# Integrated Bioprocess Design and Development Strategies for The Production of Optically Pure D- Lactic Acid : A Sustainable Approach for Cassava Based Agri Food Industry Waste

A Thesis

*Submitted for the Partial Fulfillment of the Requirements for the Degree of*

## DOCTOR OF PHILOSOPHY

*by*


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**November 2020**

Dedicated to My Beloved Father

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# DECLARATION

I, hereby declare that the research findings in this thesis entitled "**Integrated Bioprocess Design and Development Strategies for The Production of Optically Pure D- Lactic Acid : A Sustainable Approach for Cassava Based Agri Food Industry Waste**" is the result of research work carried out by me under the supervision of Dr. Senthilkumar Sivaprakasam, Department of Biosciences and Bioenginering & Prof.Vimal Katiyar of Chemical Engineering Department of Indian Institute of Technology Guwahati, for the award of the degree of Doctor of Philosophy. This work has not been submitted elsewhere for any degree or membership of any institute or university to the best of my knowledge and belief. Also, due acknowledgements have been made, wherever the research findings of other researchers have been cited in this thesis.

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# CERTIFICATE

It is certified that the work described in this thesis entitled "**Integrated Bioprocess Design and Development Strategies for The Production of Optically Pure D- Lactic Acid : A Sustainable Approach for Cassava Based Agri Food Industry Waste**" by Mr. Kiran Kumar Gali 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 Department of Biosciences and Bioengineering & Chemical Engineering Department, Indian Institute of Technology Guwahati, India. This work has not been submitted elsewhere for the award of any other degree.

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# List of Notations

## Variables

| | |
|---|---|
| X | Biomass concentration ($gL^{-1}$) |
| S | Substrate concentration ($gL^{-1}$) |
| P | DLA concentration ($gL^{-1}$) |
| $\mu_{max}$ | Maximum specific growth rate ($h^{-1}$) |
| $K_{ix}$ | Substrate inhibition constant for growth of biomass ($gL^{-1}$) |
| $K_{sx}$ | Substrate limitation constant for growth of biomass ($gL^{-1}$) |
| $P_{ix}$ | Threshold DLA concentration for growth of biomass ($gL^{-1}$) |
| $P_{mx}$ | Maximum DLA concentration for growth of biomass ($gL^{-1}$) |
| $K_d$ | Death rate constant ($h^{-1}$) |
| $q_{s,max}$ | Maximum specific sugar utilization rate ($gg^{-1}\ h^{-1}$) |
| $K_{ss}$ | Substrate limitation constant for CFWEH consumption($gL^{-1}$) |
| $K_{is}$ | Substrate inhibition constant for CFWEH consumption($gL^{-1}$) |
| $P_{is}$ | Threshold DLA concentration for CFWEH consumption($gL^{-1}$) |
| $P_{ms}$ | Maximum DLA concentration for CFWEH consumption($gL^{-1}$) |
| $q_{p,max}$ | Maximum specific DLA production rate ($gg^{-1}\ h^{-1}$) |
| $\alpha$ | Growth-associated constant in Luedeking–Piret model ($gg^{-1}$) |
| $K_{sp}$ | Substrate limitation constant for DLA production($gL^{-1}$) |
| $K_{ip}$ | Substrate inhibition constant for DLA production($gL^{-1}$) |
| $P_{ip}$ | Threshold DLA concentration for DLA production($gL^{-1}$) |
| $P_{mp}$ | Maximum DLA concentration for DLA production ($gL^{-1}$) |
| $Y_{P/S}$ | DLA yield on CFWEH consumption ($gg^{-1}$) |

| | |
|---|---|
| $Y_{X/S}$ | Biomass yield on CFWEH consumption ($gg^{-1}$) |
| $R^2$ | Correlation coefficient (dimensionless) |
| t | Fermentation time (h) |
| $S_0$ | Initial substrate concentration ($gL^{-1}$) |

# Synopsis

Research related to sustainable production biodegradable plastics especially Poly lactic acid is gaining momentum worldwide due to their wide variety of sustainable applications in day to day life. The stereospecific nature and economic viability of Lactic acid monomer is crucial for the sustainability of Poly (lactic acid). This surges the concepts of “green monomers” from biorefineries especially D lactic acid (DLA). Fermentative production and its subsequent purification is the preferred method in industries for DLA compared with the chemical synthesis, which yields racemic mixtures. Enantiomeric purity of DLA is crucial factor for its industrial applications and the greatest demand exists for the optically pure isomers. Globally, utilization of sustainable feedstock’s has been preferred as cost-effective and eco- efficient strategy for value added product synthesis. The underutilized Agri-food industries are facing economical threats from its stakeholders especially cassava based industries. Development of an effective techno-economic strategy through greener process integration approaches will boost the economy. DLA production is one of the potential option that could be value added towards the generated cassava fibrous waste (CFW) at different stages. The objective of this thesis work is to address the uncherished resource potential of CFW towards enhanced DLA production by imparting process intensification at different stages of integrated bioprocessing strategies, which includes Hydrolysis, Fermentation and Purification.

The processability of CFW and its enzymatic hydrolysis was improved by a novice process intensification attempt of integrating a polymer matrix with enzyme i.e. using a tailor-made electrospun nanofibers and its application in immobilization of α – amylase enzyme. Chitosan coated nanofiber (CCN) matrix was prepared by varying the chitosan concentration from 0 to 2% (w/v) and the 2% CCN matrix exhibited an optimal

performance with enhanced tensile strength (1.262 MPa), reduced elongation (41.2%) and contact angle (128 degrees). CCN matrix was examined for the immobilization of α - amylase enzyme and the relative activities of the immobilized and free enzyme were compared. Packed bed operation at optimized conditions (solution pH of 5.0 and a solution temperature of 50 $^{o}$C) deploying CCN matrix with initial substrate concentration of 10 $gL^{-1}$ yielded a maximum conversion ratio of 0.85 and 0.99 (high residence time of 40 min and low dilution rate of 0.04 $min^{-1}$) for without and with recycling mode, respectively. The characterization studies provided a better understanding about the recalcitrant nature of CFW.

The fermentation studies were performed using elite Lactic acid bacteria (LAB) possessing feedstock inhibition capability, which was achieved by adopting process intensification step involving designed biomass approach (DBA) and Inhibition kinetic modelling. Kinetic modelling was carried out to gain insight on the dynamics of cassava fibrous waste enzyme hydrolysate (CFWEH) utilization towards DLA production. DBA was attempted to evaluate the potential of organism ability to metabolize CFWEH into high optically pure DLA. *Sporolactobacillus inulinus* (NBRC 13595) and *Lactobacillus delbreuckii* were elucidated as elite LAB for DLA production based on DBA experiments. *S. inulinus* yielded an optical purity of 99.43 % for DLA employing CFWEH as production medium. Yeast extract (2 $gL^{-1}$ ) was observed to be potential nitrogen source over peptone, tryptone and whey protein hydrolysate for kinetic modelling investigation. Kinetic parameters predicted for DLA production by *S. inulinus* were found to be maximum specific growth rate, $\mu_{max}$ - 0.36 ($h^{-1}$); growth-associated product coefficient (α = 0.47 $gg^{-1}$) and specific productivity ( $q_{P,max}$ = 1.12 $gg^{-1}h^{-1}$) respectively. Good synchronization between simulated model and experimental data was observed for biomass growth, substrate consumption and DLA production with initial sugar

concentrations ranging from 20 – 180 $gL^{-1}$. *Lactobacillus delbreuckii* based CFWEH fermentation studies predicted parameters show that the inhibitory concentration for substrate was above 99 $gL^{-1}$, also inhibition due to DLA synthesis occurred as high as 59 $gL^{-1}$ for 120 $gL^{-1}$ substrate loading. Comparative assessment of kinetic parameters concluded that *L. delbreuckii* relatively performed better than *S. inulinus* and *L. delbreuckii* was selected as potential LAB for CFW valorization to DLA. This research outcome offers the knowledge of kinetic parameters, its transformation into operational parameters, which would be helpful for sustainable synthesis of DLA. Kinetic investigation reported in this study is a novice attempt enumerating the valorization potential of CFW for the synthesis of value-added products including DLA at commercial scale in near future.

Development of a novel process intensification strategy for esterification of D lactic acid (DLA) was aimed from the fermentation broth using a novel Amano lipase. Two different enzymes, namely *Candida antarctica* (Amano CL IM) and *Pseudomonas cepacia* (Amano PS IM) as procured from Amano enzymes, Industrail enzymes division were screened for its efficiency in methyl D-Lactate (ML) production. Different solvents were used for the pre-treatment of both the enzymes and its consequent effect on ML production was analysed. Amano CL IM outperformed Amano PS IM by yielding a maximum ML conversion efficiency of 22.1% and 67.6% conversion efficiency at the end of 18 h and 100 h, respectively. Methanol to DLA ratio of 0.6 was found to be optimal. Esterification studies exhibit promising conversions on aqueous based fermentation broth.To summarize, this doctoral thesis focusses on these integrated bioprocessing strategies for process enhancement of DLA production from cassava based agro food industry residue. The work has been presented in the following chapters and explained in detail in the subsequent sections.

Chapter 1

# Introduction and Review of Literature

# Chapter 1

## 1. Introduction and Review of Literature

### 1.1. Green Monomers

Access to cheap and abundance of resources is a linchpin of modern industry and civilization. The diminishing fossil resources, the drastic requirement of energy demand and alarming global warming issues favouring for the development of sustainable technologies. The polymer which is a part of human life now a days is not an exception for green economy. In context of this new greenish touch, highly versatile and cost-effective polymers play an important and essential role towards sustainability. This surges the concepts of "green monomers" from biorefineries [1].

### 1.2. Cassava Current Scenario

Globally, utilization of sustainable feedstocks have been preferred as cost-effective and eco- efficient strategy for value added product synthesis [2]. Development of an effective techno-economical strategy for conversion of sustainable feedstock to fermentable sugars play's a key role in determining the product cost and capability to produce variety of chemicals and fuels. Cassava *(Manihot esculenta*) is the most widely cultivated root crop in tropics and is the third largest source of food carbohydrates in the tropics, after rice and maize which is grown across a broad range of agro-climatic conditions[3–5]. Cassava is a perennial tuber crop cultivated worldwide with a production of over 250 million tonnes as per FAO statistics (Figure 1.1) [6]. The different stages of cassava processing involves peeling, grating, fermenting, de-watering, frying, drying which generate 25-45 % of waste and is generally disposed by land composting method (Figure1.2). However, composting cassava fibrous waste (CFW) pose environmental

threat leading to ground water contamination. Valorization of CFW into value added products would be a viable option.

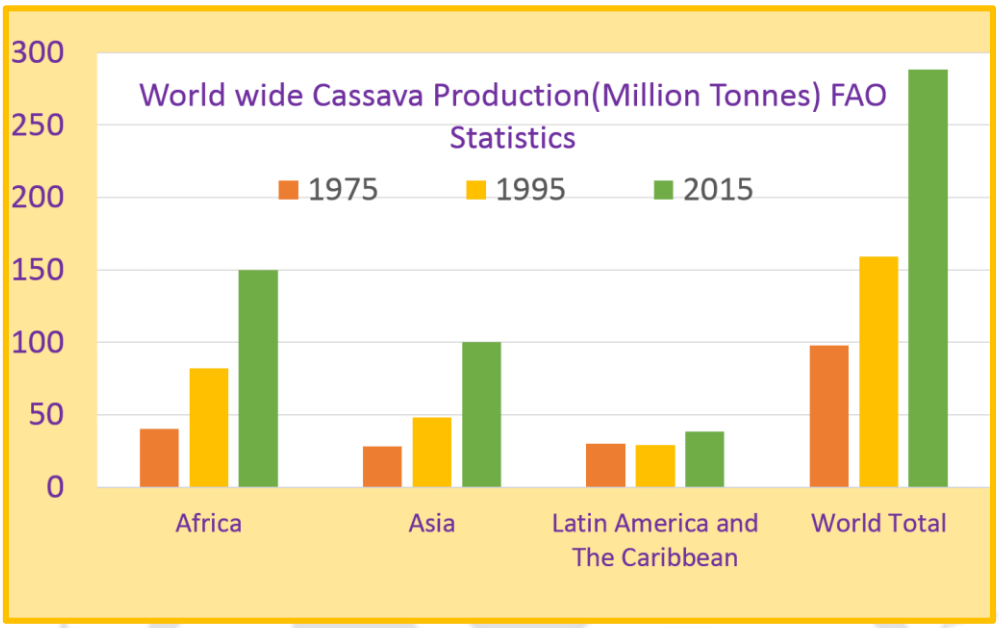



**Figure 1.1** World-wide Cassava Production statistics

Valorization of CFW into value added products would be a viable option. Cassava fibrous waste is a solid waste generated during the processing of cassava tubers in sago industries in India which contains more than 50 % by weight of starch and about 600 – 650 tons of CFW is generated in India from sago industry [7]. CFW disposed from cassava processing industries may cause serious threat to the environment because of its high organic content.

**Figure 1.2** The different stages of Cassava fibrous waste generation in Sago industry

As CFW is rich in organic content, it can be used as cheap raw material for production of various bio-chemicals. CFW is a promising raw feed stock when compared to lignocellulosic biomass because of low levels of lignin and pectin and requires minimal

pre-treatment. CFW has been used as a raw material for the production of various value added bio-products viz. glutamic acid, ethanol, pullulan, L-lactic acid. The first study of DLA by CFW was reported by Cingadi et al, 2015 [8]. While choosing a feedstock one need to consider the availability, conversion capability, economics of the stock, competition and Green House Gas savings. Considering DLA production using CFW as a feedstock not only reduces the waste generated from the cassava starch industry, but also lowers the cost of DLA production, which is one of the major components in thermostable biopolymer poly-lactic acid (PLA).

### 1.2.1. Cassava Production

Tapioca which we also know as' cassava' was once considered to be the 'poor man's crop' has now upgraded to being one of the most vital crops cultivated globally in tropical and sub-tropical regions with minimal requirements. There has been an increased demand for the production of cassava in yield per hectare mostly because of its different roles in the new and the growing industries worldwide [9]. This crop does not only serve as a healthy staple food rich in starch but it also provides food stability at the same time. This crop can be easily grown in marginal lands with well-drained soil and more moisture content. Generally this crop requires sunlight exposure for long periods of time for better starch production and can be effortlessly grown with less fertilizer. A lot of factors during the cultivation process determine the starch quantity which is obtained from cassava. The production of cassava is also highly dependent on the supply of quality stem cuttings which aids in the cultivation of more number of cassava crops. Different varieties of cassava are being produced nowadays with improved properties like enhanced starch content, less cyanide production, drought-resistant and high yield [4].

In 1960's Brazil and Africa were the major cassava producers worldwide. From early 1990's, Africa continued supplying 50 percent of cassava produced worldwide along with Nigeria [5,6]. From 2011 to 2015, the top ten countries which produced Cassava were Nigeria, Thailand, Indonesia, Brazil, Ghana, and Democratic republic of Congo, Angola, Vietnam, Cambodia and Mozambique [2]. Today Africa is still the leading producer of cassava as it is dependent on it for multiple purposes mostly as an emergency crop during drought and famine conditions while in Asia and Latin America it serves majorly as an industrial crop. Today there are more than 100 countries which produce cassava globally with a total output of 270 million tons and also satisfying the caloric demands with the increase in population [3]. The total farming area for cassava is more than 18 million hectares in Africa, Latin America and Asia.

Major cassava growing countries in Asia are India, Indonesia, Thailand and Vietnam. India has always been known for its excellent agricultural practices starting right back from the Indus Valley Civilization. India is known for growing a number of staple food crops and has now attained the title for the top ten major Cassava producing countries worldwide. Cassava production in India was initiated by the Portuguese in the 17th century as a food crop which has now upgraded to be one of the major industrial crops grown in about 13 states by the end of 20th century [4]. Kerala and Tamil Nadu are the largest producers of cassava along with emerging importance in Andhra Pradesh. It is also consumed as an important carbohydrate source in Assam, Meghalaya and Nagaland.The data is represented in the form of Table 1.1 below. The productivity, yield and production per unit area have gradually increased from 1961 to 2000. In the year between 2012-2013, the total area for tapioca cultivation in India was around 216.66 thousand hectares and the production accomplished was about 7319.13 thousand metric tons [10]. India is also known to export

cassava processed food to countries like United Arab Emirates, Saudi Arabia, Oman, European nations, Kuwait and the United States of America.

**Table 1.1** State-Wise Area and Production of Tapioca in India

| State | Area ('000 Hectares) | Production ('000 metric tones) |
|---|---|---|
| Tamil Nadu | 109.56 | 4205.82 |
| Kerala | 72.47 | 2637.20 |
| Andhra Pradesh | 16.45 | 329.02 |
| Nagaland | 6.00 | 50.00 |
| Meghalaya | 5.60 | 30.05 |
| Assam | 4.48 | 38.31 |
| Karnataka | 1.00 | 12.90 |
| Total(Including others) | 216.66 | 7319.13 |

The extraction of the starch granules from cassava is carried out in a sequential process in the large scale starch and sago manufacturing industries [11]. The cell wall is ruptured by a process called rasping, using well-designed equipment containing blades which crushes and grinds the peeled tuber crop for the separation of the starch from the root cells. This process results in the accumulation of a residual mass. Although the rasping process is implemented more than once, but still the entire starch content is not extracted completely. Cassava fibrous waste (CFW) generated during this rasping process is rich in starch and organic content and can be easily fermented to reducible sugars in presence of appropriate strains of microorganisms mostly *Lactobacillus* to produce D lactic acid. Such strategy for the production of DLA from the huge amount of cassava fibrous waste generated in industries will not only be feasible but also be a techno-economically superior business.

CFW after enzymatic hydrolysis generates huge amounts of glucose as carbon source which can be utilized entirely for DLA formation at comparatively low cost [8].

