salt precipitation and anion exchange chromatography using DEAE-Sepharose. Denaturing SDS-PAGE and activity staining were carried out to check the homogeneity of the enzyme and to determine its molecular weight. The purified cellulase was biochemically characterized. Reaction conditions of the enzyme such as optimum temperature and thermal stability, optimum pH, substrate specificity and kinetic parameters were studied. Scale-up of cellulase production was carried out in 2L laboratory scale bioreactor using the optimized medium and optimized physical parameters. The purified cellulase from the isolate was employed for bioethanol production from lignocellulosic biomass using Simultaneous Saccharification and Fermentation (SSF) process. Batch SSF experiments was carried out to evaluate the effective and comparative performance of isolated bacterial, fungal and a recombinant cellulase along with different fermentative microbes such as *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Candida shehatae* on bioethanol production. Three different methods for pretreatment like steam explosion, AFEX and phosphoric acid-acetone were employed during SSF process to make the substrate accessible for efficient enzymatic hydrolysis. Thatch grass (*Hyparrhenia rufa*), bamboo leaves (*Bambusa dendrocalmus*) and water hyacinth (*Eichhornia crassipes*) were selected for SSF

studies based on percent fraction of cellulose, hemicellulose and lignin present. Thatch grass is a weed collected from banks of river Brahmaputra near North Guwahati, Assam and is of no economic use. Water hyacinth a noxious weed and fast growing perennial aquatic plant found in ponds and lakes all over Assam, India. This plant is a typical menace infesting large areas of water bodies causing ecological and socio-economic problems which include diminution of biodiversity, blockage of rivers and drainage system, and depletion of dissolved oxygen adversely affecting the flora and fauna. Bamboo leaves which generally go as waste may also be used judiciously for bioethanol production. Owing to their high cellulose and hemicellulose and less lignin content and advantage of non competitor to food crops these three substrates were chosen for bioethanol production. Different combinations of SSF profile were studied using substrate loading of 1% and 5% both in shake flask and laboratory scale bioreactor.

Specific objectives of the present study

1. Screening, isolation and biochemical characterization of a microorganism producing cellulase.
2. Optimization of medium composition for enhanced cellulase production from *Bacillus subtilis* AS3 by statistical tools.
3. Effect of physical parameters *viz*., pH, temperature, agitation speed on growth and cellulase production from *Bacillus subtilis* AS3 using multi response surface methodology.
4. Enhanced cellulase production from *Bacillus subtilis* AS3 in a laboratory scale bioreactor using statistically designed medium.
5. Purification of cellulase, molecular weight determination and confirmation by activity staining.
6. Biochemical characterization of the purified cellulase such as optimum pH, optimum temperature, thermal stability, substrate concentration, substrate specificity and kinetics.
7. Screening and selection of the natural substrates for enzymatic hydrolysis aiming to bioethanol production.
8. Identification of efficient pretreatment method for the chosen biomass based on ethanol production by Simultaneous Saccharification and Fermentation (SSF).
9. Lignocellulosic ethanol fermentation involving recombinant and microbial released cellulases along with various fermentative microbes using screened biomass and the pretreatment method.
10. Scale up of the bioethanol production using optimized SSF process at bioreactor level.

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Chapter 2

Screening, identification, characterization and optimization of culture conditions of cellulase producing *Bacillus subtilis* AS3

2.1 Introduction

Search for efficient cellulose and lignin degrading microorganisms with high cellulase activity has recently attracted research to develop cost effective protocols for bioconversion of farm waste into bioethanol. The cellulolytic microbes are found in diverse habitats such as composting heaps, decaying plant material, feces of ruminants, soil and extreme environments like hot-springs and marine habitat (Maki *et al*. 2009; Kuhad *et al*. 2011). These microbes convert plant polysaccharides to simple sugars, which are then assimilated by the cell. A unique group of these organisms is associated closely with animals residing in the digestive tracts of ruminants or in the guts of wood degrading termites and worms (Davies, 1964; Wenzel *et al*. 2002).

The microorganisms capable of producing cellulases include protozoa, white rot and soft rot fungi (Tanaka *et al*. 2009; Shrestha *et al*. 2009; Lo *et al*. 2010), anaerobic fungi (Ljungdahl, 2008; Dashtban *et al*. 2009) and aerobic and anaerobic

bacteria (Gilkes *et al*. 1991; Thirumale *et al*. 2001; Kumar *et al*. 2004; Wang *et al*. 2009). Fungi like *Trichoderma, Aspergillus, Penicillium, Fusarium, Humicola* and *Phanerochaete* have been exploited commercially because they produce enzymes of high activity at moderate temperature (Javed *et al*. 2007; Ahamed and Vermette, 2008; Bak *et al*. 2009; Lo *et al*. 2010, de Siqueira *et al*. 2010). The prime hindrance in wider application of fungal enzymes is their slow growth and sensitivity to high temperature and pH (Chowdary *et al*. 2001). Bacteria are now being widely explored because of their short doubling period, expression of multi-enzyme complexes providing increased function and synergy, lesser feedback inhibition, capacity to colonize a wide variety of environmental niche and potential to produce enzymes under temperature and pH stress (Gilkes *et al*. 1991; Gilbert and Hazlewood, 1993; Bhat, 2000; Maki *et al*. 2009). Additionally, the expression system and domestication of bacteria is more easily manipulated compared to fungal cellulases. These attributes inducted search for fast growing bacteria able to synthesize active cellulases under harsh conditions (Maki *et al*. 2009).

Conventionally, bacterial species are differentiated based on phenotypic and biochemical characteristics (Rossello-Mora and Amann, 2001). Existence of enormous spontaneous diversity many times leads to ambiguity at strain and even species level (Rondon *et al.* 2000). This warranted the support of genotypic characterization to clearly christen a microbe. The molecular approach used to trace bacterial phylogeny is of major importance for species definition and identification (Fredricks and Relman, 1996; Rossello-Mora and Amann, 2001). An accurate and routinely used method in identifying bacterial species and trace phylogenetic relationships is 16S rRNA gene sequence determination. Universal existence of small subunit ribosomal RNA among bacteria along with species specific variability makes it possible to identify at the

genus or species level by comparing with the database in the public domain with the 16S rRNA sequence (Vandamme *et al*. 1996). In the present study the isolate identified by 16S rRNA sequence analysis as *Bacillus subtilis* was biochemically characterized. The isolate *Bacillus subtilis* was characterized by scanning electron microscopy, Gram staining, catalase test, urease test and 16S rRNA sequencing data analysis.

2.2 Materials and Methods

2.2.1 Chemicals, Reagents and substrates

Carboxymethylcellulose (CMC) (low viscosity, 50-200 cP) and lichenan were purchased from Sigma Aldrich (St. Louis, USA). Barley β-glucan was purchased from Fluka, Biochemika. Steam exploded bagasse (SEB) was gifted by Dr. A. J. Verma, National Chemical Laboratory, Pune, India. Thatch grass was collected from bank of river Brahmaputra near North Guwahati, Assam. Glucose, sucrose, lactose, glycerol and other reagents required for maintenance and enzyme production medium were analytical grade procured from Merck and Hi-Media Pvt. Ltd., India.

2.2.2 Screening and isolation of cellulolytic microorganism

The bacterial strain isolated from cow dung and identified as *Bacillus subtilis* by 16S rRNA sequence analysis was obtained as a gift from Prof. D. Goyal, Thapar University, Patiala, India. As per the protocol provided, cow dung samples were collected from different places of Patiala, India using alcohol-sterilized tools. Sterile distilled water was used to prepare 10% (w/v) suspension of samples and enumerated by serial dilution technique. Serial dilutions were prepared using sterilized saline water (0.85% NaCl). Aliquots of 100 µL from each of the 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread plated on nutrient agar medium and incubated at 37ºC. The bacterial colonies obtained were sub cultured on nutrient agar until pure cultures were obtained. Isolated and discreet colonies were picked up from the master plates, streaked on nutrient agar containing 0.5% (w/v) carboxymethylcellulose (CMC) and incubated at 37°C for 2-3 days (Ruijssenaars and Hartmans, 2001). Separate replica plates were prepared and stained with 0.1% Congo Red solution and left to stand for 20 min. The dye solution was then poured off and the plate destained with 1M NaCl for 15 min

(Teather and Wood, 1982). Finally, the plates were washed with water to remove unbound dye. Colonies showing larger halo were picked. The cultures were maintained on nutrient agar slants at 4°C and propagated by sub-culturing every 2 weeks. The cultures were also preserved as glycerol stock frozen at -80°C in 22% (v/v) final sterile glycerol concentration for long term storage.

2.2.3 Cellulase activity determination

The cellulase activity of all the isolated colonies was determined. The bacterial colony producing highest enzyme activity was selected and designated after identification as *Bacillus subtilis* AS3. The culture was grown using the medium as described in Section 2.2.5. Enzyme assay was carried out in 100 µL of reaction mixture containing 1.3% final CMC (65 µL of 2% CMC) in 50 mM glycine NaOH buffer (pH 9.2) along with 35 µL of cell free supematant and incubated at 45°C for 10 min. The cellulase activity was determined by estimating the liberated reducing sugar by Nelson-Somogyi procedure (Nelson, 1944; Somogyi, 1945). The absorbance was measured at 500 nm using a UV-visible spectrophotometer (Perkin Elmer, Model lambda-45) against a blank with D-glucose as standard. All the analyses were performed in triplicate. Protein concentration was determined by Lowry method using bovine serum albumin (BSA) as standard (Lowry *et al*. 1951) as described in Section 2.2.3.2. The specific activity (U/ mg) of the cell free supernatant was calculated by dividing enzyme activity (U/mL) with protein concentration (mg/mL).

2.2.3.1 Calculation of enzyme activity

One unit (U) of cellulase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar per min at 45°C in 50 mM glycine NaOH buffer (pH 9.2).

The cellulase activity was calculated as:

$$\text{Enzyme activity}\left(\text{U/mL}\right)=\frac{\Delta A_{500}\times C\times V}{180\times t\times v}=\left(\mu\text{mole/min/mL}\right)$$

ΔA_{500} = Optical density (OD) change at 500 nm

C = 1 OD equivalent glucose concentration (mg/mL) from standard plot

V = volume of the reaction mixture (mL)

t = time of reaction (min)

180 = molecular weight of glucose

v = volume of the enzyme source (mL) for reducing sugar estimation

2.2.3.2 Estimation of protein concentration

The protein content was estimated by the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as a reference and a concentration range from 25 µg/mL to 500 µg/mL used to plot a standard curve.

Reagents for Lowry method:

Reagent A : sodium hydroxide (0.4 g) and sodium carbonate (2.0 g) were dissolved in water and the volume made up to 100 mL.

Reagent B1 :2% sodium potassium tartarate.

Reagent B2 : 1% copper sulfate.

Reagent C : prepared fresh by mixing 1.0 mL of reagent B1 and 100 mL of reagent A followed by addition of 1.0 mL of reagent B2.

Phenol reagent : 1N phenol reagent.

To 0.2 mL of sample containing 0.01 mL cell free extract, 1.0 mL of reagent C was added. After 10 min, 0.1 mL of Folin's reagent was added and the optical density (OD) was measured after 30 min at 660 nm against a blank.

The concentration of protein was calculated as follows:

$$\text{Protein concentration}\,(\text{mg/mL}) = \frac{\Delta A_{600} \times C \times V}{v} = (\text{mg/mL})$$

ΔA_{660} = change in absorbance of the sample at 660 nm

C = 1 OD equivalent of BSA from standard plot (mg/mL)

V = Total volume (mL)

v = volume of the sample (mL)

2.2.4 Morphological and biochemical characterization of selected isolate AS3

Phenotypic characterization of the isolate AS3, based on morphology, Gram staining, catalase activity and urease test was carried out by standard methods (Johnson and Case, 1995; Cappuccino and Sherman, 2004). For gram staining, the bacterial smear was air dried and flooded with crystal violet solution for 30 sec. Extra stain was drained using running water and the smear treated with alcohol. The slide was then flooded with iodine solution for 30 sec, excess of this mordant was washed off with water and counter stained with safranin for 30 sec. The smear was washed with water before observing under a compound microscope.

Catalase activity test was performed by adding few drops of 3% H_2O_2 to 5 mL of 16 h grown bacterial culture (Kannan, 2002). *Weissella confusa* culture was treated with H_2O_2 under identical conditions and taken as negative control for catalase activity. Catalase test is performed to determine the ability of microorganisms to degrade hydrogen peroxide to free oxygen gas by producing catalase. Appearance of

bubbles of free oxygen gas indicates positive catalase test.

The urease test was performed by growing the selected strain in urea broth containing phenol red as pH indicator. Urease is a hydrolytic enzyme that dislodges the nitrogen and carbon bonds in urea and forms ammonia. Presence of ammonia creates an alkaline environment that causes phenol red to turn deep pink. Failure to develop pink colour indicates negative reaction (Krishna *et al*. 2008).

Shape, size and arrangement of cells was observed using Scanning Electron Microscope. The sample was prepared by centrifuging 1 mL of 16 h grown culture at 5,000 rpm for 10 min. The pellet was suspended in 1 mL of saline solution (0.85%, w/v) and fixed with equal volume of glutaraldehyde (2.5%, v/v) for 2-4 h. One drop of this was used to prepare a smear, dehydrated using graded concentration of alcohol and dried in a vacuum desiccator. This dried sample was attached to the SEM stub with double-sided tape and coated with 10 nm Au in a sputter coater (SCH 620, Leo) (Patel and Goyal, 2010). The surface of the sample was viewed at various magnifications in Scanning Electron Microscope (Leo1330 VP) operated at 10.0 kV.

2.2.5 Optimization of incubation time for cellulase production

Fermentation duration is crucial for enzyme production by microorganisms. The inoculum was prepared by taking a loopful of culture from the nutrient agar slant in a test tube containing 5 mL of nutrient broth with 2% (w/v) glucose and incubated at 37°C and 180 rpm in an incubator shaker (Daihan Labtech India Pvt. ltd, Model LSI-3016) for 16-18 h to reach optical density (OD) at 600 nm around 0.6~0.8. For enzyme production, 2% (v/v) of the fresh inoculum culture was transferred to two 250 mL erlenmeyer flasks each containing 50 mL modified minimal medium containing (g/L): carbon source CMC, (10); K_2HPO_4, (1); $MgSO_4.7H_2O$, (0.25); $FeSO_4.7H_2O$,

(0.25); $MnCl_2.4H_2O$, (0.5); peptone, (5) and yeast extract, (5) (Kawai *et al*. 1988). The pH was adjusted to 7.0 using 1N NaOH before autoclaving. The flasks were incubated at 37ºC with shaking at 180 rpm. 1.0 mL of sample were drawn at every 6 h interval till 72 h. The samples were centrifuged at 10,000***g*** for 10 min at 4°C to separate the cells. The cell free supernatant was analyzed for enzyme activity (U/mL) and protein concentration (mg/mL) as described in Section 2.2.3 from which specific activity (U/mg) is calculated.

2.2.6 Optimization of inoculum concentration

The bacterial culture used to inoculate the fermentation medium must be healthy, active and contain fully grown cells. erlenmeyer flasks containing 50 mL minimal medium fortified with 1% (w/v) CMC were inoculated with 0.5, 1.0 and 1.5 mL of overnight grown culture to obtain 1%, 2% and 3% (v/v) inoculum concentration, respectively. The inoculated culture was incubated at 37ºC with shaking at 180 rpm. All experiments were carried out in triplicate. After 48 h, 1.0 mL sample from each flask was withdrawn and centrifuged at 10,000***g*** for 15 min at 4°C. The cell free supernatant was analyzed for enzyme activity (U/mL) and protein concentration (mg/mL) as described in Section 2.2.3 from which specific activity is calculated (U/mg). Since the inoculum at 2% concentration resulted in maximum enzyme activity, further studies were conducted at this inoculum concentration.

2.2.7 Effect of different cellulosic substrates on cellulase production

The effect of different carbon sources on cellulase production was evaluated by separately growing the bacterial isolate *B. subtilis* AS3 in modified minimal medium (Kawai *et al*. 1988) supplemented with 1% (w/v) of CMC, lichenan, barley β-glucan, glucose, sucrose, lactose, glycerol, steam exploded bagasse (SEB) and thatch grass. The inoculum at 2% of overnight grown culture of AS3 was added to 50 mL of modified minimal medium and incubated at 37°C with shaking at 180 rpm. All the experiments were carried out in triplicate. After 48 h, 1.0 mL from each flask was centrifuged at 10,000***g*** for 15 min at 4°C and supernatant was analyzed for enzyme activity and protein concentration as described in Section 2.2.3 from which specific activity (U/mg) is calculated.

2.2.8 Optimization of the CMC concentration

To ascertain the optimum concentration of CMC supporting maximum production of cellulase, 1 mL of fresh inoculum (2% v/v) was added to 250 mL erlenmeyer flask containing 50 mL of modified minimal medium (Kawai *et al*. 1988) supplemented with different concentrations (%, w/v) of CMC ranging from 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0. The culture flasks in triplicate were then incubated at 37ºC with shaking at 180 rpm. After 48 h, 1.0 mL from each flask was withdrawn and centrifuged at 10,000***g*** for 15 min at 4°C and the supernatant analyzed for enzyme activity and protein concentration as described in Section 2.2.3 from which specific activity is calculated.

2.2.9 Effect of different nitrogen sources on cellulase production

The effect of different nitrogen sources on the growth and cellulase production of the isolate AS3 was evaluated by replacing the nitrogen source with 0.5% (w/v) each of peptone, tryptone, yeast extract, ammonium chloride and ammonium sulphate. Other experimental details were same as mentioned in Section 2.2.7. After 48 h, 1.0 mL broth sample was withdrawn, centrifuged at 10,000***g*** for 15 min at 4°C and supernatant was analyzed for enzyme activity and protein concentration as described in Section 2.2.3 from which specific activity is calculated.

2.3 Results and Discussion

2.3.1 Screening and isolation of cellulolytic microorganism

Based on the diameter of the clear zone produced after 48 h incubation, five discrete colonies were picked up and designated as AS1, AS2, AS3, S4 and S5. These isolates were examined for enzyme activity. Among these, isolate AS3 was selected for further studies as it showed the biggest clear zone and had highest enzyme activity (Fig. 2.3.1, Table 2.3.1). The isolate formed light brown flat colonies with irregular margin.

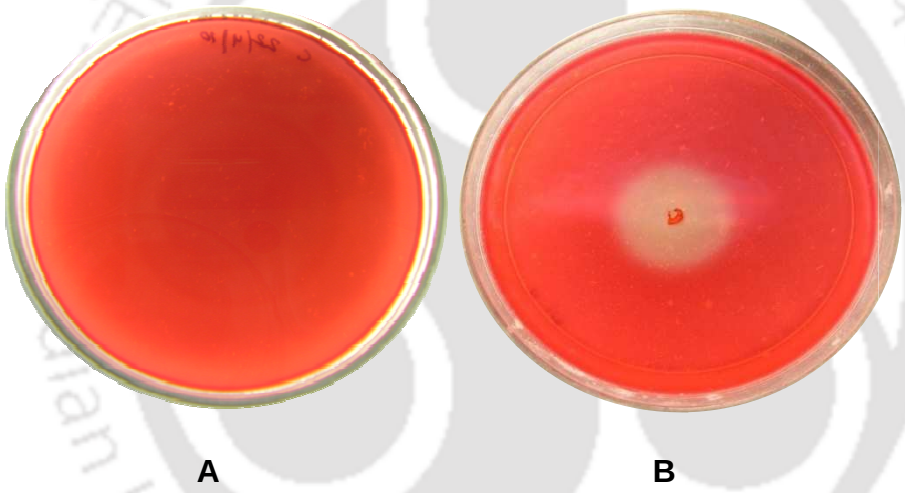

Fig. 2.3.1 Screening for cellulolytic activity of isolate AS3 after staining with 0.1% Congo Red. Plates containing nutrient agar medium with 0.5% carboxymethylcellulose incubated at 37°C. (A) Control without culture (B) Replica plate with AS3 isolate showing zone of clearance.

2.3.2 Selection of isolate based on cellulase activity

Each of the isolate was grown in enzyme production minimal medium containing 1% (w/v) CMC (Kawai *et al*. 1988) and incubated at 37°C for 48 h with shaking at 180 rpm. The cell free supernatant was analyzed for enzyme activity and protein concentration as described in Section 2.2.3. As mentioned in previous section, AS3 displayed comparatively higher enzyme activity as compared to the other isolates (Table 2.3.1). This isolate AS3 was selected and used for further characterization and studies.

Table 2.3.1 Cellulase enzyme activity of the selected isolates

Isolate	Specific activity (U/mg)
AS1	0.018 ± 0.02
AS2	0.032 ± 0.04
AS3	0.058 ± 0.01
S4	0.008 ± 0.05
S5	0.012 ± 0.04

**Values are mean ± SE (n=3)*

2.3.3 Morphological and biochemical characterization of isolate AS3

Morphological and biochemical data of the isolated strain AS3 indicated that the strain was aerobic, motile and gram positive which are characteristic of *Bacillus* spp. (Fig. 2.3.2 A). The cells appeared rod shaped under scanning electron microscope measuring 1.23 µm (Fig. 2.3.2 B). The isolate tested positive for catalase (Fig. 2.3.3A), oxidase, nitrate reduction and bile and negative for urease (Fig. 2.3.3 B), citrate utilisation and Voges-Proskauer (VP) reaction (Table 2.3.2).

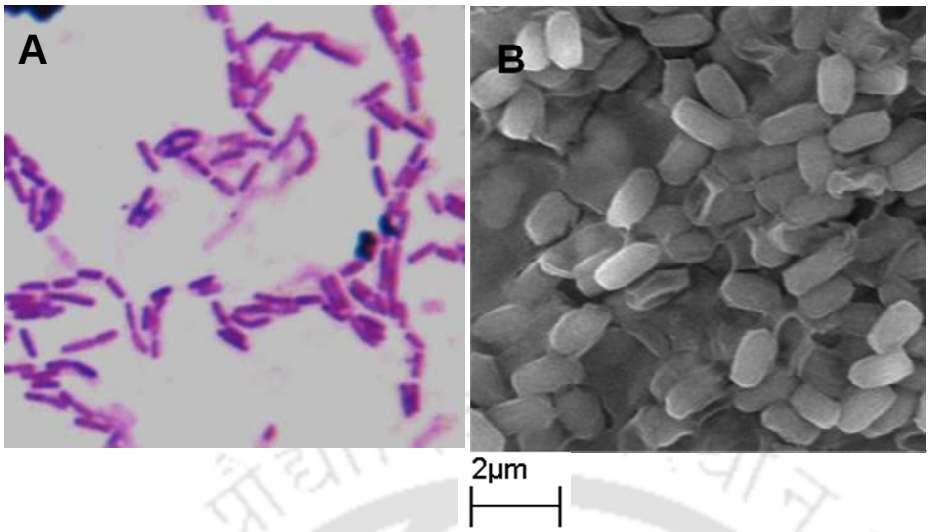

Fig. 2.3.2 (A) Gram staining (B) Scanning Electron Microscope image of the isolate AS3 showing gram positive and rod shaped appearance. (Scale-2 µm)

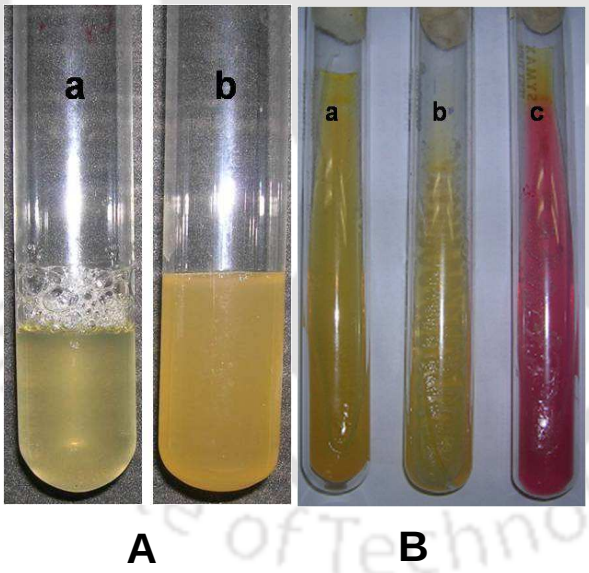

Fig. 2.3.3 (A) Catalase test showing positive nature: (a) isolate AS3 (b) *Weissella confusa*. (B) Urease test of the isolate AS3 showing urease negative nature: (a) control medium without culture (b) isolate AS3 and (c) *Proteus* sp (positive control).

Table 2.3.2 Biochemical and growth characteristics of isolate AS3

Characteristics/biochemical tests	
Colony colour	Light brown
Gram reaction	+
Morphology	Rod shaped
Growth on McConkey's agar	+
KOH	-
Oxidase	+
Catalase	+
NO_3 reduction to NO_2	+
Citrate utilisation	-
Bile Esculin	+
Voges-Proskauer (V-P) reaction	-
Urease	-

+, positive reaction; -, negative reaction

16S rRNA sequence of the isolate AS3 was compared with 16S rRNA sequences from other reference bacteria obtained from National Centre for Biotechnological Information (NCBI) genebank (http://www.ncbi.nlm.nih.gov) and Ribosomal Database Project (RDP). The phylogenetic analysis based on 16S rRNA gene sequence showed maximum homology with *Bacillus subtilis*.

Catalase activity test proved the catalase positive nature of the isolate as it could hydrolyse H_2O_2 to free oxygen gas indicated by vigorous effervescence (Fig. 2.3.3 A). It did not show urease activity as it did not change the colour of pH indicator phenol red to deep pink (Fig. 2.3.3B). Since the strain was catalase positive and urease negative, it was christened as *Bacillus subtilis* AS3 and deposited with genebank of NCBI data library under accession number EU754025.

2.3.4 Optimization of incubation time for cellulase production

Cellulase production occurred during the late growth phase of the organism after about 20 h of cultivation and reached maximum at 48 h when the organism entered early stationary phase. After this stage the enzyme activity remained static up to 60 h and declined thereafter (Fig. 2.3.4). Similar results were also reported for *Bacillus* spp. strains where maximal production was achieved after 2 to 3 days (Ray *et al*. 2007; Annamalai *et al*. 2011).

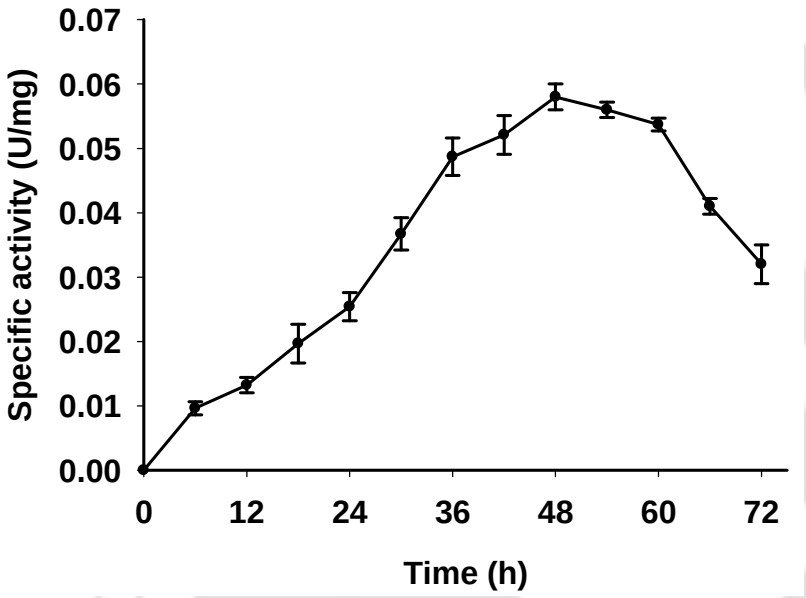

Fig. 2.3.4 Effect of incubation time on cellulase production by *Bacillus subtilis* AS3. *values are mean ± SE (n=3).*

2.3.5 Optimization of inoculum concentration

The enzyme production from *Bacillus subtilis* AS3 was maximum at 2% (v/v) of inoculum concentration compared to 1% and 3% (v/v) (Fig. 2.3.5). Ray *et al*. (2007) reported 2% inoculum concentration as best for cellulase production from *Bacillus subtilis* and *Bacillus circulans*.

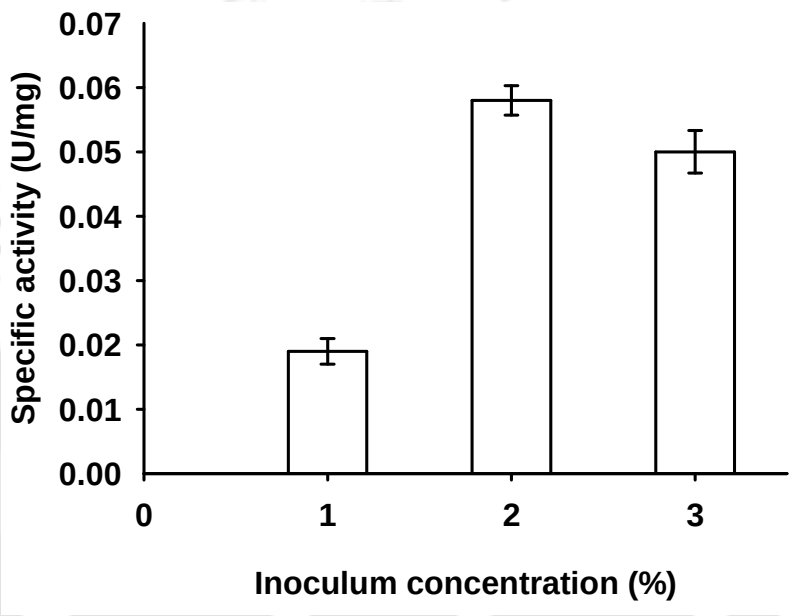

Fig. 2.3.5 Effect of inoculum concentration (%) on cellulase production by *Bacillus subtilis* AS3. *values are mean ± SE (n=3).*

2.3.6 Effect of different cellulosic substrates on cellulase production

The cellulase production was significantly influenced when cellulosic substrates with different linkages were used at 1% as substitute of CMC. It was observed that barley β-glucan (0.4 U/mg) and lichenan (0.35 U/mg) supported significantly higher activity by *B. subtilis* AS3 as compared to CMC (0.06 U/mg), simple monosaccharides such as lactose (0.045 U/mg), glycerol (0.037 U/mg), glucose (0.031 U/mg), sucrose (0.018 U/mg) and cellulosic substrates thatch grass (0.025

U/mg) and steam exploded bagasse (0.02 U/mg) (Fig. 2.3.6). There are reports of production of lichenase like β-glucanase by some strains of *Bacillus* sp. (Shikata *et al*. 1990). Most of the reports available found CMC as the good cellulase inducer for a variety of cellulolytic microbes (Domingues *et al*. 2000; Narasimha *et al*. 2006; Niranjane *et al*. 2007; Ahamed and Vermette, 2008; Annamalai *et al*. 2011). The presence of 1% CMC was found to increase the rate of cellulase synthesis by *Bacillus* spp. (Singh *et al*. 2001; Ariffin *et al*. 2008; Kim *et al*. 2009).

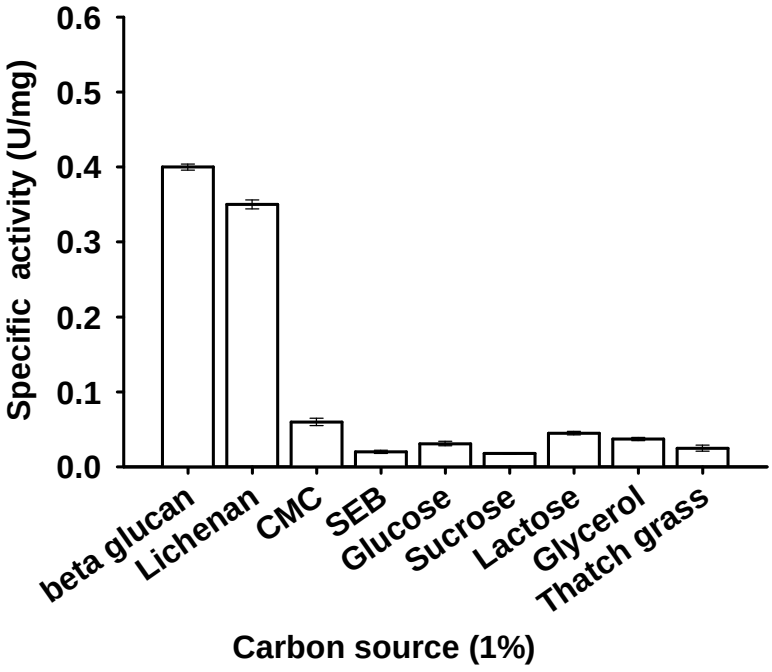

Fig. 2.3.6 Effect of carbon sources on cellulase production by *Bacillus subtilis* AS3. *values are mean ± SE (n=3).*

2.3.7 Optimization of CMC concentration for growth of *Bacillus subtilis* AS3

It was observed that 2% (w/v) CMC elicited highest specific activity of cellulase (0.08 U/mg) from *B. subtilis* AS3 (Fig. 2.3.7). Similar reports are available where 2% CMC was optimum for maximum cellulase production (Kim *et al*. 2009; Trivedi *et al*. 2011).

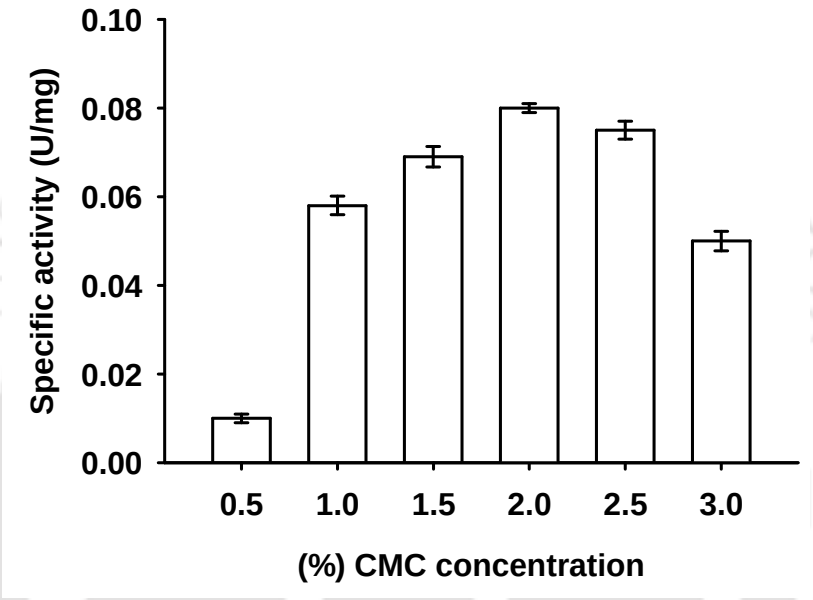

Fig. 2.3.7 Effect of CMC concentration on cellulase production by *Bacillus subtilis* AS3. *values are mean ± SE (n=3)*

2.3.8 Effect of nitrogen sources on cellulase production

The maximum cellulase activity was observed in presence of peptone followed by tryptone and yeast extract while inorganic nitrogen sources such as ammonium nitrate and ammonium sulphate had the least effect (Fig. 2.3.8). Moreover, addition of peptone and yeast extract together had synergistic effect on cellulase production. Similar results are reported earlier where organic nitrogen served better than inorganic nitrogen for cellulase production (Singh *et al*. 2001; Ariffin *et al*. 2008; Jo *et al*. 2008). Ariffin *et al*. (2008) reported 2 fold increase in cellulase production in presence of

0.2% yeast extract and ammonium sulphate by *Bacillus pumilus* EB3. Better enhancement of cellulase activity was observed in *Bacillus amyloliquefaciens* DL-3 with the addition of peptone as compared to urea and ammonium nitrate (Jo *et al*. 2008). On the other hand, Rajoka (2004) found $NaNO_3$, KNO_3 and NH_4NO_3 as best nitrogen sources for cellulase production from *Cellulomonas flavigena*. Singh *et al*. (2001) reported tryptone as the best nitrogen source for cellulase production in *Bacillus* sp.VG1.

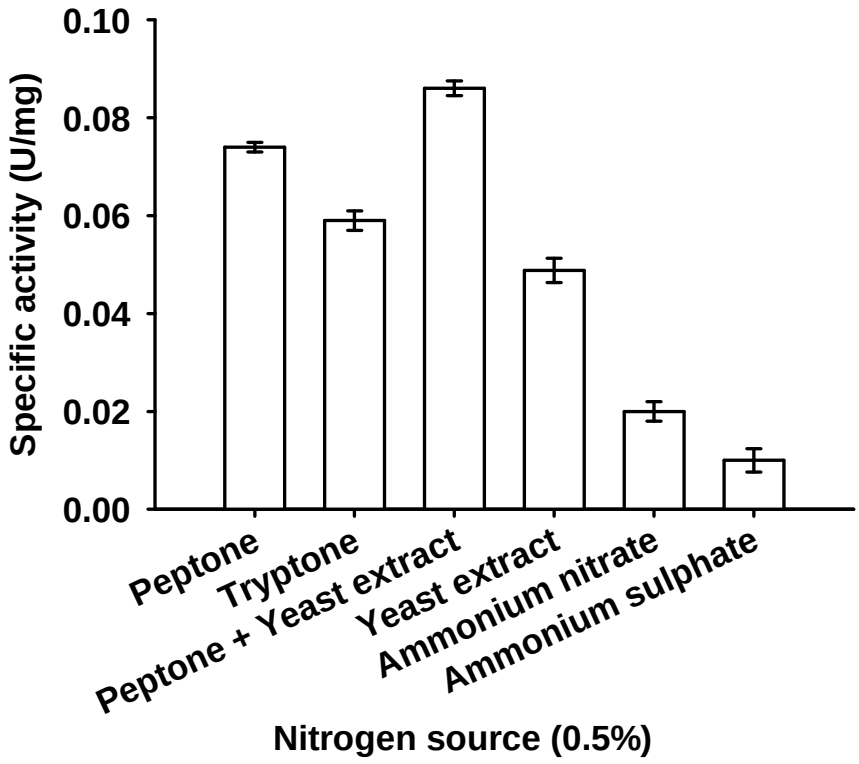

Fig. 2.3.8 Effect of nitrogen sources on cellulase production by *Bacillus subtilis* AS3. *values are mean ± SE (n=3)*.

2.4 Conclusions

Morphological and biochemical characterization of an organism are pre-requisites before embarking upon investigating it for possible commercial exploitation. Strain AS3 isolated from cow dung was found to be an efficient cellulose degrader showing a large clear zone in CMC containing plates after Congo Red staining. The strain also showed high cellulase activity. The isolate proved to be Gram positive and rod shaped and tested positive for catalase, oxidase, nitrate reduction and bile esculin and negative for urease, citrate utilisation and Voges Proskauer (VP) reaction. 16S rRNA gene sequence analysis after PCR amplification showed maximum homology with *Bacillus subtilis*. The isolated strain has been deposited in genebank of NCBI data library under accession no EU 754025. A phylogenetic tree was constructed based on 16S rRNA sequences to trace the genetic relationships of this isolate with its neighbours. The isolate was designated as *Bacillus subtilis* AS3.

Growth conditions for cellulase production were studied and compared with earlier reports. Inoculum concentration at 2% (v/v) with incubation for 48 h gave maximum cellulase production. CMC at 2% (w/v) showed maximum enzyme activity. Agro wastes like thatch grass and bagasse when used as sole carbon source, appreciably supported cellulase activity. Peptone in combination with yeast extract had synergistic effect on cellulase production. With the ability to effectively produce efficient cellulase in presence of variety of substrates and cultivation stress, the strain *Bacillus subtilis* AS3 holds out promise for commercial application, paving the way to economical production of bioethanol and other value added products in an ecofriendly way.

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Chapter 3

Optimization of medium composition for enhanced cellulase production from *Bacillus subtilis* AS3 by Response Surface Methodology

3.1 Introduction

Search for cheaper sources of economically important cellulases has recently gained momentum because of their application in the production of biofuels from farm wastes. Low yield and stability of the enzyme and difficulty in handling the conventionally used organisms like fungi led to the initiation of studies on searching and developing microbes with rapid growth and enhanced production of highly active and stable cellulases even under hostile conditions. Medium recipe plays a vital role in enhancing the enzyme production. Even small improvements can be decisive for commercial success. Development of a suitable and economical production medium requires proper selection of quality and quantity of carbon, nitrogen, phosphorous, potassium and trace elements. The proportion and interaction between these ingredients has a marked effect on the ultimate production of the cellulase. Quite a few studies were conducted earlier on the influence of medium components on cellulase production (Prasetsan and Doelle, 1987; Domingues *et al*. 2000; Rajoka, 2004; Narasimha *et al*. 2006; Ariffin *et al*. 2008; Shanmughapriya *et al*. 2010).

Statistical methodologies are generally preferred over the conventional one variable at a time technique because they help in clearly deciphering the interaction among the variables and determining their optimal levels (Pham *et al*. 1998; Ghanem *et al*. 2000; Abdel-Fattah *et al*. 2007; Alam *et al*. 2008; Han *et al*. 2009; Rashid *et al*. 2009; Youssef and Berekaa, 2009; Geetha and Gunasekaran, 2010; Deka *et al*. 2011).

Response surface methodology (RSM) is a collection of mathematical and statistical technique based on the fit of a polynomial equation to the experimental data, which describes the behaviour of a data set with the objective of making statistical predictions (Box and Hunter, 1975). It can be effectively used to study the effects of factors and ascertaining optimum levels of parameters for desired responses. Statistical methods like Plackett-Burman Design, Box-Behnken Design and Central Composite Design are often used to optimize culture medium (Plackett and Burman, 1946; Box and Wilson, 1951).

In the present study, production of an alkaline extracellular cellulase by *Bacillus subtilis* AS3 isolated from cow dung, was enhanced by medium optimization using response surface methodology. The optimization was carried out through a stepwise strategy including: (1) screening the most significant factors affecting enzyme production using a two level multifactorial Plackett-Burman design (2) optimization of the most significant components and generating a mathematical model expressing the relationship between optimized factors and cellulase production by application of central composite design and (3) verification of the model by monitoring the experimental production pattern. The effects of different carbon sources like lichenan and barley β-glucan were also tested as alternatives to carboxymethylcellulose (CMC) in optimized medium.

3.2 Materials and Methods

3.2.1 Microorganism and reagents

Bacillus subtilis AS3 (Genebank accession No. EU754025) was a gift from Prof. D. Goyal, Thapar University, Patiala, India. Carboxymethylcellulose (CMC) (low viscosity, 50-200 cP) and lichenan were purchased from Sigma Aldrich (St. Louis, USA). Barley β-glucan was purchased from Fluka, Biochemika. Ingredients required for the maintenance and enzyme production medium were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of high purity grade.

3.2.2 Sterilization and aseptic techniques

All culture media were sterilized by autoclaving at a steam pressure of 10.3 kPa (15 lb/in^2) at 121°C for 20 min. All inoculum preparations and culture transfers were carried out under aseptic conditions using laminar air flow chamber (Clean Air systems, Model CAH 1200).

3.2.3 Maintenance and sub-culturing

The culture was maintained on nutrient agar slants containing (g/L): beef extract (1.0), yeast extract (2.0), peptone (5.0), NaCl (5.0) and agar (20.0), and sub-cultured every 2-3 weeks.

3.2.4 Inoculum preparation and production of cellulase

The inoculum was prepared by taking a loop full of bacterial growth from agar slant and suspending it in a test tube containing 5 mL of nutrient broth with 2% glucose. The culture tubes were incubated at 37°C and 180 rpm in an incubator

shaker for 16-18 h to reach optical density (OD) at 600 nm around 0.6~0.8. For enzyme production, 1 mL of fresh inoculum from these tubes was transferred to 250 mL erlenmeyer flask containing 50 mL modified minimal medium (Kawai *et al*. 1988) containing CMC as carbon source and other components as per the Plackett-Burman design (Table 3.3.1) and Central composite design presented in Table 3.3.3. The pH was adjusted to 7.0 using 1N NaOH before autoclaving. For all the experimental runs the culture flasks were maintained in triplicate and incubated at 37°C with shaking at 180 rpm. After 48 h, 1.0 mL samples were collected, centrifuged at 10,000***g*** for 10 min at 4ºC and supernatant analyzed for enzyme activity.

3.2.5 Assay of enzyme activity

Assay of cellulase was carried out in 100 µL of reaction mixture containing 1.3% final concentration of CMC (65 µL of 2% CMC) in 50 mM glycine NaOH buffer (pH 9.2) and 35 µL of cell free supernatant and incubated at 45°C for 10 min. The cellulase activity was measured by estimating the liberated reducing sugar by the Nelson-Somogyi procedure (Nelson, 1944; Somogyi, 1945). Absorbance was measured at 500 nm using a UV-visible spectrophotometer (Perkin Elmer, Model Lambda-45) against a blank with D-glucose as standard. All the analyses were done in triplicate. The assay procedure is described in Chapter 2, Section 2.2.3.

3.2.6 Optimization procedure and experimental design

3.2.6.1 Screening of significant medium components by Plackett-Burman design

Plackett-Burman factorial design was employed for screening significantly influencing medium components to maximize cellulase production from *Bacillus*

subtilis AS3. Seven parameters namely CMC, K_2HPO_4, $MnCl_2.4H_2O$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, yeast extract and peptone were used for screening at –1 for low level and +1 for high level (Plackett and Burman, 1946). Table 3.2.1 shows the factors investigated and level of each factor used in the experimental design. Table 3.3.1 depicts the design matrix of the experiment. Plackett–Burman experimental design is based on the first-order polynomial model:

$$Y = \beta_o + \sum \beta_i X_i \qquad \text{(eq. 3.1)}$$

where Y, is the response (enzyme activity), β_o is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable.

Table 3.2.1 Experimental variables at different levels in Placket-Burman design for cellulase production in g/L

Variables	Symbol	Experimental values (g/L)	
		Low level (–1)	High level (+1)
CMC	X_1	2	18
Peptone	X_2	2	8
Yeast Extract	X_3	1	9
K_2HPO_4	X_4	0.5	2
$MgSO_4.7H_2O$	X_5	0.05	0.45
$FeSO_4.7H2O$	X_6	0.05	0.45
$MnCl_2.4H_2O$	X_7	0.01	0.1

This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. A total of 20 experiments were carried out in duplicate and the average of the cellulase activity was taken as the response (Table 3.3.1). The significance of each variable was determined using student's *t*-test with the help of statistical software package

MINITAB (Release 15.1, PA, USA). From the regression analysis the variables, which were significant at or above 95% level ($P < 0.05$) were considered to have greater impact on cellulase activity and further optimized by central composite design.

3.2.6.2 Central composite design (CCD) and statistical analysis

The most significant medium components were selected according to Plackett-Burman design and further optimized using central composite design (CCD) to determine the quadratic effect and two-way interaction among these variables. A 2^2 full-factorial CCD with three medium constituents, CMC, peptone and yeast extract at five coded levels, was generated by MINITAB statistical software. The experimental plan consisted of 20 runs ($=2^k + 2k + n_0$) where 'k' was the number of independent variables and n_0 the number of replicate runs at centre point of the variables. Fourteen experiments were run with six replications at the center points to evaluate the pure error. Table 3.2.2 shows the range and levels of these three factors where the levels (-1, 0 and +1) of these culture conditions were chosen in such a way that the center point values (0) represented the factor levels mostly reported in literature used for cellulase production. On the basis of the centre point values the low (–1) and high (+1) levels of the culture condition were determined in such a step change that, the centre point remain middle values of the low (–1) and high (+1) range of these factors. Furthermore, as per CCD to test all these factors in five ranges including coded value + 2 and – 2, thus the uncoded values of these respective factors were calculated by solving the following equation:

$$x_i = \frac{X_i - X_o}{\Delta X_i} \qquad i=1,2,3.....K \qquad \text{(eq. 3.2)}$$

Where, x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change. Where, default constant α = 1.682 for three factor as per the CCD design was taken.

Table 3.2.2 Experimental range and levels of independent variables in g/L

Variable	Symbol	Range and levels (g/L)				
		–2	–1	0	1	2
CMC	X_1	2.0	5.24	10.0	14.76	18
Peptone	X_2	2.0	3.22	5	6.78	8
Yeast extract	X_3	1.0	2.62	5.0	7.38	9.0

For fitting the experimental results by response surface regression procedure the following second order polynomial equation was used:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \qquad \text{(eq. 3.3)}$$

Where, Y is the predicted response, k is the number of factor variables, X_i and X_j are independent variables, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

Statistical analysis of the data was carried out by design package Design Expert 7.0 to evaluate the analysis of variance (ANOVA) and determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model. In order to verify the accuracy of the predicted model an experiment was conducted with original and optimized media.

3.2.6.3 Experimental validation of the optimized conditions

The statistically optimized medium composition for cellulase production was confirmed and validated experimentally by triplicate fermentation runs in 250 mL erlenmeyer flask containing 50 mL optimized medium and incubated at 37ºC with shaking at 180 rpm (as described in Section 3.2.4). After 48 h, 1.0 mL sample from each flask was centrifuged at 10,000***g*** for 10 min at 4°C and supernatant was analyzed for enzyme activity as described in Chapter 2, Section 2.2.3.

3.2.7 Effect of different cellulosic substrates on cellulase production

The culture was grown under same conditions except replacing 1% CMC with 1% lichenan or 1% barley β-glucan in unoptimized and 1.8% CMC replaced with 1.8% lichenan or 1.8% barley β-glucan in the optimized medium. 1.0 mL of samples were withdrawn at every 12 h interval till 60 h. The samples were centrifuged at 10,000***g*** for 10 min at 4°C and supernatant was analyzed for enzyme activity. The enzyme assay was carried out in 100 µL of reaction mixture containing

final concentration of 1.3% lichenan or 1.3% barley β-glucan in 50 mM glycine NaOH buffer (pH 9.2) and 35 µL of cell free supernatant and incubated at 45°C for 10 min as described in Section 3.2.5. The cellulase activity was calculated as described in Chapter 2, Section 2.2.3.

3.3 Results and Discussion

3.3.1 Screening of the significantly influencing medium components by Plackett-Burman design.

The experimental response (cellulase activity) embodied in Table 3.3.1 indicated that there was a wide variation ranging from 0.003 to 0.25 U/mL in twenty trials. This variation reflected the importance of medium optimization to obtain higher yield. Analysis of regression coefficients and *t*-value of seven ingredients are shown in Table 3.3.2. Generally, a large *t* value associated with a low *P* value of a variable indicates a high significance of the corresponding model term. CMC, peptone, yeast extract, K_2HPO_4 and $MnCl_2.4H_2O$ had a positive effect on enzyme production whereas; $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ proved negative. The variables with confidence levels greater than 95% were considered as significant. CMC was significant at 100% confidence level while peptone and yeast extract were found significant at 99 and 99.7% levels, respectively, for cellulase production (Table 3.3.2). None of the components had significant negative effect. Neglecting the variables which were insignificant, the model equation for cellulase activity can be written as:

$$Y_{activity} = 0.107817 + 0.083852\ X_1 + 0.020703\ X_2 + 0.024881\ X_3 \qquad \text{(eq. 3.4)}$$

Where, X_1 = CMC, X_2 = peptone, X_3 = yeast extract

CMC showed strong positive effect on enzyme production. It has been reported to be a good cellulase inducer for different cellulolytic microbes (Domingues *et al*. 2000; Ahamed and Vermette, 2008; Annamalai *et al*. 2011). The +1 level of CMC was chosen at 1.8% as higher concentration caused increased

viscosity, making separation of cells difficult. Organic nitrogen sources such as yeast extract and peptone were chosen, as these have been reported to significantly affect the cellulase production (Jo *et al*. 2008; Li *et al*. 2008). Ariffin *et al*. (2008) reported 2 fold increase in cellulase production in presence of 0.2% yeast extract by *Bacillus pumilus* EB3. Peptone was found to be a good source for enhancing cellulase production from *Bacillus licheniformis* (Annamalai *et al*. 2011).

Table 3.3.1 Plackett-Burman design in coded units for seven variables along with the cellulase activity (U/mL)

Run Order	CMC (X_1)	Peptone (X_2)	Yeast extract (X_3)	K_2HPO_4 (X_4)	$MgSO_4.7H_2O$ (X_5)	$FeSO_4.7H_2O$ (X_6)	$MnCl_2.4H_2O$ (X_7)	Enzyme activity (U/mL)
1	+1	–1	–1	–1	–1	+1	–1	0.084 ± 0.01
2	–1	–1	–1	–1	+1	–1	+1	0.003 ± 0.04
3	–1	+1	+1	+1	+1	–1	–1	0.080 ± 0.02
4	+1	–1	+1	+1	–1	–1	–1	0.226 ± 0.05
5	–1	–1	–1	+1	–1	+1	–1	0.004 ± 0.03
6	+1	+1	+1	+1	–1	–1	+1	0.252 ± 0.01
7	+1	+1	–1	+1	+1	–1	–1	0.221 ± 0.01
8	–1	+1	+1	–1	+1	+1	–1	0.026 ± 0.04
9	+1	–1	+1	+1	+1	+1	–1	0.205 ± 0.02
10	–1	–1	–1	–1	–1	–1	–1	0.006 ± 0.05
11	–1	+1	+1	–1	–1	–1	–1	0.049 ± 0.01
12	+1	+1	–1	–1	+1	+1	–1	0.168 ± 0.03
13	+1	–1	–1	+1	+1	–1	+1	0.102 ± 0.02
14	+1	–1	+1	–1	+1	+1	+1	0.197 ± 0.01
15	–1	–1	+1	–1	+1	–1	+1	0.024 ± 0.05
16	–1	+1	–1	+1	+1	+1	+1	0.003 ± 0.01
17	+1	+1	–1	–1	–1	–1	+1	0.214 ± 0.01
18	+1	+1	+1	–1	–1	+1	+1	0.249 ± 0.05
19	–1	+1	–1	+1	–1	+1	+1	0.025 ± 0.03
20	–1	–1	+1	+1	–1	+1	+1	0.022 ± 0.01

values are mean ± SE (n=3).

Pareto chart (Fig. 3.3.1) displays the absolute value of the effect of variables, which are important in the analysis of results from the design experiment. It draws a reference line (2.18) to indicate that the variables were significant with α value of 0.05. The variables which extend past this line were known to be significant at particular α (Strobel and Sullivan, 1999). On the basis of the calculated *t*-values, CMC, peptone and yeast extract had the most significant effect on the cellulase activity and were chosen for further optimization by CCD (Table 3.3.2). All other variables used in all the trials were kept at median level.

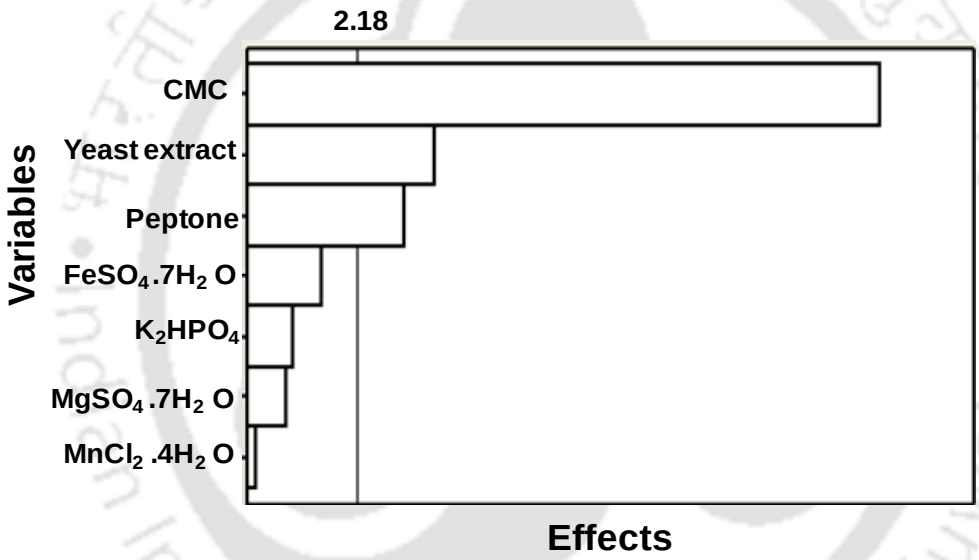


Fig. 3.3.1 Pareto chart showing the effect of nutrients on cellulase production by *Bacillus subtilis* AS3. α=0.05

Table 3.3.2 Statistical analysis of Plackett-Burman design showing coefficient values, *t* and *P* value for each variable

Variable	Coefficient	*t*-value	*P*-value	Confidence level (%)
Intercept	0.107817	15.96	0.000	100
CMC (X_1)	0.083852	12.41	0.000	100 [a]
Peptone (X_2)	0.020703	3.06	0.01	99 [a]
Yeast Extract (X_3)	0.024881	3.68	0.003	99.7 [a]
K_2HPO_4 (X_4)	0.005974	0.88	0.394	60.6 [b]
$MgSO_4.7H_2O$ (X_5)	–0.005107	–0.76	0.464	53.6 [b]
$FeSO_4.7H_2O$ (X_6)	–0.009733	–1.44	0.175	82.5 [b]
$MnCl_2.4H_2O$ (X_7)	0.001163	0.17	0.866	13.4 [b]

[a] Significant; [b] non-significant at $p > 0.1$
$R^2 = 93.76\%$, *values are mean ± SE (n=3)*

3.3.2 Optimization of medium components by CCD

At the end of screening experiments by Plackett-Burman design three factors were found to play a significant role in cellulase production. The respective low and high levels of each variable along with the CCD design with response (U/mL) are given in Table 3.3.3.

The results of the second-order response surface model fitting in the form of ANOVA are given in Table 3.3.4. To test the fit of the model equation, the regression based determination coefficient R^2 was evaluated. The nearer the values of R^2 to 1, the model would explain better for variability of experimental values to the predicted values (Khuri and Cornell, 1987). The model presented a high determination coefficient (R^2 = 0.9911) explaining 99% of the variability in the response (Table 3.3.4).

Table 3.3.3 Full factorial central composite design matrix of three variables in coded units with experimental and predicted response

Run No.	CMC (X_1)	Peptone (X_2)	Yeast extract (X_3)	Enzyme activity (U/mL)	
				Observed	Predicted
1	0	0	0	0.271 ± 0.12	0.270
2	0	0	0	0.271 ± 0.08	0.270
3	+1	–1	+1	0.330 ± 0.06	0.341
4	–1	–1	–1	0.123 ± 0.21	0.122
5	0	0	0	0.272 ± 0.18	0.270
6	0	0	0	0.261 ± 0.04	0.270
7	–2	0	0	0.119 ± 0.2	0.118
8	1	–1	–1	0.306 ± 0.09	0.313
9	0	0	0	0.274 ± 0.15	0.270
10	–1	–1	+1	0.208 ± 0.01	0.221
11	–1	+1	+1	0.214 ± 0.07	0.215
12	0	0	0	0.271 ± 0.18	0.270
13	+ 2	0	0	0.431 ± 0.23	0.422
14	+1	+1	+1	0.377 ± 0.06	0.385
15	+1	+1	–1	0.371 ± 0.01	0.364
16	0	–2	0	0.283 ± 0.03	0.269
17	–1	+1	–1	0.128 ± 0.18	0.123
18	0	0	+2	0.292 ± 0.03	0.276
19	0	+2	0	0.302 ± 0.11	0.307
20	0	0	– 2	0.168 ± 0.01	0.175

values are mean ± SE (n=3)

The coefficients of regression were calculated and the following regression equation was obtained.

$$Y_{activity} = 0.270187 + 0.151937\ X_1 + 0.019066\ X_2 + 0.050437\ X_3 + 0.000199\ X_1^2 + 0.017596\ X_2^2 - 0.045086\ X_3^2 + 0.035305\ X_1X_2 - 0.049972\ X_1X_3 - 0.005799\ X_2X_3 \quad \text{(eq. 3.5)}$$

where, Y = response (cellulase activity), X_1 = CMC, X_2 = peptone and X_3= yeast extract in coded values.

The statistical significance of Eq (3.5) was checked by *F* test, the results of ANOVA are shown in Table 3.3.4. The results demonstrated that the model is highly significant, and is evident from Fischer's *F*-test with a very low probability value (*P* model > *F* = 0.0000) (Table 3.3.4).