### 1.2.2. Cassava Utilization

Cassava being one of the staple foods for many indigenous people around the globe is more prioritized than other crops like wheat, maize, rice grain, etc., which can be related to the fact that growing cassava is more advantageous and easier than other crops which need to fulfill certain requirements for cultivation [12]. The demands for the production of cassava have increased duly with the expansion in population and urbanization. Human consumption being the major usage of cassava in the poor section of our community, its huge variety of derivatives is being marketed in various food and non-food industries. More than 60 percent of the cassava produced worldwide is consumed as flour and fermented food [5]. Starch granules extracted from the tubers are refined into fine powder and used as a raw material in the production of biofuels, biopolymers, adhesives, tanning of leather, animal feed, paper etc., and as edible items like sago, garri, chips, flour, etc [13]. Bakeries and other food and beverage industries are one of the major customers of raw starch from Sago industries for the production of jellies, candies, bread, chewing gums, syrups, etc. Chemical industries utilize raw starch obtained from cassava for the manufacturing of glucose, sucrose, lactose, dextrose, fructose, cellulose, hemicellulose and dextrin [14]. Cassava ethanol obtained from cassava serves as an excellent raw material in synthetic chemical industries and in pharmaceutical industries. Cassava also has beneficial roles on human hair, skin and health for which it has paramount usage in cosmetic industries as well.

Not only the root, cassava leaves are also consumed by a number of people due to its nutritious content and have applications as cattle feed. Cassava can be segregated as sweet

or bitter based on its cyanohydrin content. The sweet cassava is mostly consumed directly without any prior treatment and the bitter cassava is served for various industrial purposes. Human consumption of cassava is mostly trending in developing countries than the developed countries. Statistical reports have shown that cassava consumption have increased from 76 million ton to 96 million ton from 1984 to 1994. Surveys conducted have showed that the largest markets for cassava feed products are in Netherlands, Belgium, Germany, Spain and Portugal. Cassava being the 4$^{th}$ major source of starch is also leading to the expansion of starch based cassava market. Starch industries located in Asia are more functional than those located in Latin America and Africa [4].

Cassava based fermented food like Gari, Kapok pogari, Lafun, Chick-range, etc and also cassava bread is mostly prevalent in regions of West Africa. Gaplek which is dry cassava is consumed in Indonesia. Cassava in form of rice is consumed in Philliphines. Macaroni (cassava noodles), tapioca pearls and sago are consumed mostly in Asia. Beverages like Mingao and Tapioca tea are consumed in South America and Asia respectively. Fermented cassava based alcohol familiar as cassava beer is produced in regions of America and Africa [6, 7].

### 1.2.3. Economic Viability

Although the cassava crop takes around 9-11 months to grow, but the overall production is a cost-effective affair. The starch and the sago industries produce huge amounts of solid and effluent waste concurrently which has to be discarded in an appropriate manner in the environment [15][14][10][16]. As mentioned previously, disposing such waste not only causes environmental pollution but also contaminates the ground water table. Therefore, to make the manufacturing processes more economically viable, waste matter is converted into value-added products. Industries using cassava crop as a raw material for the

production of starch can easily acquire additional benefits by implementing manufacturing of other essential commodities. Pretreatment of the substrate used for fermentation is also an imperative contributor to the overall production cost for DLA which is not counted here for the reason that there is very less concentrations lignocellulose biomass present in CFW. Hence, production of DLA using CFW as feedstock will be a much economically viable approach industrially by using low cost equipment's and optimizing the process parameters for large scale production. In addition to that, sago industries in India serve as one of the major employment opportunities for manual workers in rural areas and the cost of the final products from such industries (sago and starch mostly) are extremely modest contrasting to the market price of DLA [16][17]. In general, cassava derivatives are marketed at a rate much higher than the initial investment which is made for processing of cassava as a raw material. The development of advanced tools and machinery can also make the post-harvesting processes much easier and cost-effective with respect to time and labor.

Commercial development of sago industries is being done at a very low rate. Sago industries in India are widely distributed in several districts in Tamil Nadu namely Salem, Namakkal, Dharmapuri, Erode, Tiruchirappalli, Perambalur, and Thiruvannamalai districts [8][18][19]. A lot of development is still needed in this industry related to marketing, financial and laboring limitations associated with it which needs to be solved along with implications of government policies. Waste generated from this industry can be beneficially utilized for the production of DLA, contributing the enhancement of the overall profit.

### 1.2.4. Value Addition

Cassava derived products promotes value addition. Sago industries which were considered trivial previously have now become a salient business in the modern day. These industries not only produce sago but also produce starch which is utilized as a raw material by many other industries like textile industries, paper industries, etc and even laundries in household [13][12]. Linking between the rural and urban areas will ensure better marketing of the products. The overall production cost of cassava crop is very insignificant as compared to the processing and marketing segment and the amount of waste generated from such processing units are massive which can be converted to other by-products. With the advance in research and technology, these waste generated can be converted to other marketable and essential products like biogas, ethanol, fertilizers, surfactants, adhesives etc [18][20]. Most of the sago industries dry the residual waste for utilization as animal feed. A number of organic compounds can also be produced from industrial cassava wastes under mesophilic conditions including citric acid, lactic acid, succinic acid, VFA's and biosurfactants [12][14] [5]. Biofuel production using cassava pulp has also been achieved through hydrothermal treatment. Biogas production can also be carried out from different waste from cassava processing industries and can also serve as a potential energy supplier to bio refineries. Starch based biodegradable plastics are being made for maintaining an ecofriendly environment and also for convenient usage. DLA production using CFW being a viable process is not in trend much and needs to come into limelight [21].The manual labor wage designated in such industries is not exorbitant and value addition of cassava promotes an increment in the source of income for the farmers growing them. Exportation of cassava based products is being conducted at high levels in foreign countries which also provide the wholesalers remunerative rates. Research and development have even established processes where the residual sago biomass can be even utilized for the

production of electricity [9]. Hence, agro industries establishing collaborations with research institutes will lead to development of efficient processing units and high profit with greater value-addition. Literature reports reveals that improvement in modern processing and marketing facilities will surely enhance the efficiency of such business units along with quality standardization. Proper communication, transport facilities and storage facilities along with a good planning of infrastructure will ensure more favorable outcomes [10].

### 1.3. Biomass Waste Valorization

A biorefinery is a facility that integrates biomass conversion processes and equipment to produce variety of products like fuels, value-added chemicals, heat and power from biomass [22]. It resembles petroleum refinery concept where multiple fuels and products are processed from petroleum. Abundant renewable feedstocks which are carbon neutral represents biomass for production of variety of products (Figure1.3). Present waste valorization is carried under the umbrella of biorefineries using first generation feedstocks such as corn, soybeans, and sugarcane for bioethanol and biodiesel production [23]. The use of second-generation feedstock such as lignocellulosic biomass, including forestry and agricultural residues, modern cell engineering and fast conversion technologies in biorefineries favouring sustainable economic growth with minimal or no negative impact on the environment.

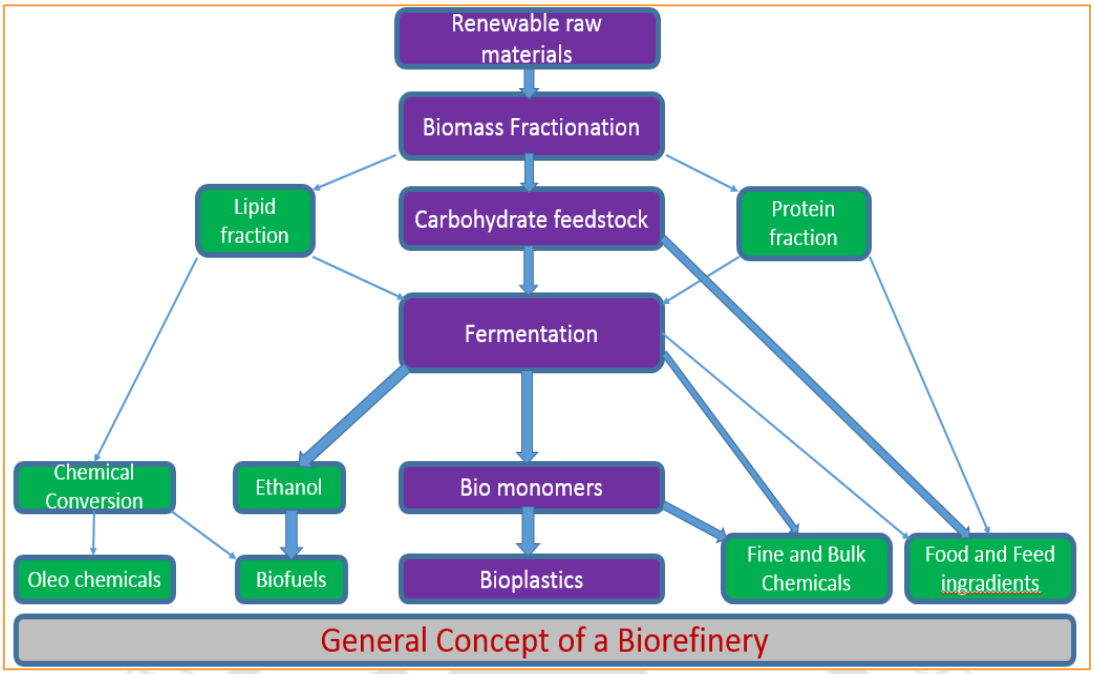



**Figure 1.3** The concept of biomass valoraization to bioplastic through biorefinery

### 1.3.1. Need

There are specific challenges that needs to be addressed in today's biorefinery industry. Particularly the recalcitrant nature of the lignocellulosic feedstock, processability of pre-treating and enzymatic hydrolysis, difficulty in scaling up makes the bio refining as uneconomical in terms of cost and time. To improve process economics, the biorefinery should utilize all components of biomass feedstock, minimize waste generation to maximize productivity which requires the integration of technologies from various areas, including new energy crops with higher biomass yields and better processability, better and cheaper enzymes for hydrolysis, novel and improved cells and catalysts for biomass conversion to fuels, chemicals, and other marketable products, and more efficient processes for the production of these biobased products at a commercial scale. This concludes that the selected lignocellulosic biomass involves the enzymatic treatment thereby processing the fermentable sugars and to purify the product towards production of optically pure DLA.

### 1.3.2. Marketability and Competetiveness:

According to recent published reports in 2020, the starch industry market is projected to grow by 47.3 million metric tons, driven by 5.3% compounded growth and liquid starch with 5.4% compounded growth. The starch extracted from different sources has different properties which are inherited during value addition and further obtained in the overall property of the final product marketed. This is mainly due to the variation in amylose content and branch chain length [11]. Cassava starch is known for its certain exceptional properties like high paste viscosity and clarity. One of the main reasons it is mostly preferred as an agro-industrial food is due to its high freeze thaw stability [12]. Marketability of cassava based products will be highly dependent on the remarkable features and quality of the final value added product and how better it competes with other products made from other starch containing raw materials (maize, sweet potato, rice grain, etc). Marketability can be enhanced by monitoring the performance of the products and their impacts on the society. In a similar manner, waste produced from cassava processing industries can also be converted into a number of value added products having high market demand. Marketability of DLA is totally dependent on its level of purity, cost and demand. Biological means for production of DLA ensures high optical purity and implementing CFW reduces overall production cost which will definitely enhance marketability [24][21].

Cassava starch also has certain limitations in their functionality which hinders its application industrially and can be also removed easily through better enzymatic treatments based on research and novel innovative strategies. The value addition also keeps on increasing the market value of the products by making it more appealing to the customers. The industries should decide upon a marketing team which will not only promote the cassava based products but also create value chains. Although there are very

less bio refineries actively functioning till date but one of the important limitations seen in industries is that none of the bio refineries are willing to set up new setups and processes from scratch using newly made technologies and mostly considers it uneconomical. So mostly such newly made technology based inventions using different raw materials remain as patents and fails to get commercialized. Therefore, it is more preferable to work on existing technological processes and upgrade it as much as possible for better profitability and efficiency [25][26].

Biomass valorization concepts are known to convert biomass into value added marketable products. Cassava based bioproduction does not only utilize the edible tuber roots of the crop but also makes use of the leaves, stems and fibrous residual wastes for conversion to essential products including DLA. Such plan of actions results in more profitable business outcomes.Bio based products manufactured through bio refineries have an advantage of being more economic and eco-friendly. With more exploration in these research fields the production cost which is one of the barriers for DLA production can be reduced many folds. Market reports have predicted that the production of high volume low value added products (e.g. DLA, succinic acid) can also be a beneficial marketing strategy and reduce the selling price of primary products [5,27,28]. The market for optically pure DLA is expanding with time for it's use in the production of PLA. Most of the industries is still dependent on non renewable resources for the chemical synthesis of DLA marketing at high price. In contrary, biological means of DLA production using fermentation method will be a better option keeping an eye on the rising global demand. Although bio refineries are capable of marketing more than one product, producing solely DLA from CFW will also be a feasible and profitable strategy [21,25–33].

Marketability is also dependent on the consistent supply of cassava crops as raw material which has to be regulated by communicating and cooperating with the producers in the field. Abundant supply of CFW is necessary for continuous production of DLA in bio refineries. Potential progress in the agricultural sector can also put a significant impact on the marketability and competitiveness of cassava based starch and other products based on the annual productivity which is also having a direct impact on cassava based industries and their waste management. In general, to achieve economies of scale, it is prioritized to have good coordination between the production team and the marketing team. Through better revolution the cassava based products can be diversified and well promoted amongst all types of customers regionally as well as globally. Lastly, government policies also need to be improved for giving more relevance to promote cultivation of cassava as a potential industrial crop. Marketing and trade developing programs can improve the partnerships between farmers and private sectors and can prove to be beneficial if implied [34].

Initiatives are being taken in India for developing the cassava market through the efforts of the R&D in private sectors. Cassava based processed foods are being manufactured and even exported to different regions. Waste generated from these plants need to have proper waste disposal schemes and the best way to get rid of them is by reusing them for other purposes. India has gained a high yield production of cassava with time owing to the extensive research which has been conducted. Indian local markets have huge sale of cassava processed foods like sago (sabudana), starch, chips, flour, wafer and papad. Private companies even market machineries for processing cassava like peelers, graters, fryers, roasters, etc [13]. But most of these industries are small scale units and still follows the age old traditional methods and lacks the implementation of advanced technological equipment. Development of more cassava based industries also generate relatively more cassava processed waste including CFW which needs to be disposed off properly.

Utilization of abundant CFW as feedstock could satisfy the increasing demand for DLA production in a cheaper mode.

### 1.3.3. Socio Environment Impact and Acceptance

Agriculture and nutrition are equally important for the socio-economic growth of developing countries. There are a lot of superstitious believes associated with the usage of cassava which have been solved and explained by researchers. Cassava although a nutritious edible food, it also has a lot of toxicity in it which needs pretreatment before consumption [35]. The local people including the farmers producing this crop mostly consume cassava directly as the cassava leaves are a good source of protein while the roots are a good source of fiber and benefits in improved digestion. Apart from that, cassava is highly rich in essential amino acids, vitamins and minerals and is also considered to be a good cholesterol-lowering and anti-diabetic agent [36][14]. Thus, cassava does not only serve as an easily accessible, low cost food crop for the lower class people but is also a good source of income for them. Similarly, with emerging bio economy the local farmers are getting exposed to more market opportunities. Cassava based bio refineries will be able to process and supply more than one value added products from biomass which itself will be a great form of a financial gain. These bio refineries will increase the number of employment opportunities especially for low class people and in rural areas which in turn can bring improvement in the quality of lives for these farmers. Bio refineries will also pave the avenue for more job opportunities to large community of the region to be considered for installation and operation.

Biomass waste based biorefineries have their main objectives to reduce environmental deterioration through pollution and improve the water and soil quality and also reduce the emission of greenhouse gases in the earth's atmosphere. Bio refineries also have a direct

impact on the agricultural sector for the improved breeding and production of cassava crop since a stable supply of the raw materials will be needed. For the establishment of a bio refinery, one major part is to get good investors for the construction of the whole business and also provide a convincing project plan. Net profit generated and the tax revenue matters are also investigated for assessing the overall economic impact. Cassava based bio refineries are also capable of manufacturing biofuels and biogas which will serve as a national energy security for countries relying totally on fossil fuels and will prove to be a good master plan for regions generating more amounts of biomass. Considering all such environmental issues along with increase in demand for energy supply with increase population, alternate options need to be considered and bio refineries should be accepted.

### 1.4. D- Lactic Acid

Poly Lactic acid (PLA) is one of the biodegradable polymer obtained from renewable resources. It is a thermoplastic aliphatic polyester produced by mostly ring opening polymerization of lactide, which is a cyclic di-ester of lactic acid or the direct condensation of lactic acid monomers (Figure1.4). In 2010, PLA had the second highest consumption volume of any bioplastic of the world [2,5,37]. The lactic acid is having two optical isomeric forms known as 'Dextro (D) rotatory and 'Levo (L)' rotatory. Enantiomeric purity is important for industrial uses and the greatest demand is for the pure isomers. Deliberate blending of the enantiomers provides an effective method to control both the physical properties of poly lactic acid and the rate of biodegradation. The subtle difference in stereochemistry has a drastic impact on mechanical properties and degradation which favours the production of lactic acid by biological processes rather than chemical processes which yield racemic mixtures. The production of lactic acid consists of two stages. The first one is the pre-treatment where characterization and enzymatic treatment of the selected starchy rich feedstock is carried out for production of fermentable sugars

[38][27][31]. The second one is the fermentation where the treated enzymatic hydrolysate is used in medium formulation for fermentative production of Lactic acid by the selected strain.