Table 3.3.4 Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of cellulase activity

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	0.133841	9	0.014871	123.94	0.000
Residual (error)	0.001200	10	0.000120		
Lack of fit	0.001087	5	0.000217	9.66	0.013
Pure error	0.000113	5	0.000023		
Total	0.135040	19			

R^2= 0.9911; Adj R^2 = 0.9831
SS, sum of squares; DF, Degree of freedom; MS, mean square

Model coefficients estimated by regression analysis for each variable is shown in Table 3.3.5. The significance of each coefficient was determined by *t*-values and *P*-values. The larger the magnitude of *t*-test value and smaller the *P*-value indicates the high significance of the corresponding coefficient (Tanyildizi *et al.* 2005). The *P* values (< 0.0001) and lack of fit (0.013) for the model suggested that the obtained experimental data were in good fit. From Table 3.3.5, it is observed that the regression coefficients of linear and quadratic terms for all the factors in the model was found to be highly significant ($P < 0.06$) except the quadratic coefficient of CMC indicated insignificance on the responses ($P > 0.9$). From the student *t*-test the regression coefficient terms for interaction between CMC and peptone and CMC and yeast extract were found to be significant ($P < 0.009$); however, interaction effects between peptone and yeast extract did not seem to be of considerable significance ($P > 0.7$) on cellulase activity. It should be noted here that such

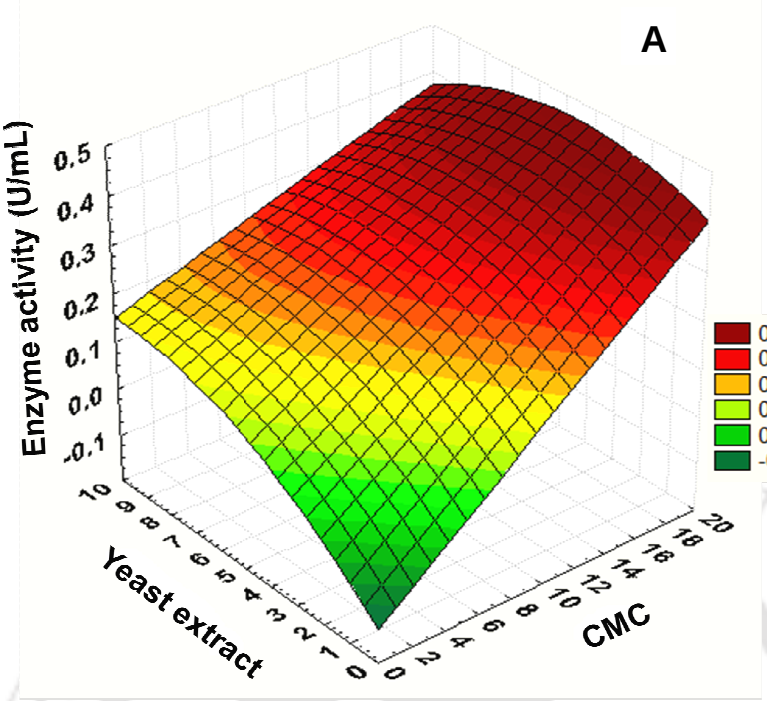
observations on significance of interaction effects between the variables would have been lost if the experiments were carried out by conventional methods (Youssef and Berekaa, 2009).

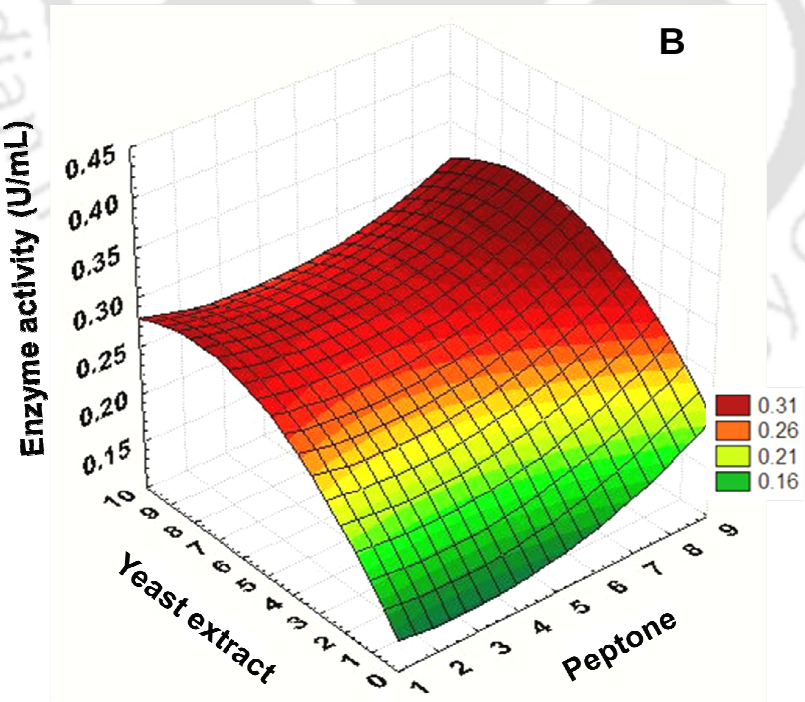
Table 3.3.5 Model coefficients estimated by multiple linear regressions

Model term	Parameter estimate	Standard Error	Computed *t*-value	*P*-value
Intercept	0.270187	0.004467	60.479	0.000
X_1 (CMC)	0.151937	0.004985	30.480	0.000
X_2 (Peptone)	0.019066	0.004985	3.825	0.003
X_3 (Yeast extract)	0.050437	0.004985	10.118	0.000
X_1^2	0.000199	0.008161	0.024	0.981
X_2^2	0.017596	0.008161	2.16	0.056
X_3^2	– 0.045086	0.008161	–5.524	0.000
$X_1 * X_2$	0.035305	0.010954	3.223	0.009
$X_1 * X_3$	– 0.049972	0.010954	–4.562	0.001
$X_2 * X_3$	– 0.005799	0.010954	–0.529	0.608

Three dimensional response surface plots were constructed by plotting the response (enzyme activity) on the Z-axis against any two independent variables, while maintaining other variables at their median levels as shown in Figs. 3.3.2 (A-C). The response surfaces having circular contour plot indicates no interaction whereas, an elliptical or saddle nature of the contour plot indicates significant interaction between the corresponding variables. Fig. 3.3.2 (A) shows that with increase in CMC concentration in the entire range surface plot is sharply ascending indicating an enhancement of enzyme activity. Similar ascending nature of surface plot was observed with yeast extract which beyond 5 g/L tended to decline indicating a decrease in enzyme activity. This proved a strong interaction between CMC and yeast extract. This was also confirmed by student *t*-test with *P*-value (P = 0.001) and *t*-value (-4.562) as shown in Table 3.3.5. Fig. 3.3.2 (B) shows that, with the increase

in yeast extract concentration to mid range, the surface showed a rise indicating enhancement of enzyme activity and beyond the mid range the surface curvature declined. No significant change in surface curvature observed with increase in peptone concentration showing an insignificant interaction. Fig. 3.3.2 (C) shows that with the increase in CMC concentration, the surface showed ascending nature indicating enhancement of enzyme activity. Peptone also followed the same pattern. This positive significant interaction was also confirmed by student *t*-test with *P*-value (P = 0.009) and *t*-value (3.223) as shown in Table 3.3.5. Therefore, in both Figs. 3.3.2 (A) and 3.3.2 (C) there was a steep enhancement in cellulase activity with increase in CMC concentration up to the maximum level of 18 g/L. The optimum levels of variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using Design Expert software. The model predicted a maximum cellulase activity of 0.49 U/mL appearing at: CMC (18 g/L), peptone (8 g/L) and yeast extract (4.8 g/L) by keeping the other components at their median levels.





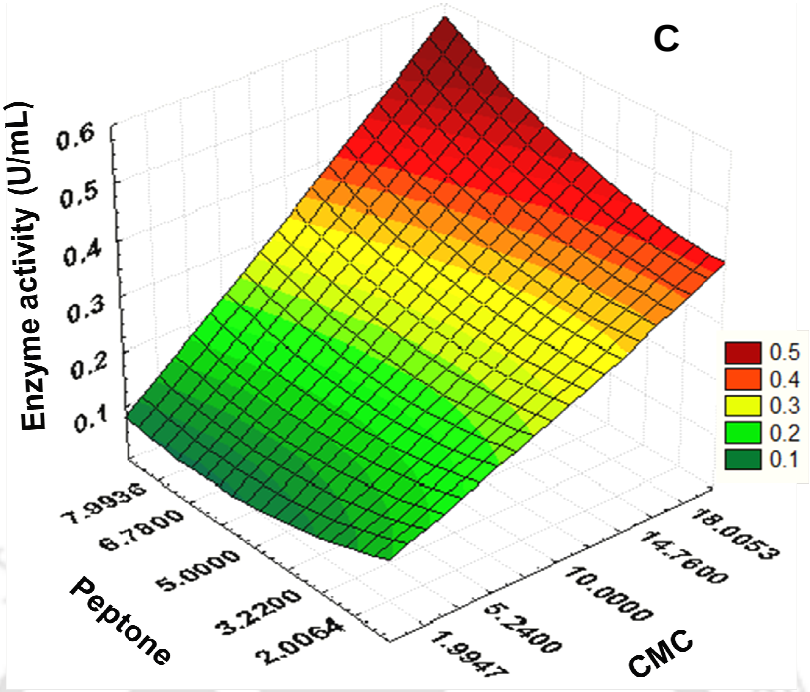


Fig 3.3.2. Three-dimensional response surface plot for alkaline cellulase production showing the interactive effects of medium components in g/L: (A) Yeast extract and CMC median level: peptone; 5g/L (B) Yeast extract and peptone median level: CMC, 10 g/L; (C) CMC and peptone, median level: Yeast extract, 5g/L.

3.3.3 Experimental validation of optimized medium composition at flask level

To validate the predicted model, an experiment was conducted in triplicate using the optimum medium composition. Cellulase activity of 0.43 U/mL was observed at this optimized medium composition. Under these optimized conditions, the predicted response for alkaline cellulase production was determined to be 0.49 U/mL and the observed experimental value was found to be 0.43 U/mL. A good correlation between predicted and experimental values justified the validity of the response model and the existence of an optimum point.

The optimized medium gave 6 fold higher enzyme activity (0.43 U/mL) as compared to unoptimised medium (0.07 U/mL) (Table 3.3.6). The enzyme activity

value obtained after medium optimization in the study was much higher than many other reported values. Li *et al*. (2008) reported maximum cellulase activity (0.26 U/mL) from *Bacillus* sp. when grown in LB medium supplemented with 1% CMC. *Bacillus* sp. DUSELR1 and *Brevibacillus* sp. DUSELG12 produced maximum cellulase activity 0.12 U/mL and 0.02 U/mL respectively, under unoptimized conditions (Rastogi *et al*. 2010). *Geobacillus* sp. reported two fold increase in cellulase production from 0.425 U/mL (basal medium) to 0.8 U/mL in presence of yeast extract and ammonium sulfate under optimized conditions (Abdel-Fattah *et al.* 2007). In another study, a much lower cellulase activity of 0.0113 U/mL was observed under optimized conditions from *Geobacillus* sp. (Tai *et al*. 2004). Rastogi *et al*. (2009) reported maximum cellulase activity of 0.058 U/mL from cell free culture supernatants of *Geobacillus* sp. Arriffin *et al*. (2006) recorded maximum FPase, CMCase and beta-glucosidase activities of 0.011, 0.079 and 0.038 U/mL respectively, by *Bacillus pumilus* EB3 produced in a 2 L stirred tank reactor. *Bacillus subtilis* CK-2 and *Bacillus megaterium* had cellulase activity of 0.26 U/mL and 0.102 U/mL respectively (Aa *et al*. 1994; Beukes and Pletschke, 2006).

3.3.4 Effect of β-glucan and lichenan on enzyme activity by replacing CMC in optimized medium

The alkaline cellulase from *Bacillus subtilis* AS3 indicated multi-substrate specificity showing activity with CMC, glucose, sucrose, lactose, glycerol, thatch grass, and steam exploded bagasse and significantly higher activity with lichenan and barley β-glucan as described in Chapter 2 Section 2.2.7. The effect of cellulosic substrates with different linkages on enzyme production was studied to examine if they can be used as alternatives to CMC. CMC in optimized medium was replaced

with lichenan and barley β-glucan as described in Section 3.2.7. It was observed that with lichenan or barley β-glucan the enzyme activity reached maximum earlier at 12 h than that with CMC which achieved maximum at 48 h (Table 3.3.6).

The optimized medium showed 3.4 and 3.5 fold increase in activity with lichenan (1.2 U/mL) and barley β-glucan (1.4 U/mL) respectively, compared to the unoptimized medium (0.35 U/mL) and 0.4 (U/mL) (Table 3.3.6).

Table 3.3.6 Variation of enzyme activity with CMC, lichenan and barley β-glucan with and without medium optimization

Carbon source	CMC	Lichenan	β-Glucan
Unoptimized medium (U/mL)	0.07	0.35	0.4
Optimized medium (U/mL)	0.43	1.2	1.4
Fold increase	6	3.4	3.5
Maximum activity (h)	48	12	12

On comparison with CMC optimized medium (0.43 U/mL) the increase with lichenan was 2.8 and β-glucan was 3.2 fold higher, respectively (Table 3.3.6). Therefore, the enzyme can be called β-1,3-1,4-glucanase (lichenase) which cleaves the β-1,3-1,4 mixed linkages.

3.4 Conclusions

Realizing the commercial importance of alkaline cellulase, an attempt to optimize the medium components for its enhanced production from *Bacillus subtilis* AS3 was made. The significant variables for enhancing alkaline cellulase production were screened and selected using the Plackett-Burman design. CMC, peptone and yeast extract significantly and positively affected the enzyme production from *Bacillus subtilis* (AS3). These three ingredients were used as variables in the response surface analysis. A 2^2 full factorial central composite design was applied to study the interaction effects of these nutrients. Under the optimal medium composition: CMC, 18 g/L; peptone, 8 g/L and yeast extract, 4.79 g/L the experimental value of enzyme activity 0.43 U/mL perfectly matched with predicted value of 0.49 U/mL showing 6 fold increase with respect to the control basal medium which showed only 0.07 U/mL enzyme activity.

Although the cellulase activity was lower as compared to the activity of fungal strains but *Bacillus subtilis* AS3 has high β-glucanase and lichenase activity that can break β-1,3-1,4 linkages as compared to carboxymethylcellulase. In comparison to optimized medium with CMC as substrate it showed 2.8 and 3.2 fold increase with lichenan and β-glucan, respectively and the activity reached maximum at 12 h in contrast to 48 h required by CMC substrate. Therefore, the isolate is a high β-1,3-1,4-glucanase (lichenase) producing strain. Having this attribute alongwith the alkaline nature and stability at variable temperature and pH, the enzyme from *Bacillus subtilis* AS3 is most likely to find wider industrial applications.

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Chapter 4

Enhanced production of alkaline cellulase from *Bacillus subtilis* by optimizing physical parameters

4.1 Introduction

Physical process parameters like temperature, pH and agitation speed that are pre-requisites for cell growth play vital role in cellulase production. These parameters need to be optimized accordingly in order to obtain high yield. Agitation speed is one of important parameter that governs dissolved oxygen level in the culture broth thereby enhancing both substrate utilisation and cell growth (Singh *et al*. 2000; Jo *et al*. 2008). However, higher agitation speed has shown to inhibit cellulase activity (Jo *et al*. 2008; Lejeune and Baron, 1995). Analogous profile in cell growth and cellulase activity with change in pH and temperature are also reported (Jo *et al*. 2008; Chipeta *et al*. 2008; Lee *et al*. 2010). Therefore, it is essential to optimize the culture conditions to improve enzyme production. The traditional "one-variable-at-a-time approach" for optimization disregards the complex interactions among various components. Statistically based experimental designs such as Placket-Burman design and response surface methodology (RSM) can be used to study the effect of various factors and to estimate their optimum levels for desired response (Latifian *et al*.

2007). Statistical design techniques have been successfully applied in many studies for example, cellulase production by *Trichoderma reesei* (Alam *et al*. 2008) and *Bacillus subtilis* AS3 (Deka *et al*. 2011), xylanase production by *Bacillus pumilus* (Nagar *et al*. 2010).

In the present study, the physical process parameters such as initial pH, temperature and agitation speed of the culture were optimized by central composite design technique using multi response analysis to enhance the alkaline cellulase activity from *Bacillus subtilis* AS3. The optimal levels of physical process parameters predicted by the model were verified both in flask and laboratory scale bioreactor.

4.2 Materials and Methods

4.2.1 Microorganism and reagents

Bacillus subtilis AS3 (Genebank accession No. EU754025) was a gift from Prof. D. Goyal, Thapar University, Patiala, India. Carboxymethylcellulose (CMC) (low viscosity, 50-200 cP) was purchased from Sigma Aldrich (St. Louis, USA). Ingredients required for the maintenance and enzyme production medium were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation and buffer preparation were of highest purity grade.

4.2.2 Inoculum preparation and production of cellulase

The inoculum was prepared by taking a loop full of bacterial growth from nutrient agar slant and suspending it in a test tube containing 5 mL of nutrient broth with 2% glucose. The culture tubes were incubated at 37°C and 180 rpm in an incubator shaker for 16-18 h to reach optical density (OD) at 600 nm around 0.6~0.8. For enzyme production, 2% of the fresh inoculum culture was transferred to 250 mL erlenmeyer flask each containing 50 mL of optimized medium which includes (g/L): CMC, (18); peptone, (8); yeast extract, (4.79); K_2HPO_4, (1); $MgSO_4.7H_2O$, (0.25); $FeSO_4.7H_2O$, (0.25); and $MnCl_2.4H_2O$, (0.5) (Deka *et al*. 2011) at different initial pH of the medium and incubated at different temperature and agitation speed as per the central composite experimental design presented in Table 4.3.1. For all the experimental runs the culture flasks were maintained in triplicate. After 48 h, 1.0 mL of samples were collected from each flask, centrifuged at 10,000***g*** for 10 min at 4ºC and supernatant analyzed for enzyme activity.

4.2.3 Assay of enzyme activity

Assay of cellulase was carried out in 100 µL of reaction mixture containing 1.3% final concentration of CMC (65 µL of 2% CMC) in 50 mM glycine NaOH buffer (pH 9.2) and 35 µL of cell free supernatant and incubated at 45°C for 10 min. All the analyses were carried out in triplicate. The assay procedure is described in Chapter 2, Section 2.2.3.

4.2.4 Cell growth measurement

Cell growth was determined by measuring absorbance at optical density of 600 nm using a UV-visible spectrophotometer (Perkin Elmer, Model Lambda-45) and the absorbance values were expressed as dry cell weight using a calibration curve of optical density (OD_{600}) versus dry cell weight (g/L) of the sample. Dry cells weight of the centrifuged sample (10,000***g*** for 10min) was measured by directly weighing the biomass after drying at 55°C to a constant weight.

4.2.5 Optimization procedure and experimental design

4.2.5.1 Optimization of culture conditions using response surface method (RSM)

In order to determine the best set of culture conditions to obtain maximum cellulase activity by *Bacillus subtilis* AS3, experiments were performed by varying the levels of culture conditions as per the central composite design (CCD). Culture conditions chosen for optimization study were pH, agitation speed (rpm) and temperature (°C), the total number of treatment combinations (experiments) was $20 = 2^k + 2k + n_0$, where 'k' was the number of independent variables and n_0 the number of replicates performed at centre point of the variables. Fourteen experiments were run with six replications at the center points to evaluate the pure error. Table 4.2.1

shows the range and levels of these three factors where the levels (–1, 0 and +1) of these culture conditions were chosen in such a way that the center point value (0) represented the level of these factors mostly reported in literature used for cellulase production. On the basis of the centre point values the low (–1) and high (+1) levels of the culture condition were determined in such a step change that, the centre point remain middle values of the low (–1) and high (+1) range of these factors. Furthermore, as per CCD to test all these factors in five ranges including coded value + α and – α, thus the uncoded values of these respective factors were calculated by solving the following equation:

$$x_i = \frac{X_i - X_o}{\Delta X_i} \qquad i=1,2,3.....K \qquad \text{(eq. 4.1)}$$

Where, x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change. Where, default alpha value for the 3 factor (α = 1.682) was chosen as per the CCD design. For fitting the experimental results by response surface regression procedure the following second order polynomial equation was used:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \qquad \text{(eq. 4.2)}$$

Where, Y is the predicted response, k is the number of factor variables. X_i and X_j are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, β_{ii} is the i^{th} quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient. The statistical

software package, MINITAB® (Release 15.1, PA, USA) was used for regression analysis of the experimental data.

Table 4.2.1 Experimental range and levels of the independent variables for CCD matrix

Variables	Symbol	–2	–1	0	+1	+2
pH	X_1	5.9	6.4	7.0	7.6	8.0
Temperature (˚C)	X_2	30	33	37	41	44
Agitation (rpm)	X_3	120	144	180	215	240

4.2.5.2 Multiple response optimization

In systems having a large number of input variables and responses, the single response analysis has serious limitations as the optimum conditions for one response may not be suitable or practical for other responses and thus the meaning of optimum becomes unrealistic. On the other hand, multiple response or desirability function are analyses where large number of responses (output variables) are measured simultaneously for each setting of a group of parameters (input variables) and are also called multi-response analysis (Derringer and Suich, 1980). The optimization methodology based on the individual desirability using a desirability function evaluates how the settings optimize a single response. Optimal settings for input variables were determined by maximizing the composite desirability. These values are combined to determine the composite or overall desirability of the multi-response system. An optimal point was where composite desirability reaches its maximum at 1.

Response parameters such as cell growth and cellulase activity were optimized applying multiple response optimization (desirability function) method by

giving higher weight to enzyme production and lower to cell growth. For this following equation was used (Derringer and Suich, 1980; Harrington, 1965):

$$d_i(\hat{y}_i) = \begin{cases} 0 & \text{if } \hat{y} < L_i \\ \left(\dfrac{\hat{y}_i - L_i}{T_i - L_i}\right)^{r_i} & \text{if } L_i \le \hat{y} \le T_i \\ 1 & \text{if } \hat{y} > T_i \end{cases} \qquad \text{(eq. 4.3)}$$

where $d_i(\hat{y}_i)$ is desirability function of a response, L_i and T_i are the lower and target values of response measured from experimental data. In the present study, while L_i for the two responses (cellulase activity and cell growth) were 0.104 U/mL and 1.65 mg/mL respectively, T_i values were set at 0.56 U/mL and 2.0 mg/mL, respectively. $\hat{y}_i$ is the value of a response predicted by the second order polynomial equations generalized before; r_i is the weight of desirability function of a response.

In this study, enzyme activity was given higher weight of 2:1 ratio as compared to cell growth. The overall desirability function (D) in turn was computed as shown below:

$$D = \left(\prod d_i^{w_i}\right)^{1/W} \qquad \text{(eq. 4.4)}$$

Where d_i is individual desirability for the i^{th} response, w_i = importance of the i^{th} response, and $W = \sum w_i$ In the present study, w_i was taken 2:1 ratio for enzyme activity and cell growth. For solving the desirability function, the statistical software package MINITAB® (Release 15.1, PA, USA) was used.

4.2.5.3 Validation of the experimental model

In order to validate the model, experiments were performed in triplicate in a batch shake flask and 2L stirred tank fermentor (Applicon, model Bio Console ADI 1025) using optimal levels of culture condition (pH 7.2; 39°C and 121 rpm) and using optimized medium as described in Chapter 3, Section 3.3.2 which includes (g/L): CMC, (18); peptone, (8); yeast extract, (4.79); K_2HPO_4, (1); $MgSO_4.7H_2O$, (0.25); $FeSO_4.7H_2O$, (0.25); and $MnCl_2.4H_2O$, (0.5) (Deka *et al*. 2011). The laboratory scale bioreactor was operated at optimal levels of culture conditions and aeration rate of 1 vvm (volume of air per volume of liquid per min) and 2% inoculum. After 48 h, 1.0 mL of samples were drawn and absorbance at OD 600 nm was measured. The absorbance values were expressed as dry cell weight using a calibration curve. The sample were then centrifuged at 10,000***g*** for 10 min at 4°C and supernatant analyzed for enzyme activity as described in Chapter 2, Section 2.2.3.

4.3 Results and Discussion

4.3.1 Optimization of culture conditions using RSM

For maximizing cellulase activity, the levels of the three important factors *viz*., pH, agitation speed (rpm), and temperature (°C) were varied using the central composite design of experiment (Table 4.3.1), and the results were analyzed in the form of analysis of variance (ANOVA). Tables 4.3.2 (a) and (b) presents ANOVA of cellulase activity and cell growth profile of the culture, respectively. The Fisher's *F* value (21.18) for cellulase activity in the model owing to regression was found to be higher than the critical *F* value ($F_{0.05}$ 9, 3 = 2.54) (Table 4.3.2 (a)). The large *F* value indicates that most of the variations in the response could be explained by the regression model equation for cellulase activity. Generally, a large *F* value with a corresponding small *P*-value indicates a high significance of the respective coefficient (Tanyildizi *et al*. 2005). The associated *P* values are used to judge whether *F* was large enough to indicate statistical significance or not. The linear and square terms of both the regression models for cellulase activity and cell growth were found to be highly significant at $P = 0.000$. In the present study, the model *F*-values of 21.18 and 37.48 for cellulase activity and cell growth respectively, indicate that the respective regression models could explain most of the variation in the responses. These findings confirmed that the second-order polynomial models for cellulase activity and cell growth were adequate in predicting both the responses. These regression model equations are presented below.

$$Y_1 = 0.436816 + 0.071256\ X_1 + 0.146789\ X_2 - 0.073141\ X_3 - 0.005283\ X_1^2 + 0.218983\ X_2^2 - 0.020083\ X_3^2 - 0.068413\ X_1X_2 + 0.014319\ X_1X_3 - 0.123355\ X_2X_3 \quad \text{(eq. 4.5)}$$

$$Y_2 = 3.18098 + 0.21897\ X_1 + 0.27103\ X_2 + 0.50635\ X_3 - 0.86722\ X_1^2 - 1.32722\ X_2^2 - 0.53722\ X_3^2 - 0.02828\ X_1X_2 + 0.03536\ X_1X_3 + 0.33941\ X_2X_3 \quad \text{(eq. 4.6)}$$

Where, Y_1 = Cellulase activity (U/mL), Y_2 = cell growth (g/L), X_1 is pH, X_2 is temperature (˚C) and X_3 is agitation speed (rpm).

Table 4.3.1 CCD showing experimental and regression model predicted cellulase activity (U/mL) and cell growth (g/L)

Run No	pH	Temp (˚C)	Agitation (rpm)	Cellulase activity (U/mL)		Cell growth (g/L)	
				Measured	Predicted	Measured	Predicted
1	0	0	0	0.429 ± 0.05	0.437	3.16 ± 0.01	3.18
2	1	–1	1	0.311 ± 0.01	0.335	2.30 ± 0.05	2.39
3	–1	1	–1	0.499 ± 0.02	0.512	1.87 ± 0.12	1.85
4	2	0	0	0.560 ± 0.01	0.503	2.74 ± 0.09	2.53
5	0	0	–2	0.500 ± 0.08	0.490	2.08 ± 0.01	2.14
6	–2	0	0	0.355 ± 0.02	0.360	1.98 ± 0.04	2.10
7	0	0	0	0.442 ± 0.05	0.437	3.23 ± 0.14	3.18
8	0	0	0	0.436 ± 0.03	0.437	3.19 ± 0.06	3.18
9	0	0	0	0.422 ± 0.06	0.437	3.16 ± 0.02	3.18
10	1	1	–1	0.532 ± 0.02	0.538	2.08 ± 0.08	2.06
11	1	1	1	0.322 ± 0.02	0.374	2.73 ± 0.05	2.93
12	0	0	0	0.448 ± 0.01	0.437	3.14 ± 0.04	3.18
13	–1	–1	–1	0.217 ± 0.07	0.202	1.88 ± 0.02	1.75
14	0	0	0	0.435 ± 0.04	0.437	3.19 ± 0.21	3.18
15	1	–1	–1	0.281 ± 0.11	0.325	1.87 ± 0.05	2.01
16	–1	1	1	0.334 ± 0.01	0.327	2.73 ± 0.02	2.67
17	0	0	2	0.386 ± 0.05	0.344	3.30 ± 0.04	3.15
18	0	2	0	0.385 ± 0.01	0.365	2.15 ± 0.18	2.13
19	0	–2	0	0.103 ± 0.14	0.071	1.65 ± 0.05	1.58
20	–1	–1	1	0.161 ± 0.03	0.192	2.00 ± 0.01	2.08

values are mean ± SE (n=3)

Table 4.3.2 (a) Analysis of variance **(**ANOVA) for optimization of cellulase activity (U/mL)

Source	df	SS	Adj MS	*F*	*P*	R^2
Regression	9	0.261363	0.029040	21.18	0.000	95.02
Linear	3	0.154385	0.051462	37.54	0.000	
Square	3	0.086877	0.028959	21.13	0.000	
Interaction	3	0.020102	0.006701	4.89	0.024	
Residual error	10	0.013708	0.001371			
Pure error	5	0.000404	0.000081			
Total	19	0.275071				

SS, sum of squares; DF, Degree of freedom; MS mean square; *F*, Fisher's *F* value (calculated by dividing the MS owing to the model by that due to error), *P* probability of incorrectly rejecting the null hypothesis when it is actually true.

Table 4.3.2 (b) Analysis of variance (ANOVA) for optimization of cell growth (g/L)

Source	df	SS	Adj MS	*F*	*P*	R^2
Regression	9	6.30943	0.70105	37.48	0.000	97.12
Linear	3	1.82418	0.60806	32.51	0.000	
Square	3	4.36800	1.45600	77.85	0.000	
Interaction	3	0.11725	0.03908	2.09	0.165	
Residual error	10	0.18702	0.01870			
Pure error	5	0.00508	0.00102			
Total	19	6.49645				

SS, sum of squares; DF, Degree of freedom; MS mean square; *F*, Fisher's *F* value (calculated by dividing the MS owing to the model by that due to error), *P* probability of incorrectly rejecting the null hypothesis when it is actually true.

Further, to determine significance of regression coefficients in the two models, the results were subjected to student's *t*-test and are presented in Table 4.3.3. From Table 4.3.3, it was observed that the regression coefficients of linear and quadratic terms for all the factors in the models for cellulase activity and cell growth were highly significant ($P < 0.007$) however, the quadratic coefficient due to pH and agitation speed for cellulase activity indicated insignificance on the responses ($P > 0.4$). From the student *t*-test of cellulase activity, the regression coefficient terms for interaction between temperature and agitation speed were found to be highly important ($P < 0.009$); however, interaction effects between pH and temperature

revealed slightly less significance ($P < 0.095$). Other coefficient terms in the models did not seem to be of considerable significance ($P > 0.7$) on cellulase activity. In case of cell growth, the regression coefficient terms for interaction between temperature and agitation speed reveal some significance ($P < 0.05$) where as no significant interaction was observed with other factors on cell growth of the culture.

Table 4.3.3 Result of Student's *t*-test for cellulase activity and the cell growth in the optimization study

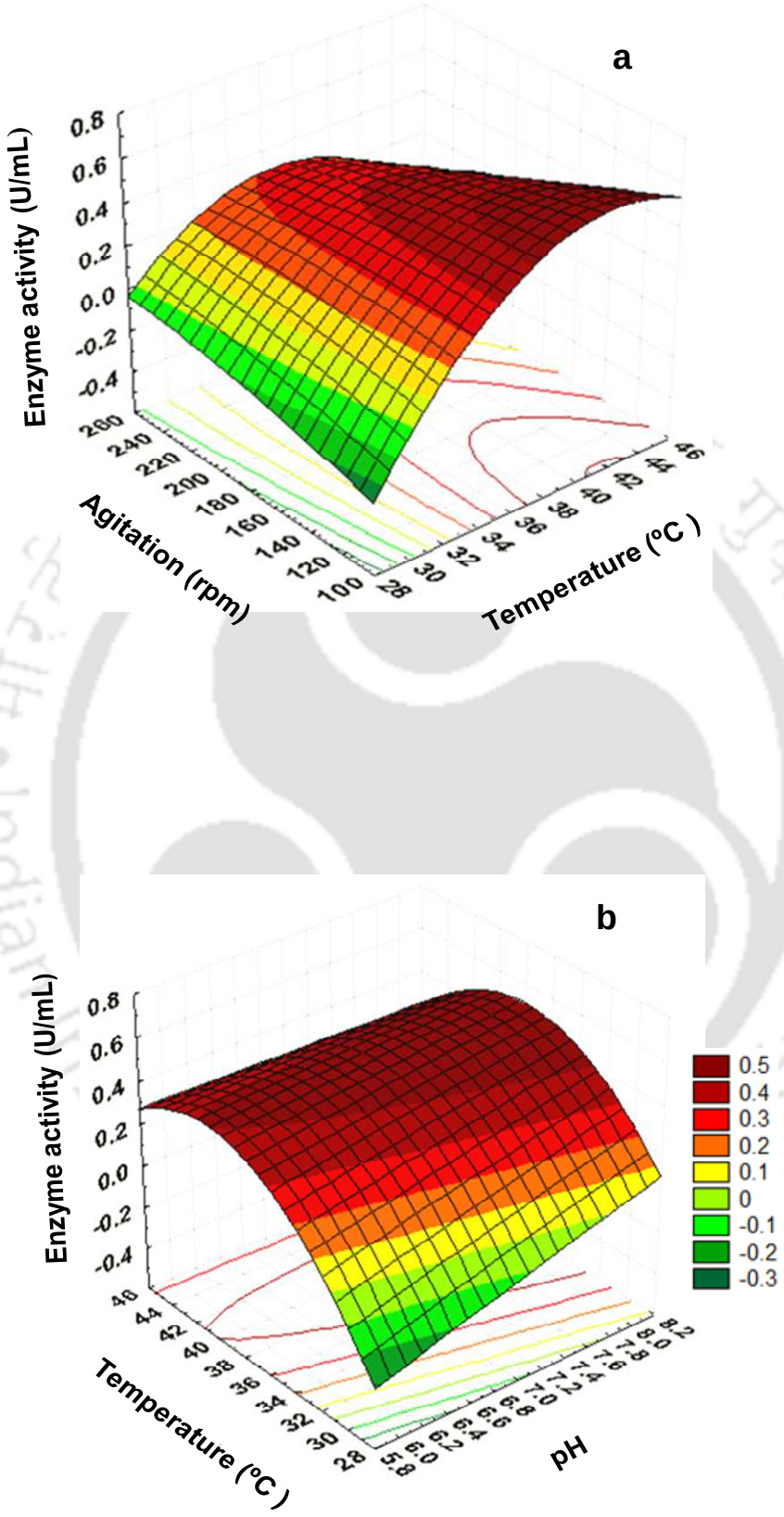
	Cellulase activity (U/mL)		Cell growth (g/L)	
Term	*t*	*P*	*t*	*P*
Constant	28.927	0.000	57.032	0.000
pH (X_1)	4.229	0.002	3.518	0.006
Temperature (˚C) (X_2)	8.712	0.000	4.355	0.001
Agitation (rpm) (X_3)	-4.341	0.001	8.136	0.000
(X_1^2)	-0.192	0.852	-8.511	0.000
(X_2^2)	-7.938	0.000	-13.026	0.000
(X_3^2)	-0.728	0.483	-5.272	0.000
$X_1 * X_2$	-1.848	0.094	-0.207	0.840
$X_1 * X_3$	0.387	0.707	0.259	0.801
$X_2 * X_3$	-3.332	0.008	2.482	0.032

t statistic is the coefficient divided by its standard error, *P* probability of incorrectly rejecting the null hypothesis when it is actually true.

In order to determine the optimal levels of the variables for maximum cellulase activity, three dimensional response surface plots as shown in Fig. 4.3.1, were constructed by plotting the response against any two of the three independent variables and by maintaining the other variable at their middle (zero) levels. Fig. 4.3.1 (a) represents the effects of temperature and agitation speed on cellulase activity at constant pH (7.0). The figure demonstrated that, although the enzyme activity was found sharply increasing with the temperature, however, beyond the agitation speed of 190 rpm a sharp decline of the enzyme activity was observed indicating a strong negative interaction between the factors. From Fig. 4.3.1 (a) it can

be revealed that higher agitation speed inhibits the enzyme activity. Fig. 4.3.1 (b) shows the interaction effect between pH and temperature on enzyme activity at constant agitation speed (180 rpm). The figure showed that enzyme activity was stridently increased with temperature, as compared to change in pH but decreased beyond pH 7.5 in contrast to change in temperature from 40-44°C revealing a negative interaction effect between these factors at their higher level. Fig. 4.3.1 (c) displays the effects of pH and agitation speed (rpm) on cellulase activity at constant temperature (37ºC). The surface plot was found to be curvilinear clearly revealing no significant change in enzyme activity with change in the culture conditions and the optimum was observed near the central values of pH and agitation speed.

To illustrate the above mentioned interaction effect between the variables in the study, typical contour plots between temperature and agitation speed and that between pH and temperature are depicted in bottom of the response surface plots of Figs. 4.3.1 (a) and (b). In general, the contours in such plots help in proper identification of the type of interactions between test variables; the surface confined in the smallest curve of such contour diagram can also be used to predict optimum response of the system. Hence, from the given plot in Fig. 4.3.1 (a), the corresponding coordinates in the region of the contour diagram gave the optimum values of the respective factors. Also, the response surface contour plots of mutual interaction between the variables, *viz*., temperature and agitation speed and that between pH and temperature, Figs. 4.3.1 (a) and (b), respectively were found to be elliptical, indicating significant interaction between these pairs of factors. Besides the two contour plots showing interaction between the variables, response surface contours drawn between pH and agitation speed was circular indicating non-significant nature of their interactions (Fig. 4.3.1 (c)).


a
Enzyme activity (U/mL)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
Agitation (rpm)
260
240
220
200
180
160
140
120
100
Temperature (°C)
28
30
32
34
36
38
40
42
44
46
b
Enzyme activity (U/mL)
Temperature (°C)
pH
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3

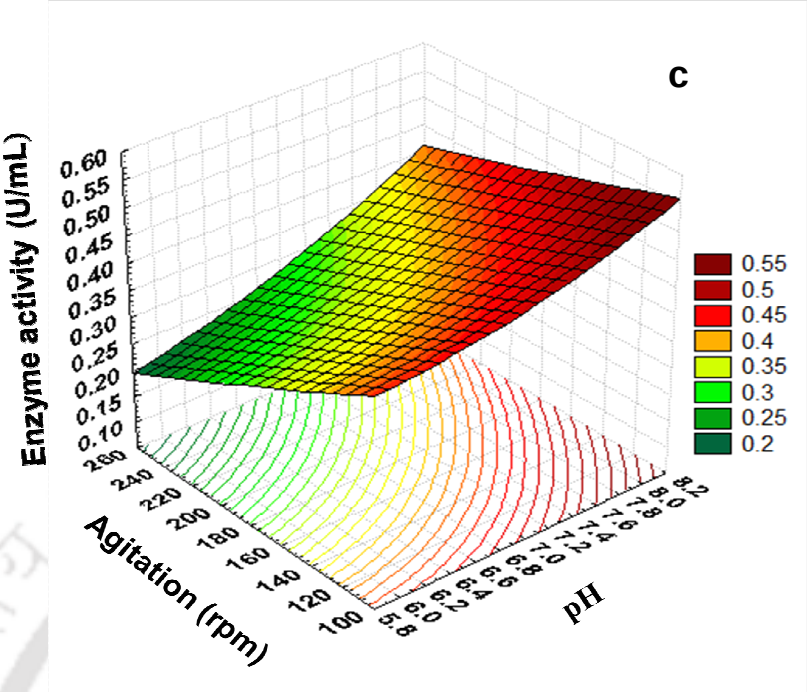


Fig. 4.3.1 Three dimensional response surface plots for cellulase activity showing the interaction effect between (a) temperature and agitation (b) pH and temperature (c) pH and agitation

Table 4.3.1 presents the experimental along with the model predicted values of cellulase activity and cell growth of the culture, which clearly shows that both the experimental and predicted values were in close agreement with each other. And in order to illustrate the accuracy of the models in predicting the responses the normal probability plot of the residuals for cellulase activity and cell growth are also depicted in Figs. 4.3.2 (a) and (b), respectively. The normal probability plot is to identify and explain the departures from the assumptions that errors are normally distributed, independent of each other and the error variances are homogenous. An excellent normal distribution established the normality assumption and the independence of the residuals (Sanjeeviroyar *et al*. 2009). A linear pattern in these

two plots clearly demonstrates that the error in the data is negligible and both the models best fitted the entire experimental data obtained (Draper and Smith, 1981).

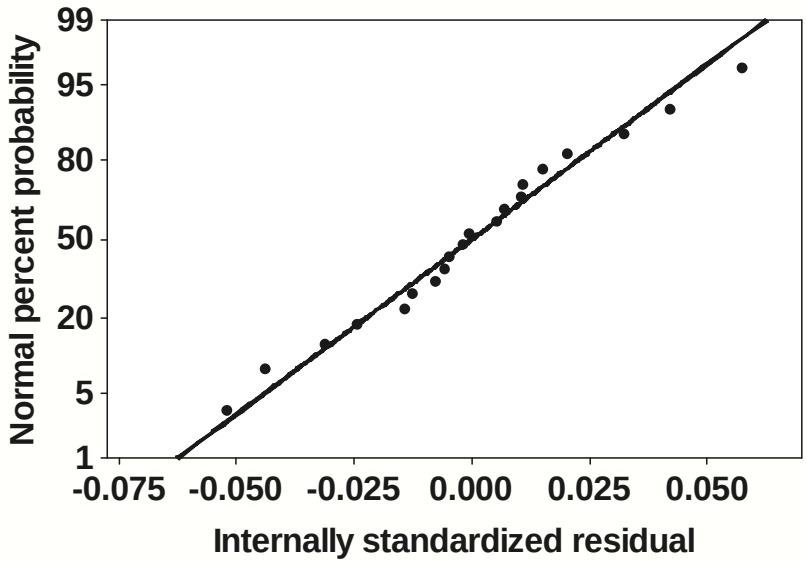


Fig. 4.3.2 (a) Normal probability plot of the residuals for cellulase activity by *Bacillus subtilis* AS3. R^2= 0.9502

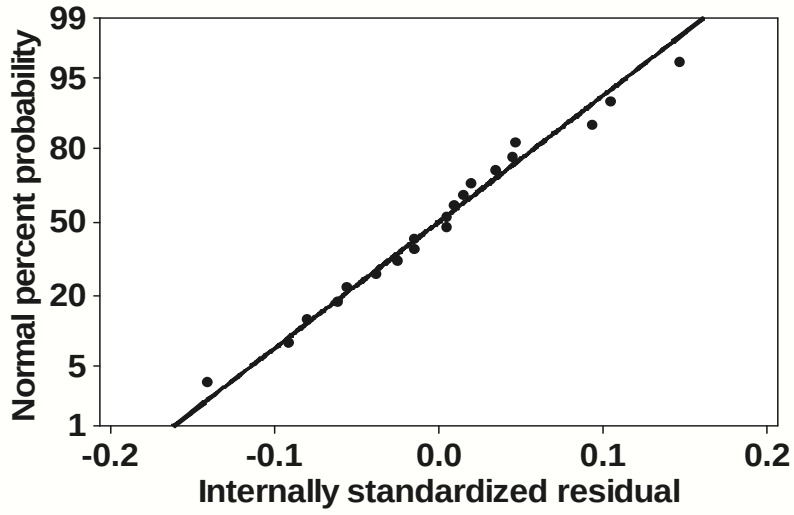


Fig. 4.3.2 (b) Normal probability plot of the residuals for cell growth of *Bacillus subtilis* AS3. R^2= 0.97

The method of desirability function was applied in order to determine the optimal levels of each variable for maximizing cellulase activity. It was reported earlier that higher cell growth was achieved at favorable culture conditions such as agitation speed, pH and temperature however, at the higher biomass concentration cellulase activity is inhibited (Jo *et al*. 2008; Lejeune and Baron, 1995; Chipeta *et al*. 2008). Similar observation was also obtained in the present study as a result enzyme activity was given higher weight of 2:1 ratio as compared to cell growth. Hence, in order to achieve maximum cellulase activity biomass growth need to be minimized. The desirability function study in this multiple response optimization method shown in Fig. 4.3.3 reveals that the overall desirability functions for cellulase activity and cell growth were close to 1 indicating the fact that the function increases linearly towards the desired target values of the two responses (Derringer and Suich, 1980; Jahani *et al*. 2008). In addition, individual desirability values of the two responses were calculated; while the value for cell growth was computed to be 1 with a maximum predicted response of 2.01 mg/mL, the value for cellulase activity was also found to be 1 with maximum predicted value of 0.56 U/mL. Thus, using the desirability function method for optimizing both the responses optimum values of the culture conditions were estimated to be: pH 7.2; temperature; 39˚C, agitation speed 121 rpm.

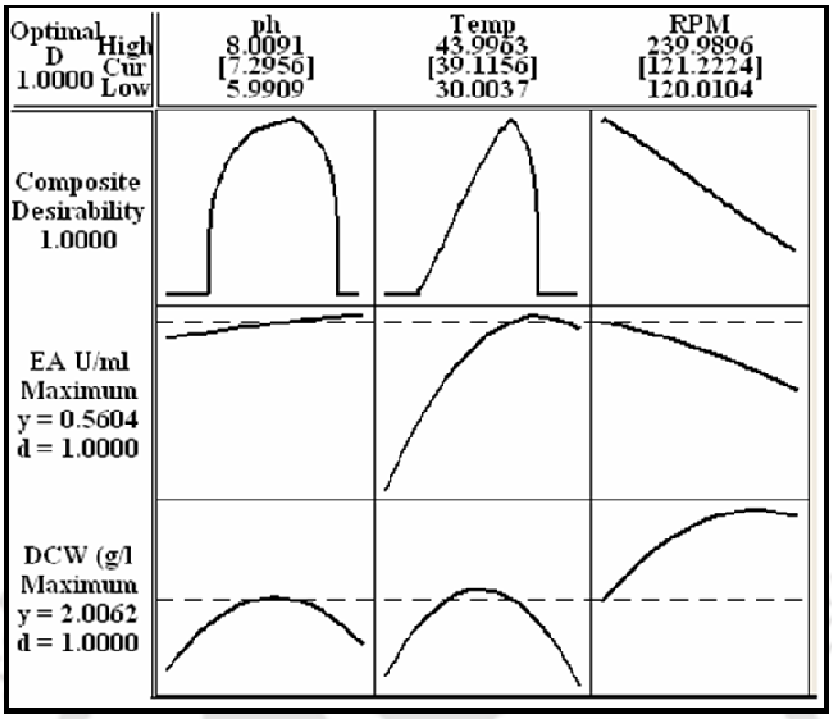


Fig. 4.3.3 Desirability function plot showing the optimum level of physical process parameters

Agitation speed is one of the important culture parameters that maintains homogenous conditions, disperses dissolved oxygen into smaller bubble thereby increasing the interfacial area and oxygen mass transfer rate for enhancing both substrate utilization and microbial activity (Singh *et al.* 2000). In this study the agitation speed was found to be optimum at 121 rpm. Other authors also reported similar optimum value of the parameter using *Bacillus spp.* (Rastogi *et al*. 2009; Rastogi *et al*. 2010). However, any further increase in agitation speed more than 121 rpm did not improve the enzyme activity by the culture in the present study, which may be attributed to increased shear stress on the cells thus leading to reduced enzyme production (Purkarthofer *et al*. 1993). Similar observations are also reported using *Bacillus amyloliquefaciens* (Jo *et al*. 2008), *Trichoderma reesei* (Lejeune and

Baron, 1995) and *Thermomyces lanuginosus* (Singh *et al*. 2000) where cellulase production declined at higher agitation rates.

Temperature is also one of the most important parameters that influences enzyme activity and is essential for a fermentation process (Rastogi *et al*. 2010). In the present study it was observed that when the culture temperature increased to an optimum level of 39ºC an enhancement in cellulase activity was achieved. Similar observations on enhancement of cellulase activity were reported in other papers where optimum temperature of medium for production of cellulase by *Bacillus subtilis* CY5 and *Bacillus circulans* were 40°C (Ray *et al*. 2007) and for *B. amyloliquefaciens* DL-3 (Jo *et al*. 2008) and *Bacillus pumilus* EB3 (Ariffin *et al*. 2008) it was 37°C which is, within the range, as obtained in the present study. However, temperature above and below the optimum level inhibited the cellulase activity by the microorganism probably due to inhibition of the multi-enzyme complex system of the cell (Sohail *et al.* 2009). For instant at low temperature substrate transport across the cells are suppressed and lower product yield are attained (Rajoka, 2004). Similarly, at higher temperature, the requirement of energy for maintenance of cellular growth and metabolism are high due to thermal denaturation of enzymes of the metabolic pathway resulting in reduced enzyme production (Rajoka, 2004).

pH of the growth medium influences many enzymatic reactions by affecting the transport of chemical products and enzymes across the cell membrane (Liang *et al*. 2010). Our results also confirmed that medium pH is an important factor affecting cellulase activity. The optimum pH for maximum production of cellulase found in this study was 7.2. Similar finding was also reported by Arriffin *et al*. (2008); Immanuel *et al*. (2006) and Rastogi *et al*. (2009) for cellulase production.

At the optimum values of physical parameters *viz*., pH 7.2; temperature 39˚C; agitation speed 121 rpm, the fermentation by *Bacillus subtilis* AS3 showed 33% enhancement in cellulase activity as compared to unoptimized parameters.

4.3.2 Validation of the model

The cellulase activity was experimentally verified in batch shake flask and at 2L stirred tank fermentor using optimized medium (Deka *et al*. 2011) and optimized physical parameters of culture conditions as described in Section 4.3.1. The maximum cellulase activity and cell growth by *Bacillus subtilis* AS3 was 0.57 U/mL and 2.1 mg/mL in shake flask (Fig. 4.3.4 (a)) which is in very good agreement with the value predicted by the model (0.56 U/mL and 2.01 mg/mL). The cellulase activity with un-optimized physical parameters and optimized medium was 0.43 U/mL (Deka *et al*. 2011). This showed 33% enhancement of cellulase activity after physical process parameter optimization (Table 4.3.4). Scale-up of batch cultivation from shake flask to bioreactor containing 1.0 L of the same optimized medium and at optimized culture conditions yielded maximum cellulase activity of 0.75 U/mL (Fig. 4.3.4 (b)). A significant increase of 32% was observed due to controlled pH and maintenance of aeration in the fermentor throughout the cultivation which is not possible in shake flask (Fig. 4.3.4 (b), Table 4.3.4). Shake flask experiments have limitations to control pH and dissolved oxygen level in the broth as compared to the fermentor. It was observed that in shake flask the pH of the culture medium showed variations with initial decline and then increasing trend at the end of cultivation (Fig. 4.3.4 (a)). The similar trends of pH variation have been observed with various other *Bacillus* strains (Heck *et al*. 2002). pH control during fermentation was reported to be essential for increased cellulase production (Heck *et al.* 2002). In scale up in

bioreactor it was observed that with pH controlled at 7.2 showed maximum activity of 0.75 U/mL after 48 h of fermentation (Fig. 4.3.4 (b)). The cell growth also showed a similar profile as cellulase production, and reached its highest value at the late log phase (Fig. 4.3.4 (b)). The cell growth and cellulase activity data inferred the growth associated production of cellulase.

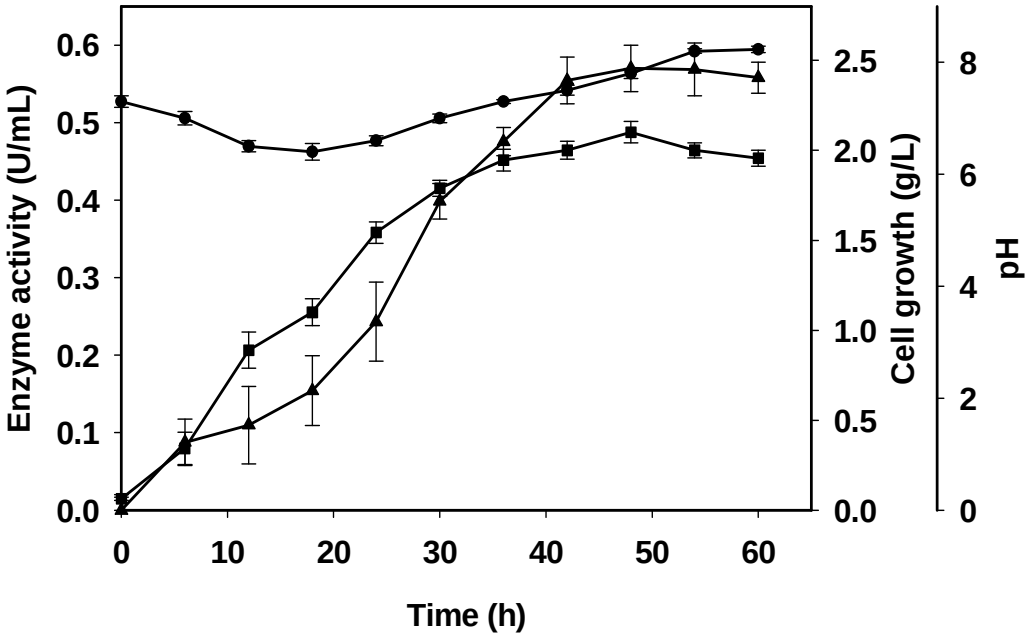


Fig. 4.3.4 (a) Cellulase production, cell growth and pH profile of *B. subtilis* AS3 in shake flask containing optimized medium and optimized physical parameter (▲) enzyme activity (U/mL), (■) Cell growth (g/L), (●) pH with time (h).

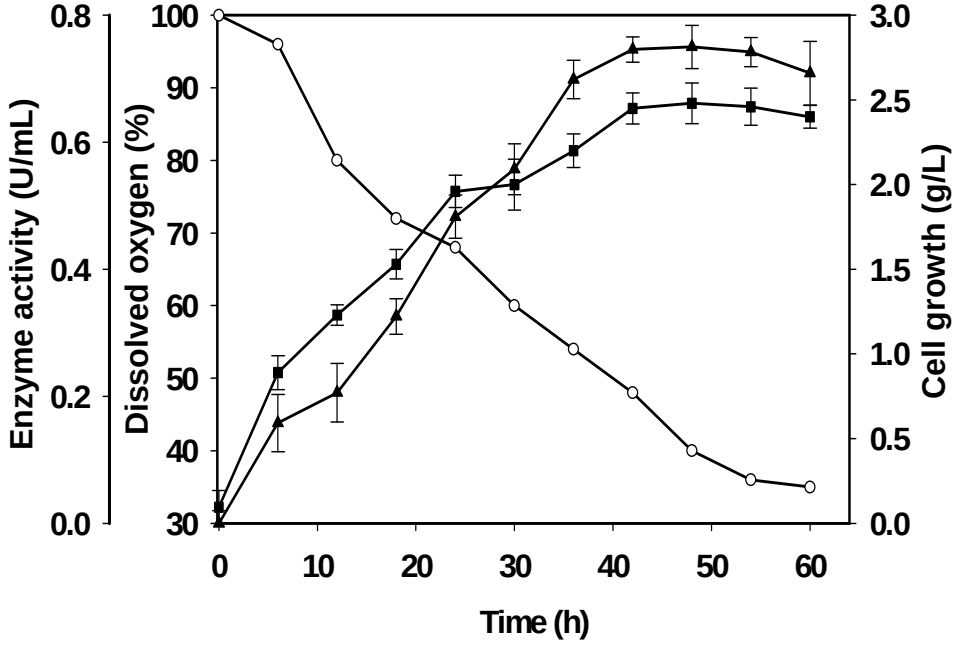


Fig. 4.3.4 (b) Cellulase production and cell growth of *B. subtilis* AS3 with controlled pH at 7.2 in fermentor containing optimized medium and optimized physical parameter (▲) enzyme activity (U/mL), (■) Cell growth (g/L), (●) pH with time (h), (°) Dissolved oxygen (%) with time (h).

Table 4.3.4 Cellulase production at different levels of optimization

Process conditions	Level of scale	Cellulase activity (U/mL)	Enhanced production (Fold)	Ref
Without optimization	flask	0.07	-	
Optimized medium	flask	0.43	6	Deka *et al.* 2011
Optimized medium + physical parameters	flask	0.57	8	In this study
Optimized medium + physical parameters	Bioreactor	0.75	11	In this study

The optimized enzyme activity value obtained in this study was much higher than many other reported values. For example, *Geobacillus* sp. and *Bacillus* sp. produced maximum CMCase activity 0.074 U/mL and 0.12 U/mL respectively, under optimized conditions (Rastogi *et al*. 2009; Rastogi *et al*. 2010). In another

study, *Brevibacillus* sp. reported maximum cellulase activity of 0.02 U/mL under optimum culture conditions (Rastogi *et al*. 2009). *Bacillus pumilus* EB3 and *Bacillus megaterium* recorded maximum cellulase activities of 0.076 U/mL and 0.102 U/mL, respectively, under optimized conditions in a 2L stirred tank reactor (Beukes and Pletschke, 2006 and Ariffin *et al*. 2008). Overall, the results of the present study clearly showed significant enhancement in cellulase activity by *Bacillus subtilis* AS3 by optimising the culture conditions and medium employing the statistical based design technique.

4.4 Conclusions

The effects of physical process parameters such as initial pH, temperature (°C), and agitation speed (rpm) on cellulase production from *Bacillus subtilis* AS3 were investigated. For optimization of cellulase activity and cell growth, central composite design of experiments followed by multiple desirability function was applied. Among the three independent variables, pH, agitation speed (rpm) and temperature (°C), the interaction effect between temperature and agitation speed was highly significant on cellulase activity. The individual optimum values of the culture conditions predicted by the model were found to be pH 7.2; temperature 39˚C and agitation speed 121 rpm. Maximum cellulase activity and cell growth predicted by the model was 0.56 U/mL and 2.01 mg/mL which is in good agreement with experimental value of 0.57 U/mL and 2.1 mg/mL which was obtained experimentally using optimized medium and optimal values of physical parameters. The cellulase activity with un-optimized physical parameters and previously optimized medium composition was 0.43 U/mL. After physical parameters optimization using optimized medium a 33% enhancement in cellulase activity (0.57 U/mL) was recorded. Using the same optimal level culture conditions in a 2L stirred tank fermentor yielded a significant 32% increase showing maximum cellulase activity of 0.75 U/mL. This study illustrates the importance of optimization of physical parameters for bioprocess development of bacterial cellulase production.

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Chapter 5

Purification and characterization of an alkaline cellulase from *Bacillus subtilis* AS3

5.1 Introduction

Cellulase belongs to an important class of industrial enzyme, therefore its efficient purification in downstream processing is an essential prerequisite for commercial exploitation. They have wide range of applications in textile, laundry, pulp and paper, fruit juice extraction and animal feed industries (Bhat, 2000; Maki *et al*. 2009). Besides, they offer tremendous potential in saccharification of lignocellulosic biomass to fermentable sugars which, in turn can be used for production of bioethanol, lactic acid, single cell protein and other industrially important chemicals (Cherry and Fidantsef, 2003; Emtiazi *et al*. 2003; Sanchez and Cardona, 2005 and Tanaka *et al*. 2006). Most of the procedures for purification of extracellular enzymes include ultrafiltration, salting out by ammonium sulfate or solvent extraction methods using acetone and ethanol (Mawadza *et al*. 2000; Huang and Monk, 2004; Singh *et al*. 2004) followed by purification in a combination of chromatographic procedures. Reports on purification of cellulase describe various

chromatographic procedures which include ion exchange and hydrophobic interaction chromatography (CM-cellulose, DEAE-cellulose, phenyl-sepharose or DEAE-Sepharose and HiTrap Q, affinity chromatography) (Apiraksakorn *et al*. 2008); affinity chromatography or gel filtration chromatography using sephadex, sephacryl, DEAE-sephadex (Kim *et al*. 2005; Bischoff *et al*. 2006; Kim *et al*. 2009; Yin *et al*. 2010; Trivedi *et al* 2011). After purification process, homogeneity of the enzyme was confirmed by either polyacrylamide gel electrophoresis or iso-electric focusing (IEF) before further characterization of the enzyme. The molecular weight of the purified cellulase was estimated based on its mobility calculated with standard calibration proteins. The active form of the enzyme was confirmed by activity staining. Commercialization of cellulases depends on their stability during isolation, purification and storage as well as their robustness against solvents and surfactants (Annamalai *et al*. 2011; Trivedi *et al*. 2011). These properties are known to vary with the nature of organism from which the enzyme is produced. Therefore, the study of kinetics and catalytic behavior of enzyme purified from any new strain is essential (Wang *et al*. 2009). In the present study, the purification and biochemical characterization of cellulase from *Bacillus subtilis* AS3 is reported. The cellulase activity and molecular size were confirmed by zymogram analysis using CMC as substrate.