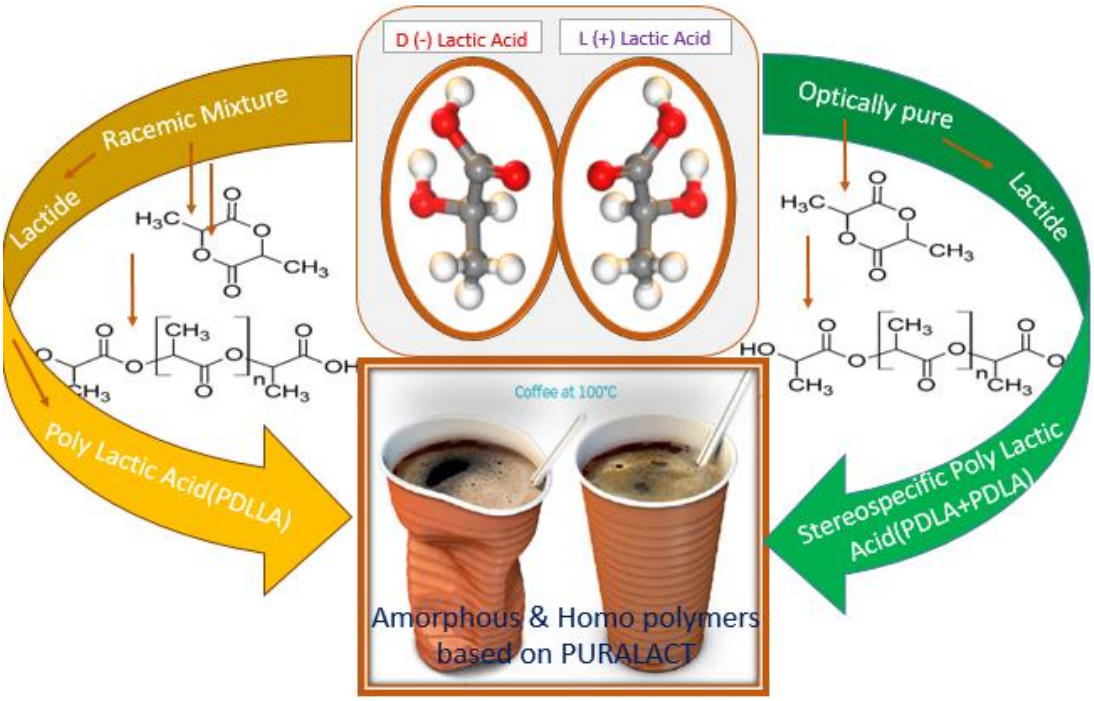



**Figure 1.4** Synthesis of Stereospecific Poly Lactic acid from optically pure monomers

D (–) lactic acid (DLA) is a versatile organic acid molecule, extensively employed in the production of thermostable biodegradable polymer, poly lactic acid (PLA). Optically pure DLA is pre-requiste for the production of PLA with improved mechanical and thermal properties[22,39–41] [15]. D lactic acid (DLA) is also an industrially important organic acid, has wide applications in chemical, pharmaceutical, agriculture, textile, and leather industries.[42] In recent years, DLA has gained a lot of attention owing to its application in thermostable biodegradable polymers production. There is increasing demand for optically pure isomers in the past two decades. According to lactic acid and PLA market analysis report 2014, the estimated global lactic acid market in 2013 was 714,200 metric tons and is forecasted to reach 1,960,100 metric tons by 2020. The worldwide PLA

production in 2013 was estimated as 360,800 metric tons and is expected to reach 1,205,300 by 2020; meanwhile there is a need for significant cut in the product cost almost to half of its current value ($ 2.2/kg) [22,39–41] [15]. Microbial fermentation process is the attractive techno-economic strategy to produce optically pure DLA compared to racemic mixture yielding expensive chemical synthesis. However, choice of raw feedstock and its preprocessing requirements for microbial fermentation decides the fate of the final product cost. Hence, a low-cost feedstock with minimal pretreatment would be the suitable choice for economic DLA production. DLA can be produced through microbial fermentation by using fermentable sugars (glucose, fructose and sucrose) and starchy materials (potato, corn and maize) as feedstocks. However, they are utilized as staple foods in different parts of world and could not be used as feedstock for DLA production. Alternatively, renewable, non-edible low cost, feedstocks such as cellulose, cellulose containing biomass (horticulture/agricultural/forest residues) could be considered for DLA production [42][43].

Therefore, selection of a suitable raw material could significantly influence the total cost of PLA production. Industrially, DLA can be produced through either expensive conventional chemical route using petrochemical feedstocks, which always yields racemic mixture or microbial fermentation route that can produce enantiomerically pure lactic acid. In microbial fermentation, homo-fermentative lactic acid bacteria (HFLAB) are preferred for DLA production due to their ability towards effective conversion of reducing sugar solely into DLA. Microbial production of DLA is significantly influenced by the cost of raw material. Renewable and low cost feedstocks need to be employed for economical fermentative production of DLA and was discussed in previously published literatures [17,18]. Renewable agricultural waste resources are gaining significance as potential feedstocks for production of DLA owing to their abundant availability and low cost.

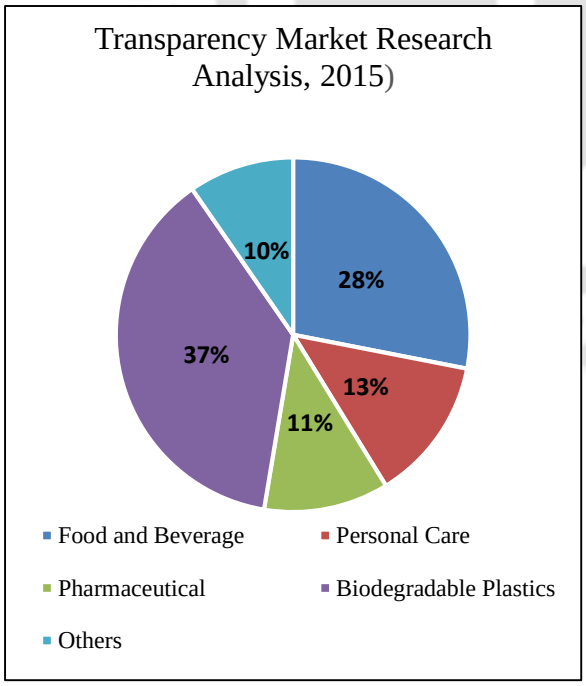
Furthermore, lactic acid bacterial (LAB) growth was inhibited at high DLA concentrations as a result of product inhibition phenomenon. However, the presence of inhibitory compounds (e.g. lignin, pectin and recalcitrant chemicals), multi-stage cost intensive preprocessing requirements, staple nature and seasonal availability hinders their application in fermentation process.

### 1.4.1. Market Demand and Stakeholders

As per the transparency market research analysis global lactic acid market is 284,000 tons per annum in 2013 and the projected values are 375,000 tons per annum by 2020 [43] where production technology and usage is mostly dominated by the Europe, America and Asia pacific regions [6]. Lactic acid is having a wide variety of applications in food, pharma and cosmetic industries. Table 1.2 and Fig 1.5 gives information regarding global lactic acid volume, price ranges and different application areas. Growing demand for poly-lactic acid (PLA) will reach to about 1,960,100 metric tons by 2020, due to its application in various industrial spheres like biodegradable plastics, biomedical equipments, textiles etc. Interest is more focused on Poly DL-Lactic acid (PDLLA), an hetero-polymer crosslinking between alternate D and L monomers [21]. Growing demand for poly-lactic acid (PLA) will reach to about 1,960,100 metric tons by 2020, due to its application in various industrial spheres like biodegradable plastics, biomedical equipments, textiles etc. Interest is more focused on PDLLA, an hetero-polymer crosslinking between alternate D and L monomers. This offers 40 – 50 °C higher melting temperature and a variation of heat deflection temperature from 60 – 190 °C than either PLLA or PDLA homo-polymers alone. For this reason, there is a huge demand for optically pure DLA in last decade. DLA is used for more specialty applications and is opening up new markets such as in disposables, semi durables and durables[42].

**Table 1.2** Lactic acid production volume and cost analysis by Cellulac

| Product | Category | Current volume (ton pa) | Approximate price (per ton) | Segment value at median price | Projected growth |
|---|---|---|---|---|---|
| Lactic Acid | Speciality chemical or ingredient in food and solvents | 320,000 | $1,300 -$2,300 | $576m | 20% pa until 2016 |
| | Speciality chemical or ingredient in bioplastic production | 1,80,000 | $1,300 -$5,000 | $567m | 28% pa until 2025 |
| Poly Lactic Acid | Substitute for fossil based polymers | 1,00,000 | $2,300 -$6,000 | $415m | 28% pa until 2025 |



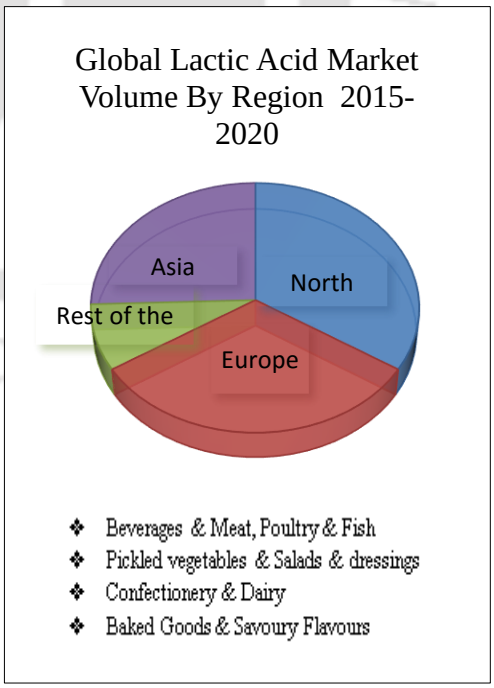




**Fig 1.5** Lactic acid production volume distributed to different sectors and regions

DLA can be purified to polymer grade lactic acid with improved temperature properties, and is now used for not only biodegradability, but also durability. An example is television screen casing, made from PLA (using D lactic acid – PDLA). There are few industries producing DLA from raw materials like corn and cellulosic materials. Raw material is one of the major cost factors in the production of the bulk chemical such as DLA. Utilization of high energy food crop such as corn cannot serve as a suitable option for the production of PLA.

### 1.4.2. Feedstock Availability and Processing Difficulty :

Globally, utilization of sustainable feedstocks have been preferred as cost-effective and eco- efficient strategy for value added product synthesis. Development of an effective techno-economical strategy for conversion of sustainable feedstock to fermentable sugars play's a key role in determining the product cost and capability to produce variety of chemicals and fuels. Cassava (*Manihot esculenta*) is the most widely cultivated root crop in tropics and is the third largest source of food carbohydrates in the tropics, after rice and maize which is grown across a broad range of agro-climatic conditions [44]. The different stages of cassava processing involves peeling, grating, fermenting, de-watering, frying, drying which generate 25-45 % of waste and is generally disposed by land composting method. However, composting cassava fibrous waste (CFW) pose environmental threat leading to ground water contamination. Valorization of CFW into value added products would be a viable option. Cassava fibrous waste is a solid waste generated during the processing of cassava tubers in sago industries in India which contains more than 50 % by weight of starch and about 600 – 650 tons of CFW is generated in India from sago industry [15]. CFW disposed from cassava processing industries may cause serious threat to the environment because of its high organic content. As CFW is rich in organic content, it can be used as cheap raw material for production of various bio-

chemicals. CFW is a promising raw feed stock when compared to lignocellulosic biomass because of low levels of lignin and pectin and requires minimal pre-treatment. CFW has been used as a raw material for the production of various value added bio-products viz. glutamic acid, ethanol, pullulan, L-lactic acid. The first report about fermentative production of DLA utilizing CFW was attempted by our research group Singadi et al, 2015 [8]. While choosing a feedstock one need to consider the availability, conversion capability, economics of the stock, competition and Green House Gas savings. Considering this DLA production using CFW as a feedstock not only reduces the waste generated from the cassava starch industry, but also lowers the cost of production of DLA, which is one of the major components in thermo stable biopolymer PLA. A recent review by Zhang et al [1,45][5] addressed the advantages and consolidated the concept of CFW biorefinery.

### 1.4.3. Critical Process Parameters and Fermentation Barriers:

The fermentation require strict control of process parameters for which detailed information needed in terms of substrate and product inhibition along with titer, optical purity and chemical purity which will give an understanding of the range of operating conditions and parameters to run an effective production process [46]. The temperature at a range from 35 to 45 $^{o}$C and pH ranging 5 to 6.5 during a typical LA fermentation, a drop in pH below a critical value (due to LA production) has an inhibitory effect on the metabolic activities of the strains. In the conventional operations suitable bases and salts are added to neutralize the LA to minimize the negative effects of undissociated LA accumulation in industrial processes [20][18]. However, the neutralization of LA during fermentation has major disadvantages as additional operations are required to regenerate undissociated LA from its salt and to dispose of or recycle the neutralizing cation. Calcium hydroxide is used as a conventional neutralizing agent in the fermentation reaction

producing calcium lactate. Sulfuric acid is used to liberate LA from calcium lactate generating calcium sulfate as solid waste, which is currently disposed as gypsum. Another process for the preparation of LA without the formation of gypsum is using other neutralizing agents, like ammonia and magnesium hydroxide. When ammonia is used as neutralizer, ammonium lactate is formed. Hence, formation of gypsum was efficiently prevented, but has the disadvantage that the formed ammonium lactate is difficult to thermally cleave to obtain the desired LA [5,27,28] [47]. Magnesium hydroxide can also be used to neutralize the LA, which forms magnesium lactate. Magnesium lactate is brought to react with water-miscible organic amine to form an organic amine-lactic acid complex and magnesium hydroxide, after which magnesium hydroxide is precipitated and separated from the complex which can thermally decomposed to liberate LA All the extra operations and expense could be reduced if undissociated LA could be accumulated by microorganisms able to grow and metabolize at low pH levels [21] [22-24].

### 1.4.4. Purification Hurdles and Energy Accountability:

The overall cost of the final product is influenced strongly by the downstream process and purification methods. A number of methods have been generated so far by which DLA can be separated from the fermentation broth to obtain its pure form which includes neutralization and precipitation, solvent and reactive extraction, electro dialysis, membrane based extraction, adsorption, salting out extraction and molecular distillation. It is a laborious task to separate lactic acid from water although the difference between their boiling points is quite high [48]. Precipitation method is mostly preferred for industrial use while membrane technologies can be applied for easy scalability and custom made products [49,50]. Each of these methods has their own advantages and disadvantages. Most of these methods consist of a number of steps and the final yield obtained is very low. Large amounts of reagents are also required which also leads to

increased cost of the final product and therefore to make the process more economically viable it is necessary to develop new innovative ideas for conducting effortless separation and recovery of DLA with high yield and purity so that it can have application in bio based refineries.Figure 1.8 gives a clear cut understanding the of DLA production process hindering parameters for assessing the posiible process integration aspects further.

For running of such biorefineries constant power supply is necessary. Energy management is vital to keep a track on the overall power consumption taking place. Equipment's should be regularly monitored and improved for reduced energy consumption [39][51]. Very large scale industries form energy management teams headed by an energy manager which conducts energy audits and further takes necessary actions. Another acceptable strategy for maintaining energy supply in bio refineries is by making use of nonrenewable sources as alternatives like biogas and solar energy.

### 1.4.5. Existing Technology and Bioprocessing Strategies:

D lactic acid has been reported to be produced by several species of lactic acid bacteria, in particular *Lactobacillus delbrueckii*, *Lactobacillus coryniformis subsp. torquens*, *Leuconostocmesenteroides subsp. mesenteroides*, *Leuconostocmesenteroides subsp. dextranicum*, *Leuconostoccarnosum*, *Leuconostocfallax* as well as genetically modified *Lactobacillus plantarum* It is well known that LAB frequently require various kinds of micronutrients for it fastidious growth; however, it is expected that the LAB isolated from dairy waste would grow and produce lactic acid in the waste without the need for additional growth factors because the bacteria is living in the sludge in the first place (Nakasaki *et al.*, 1999). Table1.3 [32,52–57][57,58][59,60][61–65][66][8,43,67–72] gives an understanding of wide variety of microorganisms reported for the utilization of different feedstocks from renewable sources are producing a wide vaiety of value-added processes

capable of converting these materials, which are otherwise considered to be wastes, into valuable products through processes with techno-economic feasibility. LAB ferment sugars via homo-, hetero-, or mixed acid fermentation. Homofermentative LAB producessor lactic acid as main product from sugars. However, the literature results highlight the need of a promising strategy for effective substrate utilization, combating salt stress and elimination of product inhibition. In nut shell, the quest for the production of high-titer optically pure DLA in short time with low cost drive is the need to formulate the proposal. There are some renowned world wide commercial producers of lactic acid in the market namely Purac, Galactic , Mushashino and some other which are producing based on the traditional technologies available.

There has been more emphasis on few major challenges which has to be faced while producing DLA. The four main criterions which are required to be fulfilled are: i) purity, (ii) acid tolerance, (iii) carbon source, and (iv) industrial parameters. A number of host organisms have been rationally metabolically engineered to produce lactic acid with >99% optical purity. and similarly organisms like Lactic acid bacteria which are wild and natural ability have been modified by increasing acid stress tolerance due to which cell survivability increased at very low pH conditions enhancing overall lactic acid production. A summary of all host organisms studies used for DLA production is provided in Table1.3. [32,52–57][57,58][59,60][61–65][66][8,43,67–72]

CFW is enriched in starch which needs to be enzymatically converted to glucose at the first step. This glucose gets converted to DLA in the second step through microbial fermentation. The existence of lactic acid bacteria can be considered as a wealth in nature due to its large number of applications. Amylolytic lactic acid producing bacteria's are capable of doing the conversion from starch to lactic acid directly through a single step.

But the whole metabolic process becomes an overburdening task for the organism due to which the end product yields and productivity decreases and is not considered suitable for industrial scale applications. Analogously, homofermentative lactic acid bacteria (Figure 1.6 ) in general will be more preferred for production of DLA from enzymatically hydrolyzed sugar through the Embden Meyerhoff pathway (EMP) and the end product obtained are more efficient and of high optical purity which is highly essential for PLA synthesis. Apart from that it is also necessary for the genetically manipulated (GM) host organisms to maintain stability throughout without getting mutated and also not cause any harmful outcomes in nature, so that it can be highly recommended for commercial use. This approach is unsure in terms of GM systems. There is an alternative approach in terms of Designed Biomass Study for this case which is represented schematically in Figure 1.7 for better understanding.

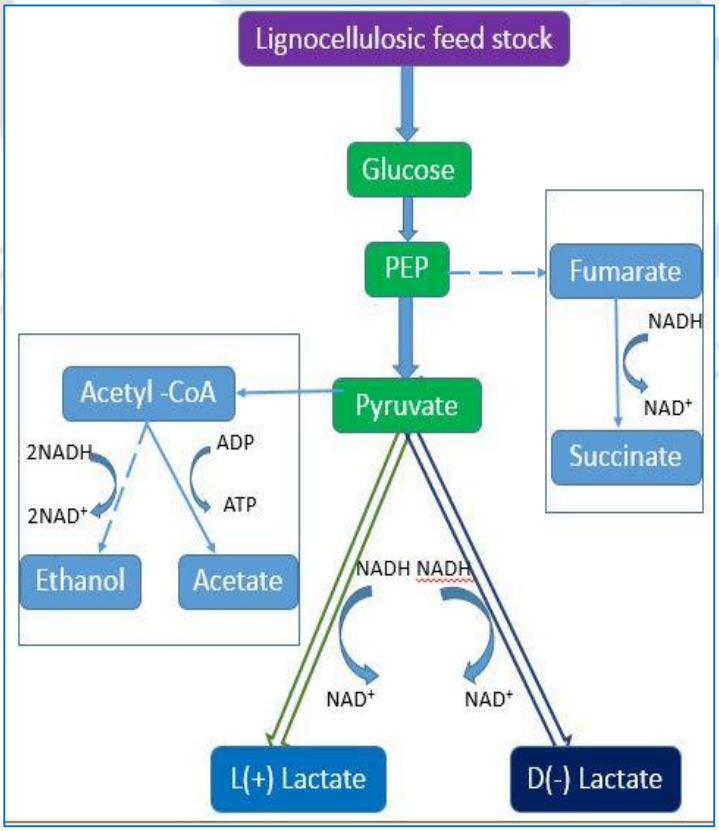



**Figure 1.6** Lactic acid Pathway from Lignocellulosic feedstock

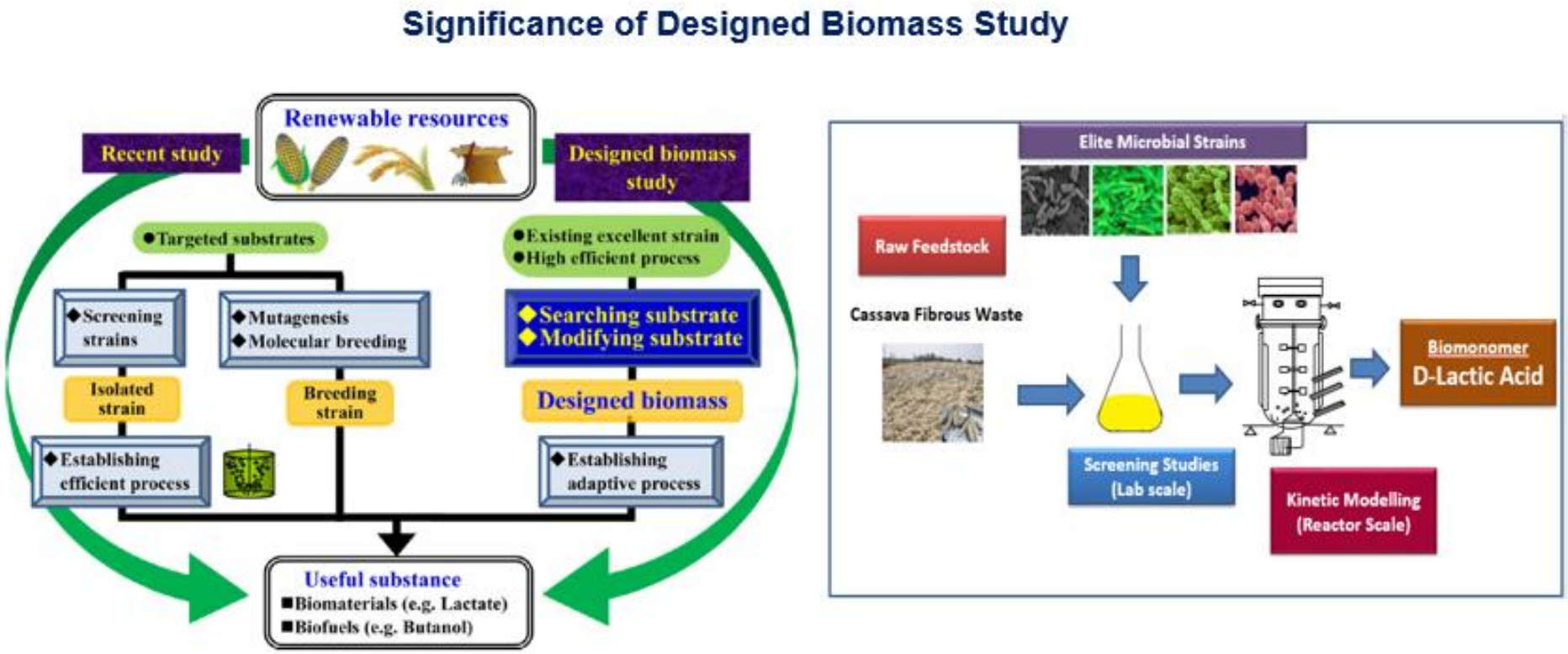



**Figure 1.7** Designed biomass approach and selection of elite strain for fermentation from renewable feedstock

**Table1.3** Reported studies of D lactic acid in the literature on Feedstocks, Fermentation and Process strategies

| S.No | Organism used | Carbon source | Nitrogen source | Mode of operation | DLA Titer ($gL^{-1}$) | Optical purity (%) | $r_p$ ($gL^{-1}.h^{-1}$) | $Y_{p/s}$ ($gg^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Lactobacillus delbrueckii* ATCC 9649* | Glucose | Yeast extract | Batch | 117.0 | - | 6.46 | 0.76 | [73] |
| 2 | *Lactobacillus bulgaricus* Lb-12 | Lactose | Yeast extract | Batch | 40.9 | - | - | - | [32] |
| 3 | *Lactobacillus coryniformis subsp. torquens* ATCC 25600 | Cellulose | Yeast extract, meat extract & peptone | Batch | 25.0 | 100 | 0.5 | 0.89 | [53] |
| 4 | *E. coli* W3110* | Sucrose<br>Molasses | Tryptone & yeast extract | Shake flask | 51.2<br>48.7 | >99.8 | - | - | [54] |
| 5 | *Lactobacillus coryniformis ssp torquens* CECT 4129T | Glucose | Corn steep liquor, yeast extract and peptone | Shake flask | 59.0 | - | 0.6 | - | [74] |
| 6 | *Lactobacillus delbrueckii subsp. delbrueckii* IFO 3202 | Rice bran | Yeast extract, meat extract & peptone | Batch | 28.0 | 95 | 0.8 | 0.78 | [56] |
| 7 | *Saccharomyces cerevisiae** | Glucose | Yeast extract & peptone | Shake flask | 61.5 | 99.9 | 0.9 | 0.61 | [57] |
| 8 | *Lactobacillus delbrueckii* JCM 1148 | Sugarcane Molasses<br>Sugarcane Juice<br>Sugar beet juice | - | Batch | 107<br>120<br>84 | 97.2<br>98.3<br>97.6 | 1.48<br>1.66<br>1.16 | 0.9<br>0.95<br>0.88 | [58] |
| 9 | *Corynebacterium glutamicumn** | Glucose | Mineral salts | Batch | 120.0 | 99.9 | 4.0 | 0.87 | [59] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 10 | *Lactobacillus plantarum* NCIMB 8826* | Glucose &Raw corn starch | Beef extract | Batch | 82.0<br>73.2 | 99.7<br>99.6 | 4.54<br>3.86 | 0.89<br>0.86 | [75] |
| 11 | *Lactobacillus plantarum* NCIMB 8826* | Xylose | Yeast extract & peptone | Batch | 41.2 | 99.2 | 0.686 | 0.89 | [75] |
| 12 | *Lactobacillus plantarum* NCIMB 8826* | Arabinose | Yeast extract & peptone | Batch | 38.6 | 99.9 | 1.42 | 0.82 | [75] |
| 13 | *Sporolactobacillus inulinus* ATCC 15538* | Glucose | Yeast extract & peptone | Batch | 93.4 | - | - | - | [61] |
| 14 | *Sporolactobacillus* ssp. CASD | Glucose | Peanut meal | Fed-batch Single Pulse Multiple pulses | 207<br>226 | 99.3<br>99.3 | 3.8<br>4.4 | 0.93<br>0.84 | [62] |
| 15 | *Lactobacillus delbrueckii* subsp. lactis QU 41* | Glucose | Yeast extract, meat extract & peptone | Batch & Continuous with cell recycle | 86.4<br>20.7 | >99.9 | 0.52<br>18.0 | 1.010<br>1.03 | [63] |
| 16 | *Escherichia coli* CICIM B0013–070B* | Glucose | Mineral salts | Batch | 122.8 | - | 4.32 | 0.89 | [76] |
| 17 | *Klebsiella oxytoca* M5a1* | Glucose<br>Sugarcane molasses<br>Malto dextrin derived from cassava starch | Peptone& yeast extract | Shake flask | 11-13<br>22-24<br>33-34 | - | 0.36-0.38<br>0.23-0.25<br>0.34-0.35 | 0.64-0.71<br>0.8 - 0.87<br>0.91-0.92 | [65] |
| 18 | *Lactobacillus coryniformis* subsp.*torquens* | Dry biomass of the microalga, Hydro dictyon reticulatum | Yeast extract & peptone | Batch | 36.6 | 95.8 to 99.6 | 1.02 | 0.458 | [66] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 19 | *Sporolactobacillus laevolacticus* DSM442 | Glucose | Cotton seed hydrolysate | Fed -batch | 144.4 | 99.3 | 4.13 | 0.96 | [77] |
| 20 | *Lactobacillus delbrueckii* subsp lactis ATCC 4797 | Casein whey permeate | casein hydrolysate | Batch | 24.3 | >98 | 0.38 | 0.49 | [68] |
| 21 | *Escherichia coli* HBUT-D* | Glucose | Yeast extract | Batch | 127.0 | 99.5 | 6.35 | 0.93 | [69] |
| 22 | *Sporolactobacillus inulinus* Y2-8 | Corn flour hydrolyzate | Yeast extract | FBB-batch<br>FBB-fed-batch | 145.8<br>218.8 | 99.0 | 1.62<br>1.65 | 0.96<br>- | [78] |
| 23 | *Lactobacillus delbrueckii delbrueckii* NBRC 3202 | CFW hydrolysate | Yeast extract | Batch | 16.15 | 0.5 | 0.9 | 98.05 | [8] |
| 24 | *S. cerevisiae* NBRC 10505 | Cane Sugar | Peptone& yeast extract | Continuous | 46.6-52.1 | 99.9 | 7.1-8.1 | 0.54 | [70] |
| 25 | *Sporolactobacillus inulinus* NBRC 13595 | Palmyra palm jaggery | Whey protein hydrolysate | Batch | 189.0 | >98 | 5.25 | 0.94 | [71] |
| 26 | *Sporolactobacillus inulinus* YBS1-5 | Corncob residue | Cottonseed meal | Fed -batch | 107 | 99.2 | 1.19 | 0.85 | [72] |
| 27 | *Lactobacillus plantarum* NCIMB 8826 | Corn stover | Soybean meal extract | Fed -batch | 61.4 | >99.0 | 0.32 | 0.77 | [43] |

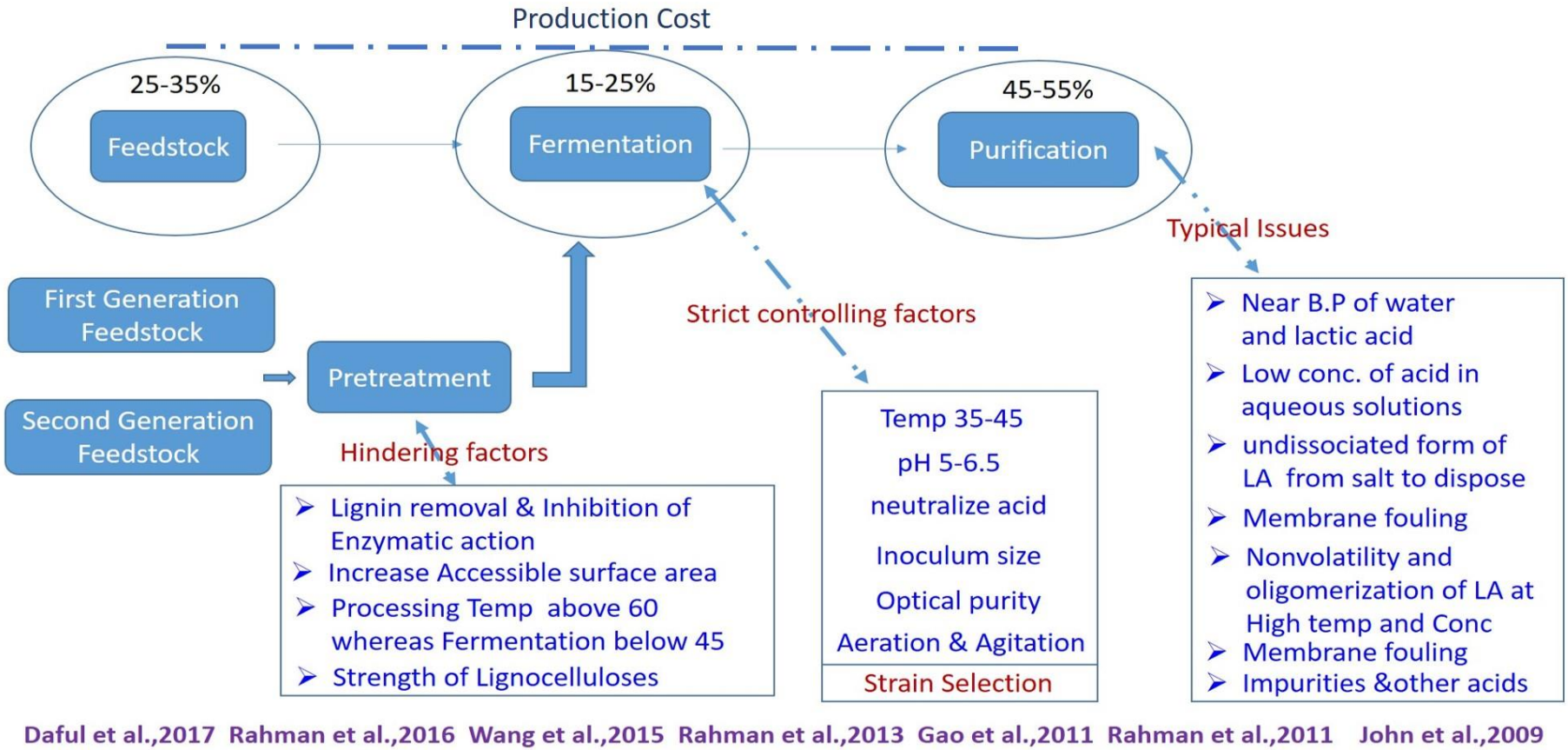



**Figure 1.8** Typical DLA production process hindering factors and cost structure of D lactic acid

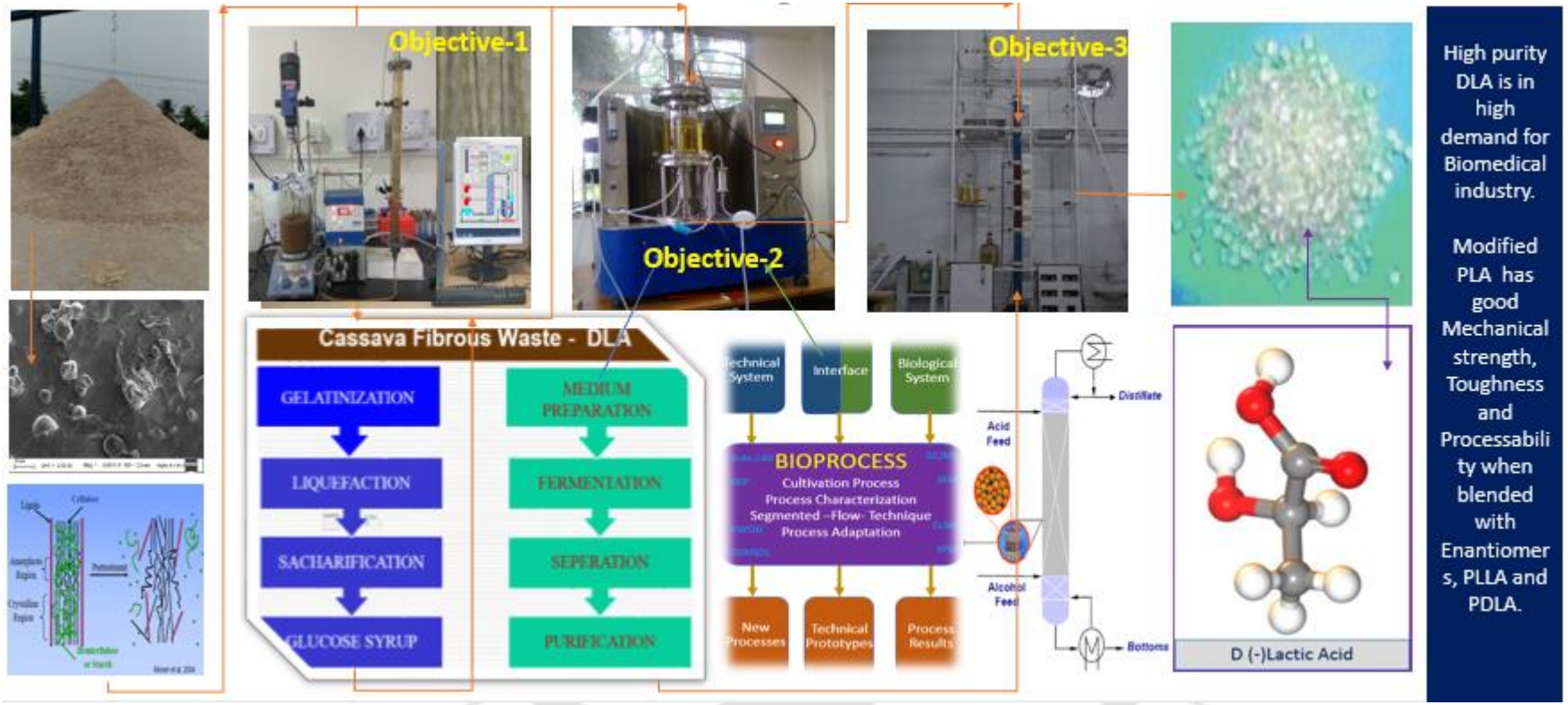



**Figure 1.9** Integrated bioprocess approach for optically pure D lactic acid

### 1.5. Research Gap

There is an economical barrier for the existing industry which can be addressed by process intensification through integrated bioprocess. The possible process strategies are mainly in the area of pretreatment, fermentation and downstream processing to achieve the desired techno ecomnomic feasibility in terms of large scale. From literature it is observed that effective pretretment with enzyme reusability is lacking for high scale continuous hydrolysate production which can be possible with the integration of a polymer matrix with the enzyme. Due to the lack of process operating parameters especially in terms of hydrolysate inhibition towards microbial growth and inhibition kinetics. This gap can be addressed by integrating designed biomass approach with kinetic modelling assessment for better optical purity, titer. There also exist a gap in terms of chemical purity towards purifying the DLA. The exisiting purification technology is more energy consuming. This can be solvable in choosing a robust biocatalyst for integrating in the separation process by solvent engineering approach. The thesis objectives are framed based on these methodologies which will address the key issues in a sustainable manner. There by improvising yield and cost-effectiveness of production. The increasing economic and ecological pressures are forcing the chemical and biological production processes to implement one pot process to replace two stage reactions. Thus, bioprocesses in general, but especially downstream processing (i.e. the recovery and purification of the product), are faced with a strong demand for intensification and integration of process steps to increase yield and productivity, to reduce process time and to cut down the running costs and capital expenditure. There are reports on lactic acid production by integrated processing and additionally new approaches to enhance efficiency. Improving the process robustness, detailed process knowledge, easy strategies for process development, effective

trouble shooting and better understanding of system leads to successful process integration. The thesis objectives are represented in a schematic way in Figure 1.9.