5.2 Materials and Methods

5.2.1 Microorganism and reagents

Bacillus subtilis AS3 (Genebank accession No. EU754025) was a gift from Prof. D. Goyal, Thapar University, Patiala, India. Carboxymethylcellulose (CMC) (low viscosity, 50-200 cP), Lichenan and Laminarin was purchased from Sigma Aldrich (St. Louis, USA). Avicel, Hydroxyethylcellulose and Barley β-glucan were purchased from Fluka, Biochemika. Steam exploded bagasse (SEB) was gifted by Dr. A. J. Verma, National Chemical Laboratory, Pune, India. DEAE-Sepharose was procured from GE healthcare. All other chemicals and reagents used in the study were analytical grade procured from Merck and HiMedia Laboratories (India)

5.2.2 Enzyme activity assay

Assay of cellulase was carried out in 100 µL of reaction mixture containing 1.3% final concentration of CMC (65 µL of 2% CMC) in 50 mM glycine NaOH buffer (pH 9.2) and 35 µL of cell free supernatant and incubated at 45°C for 10 min. The cellulase activity was measured by estimating the liberated reducing sugar by the Nelson-Somogyi procedure (Nelson, 1944; Somogyi, 1945). Absorbance was measured at 500 nm using a UV-visible spectrophotometer (Perkin Elmer, Model Lambda-45) against a blank with D-glucose as standard. All the analyses were carried out in triplicate. The assay procedure is described in Section 2.2.3 of Chapter 2. Relative activity is expressed as percentage of the maximum enzyme activity under standard assay conditions.

5.2.3 Protein determination

The total protein content of the cell free extract was estimated by the method of Lowry *et al*. (1951). Bovine serum albumin ranging from 25 µg/mL to 500 µg/mL concentration was used as a reference to plot a standard curve. The details are given in Section 2.2.3 of Chapter 2. The specific activity (U/mg) of the enzyme was calculated by dividing enzyme activity (U/mL) with protein concentration (mg/mL).

5.2.4 Inoculum preparation and production of cellulase

The inoculum was prepared by taking a loop full of culture from the nutrient agar slant in a test tube containing 5 mL of nutrient broth with 2% (w/v) glucose and incubated at 37°C and 180 rpm in an incubator shaker for 16-18 h to reach optical density (OD) at 600 nm around 0.6~0.8. For enzyme production, 2% (v/v) of the fresh inoculum culture was transferred to two 250 mL erlenmeyer flasks each containing 50 mL optimized medium (Deka *et al*. 2011). The pH was adjusted to 7.2 using 1N NaOH before autoclaving. The flasks were incubated at 39ºC with shaking at 121 rpm (optimized). After 48 h, 1.0 mL samples were collected, centrifuged at 10,000***g*** for 10 min at 4ºC and the cell free supernatant was used as the crude enzyme for further purification.

5.2.5 Purification

5.2.5.1 Ammonium sulphate precipitation

All purification steps were performed at 4°C. After 48 h of cultivation at 39°C with shaking (121 rpm) the culture broth was centrifuged at 10,000***g*** for 15 min. Enzyme in the cell-free supernatant portion of the culture was precipitated by addition of ammonium sulphate ($(NH_4)_2SO_4$) with constant stirring. Ammonium sulphate

fractions of 0-40% and 40-80% (w/v) were collected by centrifugation at 10,000***g*** for 30 min and the enzyme pellet obtained was dissolved in minimal volume of 50 mM Tris-HCl (pH 8.0) buffer. The dissolved pellet was dialyzed against the same buffer with three changes. Dialysis was performed using molecular weight cut off membrane of 5 kDa (HiMedia, India).

5.2.5.2 Ion exchange chromatography

The enzyme extract (dialysate) was further purified using Fast Protein Liquid Chromatography, FPLC (GE Healthcare). 20 mL of the dialysate (0.5 mg/mL, specific activity 1.16 U/mg) was loaded on to diethylaminoethyl (DEAE)-Sepharose column (1.5 x 20 cm). Before loading the sample, the column was pre-equilibrated with 50 mM Tris-HCl buffer pH 8.0. The column was washed with two column volume of the same buffer and the adsorbed protein was eluted with a linear gradient of 0-0.8 M NaCl in equilibration buffer at a flow rate of 1 mL/min. Each fraction of 3 mL was collected for estimation of protein concentration (absorbance 280 nm) and CMCase activity (U/mL). The active fractions containing cellulase activities were pooled and stored at 4ºC for further analysis. SDS-PAGE of the active fractions were carried out to check the homogeneity of the enzyme and to determine its molecular weight. All the purification steps were performed at 4ºC.

5.2.5.3 Analysis of purification by SDS-PAGE

To check the purity of enzyme, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970). 10% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel were used. The loading dye buffer contained 0.0625 M Tris-HCl buffer (pH 6.8), 2.3%

(w/v) SDS, 10% (w/v) glycerol, 5% (w/v) β-mercaptothanol and 0.05% (w/v) bromophenol blue. The purified enzyme sample was mixed with 5x loading dye buffer in the ratio of 4:1. The sample mixture was subjected to heat denaturation for 5 min and centrifuged at 12, 000 rpm for 1 min. The crude and column purified cellulase from different steps of purification were loaded on three identical 10% acrylamide gel and the electrophoresis was carried out using 1x running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl pH 8.3) with a current of 2.5 mA per lane. The first two gels were loaded with same samples of crude supernatant and ammonium sulphate purified cellulase but stained with silver staining (Hames and Rickwood, 1996) and 0.25% (w/v) Coomassie brilliant blue (CBB) R-250, respectively. The third gel was loaded with column purified cellulase fractions and visualized by silver staining protocol. Molecular mass marker proteins used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa) purchased from Bangalore Genei, India, was used as standard for SDS-PAGE.

5.2.5.4 Zymogram analysis

Zymogram analysis was performed according to the method of van Dyk *et al*. (2010). It was carried out by using 0.2% CMC (w/v) polymerized within 12% SDS-PAGE gel. After electrophoresis, the lane containing the marker proteins was excised from rest of the gel and visualized by silver staining. Rest of the gel was immersed in 50 mM glycine-NaOH buffer (pH 9.2) containing 2.5% (v/v) Triton X-100 at shaking condition for 30 min with three changes to remove SDS. Subsequently, Triton X-100 was removed by washing the gel three times with 50 mM glycine-NaOH buffer (pH 9.2). The gel was then incubated in the same buffer at 45°C for 12 h. After incubation,

the gel was stained with 0.3% (w/v) Congo Red for 30 min and then destained with 1M NaCl until zone of clearance was visualized within the gel which confirmed cellulase activity. Gels were then counterstained with 1N HCl for better resolution. The molecular weight of the purified cellulase was estimated based on its mobility calculated with standard calibration proteins using Hendricks plot (Hendrick and Smith, 1968).

5.2.6 Optimization of reaction conditions for maximum cellulase activity

5.2.6.1 Effect of temperature on enzyme activity and stability

The optimum temperature for assay of the enzyme was determined by incubating the mixture of 20 µL purified enzyme (1.16 U/mg, 0.5 mg/mL) with 80 µL of 2% (w/v) CMC in 50 mM glycine–NaOH (pH 9.2) buffer at different temperatures ranging from 20°C to 80°C for 10 min. 100 µL of reaction mixture was analyzed for enzyme activity as described in Section 5.2.2.

The thermal stability of the enzyme was determined by incubating 50 µL purified enzyme (1.16 U/mg; 0.5 mg/mL) in 50 mM glycine–NaOH buffer (pH 9.2) at various temperatures ranging from 20°C to 80°C for 30 min and 60 min. Aliquots of 20 µL were withdrawn at different time intervals and the residual activity was determined according to the method described in Section 5.2.2.

5.2.6.2 Effect of pH on enzyme activity

The optimum pH of the purified cellulase was determined by incubating 100 µL reaction mixture containing 20 µL purified enzyme (1.16 U/mg, 0.5 mg/mL) and 80 µL of 2% CMC in the presence of appropriate buffers at 45°C for 10 min. The buffers used are: 50 mM sodium-acetate buffer prepared by mixing sodium acetate

and acetic acid in two different ratios to obtain pH of 4.0 and 5.0; 50 mM sodium phosphate buffer prepared by mixing two different ratios of sodium dihydrogen phosphate and disodium hydrogen phosphate to obtain pH of 6.0 and 7.0; 50 mM Tris-HCl buffer prepared by mixing two different volumes of HCl to 50 mM Tris to obtain pH of 8.0 and 8.8; 50 mM glycine-NaOH buffer prepared by mixing different volumes of NaOH to 50 mM glycine to obtain pH of 9.0, 9.2, 9.5, 9.8, 10.0 and 11.0 and 50 mM KCl –NaOH buffer (pH 12.0).

5.2.6.3 Substrate specificity of the enzyme

The substrate specificity of the enzyme was determined by incubation of 20 µL of purified enzyme (1.16 U/mg, 0.5 mg/mL) with 80 µL 2% (w/v) of the cellulosic substrates: CMC, hydroxyethyl cellulose, lichenan, laminarin, avicel, steam exploded bagasse and barley β-glucan in 50 mM glycine-NaOH buffer (pH 9.2) at 45°C for 10 min. The total reaction mixture was 100 µL. The amount of reducing sugar produced by the reaction was measured by assay method as mentioned in Section 5.2.2.

5.2.6.4 Kinetic parameters

The enzyme-substrate (CMC) reaction was characterized in terms of Michaelis–Menten kinetic constants (K_m and V_{max}) (Michaelis and Menten, 1913) using the Lineweaver–Burk plot (Lineweaver and Burk, 1934) by assaying the enzyme at CMC concentration ranging from 0.1 mg/mL to 7.0 mg/mL in 50 mM glycine-NaOH buffer (pH 9.2) at 45°C for 10 min. The data were analyzed using GraphPad Prism software (GraphPad Software, Inc. USA).

5.3 Results and Discussion

5.3.1 Purification of cellulase

The purification of cellulase from cell-free supernatant was carried out by ammonium sulphate precipitation followed by ion exchange chromatography using DEAE-Sepharose. After 48 h of incubation the culture broth was centrifuged at 10,000***g*** at 4°C for 15 min. The crude enzyme obtained as a cell free supernatant had specific activity of 0.34 U/mg (Table 5.3.1). This was precipitated with addition of ammonium sulphate and maximum cellulase activity observed in fractions precipitated at 80% saturation. After ammonium sulphate precipitation the enzyme gave specific activity of 1.16 U/mg with 3.4 fold purification and 7.73% yield (Table 5.3.1).

Table 5.3.1 Purification of the cellulase from *Bacillus subtilis* AS3. Enzyme was assayed at pH 9.2 and 45ºC using CMC as substrate.

Purification step	Vol (mL)	Enzyme activity (U/mL)	Total units (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Overall Yield (%)	Fold purification
Crude	250	0.6	150	1.8	450	0.34	-	-
80% $(NH_4)_2SO_4$	20	0.58	11.6	0.5	10	1.16	7.73	3.4
DEAE-Sepharose chromatography	42	0.2	8.4	0.06	2.52	3.33	5.6	9.8

20 mL of ammonium sulphate precipitated enzyme (1.16 U/mg, 0.5 mg/mL) was subjected to DEAE-Sepharose chromatography. Purification by ion exchange chromatography resulted in fraction number 19-32 of each 3 mL fraction size showing high protein content (OD 280 nm). These 14 fractions of 3 mL each (total volume = 42 mL) were pooled for estimation of protein concentration and CMCase activity (Fig. 5.3.1). Fractions with higher CMCase activity were pooled for further work (Fig.

5.3.1). The purified enzyme showed specific activity of 3.33 U/mg with ~ 9.8 fold increase and a final yield of 5.6% (Table 5.3.1).

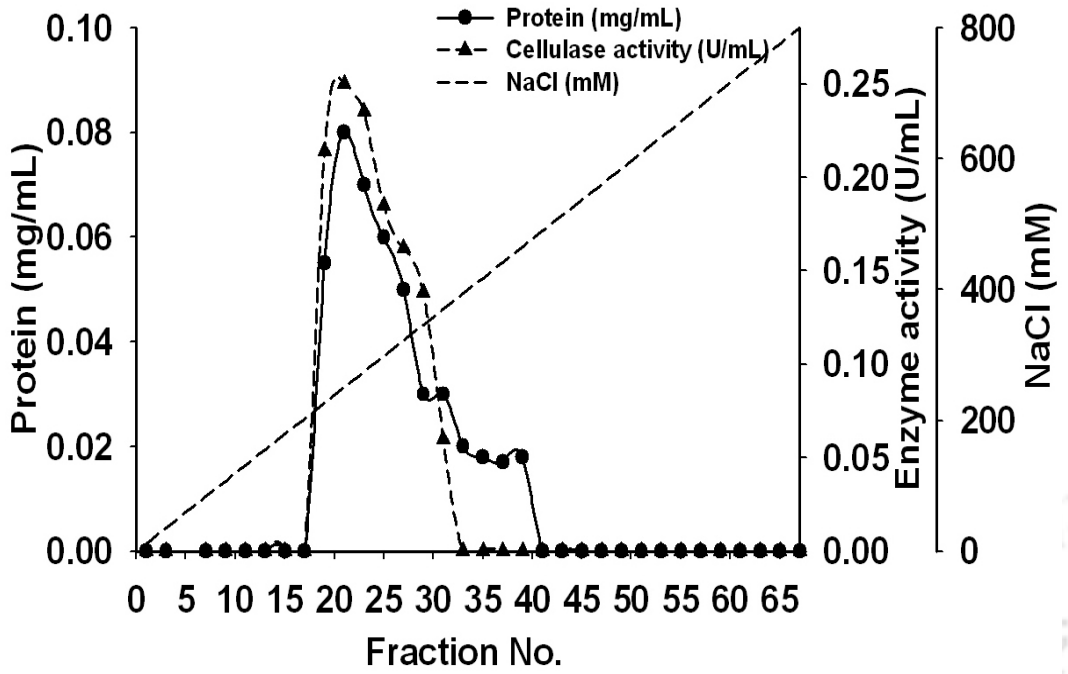

Fig. 5.3.1 Chromatogram of cellulase from *Bacillus subtilis* AS3 on DEAE-Sepharose chromatography (1.5 x 20 cm) equilibrated with 50 mM Tris-HCl buffer pH (8.0) and eluted with linear gradient of 0-800 mM NaCl (●) Protein concentration (mg/mL), (▲) cellulase activity (U/mL), (– – -) NaCl (mM).

5.3.2 SDS-PAGE analysis of fractions of ion exchange chromatography

The fractions (19-32) obtained after ion exchange chromatography showing cellulase activities were analyzed by 10% SDS-PAGE to check the purity of cellulase. Fig. 5.3.2 (A), (B) and (C) showed the different steps of purification of cellulase from *Bacillus subtilis* AS3 which includes crude supernatant, ammonium sulphate purified and DEAE-Sepharose column purified fractions. Fig. 5.3.2 (A) and (B) are identical gels with same samples having crude supernatant and ammonium sulphate purified

enzyme but Fig. 5.3.2 (A) stained with silver stain and Fig. 5.3.2 (B) with Coomassie brilliant blue. Fig. 5.3.2 (C) includes SDS-PAGE with column purified fractions and visualized by silver stain. SDS-PAGE analysis of the column purified fractions showed the presence of multiple protein bands (Fig. 5.3.2 (C)). A prominent protein band of approximately 30 kDa molecular size from column purification was obtained (Fig. 5.3.2 (C)). This was also confirmed by activity staining as described in the Section 5.3.3.

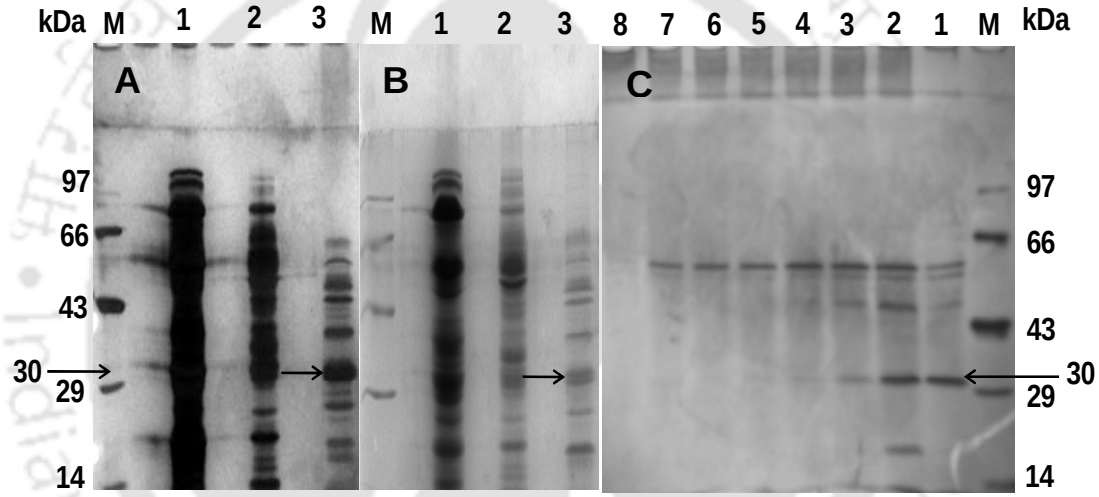

Fig. 5.3.2 10% SDS-polyacrylamide gel electrophoresis showing the purification steps of cellulase from *Bacillus subtilis* (AS3). M: Molecular mass markers. (A) Silver stained gel showing Lane 1-2 crude supernatant; Lane 3, ammonium sulphate precipitated enzyme; (B) Coomassie brilliant blue stained gel showing Lane 1-2 crude supernatant; Lane 3, ammonium sulphate precipitated enzyme; and (C) Silver stained gel showing Lane 1-8 (Fraction No. 19, 21, 23, 25, 27, 29, 31, 32) column purified fractions after ion exchange chromatography using DEAE-Sepharose.

5.3.3 Molecular size characterization of cellulase by zymogram analysis

The purified cellulase obtained from ion exchange chromatography using DEAE-Sepharose was run on SDS-denaturing PAGE gels. The location of activity staining was determined with a silver stained gel as control. A clear hydrolysis activity zone observed which corresponded to approximately 30 kDa molecular size that appeared on the gels stained with silver staining (Fig. 5.3.3).

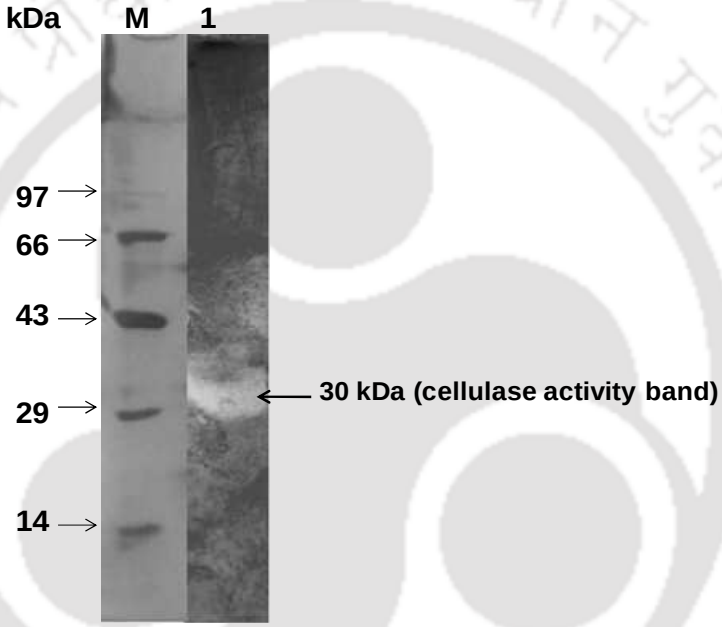

Fig. 5.3.3 12% SDS-PAGE with 0.2% CMC. Lane M: Molecular mass marker stained with silver stain, Lane 1 zymogram of purified cellulase from *Bacillus subtilis* AS3 stained with 0.3% Congo Red, destained with 1M NaCl and counter stained with 1N HCl.

A standard graph was plotted between relative mobility on x-axis and log molecular weight on y-axis for calculation of molecular weight of the purified cellulase (Hendrick and Smith, 1968) (Fig. 5.3.4). The comparison of activity staining and silver staining gels identified the presence of cellulase showing approximate molecular weight of *Bacillus subtilis* (AS3) as 30 kDa. These results were in

accordance with few literature reports where molecular masses of cellulases from *Bacillus* sp. have been found to be within the range. *Bacillus subtilis* YJ1 (Yin *et al*. 2010) showing molecular mass of 32.5 kDa and *Bacillus licheniformis* showing 37 kDa (Bischoff *et al*. 2006).

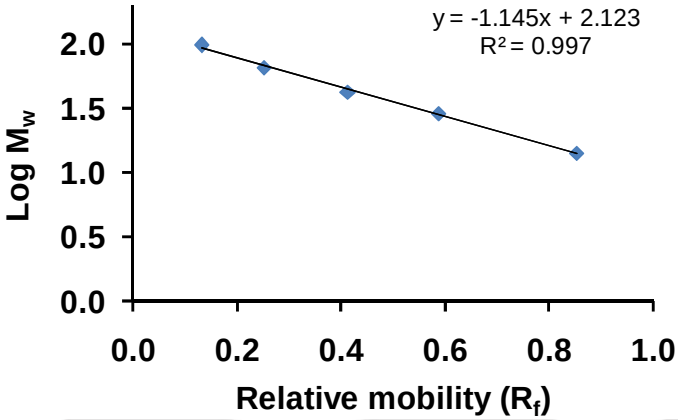

Fig. 5.3.4 Determination of molecular weight (M_w) of an unknown protein by SDS-PAGE. Plot showing R_f versus log M_w to determine molecular weight of the purified cellulase from *Bacillus subtilis* (AS3). The relative mobility of molecular weight marker proteins *viz.* phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa) was determined and plotted in the graph and M_w of unknown protein is calculated by interpolation using this graph.

5.3.4 Effect of temperature on activity and stability of the purified cellulase

The optimum temperature for the reaction with CMC as substrate was detected at around 45°C at pH 9.2 in 50 mM glycine-NaOH buffer and retaining more than 60% of the residual activity at 80°C (Fig. 5.3.5). Reports of a thermophilic *Bacillus* strain has been shown to produce maximum cellulase activity at 50°C, however, activity sharply decreased beyond this temperature (Li *et al*. 2008). Most of the alkaline cellulases from *Bacillus sp* show an optimum activity from 40°C to 60°C

(Kim *et al*. 2005).

The thermal stability analysis revealed that the enzyme is highly stable in the temperature range 20°C to 45°C. At 60°C about 40% activity is retained after incubation for 60 min. At 80°C the enzyme completely loses its activity after incubation for 60 min (Fig. 5.3.5). Similar results were observed with most alkaline cellulases showing thermal stability in the range of 40 to 60°C (Kim *et al*. 2005). In contrast, alkaline cellulases from *Marinobacter* sp. MS 1032 showed stability at 37°C and inactivated rapidly at temperatures higher than 40°C (Shanmughapriya *et al*. 2010).

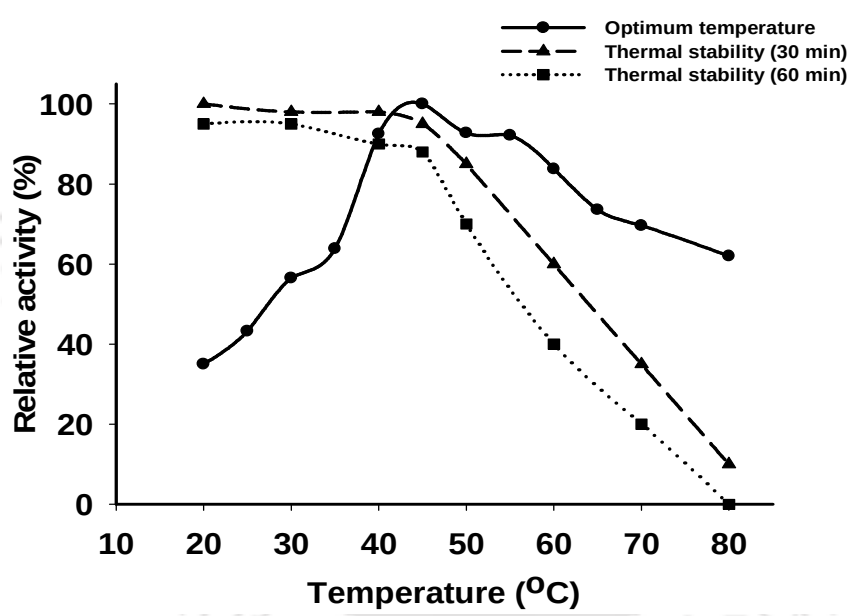

Fig. 5.3.5 Effect of temperature on activity and stability of purified cellulase from *Bacillus subtilis* (AS3) (●) optimum temperature, (▲) thermal stability (30 min), (■) thermal stability (60 min). For the optimal temperature of cellulase, the enzyme was incubated at pH 9.2 with 1% (w/v) CMC at different temperatures ranging from 20-80°C. For thermal stability the enzyme was incubated at different temperatures ranging from 20°C to 80°C for 30 min and 60 min interval. The residual enzyme activity was determined at pH 9.2, 45ºC. Relative activity is expressed as a percentage of the maximum enzyme activity under standard assay conditions.

5.3.5 Effect of pH on activity of the purified cellulase

The cellulase activity was maximum at 50 mM glycine NaOH (pH 9.2) buffer and more than 70% of original activity was retained at pH 8.0 and pH 10.0 (Fig. 5.3.6). This shows that the enzyme is more active at higher pH range. The present study correlate well with earlier reports for alkaline extracellular cellulases from *Marinobacter* sp. MS 1032 (Shanmughapriya *et al*. 2010) and *Bacillus* sp. HSH-10 (Kim *et al*. 2005) showing optimum pH 9.0 and retained about 65% activities at pH 12.0.

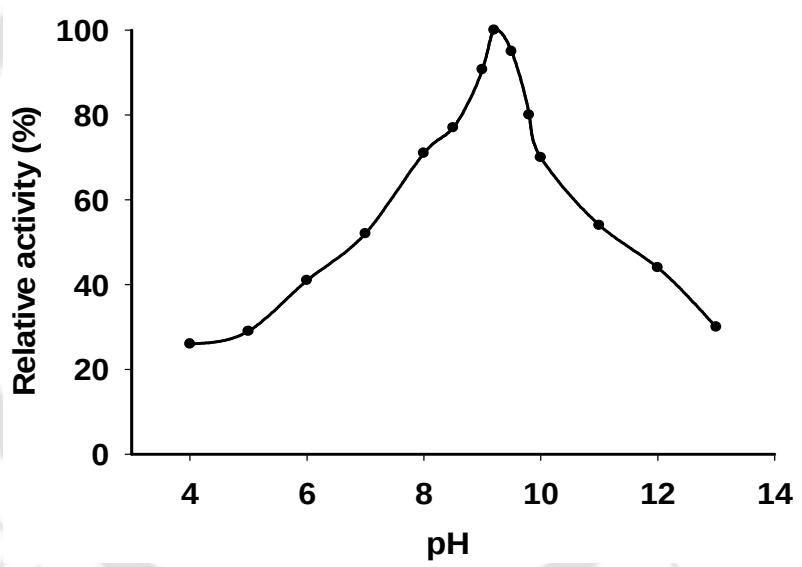

Fig. 5.3.6 Effect of pH on activity of purified cellulase from *Bacillus subtilis* (AS3). The enzyme was incubated at 45°C for 10 min with 1% (w/v) CMC dissolved in 50 mM sodium-acetate buffer pH (4.0-5.0), 50 mM sodium phosphate buffer pH (6.0-7.0), 50 mM Tris-HCl buffer pH (8.0 and 8.5), 50 mM glycine-NaOH buffer pH (9.0, 9.2, 9.5, 9.8, 10.0 and 11.0) or 50 mM KCl–NaOH buffer pH (12.0 and 13.0). Relative activity is expressed as percent of the maximum enzyme activity under standard assay conditions.

In contrast *Bacillus* sp. strains KSM-N252 and KSM-635 showed optimum pH of 9.0 but retained only 0-20% activities at pH 12 (Ito, 1997) and *Bacillus flexus* showed optimum pH 10.0 and retained about 45% activity at pH 12.0 (Trivedi *et al*. 2011).

5.3.6 Substrate specificity of the enzyme

The purified enzyme showed multi-substrate specificity with significantly higher activity with lichenan (β-1,3-1,4 linkage) and barley β-glucan (β-1,3-1,4 linkage) of 6.7 and 7.0 U/mg, respectively, as compared to CMC (β-1,4 linkage), laminarin (β-1,3 linkage), hydroxyethylcellulose and steam exploded bagasse showing activity of 3.1, 0.9, 2.2, 0.9 U/mg, respectively (Table 5.3.2). It showed negligible activity against Avicel (0.1 U/mg) and filter paper (0.3 U/mg) (Table 5.3.2). These values are found superior to many reported literature values. For example *Thermotoga maritima* MSB8 showed maximum specific activity of 6.1 and 6.2 U/mg for lichenan and barley β-glucan, respectively and low level of specific activity against CM-cellulose (0.23 U/mg) at neutral pH (Khan *et al*. 2007). There are reports of production of lichenase like β-glucanase by some strains of *Bacillus* sp. (Shikata *et al*. 1990), but very few alkaline lichenases active at pH 9.0 have been found so far.

Barley β-glucan and lichenan containing 1,3-1,4 mixed linkages are found to be preferred substrates compared to CMC which is a β-1,4 linked cellulose. Therefore, the enzyme seems to be a non typical endo (1,4) β-glucanase with enhanced β-glucanase (lichenase) activity. β-1,3-1,4-Glucanases has been used industrially in the brewing and feedstuff industries. Considering this property the enzyme can be used for various industrial applications.

Table 5.3.2 Activity of the cellulase from *Bacillus subtilis* AS3 on various substrates. 20 µL of purified enzyme (0.5 mg/mL) was incubated with 2% (w/v) of different cellulosic substrates in 50 mM glycine-NaOH buffer (pH 9.2) at 45°C for 10 min.