### 1.6. Conclusion

The concept of biorefinery is yet to develop and will be a boon for waste disposal alternatives in future. Production of value added products from waste material is not a myth but needs more limelight to be a reality. In a similar manner, rising environmental issues with time needs effective solutions serving basic human needs simultaneously. DLA production needs a boost implementing eco friendly strategies for which CFW can be a viable solution aiding in waste management concurrently.

### 1.7. Objectives

Based on state-of-art literature in DLA production, the objectives of the thesis are elucidated as follows :

- Electrospun chitosan coated polylactic acid nanofiber: A novel immobilization matrix for α – amylase and its application in hydrolysis of cassava fibrous waste
- Cost-effective waste valorization of cassava fibrous waste into enantiomerically pure D lactic acid: Process engineering and kinetic modeling approach
- Bioprospecting of cassava fibrous waste as a precursor for stereospecific lactic acid production: Inhibition insights for value addition and sustainable utilization
- Process engineering strategy for D lactic acid purification: A novel biocatalytic enzyme coupled with solvent engineering and esterification

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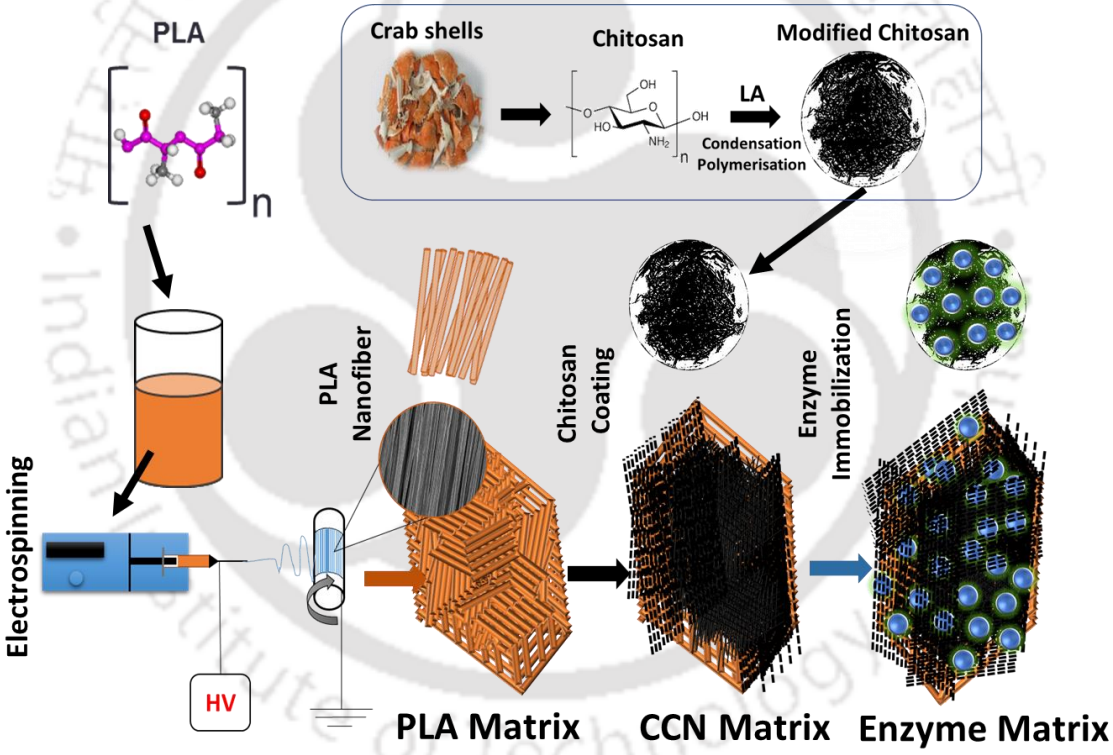
# Electrospun chitosan coated polylactic acid nanofiber: A novel immobilization matrix for α – amylase and its application in hydrolysis of cassava fibrous waste



# Chapter 2

## Electrospun chitosan coated polylactic acid nanofiber: A novel immobilization matrix for α – amylase and its application in hydrolysis of cassava fibrous waste

**Abstract**

This present study addresses the uncherished resource potential of CFW from the cassava processing industry towards production of fermentable sugars using enzymatic hydrolysis process. However, the viscous nature of the gelatinized CFW poses serious processability issues, which was overcome by a novice attempt of using tailor-made electrospun nanofibers and its application in immobilization of α – amylase. Characterization studies (FESEM, FTIR, Thermogravimetric analysis), rheological and proximate analysis of CFW revealed its suitability as the potential feedstock. Chitosan coated nanofiber (CCN) matrix was prepared by varying the chitosan concentration from 0 to 2% (w/v) and the 2% CCN matrix exhibited an optimal performance with enhanced tensile strength (1.262 MPa), reduced elongation (41.2%) and contact angle (128 degrees). CCN matrix was examined for the immobilization of α - amylase enzyme and the relative activities of the immobilized and free enzyme were compared. Packed bed operation at optimized conditions (solution pH of 5.0 and a solution temperature of 50 $^{o}$C) deploying CCN matrix with initial substrate concentration of 10 $gL^{-1}$ yielded a maximum conversion ratio of 0.85 and 0.99 (high residence time of 40 min and low dilution rate of 0.04 $min^{-1}$) for without and with recycling mode, respectively.

## 2.1. Introduction

Amongst all the root and tuber crops, cassava is one of the oldest crops well known for its use in food and beverages. Cassava is cultivated in more than 100 countries to cater the caloric need of millions of people. Further, cassava cultivations are practised in tropical areas due to its large yield of 10 tons/hectare and minimal requirement of nutrient input and its sufficiency to grow on arid soils [1]. Swiftly cassava has evolved as an industrial cash crop, the starch in the cassava is the critical component that interest's both producers as well as the companies. While in processing one ton of cassava root, it was reported that at least another ton of cassava bagasse was refused from the starch extraction process [2]. The refused cassava bagasse contains nearly 50% (by dry weight) of its composition with starch [3], which is used as a low-grade cattle feed to a certain extent [4].In contrast, during the rainy season, a significant portion of this refused source is considered as cassava waste and are landfilled without any modification [5]. Instead, this refused source could be utilized as value-added feedstock in the production of fermentable sugars by hydrolysis and its subsequent application for synthesis of value-added products like succinate, Polyhydroxybutyrate, bioethanol and biobutanol by microbial fermentation [6]. Different waste streams generated during cassava processing were utilized as feedstock for the production of various biotechnological products viz. succinate, polylactic acid and bioethanol [6] [7]. But, the processability of CFW for the enhanced production of fermentable sugars is not dealt extensively in the literature report till date.

Acid, alkaline and enzymatic hydrolysis are three different pre-treatment methods primarily used for production of fermentable sugars from biomass feedstock [7,8]. Though acid and alkaline hydrolysis methods yield consistent hydrolysis rates, they are often limited due to the co-generation of recalcitrant compounds, which inhibit microbial activity in the subsequent fermentation operation [7,8]. Enzymes are specific in action and

have enormous potential in terms of hydrolysis in many industries such as leather, jute etc. Also these, enzymatic processes in terms of hydrolysis don't generate any kind of recalcitrant by-products. However, the cost factor associated with the enzymatic hydrolysis is a major hurdle to realize its true potential in large scale applications [9]. Instead of using free enzymes on a single-use basis, reusing the enzymes is an attractive option and widely in use. To be specific, saccharification of starchy biomass using α - amylase enzyme is used for several cycles because of its enormous reuse potential [10]. Immobilization is the successful proven technique used in enzyme reuse and thereby making the process affordable at an industrial scale [11]. Immobilization of enzyme in an alginate matrix is a well-established method and used for decades in saccharification of starchy biomass. But, the processing of gelatinized form of viscous CFW is a challenging task owing to low mechanical strength of sodium alginate and high risk of enzyme loss through the large range of pore sizes present in the alginate matrix [11]. Wherefore, there is a need for tailor-made immobilization matrix to handle the starchy cassava solution. To overcome this bottleneck, chitosan support was preferred as a next immediate alternative for alginate. Chitosan, one of the most abundant biopolymer derived from the crustacean shells [12], possess better tensile strength and antimicrobial properties that are highly essential for saccharifying the gelatinized viscous cassava starch solution. Tensile strength is a desirable property for regulating high inflow of gelatinized viscous cassava starch solution in a packed bed operation and antimicrobial property facilitates the removal of contaminant microbes in the feedstock stream and eventually in the fermentation unit [13].

Moreover, immobilization support with chitosan comprises of amine group that can facilitate enzyme linkage through the ion exchange mechanism [14]. If the enzyme activity drops, the spent enzymes can be reversibly separated from the chitosan support and replaced with a new batch of the enzyme for the next cycle of operation in order to sustain

the process economy[15]. In this present study, the drop in enzyme activity associated with loss of active enzymes in the spent was significantly reduced by development and application of chitosan-polylactic acid nanocomposite as immobilization support. To the best of our knowledge, there is no study reported in literature addressing the processability aspect of CFW, and the use of Chitosan (CH) coated Polylactic acid (PLA) based nanofiber as an immobilization support for the saccharification of gelatinized CFW.

In this present chapter, the unification of PLA and chitosan can be adopted to develop a tailor-made approach which suits the present process by the addition of an advanced electrospinning process. As the addition of chitosan coating to the electrospun PLA may precisely control and improve the properties of enzyme attachment and its accessibility towards the feedstock. This aids in the development of versatile chitosan coated nanofiber (CCN) matrix onto which α - amylase was immobilized for saccharification of gelatinized viscous CFW. To achieve this, the present study is classified into three sections, wherein the first section deals in determining the processability aspect of the CFW by comparing it with standard starch using various characterization techniques viz. Field emission scanning electron microscopy (FESEM), Thermogravimetric analysis (TGA), X-ray diffraction (XRD) and rheology analysis. Thereafter, novel electrospun CCN matrix was characterized based on tensile strength, elongation property and contact angle analysis to assess its suitability in the saccharification process. The tailor-made CCN matrix was immobilized with α - amylase and enzymatic hydrolysis of CFW was investigated using shake flask and packed bed experiments.

## 2. Materials and Methods

### 2.2.1. Chemicals

All the chemicals are of analytical grade and are procured from Merck, India and Hi-Media, India. α - amylase used in the present study was procured from Rich core Enzymes, Banagalore, India. Polylactic acid (PLA) granules (grade: 2003D) was purchased from NatureWorks® LLC, USA. The number average molecular weight (Mn), weight average molecular weight (Mw) and polydispersity index (PDI) were determined by gel permeation chromatography (GPC) (autosampler SIL-20A HT; liquid chromatography, LC-20AD; degassing unit, DGU-20A3R; refractive index detector, RID-10A; Agilent two PL gel 5 µm MIXED-D column in series with column oven, CTO-20A) (Shimadzu, Japan) as ~135 kDa, ~250 kDa and 1.82, respectively. Chloroform and N, N-Dimethylformamide (DMF) used for polymer dissolution is of analytical grade were purchased from Merck, India. Millipore water was used throughout the study both for experiments and assays.

### 2.2.2. Feedstock processability analysis

CFW used in the study is the bio-cake obtained from the various filtration stages of cassava processing of a Sago industry located at Salem, Tamil Nadu, India. CFW was dried in a hot air oven at 100 $^{o}$C for about 6-8 hours. Thereafter, the moisture removed CFW was size reduced using roll crusher and a hammer mill to get fine powders. These fine powders were sieved in a rotatory shaker using a screen stack having standard meshes. CFW with its size ranging under 75 µm, was considered further for its processability estimation using various characterization techniques.

In order to choose a proper pre-treatment technique i.e. enzymatic hydrolysis or acid hydrolysis, the CFW is examined for its recalcitrance using XRD, FTIR, FESEM, and rheology analysis. In case of all the spectral examination, comparison of CFW was made

with pure starch obtained from Himedia, India. Further, the heavy metal composition of the CFW was analyzed using atomic absorption spectrophotometer (AAS) analysis. X-ray diffraction (XRD) analysis of the samples was carried out using powder XRD (TTRAX III, Rigaku, Japan) instrument scanned at a scan rate of 0.05 degree per second over a 2-theta range of 5 to 60$^{o}$. Fourier transform infrared (FTIR) spectroscopy analysis was carried out under attenuated total reflectance (ATR) mode using an FTIR spectrophotometer (Frontier1, PerkinElmer, USA), and the samples were scanned over the wavelength range of 400 – 4000 $cm^{-1}$.

Thermogravimetric analysis (TGA) was carried out using TGA (TGA-4000, PerkinElmer, USA) instrument, and the samples were analyzed over a temperature range of 30 to 700$^{o}$C with a heating rate of 10$^{o}$C/min. Unlike the other characterization techniques cited above, FESEM analysis was carried out with a pre-treatment step wherein the samples were gold coated using a sputtering instrument, and the gold-coated samples were stuck on a double-sided carbon take pasted on a stainless steel stub. This stub was thereafter mounted on FESEM (Sigma, Zeiss, USA) instrument and analyzed at an operating voltage of 2-4 kV. Rheological analysis was carried using a rheometer (MCR 120, Anton paar) under different temperature in an oscillatory shear mode. All the rheological analysis was carried out under fixed strain and frequency sweep in the range of 0.1 to 500 Hz. Unlike the micron-sized powdery CFW used for all the aforementioned characterization, gelatinized CFW solution was used for rheology analysis. Gelatinisation of CFW is detailed in Section 2.3.

Metal composition analysis was carried out using atomic absorption spectrophotometer (Spectra AA 220 FS, Varian, Netherland). 0.2 g of CFW was digested with a mixed acid solution comprising potassium dichromate in 7 N sulphuric acid

solution. This acid digestion was carried out in a COD digester wherein the temperature was gradually increased from 150 to 550 $^{o}$C. The resulting digestate was filtered using nylon membranes and taken for AAS analysis. The concentration of metal samples was identified by comparison with different standard metal solutions.

### 2.2.3. Preparation and characterization of CCN matrix

Chitosan used for coating was modified as reported elsewhere in the literature[16,17] in order to get better adhesion of chitosan on the PLA nanofibre. Inlet stream for the electrospinning set up (E-spin Nanotech) comprises a mixture of 9% (w/v) of PLA which is a base polymer for matrix was dissolved in a binary solution of Chloroform and Dimethylformamide in the ratio of 9:1. The aforementioned solution mixture were taken into the electrospinning setup through an automated syringe pump operated at a flowrate of 1 mL/h. Further, the electrospinning setup was operated at 12 kV, and the nanofiber mat was drawn by placing a collector at a distance of 15 cm from the needle. Various nanofiber mats are produced. Modified chitosan at different concentrations viz. 0.5%, 1% and 2% (w/v) were dissolved in 75% (v/v) ethanol solution. The produced PLA nanofiber mats were dip-coated in the prepared chitosan solution comprising of 0.5%, 1% and 2% chitosan, and referred hereafter as 0.5% CCN matrix, 1% CCN matrix and 2% CCN matrix, respectively.

The tensile test was carried out using TST 350 micro-tensile stage (Linkam, U.K.) built-in 200 N load cell with micro-Newton resolution. CCN matrix mats with varying concentration of chitosan were subjected tensile load with a sample elongation rate of 3 mm/min. Samples were prepared according to the ASTM standard D638, in which the (breadth) x (thickness) of the sample was fixed as 7 x 15 mm and the gauge length is adjusted (~50 mm). The ultimate strength (MPa) and elongation rate (%) of various CCN

matrix were calculated from the stress-strain curve using the following Eqs. (2.1) and (2.2), respectively:

$$\text{Ultimate strength } (\tau) = \frac{\text{Maximum force } (F_{max})}{\text{Cross-sectional area } (A)} \qquad \text{(Eq.2.1)}$$

$$\text{Elongation } (\%) = \frac{\text{Change in length } (\Delta L)}{\text{Original length } (L)} \text{ x } 100 \qquad \text{(Eq.2.2)}$$

FESEM analysis of the nanofiber matrix was carried out with a similar procedure mentioned as in feedstock processability section 2. 1. However, the powder samples in feedstock processability analysis were replaced with tiny films of 0.5 x 0.5 mm square sample CCN matrix mat dried at 40 $^{o}$C overnight. The water affinity of the chitosan coated PLA nanofiber mat was identified by water contact angle. A drop of water about 1µl automated from a syringe pump was placed over the nanofiber mat. Thereafter, a contact angle goniometer (GmbH DSA25, KRUSS, Germany) with integrated software gave the angle of contact by analysing the contour.

### 2.2.4. Immobilization of α - amylase onto CCN matrix for CFW hydrolysis

The CCN matrix with optimized chitosan concentration was considered for immobilization of α - amylase enzymes. 0.15 mg.mL$^{-1}$ of α - amylase solution was prepared with a phosphate buffer solution having a pH of 7.0 and incubated for 18 h under an agitation rate of 100 RPM and solution temperature of 20 $^{o}$C. Enzyme loading into CCN matrix was assessed by measuring the initial and final concentration of the protein measured using Folin-Ciocalteu's phenol reagent [18]. Finally, the enzyme immobilized CCN matrix was suspended in a 50 mM buffer solution (pH 7.0), and the resulting mixture was preserved at 4 $^{o}$C until considered for further use.

Enzyme hydrolysis was carried out by taking 7% (w/v) of CFW powder (75 µm) in 0.1 M sodium acetate buffer. Before enzyme hydrolysis, the CFW was gelatinized by

heating at 75 °C for 45 min. In shake flask studies, the gelatinized CFW solution was treated with both free as well CCN immobilized enzyme matrix. Activity assay was carried out, both using free enzyme and CCN immobilized enzyme matrix. Free enzyme hydrolysis was carried out by mixing 0.2 $mg.mL^{-1}$ of α - amylase solution in 1% (w/v) gelatinized starch mixed in 0.1 M sodium acetate buffer, and the mixture was incubated at 60 °C for 36 h. Likewise, hydrolysis using immobilized enzyme was carried out with similar conditions as in the case of free enzyme hydrolysis except for a replacement of free α - amylase solution with 50 $mg.mL^{-1}$ of α - amylase immobilized CCN matrix. At the end of hydrolysis, the release of glucose from gelatinized CFW solution was measured using glucose oxidase and peroxidase (GOD-POD) method. One unit of α - amylase activity (U) was defined as the amount of α - amylase consumed to form 1 mg of glucose on the given assay conditions. Recyclability analysis was carried out only in the case of the immobilized enzyme. α - amylase loaded CCN matrix was separated at the end of the hydrolysis experiment and the same was reintroduced in the next cycle to find the reduction in hydrolytic efficiency. After every cycle enzyme loaded CCN matrix was cleaned with a phosphate buffer solution. Performance analysis of the enzymatic hydrolysis on all the case was reported as relative activity by using Eq. (2.3):

$$\text{Relative activity} = \frac{\alpha - \text{amylase activity}}{\text{Maximum } \alpha - \text{amylase activity}} \times 100 \qquad \text{(Eq.2.3)}$$

Investigation of continuous enzymatic hydrolysis in a packed bed column is carried out after the shake flask experiments. Wherein 1 g of enzyme immobilized CCN matrix was packed in a glass column having an inner and outer diameter of 8mm and 10 mm, respectively. Gelatinized CFW solution was passed through the packed at a flowrate of 1 mL/min, and the conversion efficiency was analysed by using the following Eq. (4):

$$\text{Conversion ratio} = \frac{C_{ig} - C_{og}}{C_{ig}} \qquad \text{(Eq.2.4)}$$

Where, $C_{ig}$ and $C_{og}$ are the initial and final concentration of glucose (mg/L), respectively. All the analysis was carried in triplicate, and the mean ± standard deviation was provided.

## 2.3. Results

### 2.3.1. Feedstock processability

Morphological analysis of the CFW was examined through FESEM and compared with the morphology of the pure starch (Figure 2.1). Pure starch was observed to be uniform and granular, and the average size of the starch particle was measured to be 8.9 µm. While in the case of CFW, though spherical starch particle was observed, they were limited to very few and on contrast, most of the starch particles remain shredded having no regular shape.

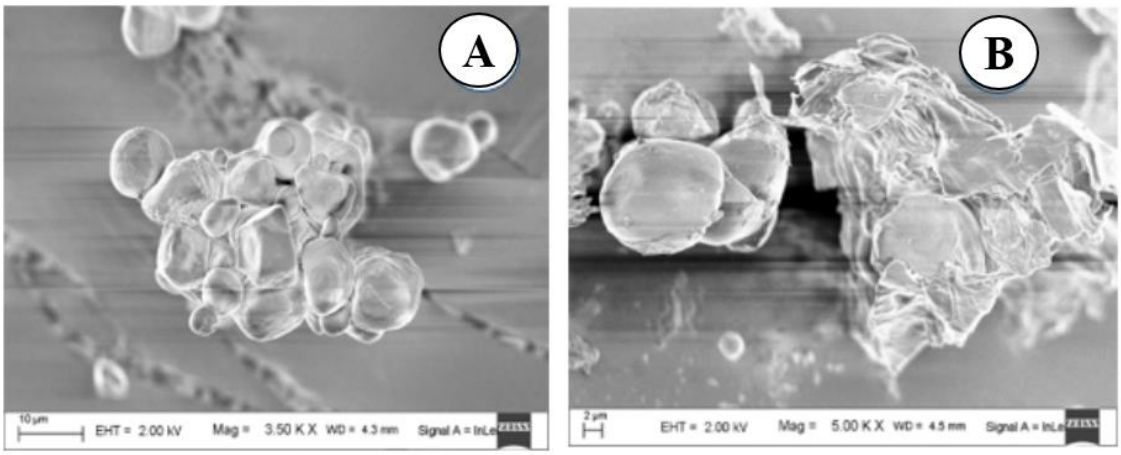



**Figure 2.1** FESEM image of (a) pure starch and (b) cassava fibrous waste

Thus, the average size of the well-defined starch particle in the CFW was measured to be 9.4 µm. XRD spectra of the CFW was compared with pure starch in Figure 2.2.

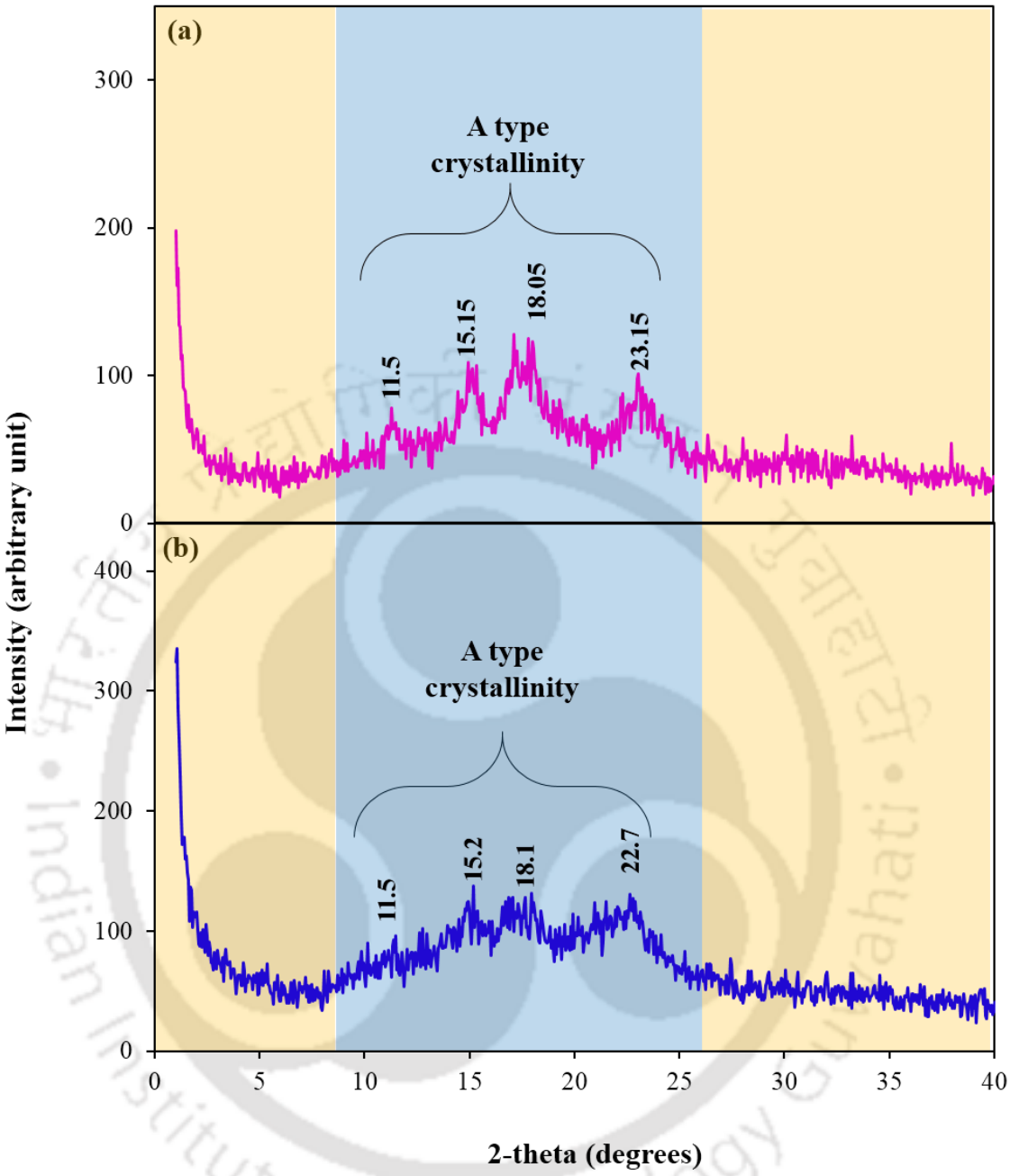



**Figure 2.2** XRD spectra of (a) pure starch and (b) cassava fibrous waste.

In general, both CFW and pure starch matched closely and resembled a semi-crystalline structure. Based on the characteristics and unique XRD pattern, starch can be classified as starches revealing A-type crystallinity ($C_a$), starches revealing B-type crystallinity ($C_b$),

and starches revealing C-type crystallinity ($C_c$). These variations in crystallinity type can change with respect to the plant source and processing conditions. For instance, starches of cereal origin reveals $C_a$, root starches reveals $C_b$, and smoot peas or edible beans reveal $C_c$ type starch [19]. Starch exhibiting b-type crystallinity can be identified by the characteristic XRD peaks observed around 5.6°, 15°, 17° and 20° alongside a doublet peak can be observed between 22-24°. While the starch elucidating A-type crystallinity depicts a similar pattern as that of the B-type starch, but A-type starch is devoid of a peak at 5.6°. Instead of a doublet peak, a characteristic peak can be observed around 23°. The C-type starch exhibits broad peaks at 17° and 23° along with that minor peaks at 5.6° and 15°. Further, it was recognized that the A-type starch comprises of glucose units of shorter chains, and B-type starch comprises longer branch chains [20,21]. In the present study, both pure starch and CFW revealed A-type starch XRD pattern with characteristic peaks observed at around 11°, 15°, 18° and 23°. A similar result for A-type XRD pattern for cassava was reported in a thermal degradation study involving various starch sources [22]. The pure starch used for comparison was presumed to be obtained from the corn source, and the XRD pattern revealed A-type starch material as reported elsewhere in the literature [23]. FTIR is used to identify the functional groups, and FTIR spectra of both pure starch and cassava starch were presented in Figure 2.3. A similar FTIR spectra were identified for both pure and cassava starch dealt in the present study. The peaks identified at 1008 $cm^{-1}$, 1154 $cm^{-1}$ and 1336 $cm^{-1}$ can be attributed to vibration peak of C-O in alcohol hydroxyl group, vibration peak of C-O group, and $CH_2$ symmetrical stretching vibrations in the carbohydrate sample, respectively [25].

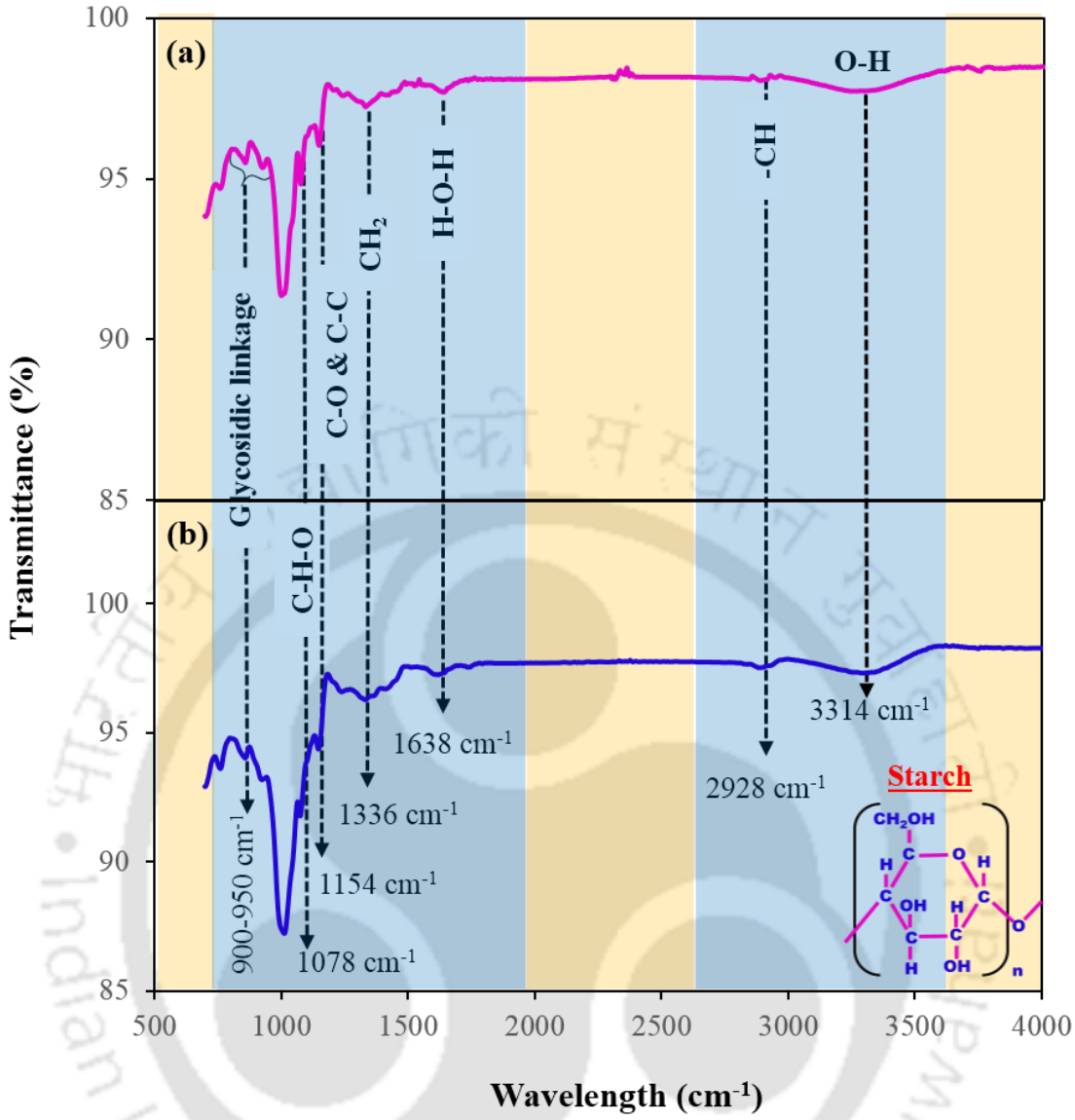



**Figure 2.3** FTIR spectra of (a) pure starch and (b) cassava fibrous waste. Inset: Starch structure

These peaks are reported as the characteristic peaks of starch and native to all kinds of starch samples. Further, the strong peak observed at 1008 $cm^{-1}$ is responsible for the amorphous structure of starch, while the ordered structure of starch arises due to the C-O and $CH_2$ peak observed at 1154 $cm^{-1}$ and 1336 $cm^{-1}$ [25]. The characteristic peak observed at 1638 $cm^{-1}$ is due to bound water molecule present in the starch, and the same explains the hydrophilic nature of the starch. The extremely broad peak observed at 3314 $cm^{-1}$ was related to stretching vibrations that arose due to the hydroxyl group present in the starch

sample. Apart from the peaks mentioned above, cluster peaks observed around 900 - 950 $cm^{-1}$ was due to the Glycosidic linkages connecting the glucose units present in the starch [26]. In addition to this –CH stretch in the starch samples was also revealed at around 3314 $cm^{-1}$. All the aforementioned functional groups can be visualized from the simplified starch structure provided as an inset in Figure 2.3.

Thermogravimetric analysis (TGA) was carried out to determine the thermal stability of both the CFW and pure starch. TGA plots for CFW and pure starch were shown in Figure 2.4. The first weight loss step observed in the TGA plot around 75 – 150 $^{o}$C, which could be attributed to the loss of free and bound moisture/water present in the starch [27].