Substrate	Specific activity (U/mg)
Avicel	0.1
Filter paper	0.3
Steam exploded bagasse	0.9
Laminarin	0.9
Hydroxyethylcellulose	2.2
CMC	3.3
Lichenan	6.7
β-glucan (barley)	7.0

5.3.7 Kinetic characterization of cellulase

The kinetic parameters K_m and V_{max} of the purified enzyme were determined using various concentration of CMC as substrate. The apparent value of Km of cellulase was 0.13 mg/mL and Vmax was 3.38 U/mg (Fig. 5.3.7). The K_m obtained was lower than those reported for other *Bacillus* cellulase with K_m values of 0.59 mg/mL (Sharma *et al.* 1990); and 3.1 mg/mL (Rastogi *et al*. 2010) using CMC as substrate. On the other hand V_{max} values was higher than reported for other *Bacillus* sp with V_{max} values of 0.93 and 1.7 mmol glucose min^{-1} mg $protein^{-1}$ (Mawadza *et al*. 2000) and 0.56 U/mL (Rastogi *et al*. 2010). Therefore, cellulase from the *Bacillus subtilis* AS3 displayed more affinity for CMC.

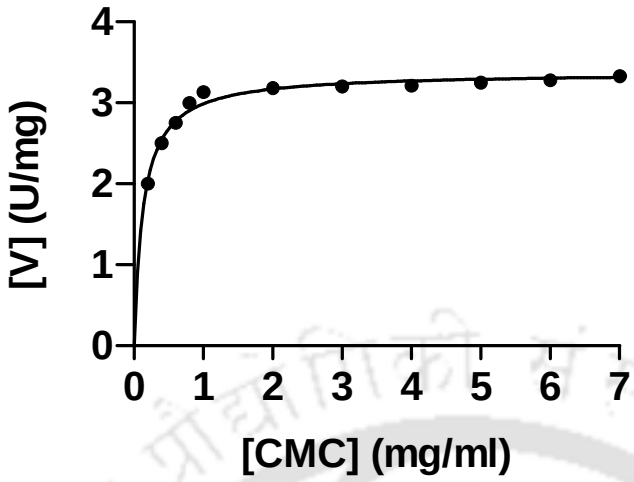

Fig. 5.3.7 Kinetics of the purified cellulase with varying concentrations of CMC (0.1-7.0 mg/mL). Plot of initial velocity of reaction (v) *vs* initial CMC concentration (S). The reaction was performed with varying concentrations of CMC in 50 mM glycine-NaOH buffer (pH 9.2) at 45°C.

5.4 Conclusions

An extracellular alkaline carboxymethylcellulase (CMCase) was purified from the culture supernatant of *Bacillus subtilis* by salt precipitation followed by anion exchange chromatography using DEAE-Sepharose. The purified enzyme showed specific activity of 3.33 U/mg with 9.8 fold increase and a final yield of 5.6%. The molecular mass of this cellulase was found to be approximately 30 kDa. The enzyme showed multi-substrate specificity, with significantly higher activity with lichenan and β-glucan and lower activity with laminarin, hydroxyethylcellulose, CMC and steam exploded bagasse and negligible activity with crystalline substrate such as avicel and filter paper. The purified enzyme was optimally active at pH 9.2 and 45ºC. The enzyme was stable in the temperature range 20°C to 45°C. It retained more than 80% of original activity in the pH range 6-10 and 70% of activity was retained at pH 12. The enzyme showed a K_m of 0.13 mg/mL and V_{max} of 3.38 U/mg using carboxymethylcellulase as substrate at 45°C and pH 9.2. The data showed that the cellulase produced by the isolate is considerably stable at higher pH and temperature well suited to harsh conditions of lignocellulose degradation. Hence, cellulase isolated from *Bacillus subtilis* AS3 is alkaline in nature, thermostable and active over a wide range of pH, showing significant activity for lichenan and β-glucan and can be used for various applications. Therefore, considering the versatile nature of the enzyme, the focus will be on to utilize for alternative fuel generation such as bioethanol at laboratory scale.

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Chapter 6

Bioethanol production from thatch grass (*Hyparrhenia rufa*) by Simultaneous Saccharification and Fermentation involving recombinant and microbial released cellulases and different fermentative microbes

6.1 Introduction

The exhaustion of fossil fuel reserves and increasing problem of greenhouse gas effects due to its overuse have stimulated a worldwide interest in alternative, non-petroleum based sources of energy (Alan *et al*. 1999). The use of bioethanol as an alternative fuel derived by fermentation process will significantly reduce the consumption of crude oil and eventually the net carbon dioxide emissions. The choice of feedstock for bio-ethanol production is a major concern since the biomass either directly or indirectly competes with the food crops. Crops like corn and sugarcane are well established for commercial bioethanol production. However, it is being less favoured owing to their primary importance as a source of food besides adding to the cost of production. Lignocellulosic biomass such as crop residues and agricultural wastes are abundantly available and much cheaper considered against food crops to be used as raw material for bioethanol production (Fujii *et al.* 2009, Wyman, 2007;

Sanchez and Cardona, 2008). They contain lot of fermentable carbohydrates in the form of hexose and pentose polymers which have huge potential as an alternative for renewable energy resource (Zaldivar *et al*. 2001). The major rate limiting step in efficient economic conversion of such substrates to liquid biofuels is efficacy of hydrolytic enzymes and degree of crystallinity of the lignocellulosic biomass. It is therefore important to employ more effective hydrolytic enzymes with maximum release of utilizable sugars for bioethanol production. Pretreatment breaks down the lignocellulosic complex composed of cellulose and lignin moieties bound by hemicellulose chains and make the substrate accessible for enzymatic hydrolysis (Lee *et al*. 1994; Mosier *et al*. 2005). The basic purpose of pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity and enzymatic hydrolysis (Lynd *et al*. 2002; Kumar *et al*. 2009).

T. reesei cellulases have been under use for lignocellulose degradation for few decades but the major impediments in use of fungal cellulase are slow growing, absence of prominent β-glucosidase activity, and sensitive to high temperature. *Clostridium thermocellum* contains genes coding for exocellular multienzyme complexes called cellulosomes exhibiting endoglucanase and exoglucanase activity (Taylor *et al*. 2005). The cellulosome displays 50-fold higher specific activity against crystalline cellulose in comparison to *Trichoderma reesei* (Fontes and Gilbert, 2010). Glycoside hydrolase (GH5) family 5 gene from *Clostridum thermocellum* belongs to a set of enzymes with varying substrate specificity having high cellulase activity (Bharali *et al*. 2005). These cellulolytic enzyme systems cloned and expressed in a suitable vector can be utilized for lignocellulose degradation.

Bacillus species produce a number of extracellular polysaccharide hydrolyzing enzymes and can withstand harsh conditions such as high temperature, sugar, salt and ethanol concentrations during lignocellulose degradation (Maki *et al*. 2009). *Bacillus subtilis* (AS3) produce a thermostable cellulase system which remain active in varying pH conditions and wide substrate specificity (Deka *et al*. 2011). The optimum temperature for *Bacillus subtilis* activity has been determined to be somewhat close to that of recombinant and fungal cellulases (Deka *et al*. 2011).

The reducing sugars released after enzymatic hydrolysis are consumed by yeasts like *Saccharomyces cerevisiae* and *Candida shehatae* and bacterium such as *Zymomonas mobilis. Saccharomyces cerevisiae* provides a better choice for production of ethanol in large scale fermentation, due to their inherent ability to utilize various substrates, high ethanol tolerance and its robustness to withstand range of metabolic inhibitions (Casey and Ingledew, 1986). While, both *S. cerevisiae* and *Z. mobilis* are known to utilize hexoses predominantly, the key enzymes for ethanol fermentation: alcohol dehydrogenase and pyruvate decarboxylase was reported to be best expressed in *Z. mobilis* (Sprenger, 1996). However, it cannot utilize pentose sugars which are also major components of lignocellulosic biomass. Yeasts like *C. shehatae* having key enzymes xylose reductase and xylitol dehydrogenase can assimilate pentose sugars (arabinose and xylose) to ethanol by the pentose phosphate pathway (Sanchez and Cardona, 2008; Kadam and Schmidt, 1997; Wang and Jeffries, 1990).

Among all processes involved for bioethanol production from lignocellulosic biomass, simultaneous saccharification and fermentation (SSF) appears as a promising alternative to separate hydrolysis and fermentation. In this process, the enzymatic

hydrolysis of cellulose and the fermentation of reducing sugars (glucose) are performed in one single step, which means a reduction in the end-product inhibition of the enzymatic complex (Stenberg *et al*. 2000 and Xiao *et al*. 2004). SSF process first described by Takagi *et al*. (1977), scores over separate hydrolysis and fermentation presenting higher saccharification rate and ethanol yield by eliminating end product inhibition and decreased risk of contamination.

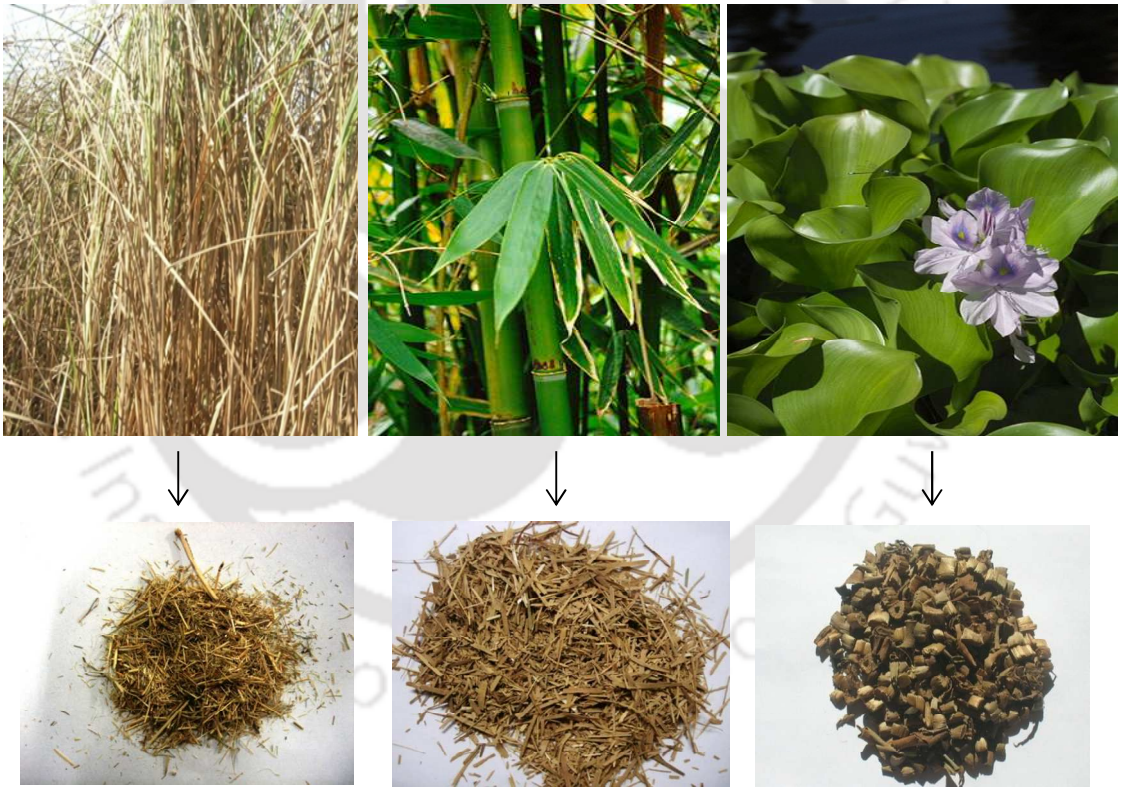
The present study was carried out to compare the hydrolytic performance of *Trichoderma reesei* cellulase, recombinant cellulase (GH5) and thermostable cellulase obtained from *Bacillus subtilis* AS3 in batch SSF process. Three different pretreatments *viz*., steam explosion, phosphoric acid-acetone and AFEX along with different fermentative microbes like *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Candida shehatae* were employed using thatch grass in batch SSF process at shake flask and bioreactor level.

6.2 Materials and Methods

6.2.1 Reagents and substrates

Ampicillin, potassium dichromate ($K_2Cr_2O_7$) and components for LB and GYE medium and other reagents were of analytical grade procured from Merck and HiMedia laboratories (India). Carboxymethylcellulose (low viscosity, 50-200 cP) was purchased from Sigma Aldrich (St. Louis, USA).

Three different lignocellulosic substrates thatch grass (*Hyparrhenia rufa*), bamboo (*Bambusa dendrocalmus*) leaves and water hyacinth (*Eichhornia crassipes*) were used for SSF study (Fig. 6.2.1).



A Thatch grass (*Hyparrhenia rufa*)

B Bamboo leaves (*Bambusa dendrocalmus*)

C Water hyacinth (*Eichhornia crassipes*)

Fig. 6.2.1 Lignocellulosic substrates selected for simultaneous saccharification and fermentation study: (A) Thatch grass (B) Bamboo leaves (C) Water hyacinth.

Thatch grass is a weed collected from banks of river Brahmaputra near North Guwahati, Assam. Bamboo leaves and water hyacinth generally go as wastes were collected from campus of IIT Guwahati (Fig. 6.2.1). These substrates were selected based on the percent fraction of cellulose, hemicellulose and lignin present in its biomass. They are abundantly available in North-east India with advantage of non-competitor to food crops and could be used suitably for bioethanol production. The selected substrates are cut into small pieces of length 4-5 cm. Prior to pretreatment, they were washed with water to remove unwanted dust particles and then dried at 80°C in hot air oven. The substrates were further grinded in a mixer and passed through a 1 mm mesh size sieve and ready to undergo pretreatment.

6.2.2 Microorganisms and culturing conditions

Trichoderma reesei (MTCC 164) and *Zymomonas mobilis* (MTCC 2427) were procured from IMTECH, Chandigarh whereas, *Saccharomyces cerevisiae* (NCIM 3215) and *Candida shehatae* (NCIM 3500) was procured from National Chemical Laboratory, Pune, India. *Bacillus subtilis* AS3 (Genebank accession No. EU754025) isolated from cow dung was a gift from Prof. D. Goyal, Thapar University, Patiala, India. The recombinant family 5 glycoside hydrolase (GH5) gene was cloned and expressed earlier by Prof. Arun Goyal as reported elsewhere (Taylor *et al*. 2005) and is now commercially available with NZY Tech, Lda, Lisbon, Portugal.

Bacillus subtilis inoculum was prepared by taking a loop full of culture from the nutrient agar slant in a test tube containing 5 mL of nutrient broth with 2% (w/v) glucose and incubated at 37°C and 180 rpm in an incubator shaker for 16-18 h (to reach optical density (OD) at 600 nm = 0.6~0.8). For enzyme production, 2% (v/v) of

the fresh inoculum culture was transferred to two 250 mL erlenmeyer flasks each containing 50 mL optimized media (Deka *et al*. 2011). The pH was adjusted to 7.2 using 1N NaOH before autoclaving. The flasks were incubated at 39ºC with shaking at 121 rpm. After 48 h, 1.0 mL samples were collected, centrifuged at 10,000***g*** for 10 min at 4ºC and the cell free supernatant was used as the crude enzyme for SSF experiment.

Trichoderma reesei was maintained in potato dextrose medium at 4°C (Brijwani *et al*. 2010). 1 mL of spore suspension (5 x 10^7 spores/mL) was inoculated to 100 mL of Potato dextrose broth and incubated at 28°C with shaking at 120 rpm for 48 h. 1.0 mL of culture broth was centrifuged at 10,000 rpm for 15 min and the cell free supernatant obtained was filtered twice and 1 mL of the filtrate was used as the crude enzyme for SSF experiment.

Saccharomyces cerevisiae was maintained in MGYP agar slants containing (g/100 mL): Malt extract (0.3), Glucose (1), Yeast extract (0.3) and Peptone (0.5) (Wickerman, 1951) and stored at 4°C. A loop full of these slant culture was transferred to GYE medium containing (g/100 mL) Glucose (1), Yeast extract (0.1) supplemented with KH_2PO_4 (0.1), $(NH_4)_2SO_4$ (0.5) and $MgSO_4.7H_2O$ (0.05) and incubated at 30°C with shaking at 120 rpm for 48 h. 1 mL of actively growing aerobic culture (3.6 x 10^8 cells/mL) was transferred to the fermentation medium for SSF experiment.

Candida shehatae was maintained in MGYP agar slants containing (g/100 mL): Malt extract 0.3, Glucose 1, Yeast extract 0.3 and Peptone 0.5 (Wickerman, 1951) and stored at 4°C. A loop full of these slant culture was transferred to GYE medium containing (g/100 mL) Glucose (1), Yeast extract (0.1) supplemented with

KH_2PO_4 (0.1), $(NH_4)_2SO_4$ (0.5) and $MgSO_4.7H_2O$ (0.05) and incubated at 30°C with shaking at 120 rpm for 48 h. 1 mL of actively growing culture of *C. shehatae* (2.1 x 10^8 cells/mL) was transferred to the fermentation medium for SSF experiment.

Zymomonas mobilis inoculum was prepared by growing the strain in the medium containing (g/100 mL) glucose (2); Yeast extract (1); KH_2PO_4 (0.1); $(NH_4)_2SO_4$ (0.1) and $MgSO_4.7H_2O$ (0.05) (Santos *et al*. 2010). The pH was adjusted to 6.0 and incubated at 30°C with shaking at 120 rpm for 48h. 1.0 mL of actively growing aerobic culture (2.1 x 10^6 cells/mL) was transferred to the fermentation medium for SSF experiment.

Recombinant *E. coli* BL21 (DE3) cells transformed with plasmid containing glycoside hydrolase family 5 gene from *Clostridium thermocellum* inserted in an expression vector pET21a (Bharali *et al*. 2005) was used as source of recombinant cellulase enzyme and maintained in LB medium with ampicillin (100 µg/mL) as glycerol stock at –80°C (Taylor *et al*. 2005).

6.2.3 Production of recombinant cellulase (GH5)

1% of *E. coli* Bl-21 cells from glycerol stock were inoculated into 5 mL of LB medium containing 100 µg/mL ampicillin and incubated at 37°C for 16 h at 180 rpm. 1% of the culture inoculum was transferred to 250 mL of LB medium in 500 mL flask containing 100 µg/mL ampicillin and was incubated at 37°C, 180 rpm till mid-exponential phase to reach optical density (OD) at 600 nm around 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to this mid-exponential to a final concentration of 1 mM and incubated further 8 h for protein induction (Taylor *et al*. 2005). The cells were collected by centrifugation (9,000***g***, 4°C, 15 min) and the cell

pellet was resuspended in 50 mM sodium phosphate buffer (pH 6.0). The recombinant enzymes were expressed as soluble protein. The cell extract containing soluble enzyme was sonicated in an ice bath for 15 min followed by centrifugation at 13,000***g***, 4°C for 30 min. The supernatant was used as the enzyme source for SSF experiment.

6.2.4 Pretreatment of substrate

6.2.4.1 Steam explosion

One g of powdered thatch grass was soaked in deionized water (0.6 mL/g of dry biomass). The slurry was autoclaved at 15 psi and 121°C for 15 min followed by sudden steam depressurization by fully opening the steam exhaust valve (Sharma *et al*. 2007). It was then used for SSF studies.

6.2.4.2 Ammonia fibre explosion (AFEX)

One g of powdered thatch grass was mixed with 2.5 mL of aqueous ammonia solution in a crucible. The slurry was then kept at 170°C for 1 h in digital muffle furnace (Dale *et al*. 1996; Garlock *et al*. 2011) and finally used for SSF studies.

6.2.4.3 Phosphoric acid-acetone

One g of powdered thatch grass mixed with 8 mL of concentrated phosphoric acid (of not less than 85% concentration) was kept at 50°C at 120 rpm for 1 h. The slurry was and then poured into 24 mL of chilled acetone and mixed thoroughly. The mixture was centrifuged at 8000 rpm for 10 min. The pellet was collected and washed 3 times with distilled water. During the third washing, pH was adjusted to 5-6 using NaOH (Li *et al*. 2009).The pretreated substrate was then used for SSF studies.

6.2.5 Simultaneous saccharification and fermentation using 1% (w/v) thatch grass at shake flask level

In the first step, hydrolytic efficiency of *Trichoderma reesei* cellulase, recombinant cellulase (GH5) and *Bacillus subtilis* AS3 cellulase alongwith three different pretreatment methods and fermentative microbes like *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Candida shehatae* are compared in batch SSF process. Overall, twenty seven SSF trials were executed in nine batches. SSF studies were carried out using one gram of the pretreated thatch grass autoclaved in a 250 mL erlenmeyer flask separately containing 100 mL working volume of sodium phosphate buffer (20 mM, pH 6.0) supplemented with 0.1% (w/v) each of yeast extract along with peptone and used as the fermentation medium.

The first batch of SSF experiment comprised fermentation medium with 1% (w/v) steam exploded thatch grass along with1 mL of *Trichoderma reesei* cellulase (12.9 U/mg, 0.82 mg/ml) for hydrolysis and 1 mL of *S. cerevisiae* (3.6 x10^8 cells/mL) as the fermentative organism. Similar enzymatic and fermentative combination was applied for 1% (w/v) acid-acetone and AFEX pretreated thatch grass.

The second batch of fermentation medium contained 1% steam exploded thatch grass, 1 mL of crude recombinant cellulase (GH5) (5.6 U/mg, 0.44 mg/mL) as the saccharifying enzyme with 1 mL of *S. cerevisiae* (3.6 x10^8 cells/mL) as bioethanol producers. Recombinant *E. coli* BL21 (DE3) cells transformed with plasmid containing glycoside hydrolase family 5 gene from *Clostridium thermocellum* inserted in an expression vector pET21a (Bharali *et al*. 2005) was used as the source of recombinant cellulase (GH5) enzyme. Recombinant cellulase (GH5) production has been described in Section 6.2.3.Similar hydrolytic enzymes and bioethanol producer

was used for 1% (w/v) acid-acetone and AFEX pretreated thatch grass.

The third batch of SSF medium having 1% steam exploded thatch grass involving 1 mL of *B. subtilis* cellulase (3.3 U/mg, 0.5 mg/mL) for hydrolysis and 1 mL of *S. cerevisiae* (3.6 x10^8 cells/mL) as the bioethanol producer. Likewise, SSF using 1% (w/v) acid-acetone and AFEX pretreated thatch grass were carried out using same group of enzymes and fermentative microbe. The three batch of SSF experiments are compared with the aim to determine the best pretreatment and better performing hydrolytic enzymes.

The fourth batch comprised of *T. reesei* (12.9 U/mg, 0.82 mg/ml) cellulase for saccharification and *Z. mobilis* inoculum (2.1 x 10^6 cells/mL) for fermentation. This is employed equally for 1% (w/v) steam exploded, acid-acetone and AFEX pretreated thatch grass.

The fifth batch of SSF medium consisting of 1% (w/v) steam exploded thatch grass contained 1 mL of crude recombinant cellulase (GH5) (5.6 U/mg, 0.44 mg/mL) for hydrolysis and 1 mL of *Z. mobilis* inoculum (2.1 x 10^6 cells /mL) as the fermentative microbe. Same enzyme and fermentative microbe were included in SSF using 1% (w/v) acid-acetone and AFEX pretreated thatch grass.

The six batch of SSF medium encompassing 1% steam exploded thatch grass along with 1 mL of *B. subtilis* cellulase (3.3 U/mg, 0.5 mg/mL) for hydrolysis and 1 mL of *Z. mobilis* inoculum (2.1 x 10^6 cells /mL) as the bioethanol producer. Similar mixture of enzyme and fermentative microbe were engaged during SSF using acid-acetone and AFEX pretreated thatch grass. The fourth, fifth and sixth batches are compared with the previous batches with the purpose of determining the superior hydrolytic enzyme and fermentative microbe.

The seventh batch comprised SSF medium with mixture of 1mL of *T. reesei* cellulase (12.9 U/mg, 0.82 mg/ml) and 0.5 mL each of *S. cerevisiae* (3.6 x10^8 cells/mL) and *C. shehatae* (2.1 x 10^8 cells/mL) inoculum. This combination of enzyme and fermentative microbe is employed identically for 1% (w/v) steam exploded, acid-acetone and AFEX pretreated thatch grass.

The eight batch of SSF medium comprising 1% steam exploded thatch grass were carried out employing1 mL of crude recombinant cellulase (GH5) (5.6 U/mg, 0.44 mg/mL) and 0.5 mL each of *S. cerevisiae* inoculum (3.6 x10^8 cells/mL) and *C. shehatae* (2.1 x 10^8 cells/mL) as the bioethanol producers. SSF experiments with 1% (w/v) acid-acetone and AFEX pretreated thatch grass used similar combinations of enzyme and fermentative microbes.

The ninth batch of SSF medium having 1% (w/v) steam exploded thatch grass was carried out involving 1 mL of *B. subtilis* cellulase (3.3 U/mg, 0.5 mg/mL) for hydrolysis and using 0.5 mL each of *S. cerevisiae* inoculum (3.6 x10^8 cells/mL) and *C. shehatae* (2.1 x 10^8 cells/mL) as fermentative microbes. Similarly, SSF using 1% (w/v) acid-acetone and AFEX pretreated thatch grass were carried out using identical enzymatic and fermentative combination. The seventh, eight and ninth batches are compared with the preceding batches to determine overall the best pretreatment method, best hydrolytic enzymes and best bioethanol producers.

Each of the twenty seven flasks (triplicate sets of each nine batches) was incubated at 30°C at 120 rpm for 72 h. 1.5 mL of sample was collected at every 6 h till 72 h. The dynamic profile of the SSF was obtained by estimating various fermentation parameters like cell OD, reducing sugar (g/L), specific activity (U/mg) and ethanol content (g/L).

6.2.6 Simultaneous saccharification and fermentation using AFEX pretreated 5% (w/v) thatch grass in shake flask level

A higher substrate concentration of 5% (w/v) AFEX pretreated thatch grass was used for best SSF combination involving *B. subtilis* as hydrolyzing enzyme and mixed culture of *S. cerevisiae* and *C. shehatae* as bioethanol producer. 5 g of AFEX pretreated thatch grass was autoclaved in a 250 mL erlenmeyer flask containing 100 mL working volume of sodium phosphate buffer (20 mM, pH 6.0) supplemented with 0.1% (w/v) each of yeast extract and peptone used as the fermentation medium.5 mL of *B. subtilis* cellulase (3.3 U/mg, 0.5 mg/mL) for hydrolysis along with 2.5 mL of *S. cerevisiae* (3.6 x10^8 cells/mL) and 2.5 mL of *C. shehatae* (2.1 x 10^8 cells/mL) as the fermentative microbes were added to the fermentation medium. The flasks were incubated at 120 rpm and 30°C. 1.5 mL of sample was collected at every 6 h till 72 h with the estimation of fermentation parameters like cell OD, reducing sugar (g/L), specific activity (U/mg) and ethanol content (g/L).

6.2.7 Simultaneous saccharification and fermentation using AFEX pretreated 5% (w/v) thatch grass in bioreactor

Bioethanol production in batch mode was scaled up in a 2 L capacity bioreactor (Applicon, model Bio Console ADI 1025) using 5% (w/v) AFEX pretreated thatch grass with a working volume of 1 L sodium phosphate buffer (20 mM, pH 6.0) supplemented with 0.1% (w/v) each of yeast extract and peptone. 50 mL of *B. subtilis* cellulase (3.3 U/mg, 0.5 mg/mL) was used for saccharification alongwith 25 mL of *S. cerevisiae* (3.6 x10^8 cells/mL) and 25 mL of *C. shehatae* (2.1 x 10^8 cells/mL) were added to the fermentation medium for bioethanol production. Agitation rate of 120 rpm, temperature of 30°C and 1 vvm (volume of air per volume of liquid per min)

aeration rate were maintained by a mass flow controller. Cell growth was monitored by a spectrophotometer (Varian, model Cary50) at 600 nm. The online process parameters like temperature (°C), pH and stirring rate (rpm) were recorded. The various offline parameters such as cell OD, reducing sugar (g/L), specific activity (U/mg) and ethanol content (g/L) were estimated at every sampling time period of 6 h.

6.2.8 Analytical methods

6.2.8.1 Assay of enzyme activity

The enzyme assay of crude recombinant (GH5) was carried out by incubating the enzyme with CMC for 10 min at 50°C. The reaction mixture (100 µL) contained 10 µL of enzyme and 1.0% final concentration of CMC in 20 mM sodium acetate buffer (pH 4.3). In case of *B. subtilis* AS3 the assay of cellulase was carried out in 100 µL of reaction mixture containing 1.3% final concentration of CMC (65 µL of 2% CMC) in 20 mM Glycine NaOH buffer (pH 9.2) and 35 µL of cell free supernatant and incubated at 45°C for 10 min. The cellulase activity was measured by estimating the liberated reducing sugar following Nelson-Somogyi procedure (Nelson 1944; Somogyi 1945). The absorbance was measured at 500 nm using a UV-visible spectrophotometer (Perkin Elmer, Model Lambda-45) against a blank with D-glucose as standard. One unit (U) of cellulase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar (glucose) per min at 50°C.

6.2.8.2 Estimation of protein concentration

The protein concentration was determined by Bradford method using bovine serum albumin (BSA) as standard (Bradford, 1976).

6.2.8.3 Ethanol estimation

For ethanol content estimation, dichromate method was used where ethanol produced was converted to acid by reaction with dichromate (Seo *et al*. 2009). The cell free culture was diluted 10 times (reaction volume 10 mL) to which 2 mL of potassium dichromate ($K_2Cr_2O_7$) (3.37 g/100 mL) was added and absorbance was measured on spectrophotometer (Perkin Elmer, Model Lambda-45) at 600 nm.

6.2.8.4 Structural carbohydrates estimation

Cellulose, hemicellulose and lignin were determined by standardized methods of National Renewable Energy Laboratory (NREL), USA (Sluiter *et al*. 2008). 0.3 g of cellulosic substrate (leafy biomass) was mixed with 3 mL of 72% H_2SO_4 and incubated at 30°C for 1 h. To the mixture, 84 mL of distilled water was added to bring down concentration of H_2SO_4 to 4% (v/v) and was further autoclaved at 121°C and 15 psi pressure for 1 h followed by vaccum filtration. The residue collected after filtration was weighed which is acid insoluble lignin. The pH of the collected filtrate was neutralized by addition of 1 M $CaCO_3$solution. Finally, the filtrate was assayed for reducing sugar which is glucose from where cellulose is calculated (1 g cellulose = 1.1 g of glucose). The remaining content was hemicellulose.