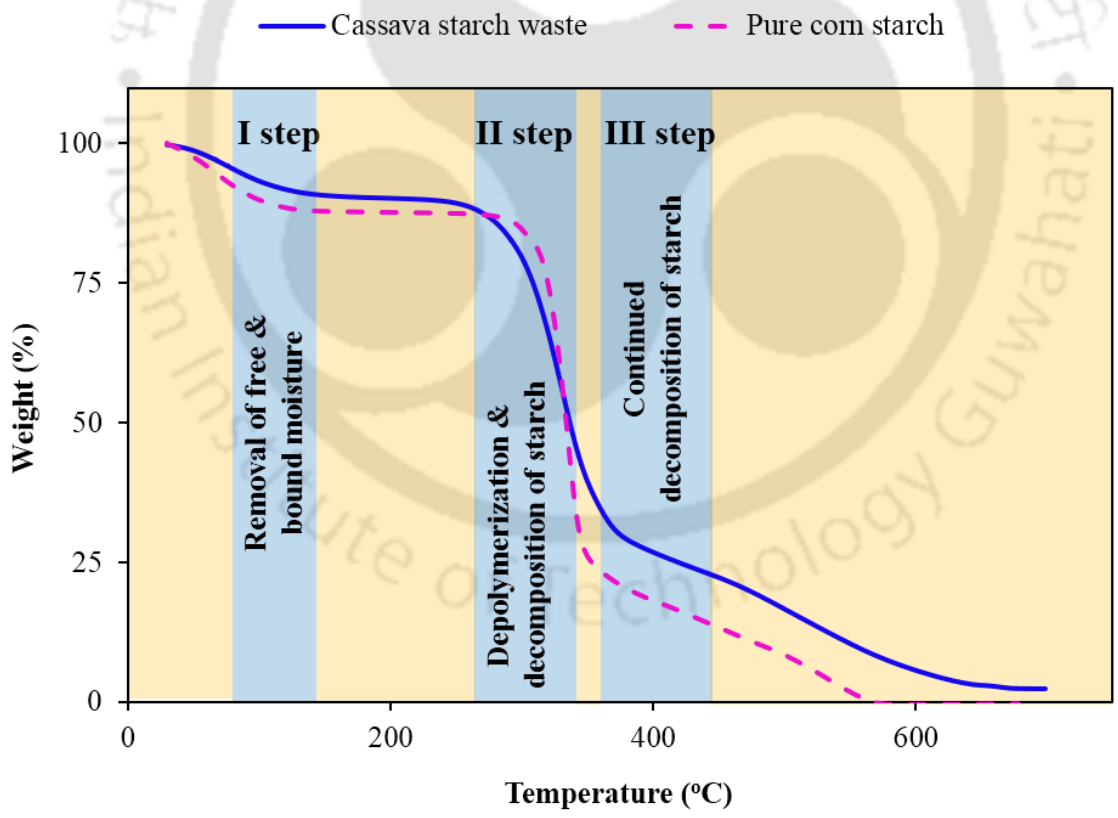



**Figure 2.4** TGA thermogram of pure starch and cassava fibrous waste

The mass was stable till 250 $^{o}$C, and a secondary weight loss step was observed between 255 – 355 $^{o}$C; which corresponds to a major weight loss, and it is connected with the disintegration of the starch material. Further, weight loss could be correlated to the loss of poly hydroxyl groups and depolymerisation-cum-decomposition[28]. Beyond 350 $^{o}$C, yet another degradation step was observed, which was due to the continual degradation of the starch material. Thus, the 50% mass loss temperature for CFW and pure starch was found out to be 333 $^{o}$C.

Rheological analysis of the gelatinized CFW was carried out to identify the nature of starch suspension in aqueous solution. The viscosity of the gelatinized solution was not attributed to the nature of the starch particle in itself, instead due to the swollen nature of these starch particles in the starch-aqueous system [31]. Further, the susceptibility of the starch for enzymatic hydrolysis can be examined by the rheological analysis, where a drastic reduction in the viscosity of the gelatinized starch solution upon shearing gives a clear picture on the disruptive nature of starch to release polysaccharide units. A steady increase in the shearing of the CFW resulted in a drastic reduction in the viscosity, and a steep decrease was observed (Figure 2.5a) beyond a shear rate of 100 $s^{-1}$. This declining trend is associated with the shear thinning or pseudoplastic fluid, where a continuous increase in the shear rate will result in molecular alignment and ease in the flow to give way for the reduction in viscosity[32]. Thus in Figure 2.5a & b, a steady increase in the shear stress and decline in the viscosity with an increase in shear rate concludes non-Newtonian and pseudoplastic behaviour of the present CFW solution. However, an increase in the temperature from 45 to 65 $^{o}$C resulted in a heightened viscosity profile.

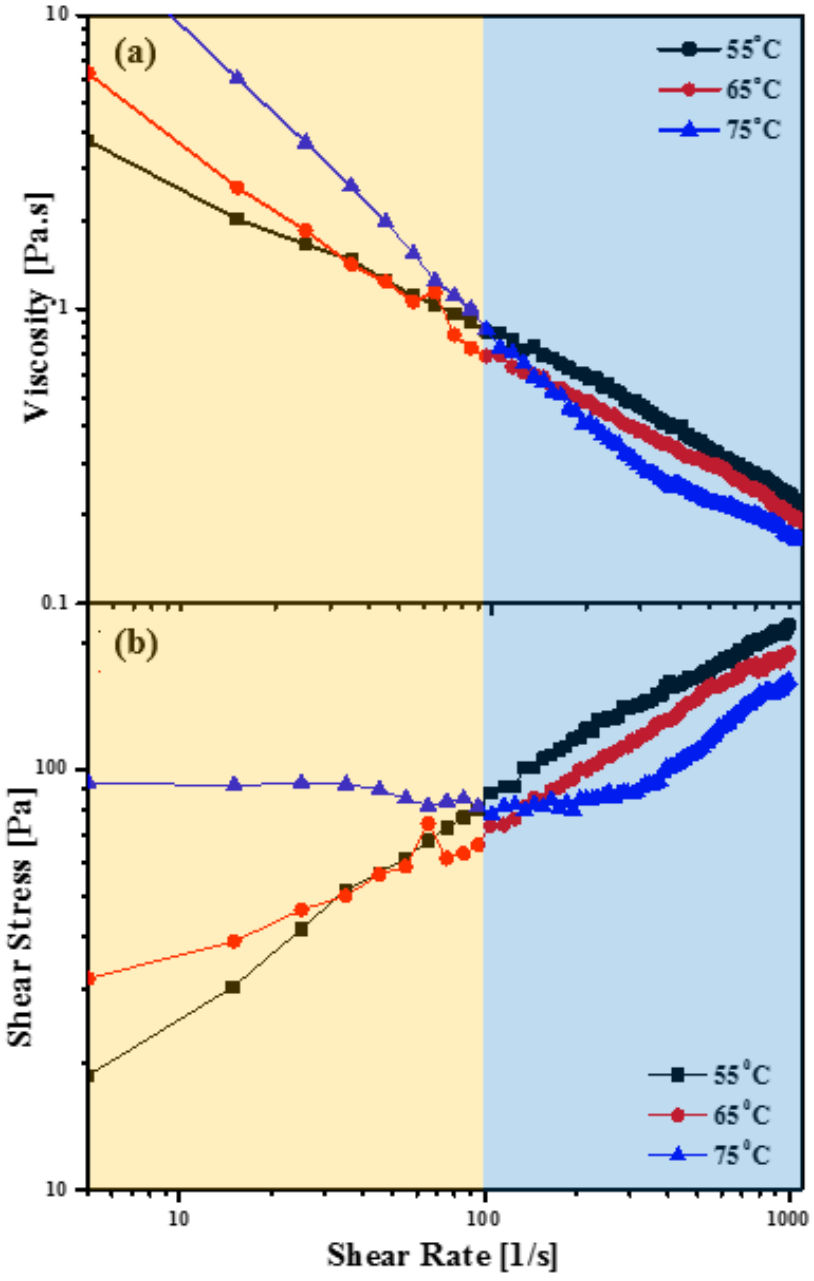



**Figure 2.5** Rheological analysis of gelatinized cassava fibrous waste with the effect of shear rate on (a) viscosity and (b) shear stress.

Zinc, calcium, magnesium, mercury, cadmium, sodium, potassium, and Nickel are the major minerals found in the CFW (Figure2.6), which corroborates with the previous published literature about cassava root [32]. However, the minerals composition observed in the present study is slightly higher than the analysis carried out on the cassava root. This can be attributed to the fact, and CFW consists of crude mixtures of refused source that

often contains an elevated amount of minerals. Moreover, the chemical composition may vary based on the species of origin, geographical region of cultivars, and even between the various plant parts of cassava [33,34].

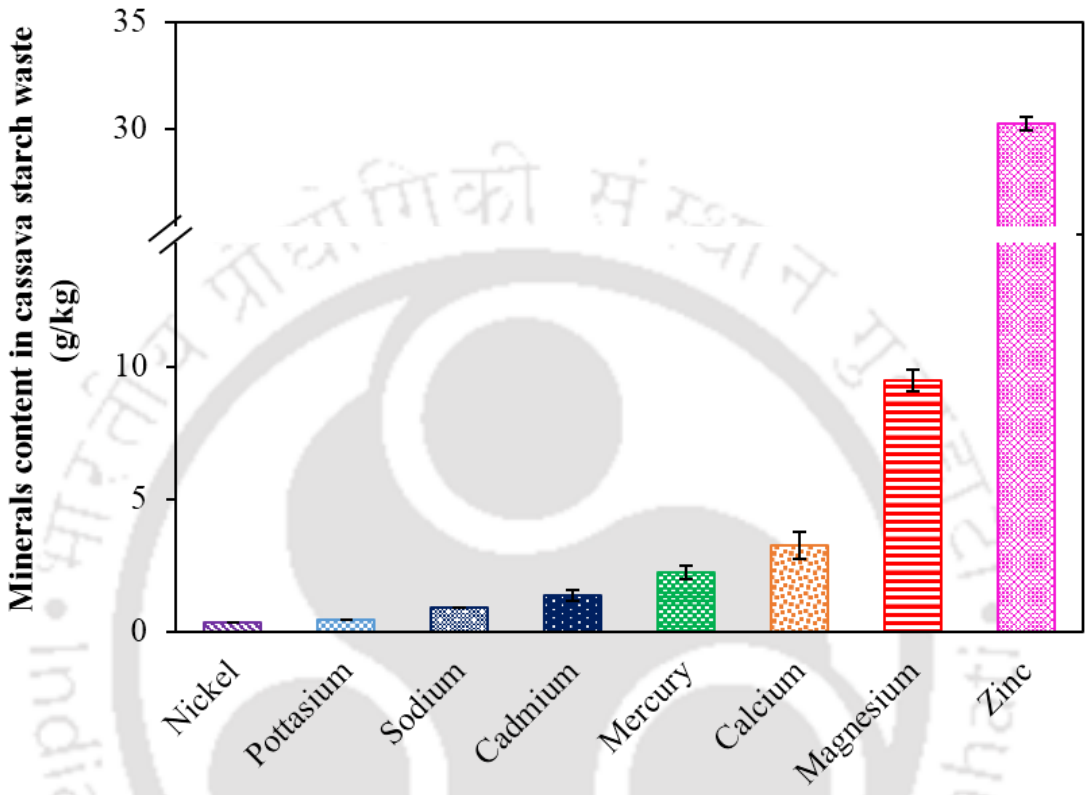



**Figure 2.6** Minerals composition of cassava fibrous waste

### 2.3.2. Characterization of Electrospun CCN matrix

The increase in chitosan concentration over the PLA nanofiber, and the properties of the resultant nanofiber for enzyme immobilization need to be investigated. The decrease in contact angle of the matrix of the CCN matrix attributed to the decrease in hydrophobicity with the increase in chitosan coating concentration (Figure 2.7). The modified chitosan has better compatibility with the PLA matrix and is soluble in variety of organic solvents [16,17]. This modification enabled the solubility of chitosan in ethanol and used for coating over the CCN matrix. The decrease in the contact angle of the CCN matrix along with an increase in coating concentration was due to the enhancement in the smoothness

of the CCN matrix. This enhancement in the smoothness was brought about by filling up of chitosan on the air pockets of the CCN matrix.

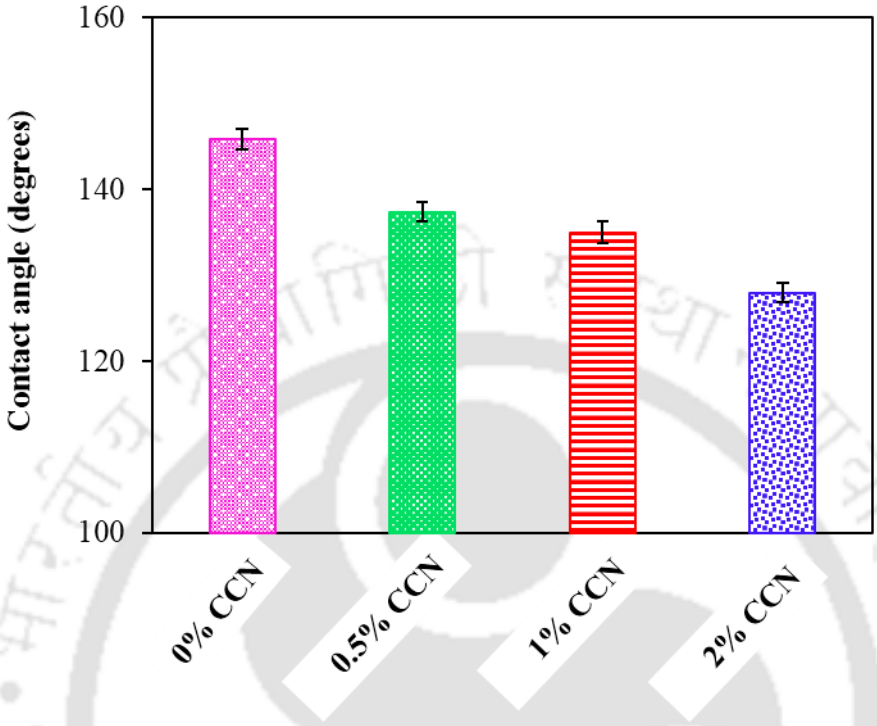



**Figure 2.7** Contact angle analysis of CCN matrix with varying concentration of chitosan

The roughness and air pockets were observed to contribute for the increased contact angle on the matrix [35]. Thus, the unique matrix with enhanced hydrophilicity is achieved by the combination of hydrophobic and hydrophilic polymer carried out through electrospinning technique [17]. It could be observed that the chitosan coating steadily increased the tensile strength of the CCN matrix (Figure 2.8a). Whereas the elongation marked its peak value with an addition of 2% (w/v) chitosan coating. The chitosan coating over the nanofiber matrix acted as a binder between individual strands of nanofiber that increased overall tensile strength of the matrix along the load direction compared to the neat matrix. Thus, the CCN matrix with 2% (w/v) chitosan concentration was optimized to be the best performing material with a contact angle, tensile strength and percentage elongation of 128$^{o}$, 1.262 MPa and 41.2%, respectively.

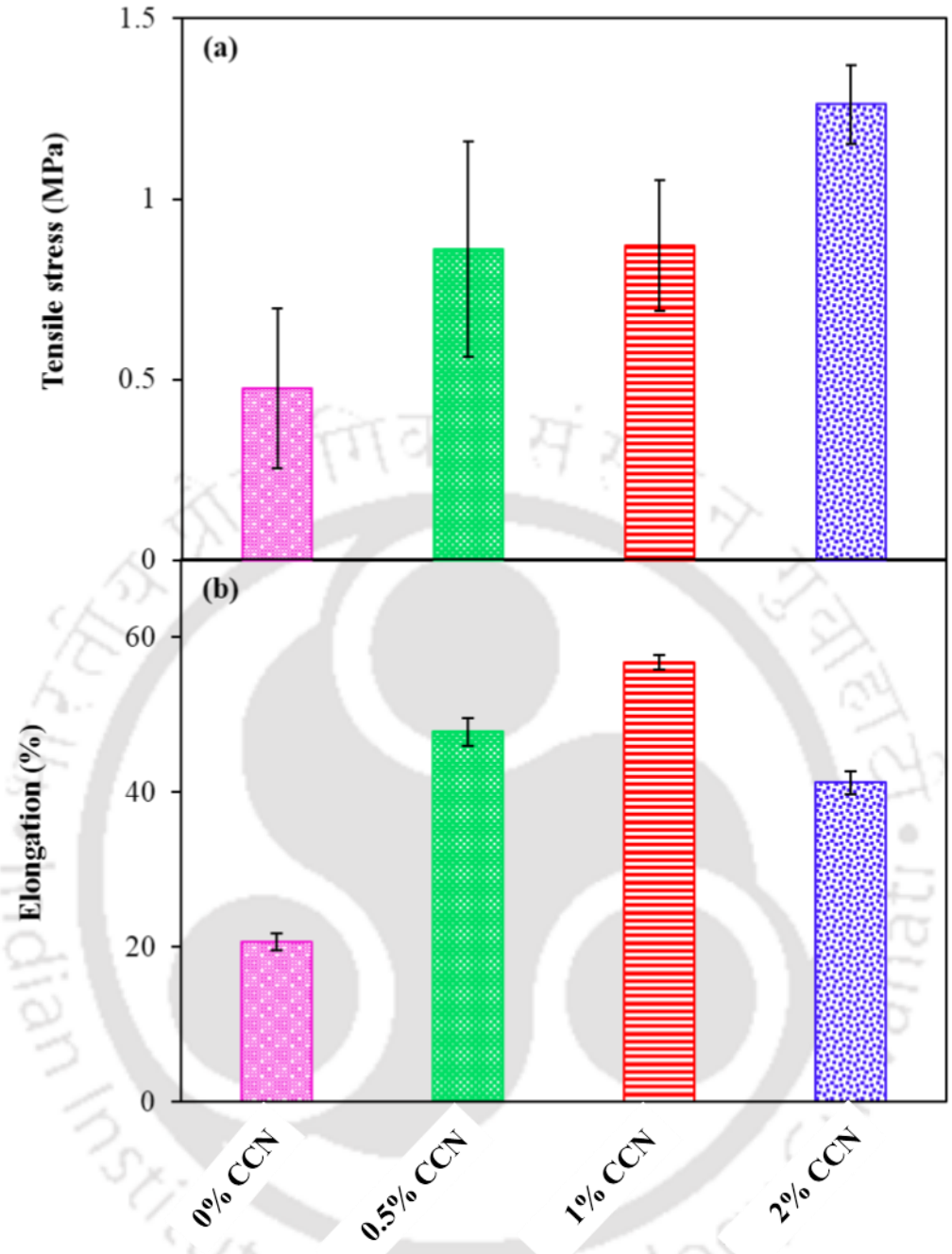



**Figure 2.8** (a) Tensile strength and (b) elongation analysis of CCN matrix with varying concentration of chitosan

### 2.3.3. Immobilization of α - amylase onto CCN matrix and its use in CFW hydrolysis

2% CCN matrix was found to exhibit optimal performance and was loaded with α - amylase in order to investigate its potential in the hydrolysis of CFW. Morphological analysis of PLA nanofiber using FESEM has demonstrated the fibrous nature of the present nanocomposite and almost all the fibers were under 1 µm diameter, and fibers in the nano range were also observed in the FESEM images (Figure 2.9a). Further, uniform coating of chitosan on the PLA nanofiber to fill the air gap was clearly visualized in Figure 9b. Finally, α - amylase loading onto the CCN matrix was visualized by the lustrous outburst of fluffy wax-like topographies on the 2% CCN matrix. Since α - amylase used for CFW hydrolysis is a thermotolerant enzyme optimization of the process temperature, and solution pH was carried out for α - amylase loaded 2% CCN matrix. This enzymatic hydrolysis using the immobilized enzymatic system was compared for its performance with the freely suspended enzymes. It can be observed from Figure 2.10a, the relative activity for both immobilized enzyme system and freely suspended system steadily increased from 30 to 50 $^{o}$C and reached its maximum relative activity at a solution temperature of 50 $^{o}$C. Thereafter, any effort to increase the solution temperature significantly reduced the relative activity of the enzymatic hydrolysis. Further, it was observed that the hydrolysis performance of the immobilized system was comparable to the performance of the freely suspended system and more than 99% relative activity was observed for both immobilized systems and freely suspended system at 50 $^{o}$C. The observation recorded in the present study at the optimum solution temperature of 50 $^{o}$C correlates with the our earlier reported study [8]. Similar strategy of overcoming the mass transfer limitations in the enzyme loaded immobilization support was reported in literature

[37]. Similar strategy of overcoming the mass transfer limitations in the enzyme using an electrically spinned PVA based nanofibers for the immobilization of naranginase.