6.3 Results and Discussion

6.3.1 Structural carbohydrates determination

The composition analysis of structural carbohydrates of thatch grass, bamboo leaves and water hyacinth are presented in Table 6.3.1. The values obtained are found to be similar to Bermuda grass, reed and rapeseed reported to having cellulose content of 47.8%, 39.5%, 27.6% and lignin content of 19.4%, 24.0%, and 18.3% (Li *et al*. 2009), respectively. On analysis, it was observed that thatch grass contained higher content of cellulose and less lignin content as compared to the other two substrates. Therefore, thatch grass was found to be more suitable for bioethanol production hence, it was used for all SSF experiments in the present study.

Table 6.3.1 Structural carbohydrate composition determination of the selected substrates

Substrates	Cellulose (%)*	Hemicellulose (%)*	Lignin (%)*
Thatch grass (*Hyparrhenia rufa*)	45.07 ± 0.41	29.06 ± 0.49	17.43 ± 0.52
Bamboo leaves (*Bambusa dendrocalmus*)	32.05 ± 0.48	34.68 ± 0.73	26.19 ± 0.40
Water hyacinth (*Eichhornia crassipes*)	20.91 ± 0.53	44.88 ± 0.64	30.04 ± 0.43

**values are mean ± SE (n=3)*

6.3.2 Pretreatment of substrate (Thatch grass)

Pretreatment breaks down the lignocellulosic matrix thereby enhancing the accessibility of the substrate for enzymatic hydrolysis (Lee *et al*. 1994; Mosier *et al*. 2005). The basic rationale of pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity and enzymatic hydrolysis (Lynd et al. 2002; Kumar *et al*. 2009). Thatch grass was used as substrate for all SSF experiments.

Three different methods for pretreatment of substrates was employed such as steam explosion, phosphoric acid-acetone and ammonia fiber explosion method (AFEX). Table 6.3.2 shows comparative chart of different pretreatment methods with different combinations of hydrolytic enzymes and fermentative microbes. Among these pretreatment methodologies, AFEX pretreatment found to be better than steam explosion and phosphoric acid-acetone technique on the basis of maximum release of sugars and high ethanol yield obtained from SSF experiments (Table 6.3.2). AFEX pretreatment removes simultaneously lignin and hemicellulose, while decrystallizing cellulose, thereby increasing the cellulose accessibility to enzymes (Jung *et al*. 2011; Garlock *et al*. 2011). It also has the advantage of minimizing the formation of sugar degradation products and inhibitors during the downstream processes resulting in higher yields of ethanol (Mosier *et al*. 2005; Teymouri *et al*. 2005). AFEX pretreatment was reported to be more effective for herbaceous crops and grasses than for hardwood and softwood (McMillan, 1994). Sun and Cheng (2002) reported 90% hydrolysis of cellulose and hemicellulose and 5% lignin after AFEX pretreatment of bermuda grass and bagasse.

The hydrolytic performance of the cellulolytic enzymes from *T. reesei*, recombinant *E. coli* and *B. subtilis* AS3 and the performance of fermentative microbes *S. cerevisiae*, Z. *mobilis* and *C. shehatae* are compared with the aim to determine the efficient enzyme and bioethanol producing microorganism in terms of release of maximum reducing sugars and ethanol yield (Table 6.3.2).

6.3.3 SSF with different pretreatments and 1% (w/v) thatch grass at shake flask level

Preliminary SSF were carried out using 1% (w/v) thatch grass to find out the most efficient of the three pretreatment strategies such as steam explosion, acid-acetone and AFEX. The pretreated thatch grass substrate was subjected to simultaneous saccharification and fermentation using cellulolytic enzymes from *T. reesei*, recombinant *E. coli* (DE3) and *B. subtilis* AS3 and fermentative microbes such as *S. cerevisiae, Z. mobilis* and *C. shehatae*.

First batch of SSF experiments involving microbial combination of *T. reesei* cellulase and *S. cerevisiae* obtained an ethanol titre of 0.78 g/L from a reducing sugar concentration of 0.88 g/L. On the other hand, phosphoric acid-acetone pretreatment gave a titre of 0.83 g/L and AFEX pretreatment achieved an ethanol titre of 0.86 g/L from reducing sugar concentration of 0.91 g/L with a yield of 0.086 (g of ethanol/ g of substrate) (Table 6.3.2).

Second batch of SSF experiments with steam exploded thatch grass gave an ethanol titre of 0.8 g/L from a reducing sugar concentration of 0.92 g/L. Phosphoric acid-acetone pretreatment gave an ethanol titre of 0.82 g/l from reducing sugar of 0.93 g/L and AFEX pretreatment resulted in ethanol titre of 0.88 g/L from reducing sugar

concentration of 0.96 g/L with a yield of 0.088 g/g with recombinant cellulase (GH5) as saccharifying enzyme and *S. cerevisiae* as bioethanol producer (Table 6.3.2).

Table 6.3.2 Ethanol production by SSF at shake flask using 1% (w/v) thatch grass with different pretreatments along with different combinations of hydrolytic enzymes and fermentative microbes

Batch No	SSF combination	Pretreatment	Reducing sugar * (g/L)	Ethanol yield# (g/g)	Ethanol titre* (g/L)
1.	*T. reesei* cellulase + *S. cerevisiae*	Steam explosion	0.88 ± 0.04	0.078	0.78 ± 0.08
		Acid-acetone	0.90 ± 0.01	0.083	0.83 ± 0.02
		AFEX	0.91 ± 0.06	0.086	0.86 ± 0.01
2.	Recombinant cellulase (GH5) + *S. cerevisiae*	Steam explosion	0.92 ± 0.01	0.080	0.80 ± 0.04
		Acid-acetone	0.93 ± 0.04	0.082	0.82± 0.05
		AFEX	0.96 ± 0.02	0.088	0.88 ± 0.03
3.	*B. subtilis* (AS3) cellulase + *S. cerevisiae*	Steam explosion	1.05 ± 0.05	0.083	0.83 ± 0.05
		Acid-acetone	1.12 ± 0.03	0.088	0.88 ± 0.01
		AFEX	1.16 ± 0.04	0.094	0.94 ± 0.08
4.	*T. reesei* cellulase + *Z. mobilis*	Steam explosion	1.1 ± 0.03	0.118	1.18 ± 0.06
		Acid-acetone	1.24 ± 0.1	0.121	1.21 ± 0.08
		AFEX	1.30 ± 0.04	0.128	1.28 ± 0.03
5.	Recombinant cellulase (GH5) + *Z. mobilis*	Steam explosion	1.45 ± 0.03	0.142	1.42 ± 0.04
		Acid-acetone	1.53 ± 0.01	0.150	1.50 ± 0.05
		AFEX	1.58 ± 0.02	0.156	1.56 ± 0.03
6.	*B. subtilis* (AS3) cellulase + *Z. mobilis*	Steam explosion	1.09 ± 0.04	0.09	0.90 ± 0.02
		Acid-acetone	1.6 ± 0.03	0.156	1.56 ± 0.01
		AFEX	1.72 ± 0.04	0.162	1.62 ± 0.07
7.	*T. reesei* cellulase + *S. cerevisiae* + *C. shehatae*	Steam explosion	1.40 ± 0.04	0.135	1.35 ± 0.06
		Acid-acetone	1.43 ± 0.12	0.140	1.40 ± 0.04
		AFEX	1.53 ± 0.08	0.150	1.50 ± 0.07
8.	Recombinant cellulase (GH5) + *S. cerevisiae* + *C. shehatae*	Steam explosion	1.56 ± 0.01	0.155	1.55 ± 0.04
		Acid-acetone	1.61 ± 0.01	0.159	1.59 ± 0.05
		AFEX	1.72 ± 0.02	0.164	1.64 ± 0.05
9.	*B. subtilis* (AS3) cellulase + *S. cerevisiae* + *C. shehatae*	Steam explosion	1.65 ± 0.03	0.161	1.61 ± 0.02
		Acid-acetone	1.68 ± 0.02	0.166	1.66 ± 0.03
		AFEX	1.98 ± 0.01	0.194	1.94 ± 0.04

**the values correspond to the maximum reducing sugar and maximum ethanol titre at a particular time. # g of ethanol/ g of substrate. values are mean ± SE (n=3)*

Third batch of SSF trial with steam exploded thatch grass gave an ethanol titre of 0.83g/L from a reducing sugar concentration of 1.05 g/L. On performing SSF with phosphoric acid-acetone pretreatment gave an ethanol titre of 0.88 g/L from reducing

sugar concentration of 1.12 g/L while AFEX pretreatment gave a maximum ethanol titre of 0.94 g/L from reducing sugar concentration of 1.16 g/L with a yield of 0.094 (g of ethanol/ g of substrate) as obtained with hydrolytic enzyme *B. subtilis* cellulase and *S. cerevisiae* (Table 6.3.2).

On comparison of the ethanol titre (g/L) obtained from first, second and third batch of SSF experiments, AFEX pretreatment was found to perform better compared to phosphoric acid-acetone and steam exploded pretreatment in all the combinations. In addition, cellulolytic enzyme from *B. subtilis* gave better saccharification efficiency over recombinant cellulase (GH5) or *T. reesei* cellulase which was apparent from amount of sugar released and ethanol yield obtained (Table 6.3.2).

The fourth batch SSF experiment involving *T. reesei* cellulase and substituting *S. cerevisiae* with bacterium, *Z. mobilis* as the fermentative microbe gave an ethanol titre of 1.18 g/L from a reducing sugar concentration of 1.1 g/L with steam exploded thatch grass. With phosphoric acid-acetone pretreatment an ethanol titre of 1.21 g/L was obtained from reducing sugar concentration of 1.24 g/L and AFEX pretreatment resulted in an ethanol titre of 1.28 g/L from reducing sugar concentration of 1.3 g/L and a yield of 0.128 (g/g) (Table 6.3.2).

Fifth batch SSF experiment comprising recombinant cellulase (GH5) and *Z. mobilis* achieved an ethanol titre of 1.42 g/L from a reducing sugar concentration of 1.45 g/L with steam exploded thatch grass. An ethanol titre of 1.5 g/l from reducing sugar of 1.53 g/L with an ethanol yield of 0.15 (g/g) was obtained from phosphoric acid-acetone pretreatment. AFEX pretreatment resulted in ethanol titre of 1.56 g/L, reducing sugar concentration of 1.58 g/L and ethanol yield of 0.156 (g of ethanol/g of substrate) (Table 6.3.2).

Sixth batch of SSF experiment involving *B. subtilis* cellulase and *Z. mobilis* obtained an ethanol titre of 0.9 g/L from a reducing sugar concentration of 1.09 g/L with steam exploded thatch grass. At the same time phosphoric acid-acetone pretreatment gave an ethanol titre of 1.56 g/L from reducing sugar concentration of 1.6 g/L. On the other hand, AFEX pretreatment resulted in a maximum ethanol titre of 1.62 g/L from reducing sugar concentration of 1.72 g/L with an ethanol yield of 0.160 (g of ethanol/g of substrate) (Table 6.3.2).

On the basis of ethanol titre (g/L) obtained in fourth, fifth and sixth batch of SSF experiments, a 3.8% increase was observed using saccharifying enzyme *B. subtilis* cellulase (1.62 g/L) as compared to recombinant cellulase (GH5) (1.56 g/L) and 26% increase as compared to *T. reesei* cellulase (1.28 g/L) using *Z. mobilis* as fermentative microbe (Table 6.3.2). The hydrolytic enzyme *B. subtilis* cellulase proved superior over recombinant cellulase (GH5) or *T. reesei* cellulase owing to its capability for maximum release of sugars obtaining high ethanol titre.

Overall comparison from first to sixth batch of SSF found a significant increase of 72% in ethanol titre using *Z. mobilis* (1.62 g/L) as bioethanol producer as compared to *S. cerevisiae* (0.94 g/L) along with *B. subtilis* cellulase as hydrolytic enzyme and AFEX pretreated thatch grass (Table 6.3.2). Though *S. cerevisiae* and *Z. mobilis* are known to be efficient ethanol producers, *Z. mobilis* have shown better yield because of its having capacity of high sugar uptake and high ethanol tolerance (Santos *et al*. 2010). In conclusion, AFEX pretreatment was the best and hydrolytic enzyme *B. subtilis* AS3 and bioethanol producer *Z. mobilis* proved efficient over the others.

Seventh batch of SSF experiment were performed using *T. reesei* cellulase along with mixed culture of *S. cerevisiae* and *C. shehatae* with the aim of utilizing both hexose and pentose sugars released during enzymatic hydrolysis of pretreated substrate. On carrying out SSF using steam exploded thatch grass an ethanol titre of 1.35 g/L, a reducing sugar concentration of 1.4 g/L was attained (Table 6.3.2). An ethanol titre of 1.4 g/L from reducing sugar concentration of 1.43 g/L was obtained from phosphoric acid-acetone pretreated thatch grass. AFEX pretreatment resulted in an ethanol titre of 1.5 g/L from reducing sugar concentration of 1.53 g/L (Table 6.3.2, Fig. 6.3.1).

Eight batch of SSF experiment with similar mixed culture of fermentative microbe and using recombinant cellulase (GH5) gave an ethanol titre of 1.55g/L, a reducing sugar concentration of 1.56 g/L with steam exploded thatch grass. Phosphoric acid-acetone pretreatment gave an ethanol titre of 1.59 g/L from reducing sugar of 1.61 g/L (Table 6.3.2). During, AFEX pretreatment a maximum ethanol concentration of 1.64 g/L from reducing sugar concentration of 1.72 g/L and an ethanol yield of 0.164 (g/g) is achieved (Table 6.3.2, Fig. 6.3.2).

Ninth batch followed the same combination of bioethanol producers using cellulase from *B. subtilis* AS3. An ethanol titre of 1.61 g/L from reducing sugar concentration of 1.65 g/L with a yield of 0.161 (g/g) was obtained from steam exploded thatch grass. The phosphoric acid-acetone pretreatment gave an ethanol titre of 1.66 g/L from reducing sugar concentration of 1.68 g/L (Table 6.3.2). A maximum ethanol titre of 1.94 g/L from reducing sugar concentration of 1.98 g/L with an ethanol yield of 0.194 (g of ethanol/g of substrate) was obtained with AFEX pretreated thatch grass (Table 6.3.2, Fig. 6.3.3).

On comparison of the ethanol titre (g/L) values obtained from seventh, eight and ninth batch of SSF, a 18% and 29% increase in ethanol titre was observed using *B. subtilis* cellulase (1.94 g/l) as compared to recombinant cellulase (1.64 g/L) and *T. reesei* cellulase (1.5 g/L) respectively, with AFEX pretreated thatch grass using mixed culture of *S. cerevisiae* and *C. shehatae* as bioethanol producers (Table 6.3.2).

The dynamic profiles of SSF combinations were studied to analyze the rate of saccharification and ethanol formation during the period of experiment. The SSF profile comprising *T. reesei* cellulase and mixed culture of *S. cerevisiae* and *C. shehatae* showed an increase in cell biomass gradually with time reaching a maximum of (A_{600}= 0.8) at 66 h of fermentation (Fig. 6.3.1).

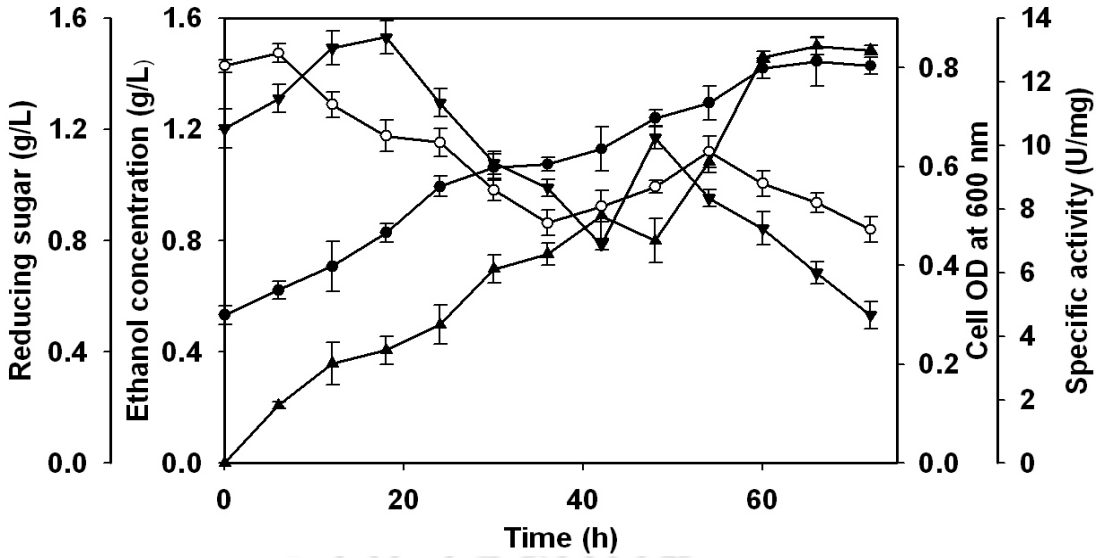


Fig. 6.3.1 SSF profile of AFEX pretreated 1% (w/v) thatch grass using *T. reesei* cellulase alongwith *S. cerevisiae* and *C. shehatae* at shake flask level. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g/L), (▼) reducing sugar (g/L) and (○) specific activity (U/mg) with time (h). Cultivation conditions**:** working volume 100 mL in 250 mL erlenmeyer flask; initial pH 6.0; temperature 30°C; shaking 120 rpm; incubation period 72 h; sampling interval every 6 h.

During the lag phase (6-12 h), high amount of reducing sugars were released due to enzymatic hydrolysis which are used by the microorganism for growth and acclimatization. A maximum reducing sugars (1.53 g/L) was achieved during 18 h of fermentation which causes inhibitory effect of enzyme activity during 30-36 h of fermentation. After 18 h there was a gradual increase in ethanol concentration over rest of the incubation period with simultaneous decrease in total sugar. Sugar concentration below a threshold level decreased the repressive effect on cellulase activity. An ethanol titre of 1.5 g/L with a yield coefficient of 0.15 (g/g) was achieved at 60 h of fermentation (Fig. 6.3.1).

The SSF profile involving recombinant cellulase (GH5) along with *S. cerevisiae* and *C. shehatae* showed a sigmoidal increase in cell biomass production reaching its peak value (A_{600}= 0.78) at 66 h of fermentation. During the initial hours (6-18 h), high activity of recombinant cellulase (GH5) released more amount of reducing sugars which is utilized for the growth of the organism (Fig. 6.3.2). After 24 h, reducing sugar concentration increases attaining a maximum concentration of 1.72 g/L during 36-42 h with concomitant decrease in specific activity of the enzyme (Fig. 6.3.2). The SSF profile of reducing sugar showed a sinusoidal behavior depicting the balance between the rate of saccharification for release of reducing sugar and rate of its subsequent utilization for growth and ethanol formation. The ethanol production was proportional to the increase in cell biomass achieving a maximum ethanol titre of 1.64 g/L at 60 h of fermentation. The ethanol yield obtained was 0.164 (g of ethanol/g of substrate) (Fig. 6.3.2).

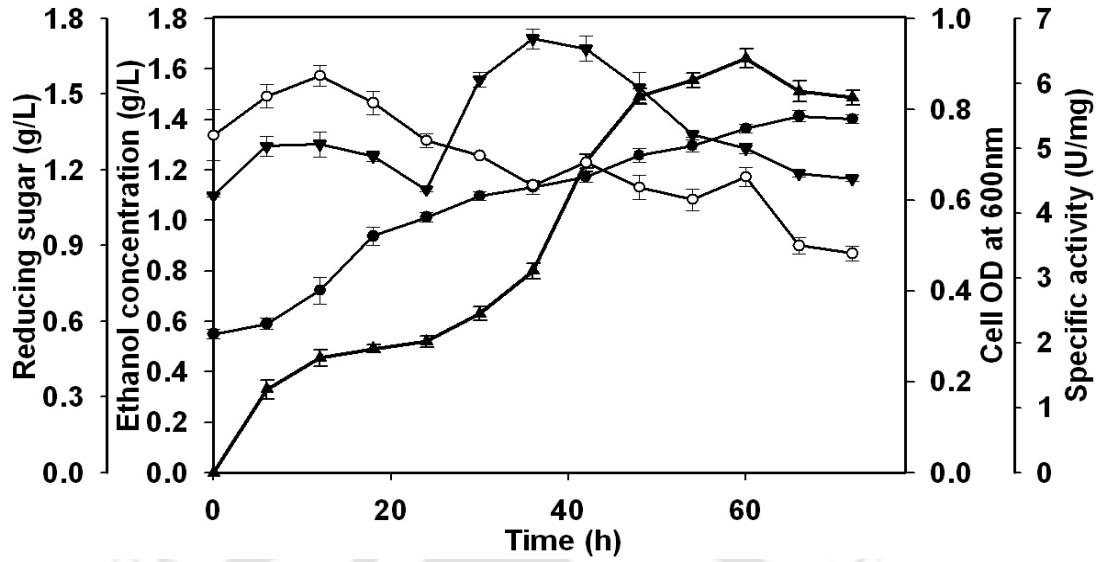


Fig. 6.3.2 SSF profile of AFEX pretreated 1% (w/v) thatch grass using recombinant cellulase (GH5) alongwith *S. cerevisiae* and *C. shehatae* at shake flask level. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g/L), (▼) reducing sugar (g/L) and (○) specific activity (U/mg) with time (h). Cultivation conditions: working volume 100 mL in 250 mL erlenmeyer flask; initial pH 6.0; temperature 30°C; shaking 120 rpm; incubation period 72 h; sampling interval every 6 h.

SSF profile involving *B. subtilis* cellulase along with *S. cerevisiae* and *C. shehatae* showed a sigmoidal increase in cell biomass attaining a maximum of 0.8 at 66 h of fermentation (Fig. 6.3.3). The sugars released after enzymatic hydrolysis of the pretreated thatch grass were initially used by the microbes for acclimatization and growth which is evident from low ethanol titre. The ethanol production increases gradually with increase in cell biomass, thereby showing a maximum ethanol concentration at late log phase during 54-66 h of fermentation (Fig. 6.3.3). The amount of reducing sugars in the fermentation broth was high during the early stage around 6-18 h due to high activity of *B. subtilis* AS3 cellulase. After 24 h reducing sugar increases attaining a maximum concentration of 1.98 g/L during 36-42 h and

simultaneous decrease in specific activity of the enzyme due to inhibition by glucose released from breakdown of cellulose. After 36 h, there was a sharp increase in ethanol concentration with gradual decline in total sugar (Fig. 6.3.3). This is due to consumption of pentose sugars by *C. shehatae* after depletion of hexose sugars giving a maximum ethanol titre of 1.94 g/L at 60 h of fermentation (Fig. 6.3.3).

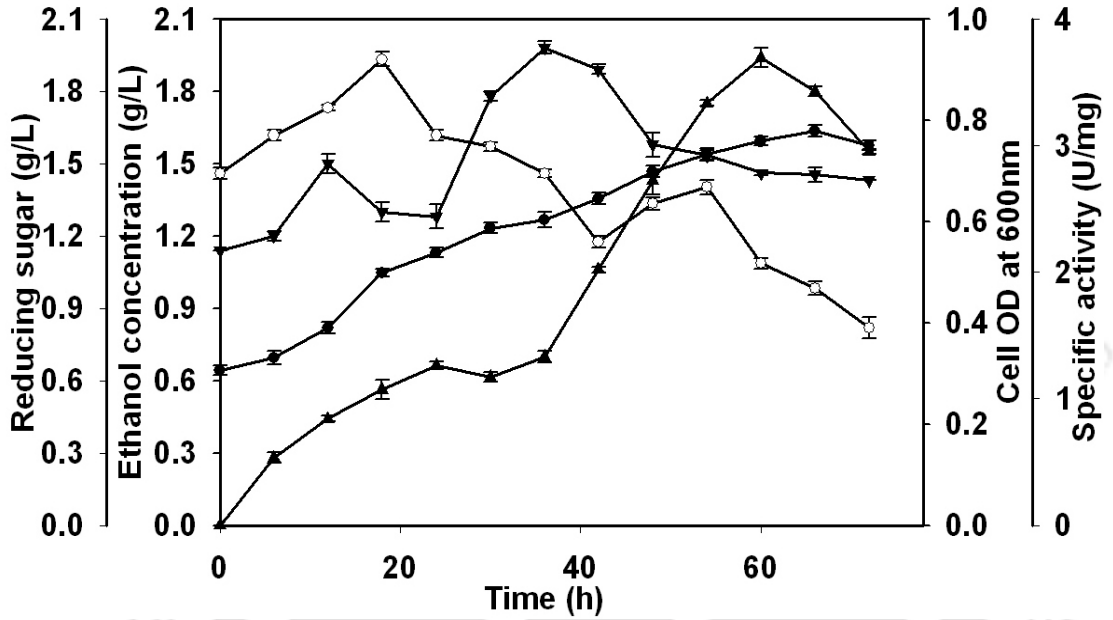


Fig. 6.3.3 SSF profile of AFEX pretreated1% (w/v) thatch grass using *B. subtilis* cellulase along with *S. cerevisiae* and *C. shehatae* at shake flask level. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g/L), (▼) reducing sugar (g/L) and (○) specific activity (U/mg) with time (h). Cultivation conditions: working volume 100 mL in 250 mL erlenmeyer flask; initial pH 6.0; temperature 30°C; shaking 120 rpm; incubation period 72 h; sampling interval every 6 h.

On overall analysis of the results of the nine batches, a 20% increase in the ethanol titre was achieved using dual combination of *S. cerevisiae* and *C. shehatae* (1.94 g/L) as compared to single culture of *Z. mobilis* (1.62 g/L) using cellulase from *B. subtilis* AS3 and AFEX pretreated thatch grass (Table 6.3.2). In conclusion, AFEX

pretreatment proved to be best in all the SSF combinations. Also the saccharification efficiency of cellulolytic enzyme from *B. subtilis* AS3 was found to be higher over recombinant cellulase (GH5) or *T. reesei* cellulase as apparent from the concentration of reducing sugars and yield of ethanol obtained. In addition, mixed combination of *S. cerevisiae* and *C. shehatae* found to be perfect bioethanol producers having potential to consume both hexose and pentose sugars simultaneously, rather than using single culture of *S. cerevisiae* or *Z. mobilis*. The SSF combination of *B. subtilis* cellulase and mixed fermentative microbes (*S. cerevisiae* and *C. shehatae*) was selected for further studies with 5% (w/v) thatch grass.

6.3.4 SSF involving *B. subtilis* cellulase along with *S. cerevisiae* and *C. shehatae* using AFEX pretreated 5% (w/v) thatch grass in shake flask

On performing batch SSF in shake flask using 5% (w/v) AFEX pretreated thatch grass along with *B. subtilis* cellulase and mixed microbial combination of *S. cerevisiae* and *C. shehatae*, yielded an ethanol titre of 11.06 g/L with a maximum released reducing sugar of 12.18 g/L (Fig. 6.3.4, Table 6.3.3). The yield of ethanol (g of ethanol/g of substrate) obtained was 0.221 (g/g). The dynamic SSF profile showed growth associated ethanol production. During the initial lag phase (6-12 h) the ethanol titre was low which gradually started increasing and reaching maximum concentration at late log phase (60-66 h) of cell growth. The highest ethanol titre was obtained at 66 h of fermentation and thereafter the ethanol concentration show decreasing trend with the cessation of cell growth. Release of reducing sugars during enzymatic hydrolysis is based on the efficacy of enzyme and pretreatment method. Initially, reducing sugar concentration was high due to high activity of the enzyme showing maximum

concentration of 12.18 g/L. After 36 h the reducing sugar showed a gradual decline with time owing to its utilisation for ethanol production. Figure. 6.3.4 shows high enzyme activity during early stages of SSF, releasing more amount of reducing sugars, causing repressive effect on the enzyme activity during 54-72 h. Sugars released are consumed by the organisms for growth during 18-30 h. After 36 h, there was a gradual increase in ethanol concentration with gradual depletion in total sugar depicting a sinusoidal behavior between the rate of sugar utilization and ethanol formation. On increasing the concentration of AFEX pretreated thatch grass from 1% (w/v) to 5% (w/v) in shake flask, a 5.7 fold increment in ethanol titre from (1.94 g/L) to (11.06 g/L) was achieved (Table 6.3.3).

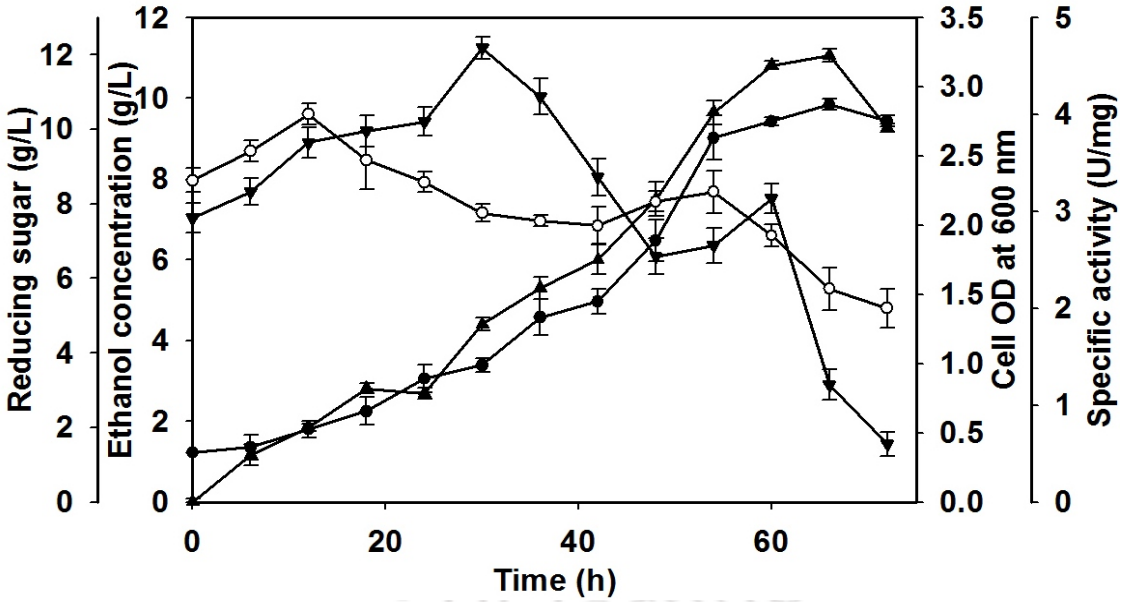


Fig. 6.3.4 SSF profile of AFEX pretreated 5% (w/v) thatch grass using *B. subtilis* cellulase along with *S. cerevisiae* and *C. shehatae* at shake flask level. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g/L), (▼) reducing sugar (g/L) and (○) specific activity (U/mg) with time (h). Cultivation conditions: working volume 100 mL in 250 mL erlenmeyer flask; initial pH 6.0; temperature 30°C; shaking 120 rpm; incubation period 72 h; sampling interval every 6 h.

6.3.5 SSF involving *B. subtilis* cellulase along with *S. cerevisiae* and *C. shehatae* using AFEX pretreated 5% (w/v) thatch grass in bioreactor

With the purpose of maximizing ethanol titre, the fermentation employed at shake flask level was scaled up in the bioreactor. The dynamic profiles of cell biomass formation, utilization of reducing sugar along with subsequent ethanol formation and specific activity of the enzyme are shown in Fig. 6.3.5. The growth profile of the organism in the bioreactor followed a sigmoidal behavior. During 8-9 h of SSF the organism remained in the lag phase. Subsequently, the organism continued to grow exponentially attaining a maximum of 7.0 (at A_{600}) at 66 h of fermentation. Later on the organism entered the stationary phase with gradual decline in ethanol production (Fig. 6.3.5). Ethanol concentration increased in accordance with increase in cell biomass illustrating direct relationship between the rate of ethanol formation with rate of substrate utilization and cell growth. The maximum ethanol concentration of 22.0 g/L was obtained after the microbe entered early stationary phase during 54-66 h. The reducing sugar profile was more or less similar to that which was observed in shake flask level showing an initial increasing trend in concentration of reducing sugars causing inhibition of enzyme activity. A maximum reducing sugar concentration of 21.0 g/L was released after enzymatic hydrolysis of the pretreated substrate (Fig. 6.3.5, Table 6.3.3). SSF profile showed a gradual decline in sugar concentration from 48-82 h with simultaneous increase in cell biomass and ethanol concentration due to consumption of pentose sugars by *C. shehatae* after the exhaustion of hexose sugars. (Fig. 6.3.5, Table 6.3.3).

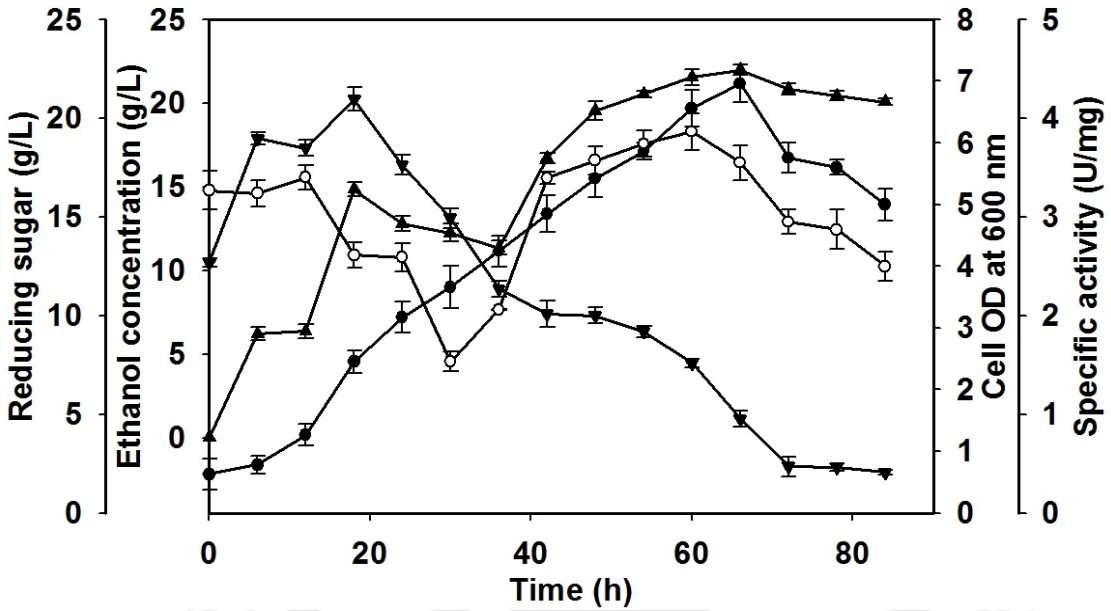


Fig. 6.3.5 SSF profile of AFEX pretreated 5% (w/v) thatch grass using *B. subtilis* cellulase along with *S. cerevisiae and C. shehatae* in a 2L laboratory scale bioreactor with working volume of 1L. (●) cell OD measured at 600 nm, (▲) ethanol concentration (g/L), (▼) reducing sugar (g/L) and (○) specific activity (U/mg). Cultivation conditions**:** Aeration rate 1 vvm, pH 6.0; temperature 30°C; shaking 120 rpm; incubation period 84 h; sampling interval every 6 h.

Performing SSF in bioreactor gave promising results due to the fact that the parameters can be constantly monitored and controlled which could not been taken care of during shake flask experiments. Aeration rate of 1 vvm was the additional parameter that was taken in to consideration while running the bioreactor. A significantly enhanced yield of ethanol (0.44 g of ethanol/g of substrate) (Table 6.3.3, Fig. 6.3.5) was obtained using 5% (w/v) thatch grass as substrate. Accordingly, rise in substrate concentration along with enzyme loadings and inoculum size has a significant augmentation in ethanol titre (Zhang *et al*. 2010).

Table 6.3.3 Ethanol production from SSF at shake flask and 2L bioreactor using AFEX pretreated 5% (w/v) thatch grass alongwith *B. subtilis* cellulase as hydrolytic enzymes and *S. cerevisiae* and *C. shehatae* as fermentative microbes.

Sl. No	SSF combination	Substrate concentration (%, w/v) and mode of SSF	Reducing sugar* (g/L)	Ethanol yield # (g/g)	Ethanol titre* (g/L)
1.	*B. subtilis* (AS3) cellulase + *S. cerevisiae* + *C. shehatae*	5% Shake flask	12.18 ± 0.07	0.221	11.06 ± 0.02
2.	*B. subtilis* (AS3) cellulase + *S. cerevisiae* + *C. shehatae*	5% Bioreactor	21.0 ± 0.04	0.440	22.0 ± 0.01

** the values correspond to the maximum reducing sugar and maximum ethanol titre at a particular time. # correspond to g of ethanol/ g of substrate. values are mean ± SE (n=3)*

Therefore, on scaling up the SSF process from shake flask to bioreactor using AFEX pretreated 5% (w/v) thatch grass and *B. subtilis* AS3 cellulase, a two fold rise in ethanol titre from 11.06 g/L to 22.0 g/L was obtained (Table 6.3.3). Similarly, a two fold increase in yield coefficient from 0.221 g/g to 0.44 g/g was also achieved on scale up in bioreactor using 5% (w/v) AFEX pretreated thatch grass (Table 6.3.3).

The values of ethanol concentration obtained in our study are comparable with other reports of the literature. An ethanol concentration of 60 g/L was obtained using commercial cellulase utilizing 30% sugarcane bagasse and *Z. mobilis* as the fermentative organism (Santos *et al.* 2010). Using co-culture of *Clostridium thermosaccharolyticum*HG8 and *Thermoanaerobacter ethanolicus* ATCC 31937 as fermentative microbe with 100 g/L banana waste as substrate, an ethanol concentration of 2.2 g/L was obtained (Reddy *et al*. 2010). An ethanol titre of 62.7 g/L using 19% dry corncorb and commercial cellulolytic enzymes in bioreactor was reported by Zhang *et al*. (2010). An ethanol concentration of 5 g/L was obtained from

5% wheat straw using crude unprocessed cellulase from *Trichoderma reesei* (Lever *et al*. 2010). A reactor level aerobic batch fermentation with optimized process conditions offered a maximum ethanol concentration of 3.36 g/L from 50 g/L of steam autoclaved pretreated sugarcane bagasse (Sasikumar and Viruthagiri, 2010).

6.4 Conclusions

SSF profiles of various combinations yield an interesting relation between the rate of saccharification, rate of sugar utilization and on the rate of ethanol formation. Different SSF trials were set up to determine the outcome of various pretreatment procedures on enzymatic hydrolysis and to find out the better fermentative microbes. Out of the three pretreatment strategies, steam explosion, AFEX and phosphoric acid-acetone, the AFEX pretreatment method proved to be the best in all the SSF combinations as revealed from the amount of ethanol titre and yield obtained during SSF process. The saccharification efficiency of *B. subtilis* cellulase was found to be superior over *T. reesei* or recombinant cellulase (GH5) as it was clear from the ethanol concentration (g/L) and reducing sugars (g/L) obtained during SSF process using 1% (w/v) thatch grass.

B. subtilis cellulase gave an ethanol titre of 0.94 g/L using AFEX pretreated thatch grass and *S. cerevisiae* as fermentative microbe which showed a 6% increase as compared to recombinant cellulase (GH5) (0.88 g/L) and 9.3% increase as compared to *T. reesei* cellulase (0.86 g/L). On the other hand, using *Z. mobilis* as bioethanol producer gave an ethanol titre of 1.62 g/L showing a significant 72% increase as compared to ethanol titre values of 0.94 g/L given by *S. cerevisiae* using *B. subtilis* cellulase as hydrolytic enzyme and AFEX pretreated 1% (w/v) thatch grass.

On performing SSF with mixed culture of *S. cerevisiae* and *C. shehatae* with AFEX pretreated thatch grass using *B. subtilis* AS3 cellulase, gave a maximum ethanol titre of 1.94 g/L with a yield coefficient of 0.194 g/g. This has shown a significant 2.1 and 1.2 fold increase in ethanol titre as compared to the values obtained from single culture of *S. cerevisiae* (0.94 g/L) and *Z. mobilis* (1.62 g/L).

It was also concluded that using dual combination of *S. cerevisiae* and *C. shehatae* with AFEX pretreated thatch grass, *B. subtilis* AS3 cellulase gave a higher ethanol concentration of 1.94 g/L resulting in 18% and 29% enhancement in contrast to ethanol titre values of 1.64 g/L obtained by recombinant cellulase and 1.5 g/L by *T. reesei* cellulase, respectively.

In conclusion, cellulolytic enzyme from *B. subtilis* AS3 showed promising saccharification rate with maximum release of utilizable sugars in the least pretreatment time. Also, mixed microbial combination of *S. cerevisiae* and *C. shehatae* found to be perfect bioethanol producers having potential to consume both hexose and pentose sugars simultaneously, rather than using single culture of *S. cerevisiae* or *Z. mobilis* as determined on basis of ethanol titre and yield.

On increasing the AFEX pretreated thatch grass from 1% (w/v) to 5% (w/v) in shake flask, using the best SSF combination involving *B .subtilis* cellulase along with *S. cerevisiae* and *C. shehatae* gave an ethanol titre value of 11.06 g/L showing a 5.7 fold enhancement in bioethanol production as compared to ethanol value of 1.94 g/L. Scaling up the SSF process from shake flask to 2L bioreactor using AFEX pretreated 5% (w/v) thatch grass gave an ethanol titre of 22.0 g/L showing a 2 fold enhancement as compared to ethanol titre values of 11.06 g/L given by shake flask under similar cultivation conditions. The continuous monitoring and controlled conditions of various parameters in the bioreactor along with supply of aeration rate of 1 vvm had an added advantage on the growth and ethanol production in the bioreactor as compared to shake flask. Therefore, with increase in substrate concentration alongwith enzyme loadings and the inoculum size had a significant effect on ethanol production.

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1. Saprativ P. Das**, Deepmoni Deka**, Rajeev Ravindran, Debasish Das and Arun Goyal **(2012)** Lignocellulosic fermentation of water hyacinth involving mixed pretreatment and different hydrolytic enzymes. "International workshop and conference on renewable energy and climate change-Exploring opportunities for sustainable development". April 05-07, 2012, Madurai Kamaraj University (abstract accepted).
2. **Deepmoni Deka**, Saprativ P. Das, Rajeev Ravindran and Arun Goyal (**2012**) Water hyacinth as a potential source of biofuel for sustainable development. **Oral presentation**, "International Conference on Environmentally Sustainable Urban Ecosystems", February 24-26, 2012, IIT Guwahati, Assam.
3. Rajeev Ravindran, Saprativ P. Das, **Deepmoni Deka**, and Arun Goyal (**2012**) Lignocellulosic biomass as a sustainable source for bioethanol production. **Paper presentation**. "International Conference on Environmentally Sustainable Urban Ecosystems", February 24-26, 2012, IIT Guwahati, Assam.
4. Rajeev Ravindran, Saprativ P. Das, **Deepmoni Deka** and Arun Goyal (**2011**) Bench scale bioethanol production involving recombinant *C. thermocellum* hydrolytic enzymes and fermentative microbes. 52nd annual conference of Association of Microbiologists of India, "International Conference on Microbial Biotechnology for Sustainable development", November 3-6, 2011, Punjab University, Chandigarh, IM 118, p 195.
5. **Deepmoni Deka**, Ashish Sharma, Dinesh Goyal and Arun Goyal (**2009**) Partial purification and characterization of an alkaline carboxymethyl cellulase from a new isolate of *Bacillus* sp. "International Conference on Emerging Trends in Biotechnology", December 2-6, 2009, Banaras Hindu University, Varanasi, India, p-179.
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1. **Deepmoni Deka**, Saprativ P. Das, Rajeev Ravindran, Mohammad Jawed, Debashish Das and Arun Goyal (**2011**) Approaches for identification of a combination of hydrolytic enzymes and fermentative microbes for bioethanol production from thatch grass. "Symposium on Carbohydrates at the Interface of Chemistry and Biology (CARBO XXVI)", November 23-25, 2011, Indian Institute of Chemical Biology, Kolkata, PP-36, p64.

2. **Deepmoni Deka**, Saprativ P. Das, Mohammad Jawed and Arun Goyal (**2010**) Cellulase production under solid state culture by an isolated fungus using agrowaste for bioconversion to ethanol. Oral presentation, "National Conference on Emerging Trends in Biopharmaceuticals: Relevance to Human Health & 4th Annual Convention of Association of Biotechnology and Pharmacy", November 11-13, 2010, Thapar University, Patiala.
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1. **Deepmoni Deka**, Shadab Ahmed, Nadeem Akhtar, Sangeeta Bharali, M. Jawed, Carlos M.G.A. Fontes, Dinesh Goyal and Arun Goyal (**2009**) Determining substrate specificity and biochemical characterization of a full length recombinant cellulase (Lic26A-Cel5-CBM11) of *Clostridium thermocellum.* ***Journal of Applied Bioscience and Biotechnology*** 5(1) 13-18.
2. Shadab Ahmed, **Deepmoni Deka**, M. Jawed, Dinesh Goyal, Carlos M.G.A. Fontes and Arun Goyal (**2009**) Biochemical characterization of a recombinant derivative (*Ct*Lic26A-Cel5) of a cellulosomal cellulase from *Clostridium thermocellum.* ***Current Trends in Biotechnology and Pharmacy*** 3, 633-640.
3. **Deepmoni Deka**, P. Bhargavi, Ashish Sharma, Dinesh Goyal, M. Jawed and Arun Goyal (**2011**) Enhancement of cellulase activity from a new strain of *Bacillus subtilis* by medium optimization and analysis with various cellulosic substrates. ***Enzyme Research***. doi:10.4061/2011/151656.
4. **Deepmoni Deka**, Saprativ P. Das, Debashish Das, Mohammad Jawed and Arun Goyal (**2012**) Reactor scale up for efficient bioethanol production involving recombinant cellulase (GH5) from *Clostridum thermocellum*. ***Fuel*** (submitted).
5. **Deepmoni Deka**, Saprativ P. Das, Naresh Sahoo, Debashish Das, Mohammad Jawed and Arun Goyal (**2012**). Enhanced cellulase production from *Bacillus subtilis* by optimising physical parameters for bioethanol production. ***Biochemical Engineering Journal*** (submitted).
6. Nadeem Akhtar, Ashish Sharma, **Deepmoni Deka**, Mohammad Jawed, Dinesh Goyal and Arun Goyal (**2012**) Characterization of cellulolytic *Bacillus* sp. from cowdung. ***Environmental Progress & Sustainable Energy*** (submitted).
7. Saprativ P. Das, **Deepmoni Deka**, Rajeev Ravindran, Debasish Das, Mohammad Jawed, Carlos M.G.A. Fontes and Arun Goyal (**2012**) Enhanced bioethanol production from water hyacinth (*Eichhornia crassipes*) for sustainable development. ***Bioresource Technology*** (submitted).

Synopsis

Introduction

Cellulases are a class of hydrolytic enzymes that break down complex cellulose into cellobiose and ultimately to simple soluble sugars such as glucose and cello-oligosaccharides as available carbon and microbial energy sources. They include three different groups of enzymes comprising of endoglucanase, exocellobiohydrolase and β-glucanase which act in coordinated manner to bring about the enzymatic hydrolysis employing the catalytic mechanism of either retention or inversion. Cellulases have versatile applications in textile, laundry, pulp and paper, fruit juice extraction and animal feed industries. In addition, they find use in saccharification of lignocellulosic wastes to fermentable sugars which can be used for the production of bioethanol, lactic acid, single cell protein and other industrially important chemicals. Bacteria are now being widely explored for cellulase production owing to their high growth rate, expression of multi-enzyme complexes, stability at extremes of temperature and pH, lesser feedback inhibition and ability to withstand variety of environmental stress. *Bacillus subtilis* are most sought after as they produce diverse range of cellulases having high yield and versatile applicability and stable under extreme conditions. With this objective, an efficient cellulase producing bacterial strain was isolated from cow dung. 16S rRNA gene sequence analysis showed maximum homology with *Bacillus subtilis*. The strain was found to be an efficient cellulose degrader showing a large clear zone in CMC containing plates after Congo

Red staining. Morphological and physiological characterization of the isolate *Bacillus subtilis* (AS3) was conducted. Statistical tools like Plackett-Burman and central composite design followed by multiple desirability function were employed for optimization of medium and culture parameters to achieve enhanced cellulase production. Validation of the model was carried out both in shake flask and laboratory scale bioreactor. Purification by two step process and biochemical characterization of purified cellulase was executed. Industrial utility of cellulase from this isolate was studied and found suitable for economically feasible bioethanol production from lignocellulosic waste. Simultaneous saccharification and fermentation process (SSF) alongwith different substrate pretreatments were employed during bioconversion process. Pretreatments employed during the batch SSF process are steam explosion, acid acetone and ammonia fibre explosion (AFEX) used in order to make the substrate accessible for enzymatic hydrolysis. The comparative performance of fungal, naturally isolated bacterial and recombinant hydrolytic enzymes was studied. The performance of various fermentative microbes like *Saccharomyces cerevisiae*, *Zymomonas mobilis* and *Candida shehatae* on bioethanol production from pretreated thatch grass using SSF process at shake flask and bioreactor level were studied.

Present work

The present work focuses on the “Optimization of production, purification and characterization of cellulase from a novel strain of *Bacillus subtilis* AS3 to explore its potential for lignocellulosic ethanol fermentation”. The thesis work comprises 6 Chapters.

Chapter 1 is the General Introduction which embodies the brief review of literature dedicated to the importance of cellulase. Concise discussion on classification and mechanism of action of cellulase is included in this chapter. A literature survey on screening and isolation of cellulolytic microorganisms and their production is presented. Different optimization strategies for cellulase production using one variable at a time method (OVAT) and response surface methodology, purification and characterization of microbial cellulase available in literature are also highlighted. The chapter also elaborates the potential applications of the cellulase in industry and environment. Application of cellulase for bioethanol production from various pretreatments of lignocellulosic wastes using batch SSF are extensively reviewed. A literature survey on different pretreatment techniques is presented. Based on the extensive literature review, the scope and importance of the present investigation and the objectives of the present research work is outlined at the end of chapter 1.

Chapter 2 describes the detailed protocol of isolation and screening of the microorganisms producing cellulase and its identification. The morphological and biochemical characterization of the isolate based on Gram nature test, catalase activity and urease test was carried out. The isolate proved to be Gram positive and rod shaped and tested positive for catalase, oxidase, nitrate reduction and bile esculin and negative for urease, citrate utilisation and Voges–Proskauer (VP) reaction. 16S rRNA gene sequence analysis and its phylogenetic tree were constructed to trace the genetic relationships of this isolate with its neighbours. The isolate was designated as *Bacillus subtilis* AS3. Growth conditions for cellulase production such as inoculum concentration, incubation time were optimized to maximize the cellulase production. Inoculum concentration at 2% (v/v) with incubation for 48 h gave maximum cellulase

production. Selection of most efficient carbon and nitrogen source were carried out by one variable at a time method (OVAT). CMC at 2% (w/v) concentration and peptone in combination with yeast extract achieved maximum cellulase production. Biomass like thatch grass and bagasse when used as sole carbon source, appreciably supported cellulase activity.

Chapter 3 describes the statistical optimization of medium composition for cellulase production from *Bacillus subtilis* AS3. The aim was to enhance the cellulase activity and determine the interaction between various medium components using response surface methodology. Statistically-based experimental tools, Plackett-Burman factorial design and central composite design were applied for the optimization experiments. Seven medium components were examined for their significance on cellulase production. Regression analysis of the experimental data found CMC, peptone and yeast extract significantly and positively affected the enzyme production. The interactive effect of these three crucial variables on cellulase production was studied using a 2^2 full-factorial central composite design. After ANOVA, a second-order polynomial equation was established to determine the relationship between the cellulase production and the three medium components. The optimal concentrations of variables for maximum cellulase production were CMC, 18 g/L; Peptone, 8 g/L and Yeast extract, 4.79 g/L. Under the optimal medium composition the experimental value of enzyme activity 0.43 U/mL nearly matched with predicted value of 0.49 U/mL showing 6 fold increase with respect to the control basal medium which showed only 0.07 U/mL enzyme activity. Thus, the statistically-based experimental designs were found very promising in enhancing cellulase activity. Moreover, replacing CMC in optimized medium the enzyme showed 2.8 and 3 fold increase with lichenan and β-glucan, respectively and the activity reached

maximum at 12 h in contrast to 48 h required by CMC substrate. Therefore, the isolate is a high β-1,3-1,4-glucanase (lichenase) producing strain that can break β-1,3-1,4 linkages. Having this attribute alongwith alkaline nature and stability at variable temperature and pH, the enzyme from *Bacillus subtilis* AS3 can find wider industrial applications.

Chapter 4 describes the effects of physical process parameters such as initial pH, temperature (°C), and agitation (rpm) on cellulase production from *Bacillus subtilis* AS3. For optimization of cellulase activity and cell growth, central composite design of experiments followed by multiple desirability function was applied. Among the three independent variables the interaction effect between temperature and agitation was highly significant on cellulase activity. To illustrate the accuracy of the models in predicting the responses normal probability plot of the residuals are also depicted. Overall desirability functions for cellulase activity and cell growth were close to 1 indicating linear increase of the function towards the desired target values of the two responses. Using the desirability function method for optimizing both the responses optimum values of the culture conditions predicted by the model were found to be pH 7.2; temperature 39˚C and agitation 121 rpm. The maximum cellulase activity 0.56 U/mL and cell growth 2.01 mg/mL predicted by the model was in consensus with values (0.57 U/mL and 2.1 mg/mL) obtained experimentally, using optimized medium and optimal values of physical parameters. After optimization a 33% enhancement in cellulase activity (0.57 U/ml) was recorded. With the scale up of cellulase production process with all the optimized conditions in bioreactor, yielded a significant 32% increase showing maximum cellulase activity of 0.75 U/mL. This study illustrates the importance of optimization of physical parameters for bioprocess development of bacterial cellulase production.

Chapter 5 describes the purification, zymogram analysis and characterization of cellulase from *Bacillus subtilis* (AS3). The purification of enzyme from cell-free supernatant was carried out by ammonium sulphate precipitation followed by ion exchange chromatography using DEAE-sepharose. The crude enzyme obtained as a cell free supernatant had specific activity of 0.34 U/mg. This was precipitated with addition of ammonium sulphate and maximum cellulase activity was observed at 40-80% saturation. After ammonium sulphate precipitation the enzyme gave specific activity of 1.16 U/mg with 3.4 fold purification and 7.7% yield. Purification by ion exchange chromatography resulted in 9.8 fold increase showing specific activity of 3.33 U/mg and 8% overall yield. The purified enzyme displayed a protein band on SDS-PAGE with an apparent molecular size of 30 kDa as confirmed by the zymogram. The enzyme showed multi-substrate specificity, showing significantly higher activity with lichenan and β-glucan as compared to CMC, laminarin, hydroxyethylcellulose and steam exploded bagasse and negligible activity with crystalline substrate such as avicel and filter paper. The enzyme was optimally active at pH 9.2 and temperature 45°C. The enzyme was stable in the pH range 6-10 and retained 70% activity at pH 12. Thermal stability analysis revealed that the enzyme is stable in temperature range of 20°C to 45°C and retained more than 50% activity at 60°C for 30 min. The enzyme had a K_m of 0.13 mg/ml and V_{max} of 3.38 U/mg using carboxymethylcellulose (CMC) as substrate. The data showed that the cellulase produced by the isolate is considerably stable at higher pH and higher temperature well suited to harsh conditions of lignocellulose degradation.

Chapter 6 illustrates simultaneous saccharification and fermentation (SSF) process involving the combination of different hydrolytic enzymes alongwith fermentation of monomeric sugars (hexose and pentose) to ethanol in one single step. The comparative hydrolytic performance of three different enzymes such as recombinant cellulase (GH5), thermostable cellulases obtained from *Trichoderma reesei* and *Bacillus subtilis* AS3 in batch SSF process were studied. Sugars released after enzymatic hydrolysis were consumed by yeasts like *Saccharomyces cerevisiae* and *Candida shehatae* and bacterium such as *Zymomonas mobilis* and their performance in bioethanol production from thatch grass (*Hyparrhenia rufa*) were investigated. SSF process employing different pretreatments along with various enzyme consortium and bioethanol producers were carried out both at shake flask and laboratory scale bioreactor. The SSF profiles with various combinations implicate an interesting relation between cell growth, specific activity of the enzyme, rate of sugar utilisation and in turn rate of ethanol formation. Three pretreatment techniques were investigated such as steam explosion, phosphoric acid-acetone and ammonia fibre explosion (AFEX). AFEX pretreatment found to perform better among the three with release of maximum amount of reducing sugars and high ethanol yield on analysis of SSF parameters.

B. subtilis cellulase gave an ethanol titre of 0.94 g/L using AFEX pretreated thatch grass and *S. cerevisiae* as fermentative microbe which showed a 6% increase as compared to recombinant cellulase (GH5) (0.88 g/L) and 9.3% increase as compared to *T. reesei* cellulase (0.86 g/L). Therefore, cellulolytic enzyme from *B. subtilis* gave better saccharification efficiency over recombinant cellulase (GH5) or *T. reesei* cellulase which was apparent from the amount of sugar released and ethanol titre obtained. On the other hand, using *Z. mobilis* as bioethanol producer gave an ethanol

titre of 1.62 g/L showing a significant 72% increase as compared to ethanol titre values of 0.94 g/L given by *S. cerevisiae* using *B. subtilis* cellulase as hydrolytic enzyme and AFEX pretreated 1% (w/v) thatch grass. On carrying out SSF using fermentative microbial combination of *S. cerevisiae* and *C. shehatae with* steam exploded thatch grass *T. reesei* gave an ethanol titre of 1.35 g/L and recombinant cellulase (GH5) gave an ethanol titre value of 1.55 g/L. A higher titre of 1.61 g/L with *B. subtilis* cellulase amid a fermentative microbial combination of *S. cerevisiae* and *C. shehatae* was obtained. Phosphoric acid-acetone pretreated thatch grass with similar mixed culture of fermentative microbes gave an ethanol titre of 1.4 g/L with *T. reesei* cellulase, 1.59 g/L with recombinant cellulase (GH5). A higher ethanol titre (1.66 g/L) was achieved with *B. subtilis* cellulase, the reducing sugar concentration being 1.68 g/L and the yield being 0.166 (g of ethanol/g of substrate). On performing SSF using AFEX pretreated thatch grass, *T. reesei* cellulase gave an ethanol concentration of 1.5 g/L and recombinant cellulase (GH5). gave ethanol titre values of 1.64 g/L. Interestingly, a maximum ethanol titre of 1.94 g/L of ethanol was achieved from reducing sugar (1.98 g/L) with a yield coefficient of 0.194 (g of ethanol/g of substrate) using *B. subtilis* cellulase and mixed fermentative consortium.

On performing SSF with mixed culture of *S. cerevisiae* and *C. shehatae* with AFEX pretreated thatch grass using *B. subtilis* AS3 cellulase, a significant 2.1 and 1.2 fold increase in ethanol titre was achieved as compared to the values obtained from single culture of *S. cerevisiae* (0.94 g/L) and *Z. mobilis* (1.62 g/L). It was also concluded that using dual combination of *S. cerevisiae* and *C. shehatae* with AFEX pretreated thatch grass, *B. subtilis* AS3 cellulase gave a higher ethanol concentration of 1.94 g/L resulting in 18% and 29% enhancement in contrast to ethanol titre values

of 1.64 g/L obtained by recombinant cellulase (GH5) and 1.5 g/L by *T. reesei* cellulase, respectively.

In conclusion, AFEX pretreatment method proved to be best among the three with the release of maximum amount of reducing sugars and high ethanol yield. Also, the hydrolytic enzyme *B. subtilis* proved superior over recombinant cellulase (GH5) and *T. reesei* cellulase owing to its ability for high saccharification rate which was evident from the amounts of sugars and ethanol titre obtained. Mixed cultures of *S. cerevisiae* and *C. shehatae* found to be more suitable as bioethanol producers having potential to consume both hexose and pentose sugars simultaneously, rather than using single culture of *S. cerevisiae* or *Z. mobilis* on analysis of SSF parameters. These SSF trials were set up to determine the outcome of various pretreatment procedures on enzymatic hydrolysis and to find out the better fermentative microbes. The best SSF combination involving *B. subtilis* hydrolytic enzymes and mixed fermentative microbes was selected for further studies with 5% (w/v) AFEX pretreated thatch grass. On increasing the AFEX pretreated thatch grass from 1% (w/v) to 5% (w/v) in shake flask, using the best SSF combination a 5.7 fold enhancement in ethanol titre from 1.94 g/L to 11.06 g/L was obtained. On scaling up the SSF process from shake flask to bioreactor using AFEX pretreated 5% (w/v) thatch grass and best SSF combination involving *B. subtilis* cellulase alongwith *S. cerevisiae* and *C. shehatae* a two fold rise in ethanol titre from 11.06 g/L to 22.0 g/L was obtained.

VITAE

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