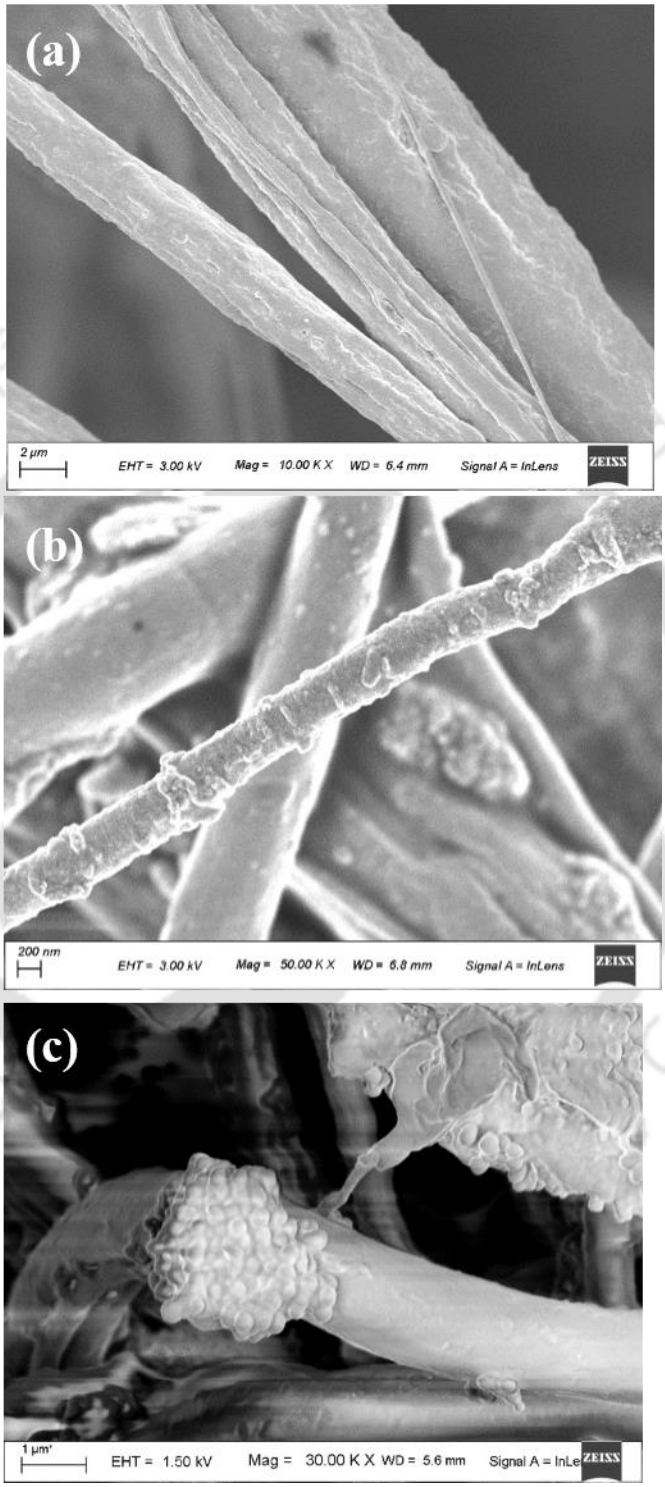



**Figure 2.9** Morphological analysis of (a) uncoated PLA nanofiber, (b) CCN matrix displaying uniform coating of chitosan and (c) α-amylase loaded CCN matrix

The enzymatic hydrolysis of CFW was carried out in the pH range of 3 - 8 to identify the optimal pH and the solution temperature is maintained at its optimal value (50 $^{o}$C). The enzymatic hydrolysis of the CFW was found to be optimum at acidic range with the solution pH of 2 (Figure 2.10b), which corroborates with the literature report[7].

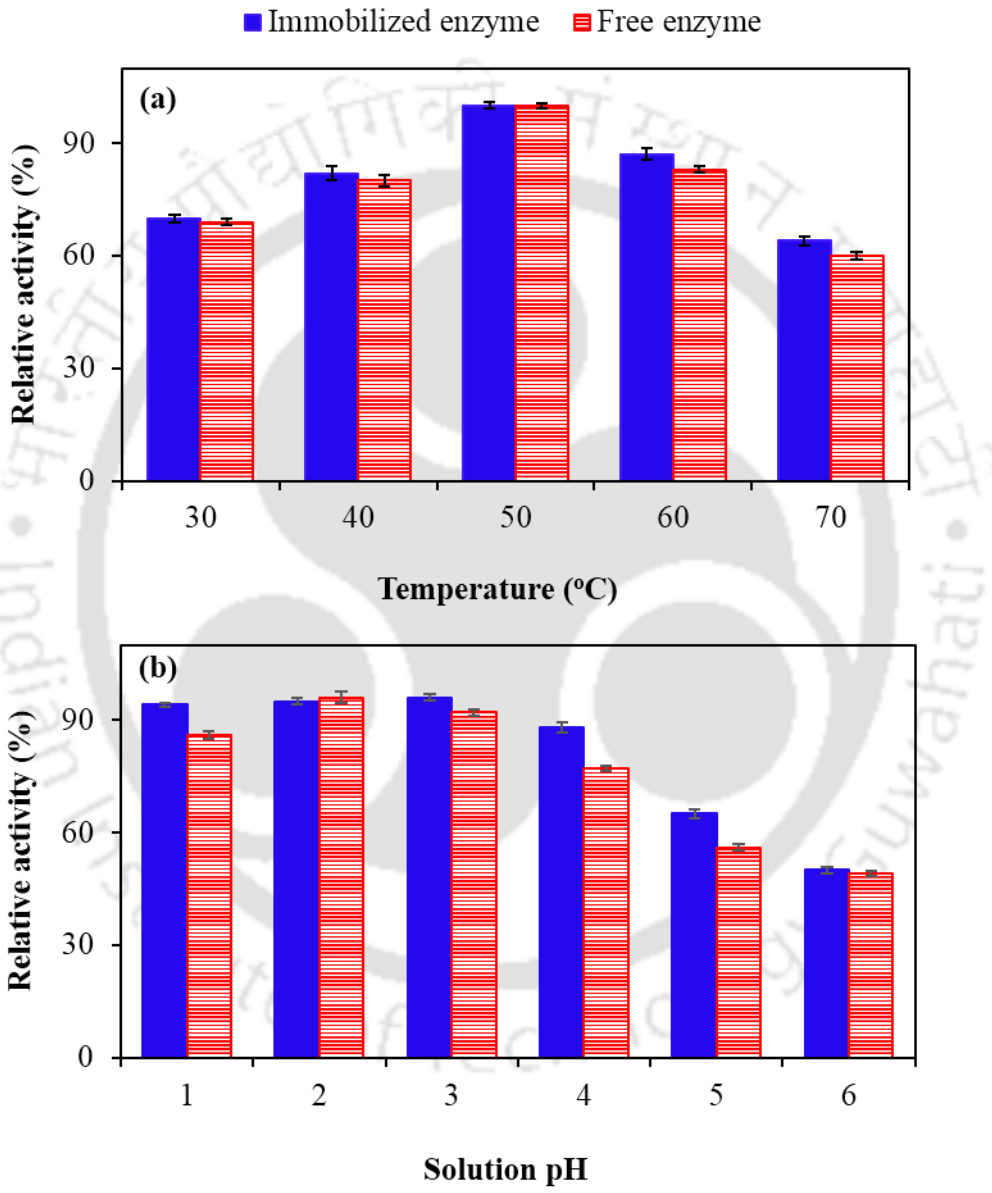



**Figure 2.10** Comparative analysis of the relative activity of enzyme in the immobilized system and freely suspended system as a function of (a) temperature and (b) pH

The key advantage of the immobilized system over the freely suspended system is on the reusability potential of the immobilized enzyme for several cycles without any significant

reduction in the hydrolytic efficiency. Thus the reusability studies were carried out by using the immobilized enzymes for six consecutive runs at optimized solution temperature (50 $^{o}$C) and pH 2. A slight reduction in the relative activity of the immobilized nanofiber was observed for each cycle of reuse. However, the relative activity was observed to be greater than 90% on all six cycles of reuse carried out in the present study (Figure 2.11).

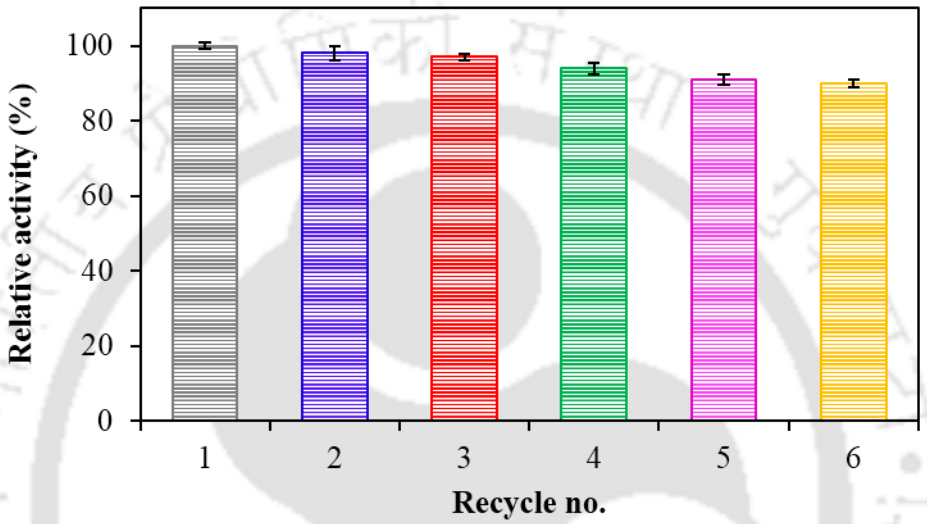



**Figure 2.11** Recyclability analysis of α-amylase enzyme loaded onto CCN matrix

Enzymatic hydrolysis of CFW was performed using a packed bed system carried out with/without recycling operation. In the case of the packed bed reactor system without recycling, the residence time of the substrate was varied by changing the flow rate of the substrate, and the conversion ratio of CFW to fermentable sugars was recorded. A maximum conversion ratio of 0.99 was observed within a residence time of 40 min. The higher conversion ratio observed at longer residence time can be attributed to the enhanced contact between the enzyme and substrate [38]. Michaelis Menten kinetic simulation was carried out using MATLAB$^{TM}$ programming using Eq. (2.5):

$$\tau = \left(\frac{\delta S_O}{v_m}\right) - \left(\frac{K_m}{v_m}\right)\ln(1-\delta) \qquad \text{(Eq.2.5)}$$

Where, $v_m$, $K_m$, $S_O$ and δ represents the maximum velocity (mmol/s), Michaelis constant (mmol), and the substrate concentration varied from 10gL$^{-1}$ to 100 gL$^{-1}$ to understand the trends of conversion by using the mathematical expressions (Eqs 2.5 and 2.6). The experimental kinetic parameters $v_m$ and $K_m$ were estimated and used in the simulation. Simulation of cassava starch hydrolysis with an inlet substrate concentration of 10 gL$^{-1}$ was carried out to obtain a clear overlay of simulated values on the experimental values. Thereafter, the conversion patterns of all other substrate concentrations were also simulated, and the results were shown in Figure 2.12a. Similar procedure was adopted in the case of a packed bed reactor system operated under recycling mode with different dilution rates (Figure 2.12b). For an initial substrate concentration of 10 gL$^{-1}$, the experimental results have shown a steady decrease in conversion ratio from 0.85 to 0.17 along with an increase in the dilution rate from 0.04 to 0.2 min$^{-1}$. This decrease in conversion ratio along with an increase in dilution rate is obvious because of the reduced contact time experienced by the enzyme over the substrate. Also, the conversion rate for other substrate concentration ranging from 20 to 100 gL$^{-1}$ were simulated (Figure 2.12b) using the Eq. (2.6):

$$\frac{1}{D} = \frac{\delta(K_m + S_o(1-\delta))}{v_m(1-\delta)} \qquad \text{(Eq.2.6)}$$

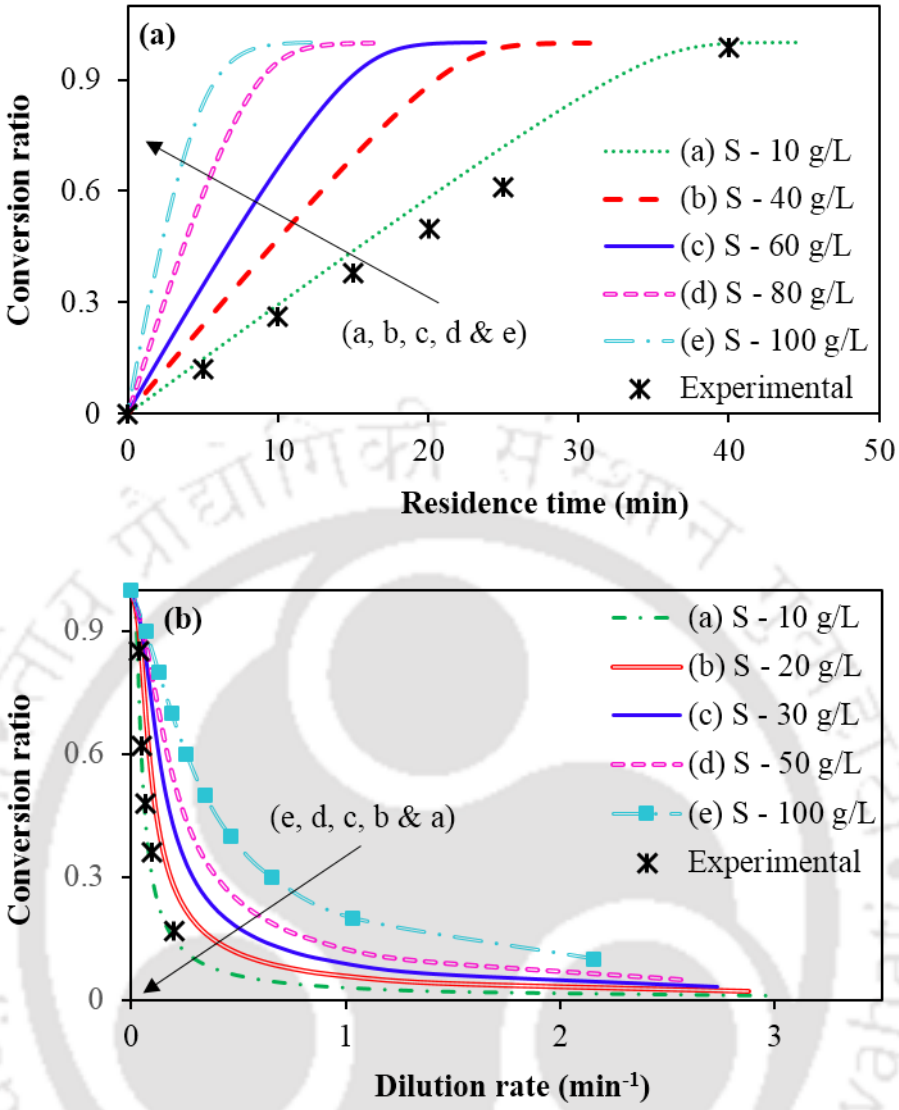



**Figure 2.12** Packed bed reactor operated: (a) without recycle and (b) recycling mode as a function of initial substrate concentration (S)

### 2.4. Discussion

The irregular morphology of the CFW could be attributed by subjecting cassava fiber to harsh treatment steps involved in its processing. Similar results were reported for native starch of well-defined structure led to retrogradation if subject to process treatment methods [18]. These irregular and shredded morphology observed in the CFW will culminate in facilitating the release of sugars from CFW upon enzymatic hydrolysis as crystallinity play a major role in biological conversion. The XRD analysis portrayed that

the CFW selected in the present study was found out to be semi-crystalline in nature and comprises of shorter glucose units, this indeed an advantage for the present enzymatic hydrolysis process as the enzyme load on splitting the longer chain polymer in the case of B-type will be significantly reduced in the A-type starch material [24]. TGA analysis outlined the similar depolymerization temperature of the CFW and pure starch implies the prevention of excessive energy burden observed in the case of other biomass [29]. TGA results substantiates that no significant differences with respect to the starch source of botanical origin and corroborates with a recent report [30] on the preparation of carboxyl methyl cellulose films with corn and cassava starch. Rheological analysis has shown the viscosity of the starch-aqueous system is due to the swollen nature of the starch, and the starch swells with an increase in temperature until its gelatinization point. Similar observation was reported in literature [30] highlighting that the normal starch, waxy starch, and high amylose starch all exhibited an increasing viscosity trend till 75 $^{o}$C beyond which the viscosity was in downtrend. Thus, the gelatinized solution of the present CFW was found to be a suitable feedstock for saccharification through enzymatic hydrolysis. Composition analysis demonstrated that the concentration of minerals was not observed to be inhibitory; instead, these minerals may support microbial activity in the fermentation, a process consecutive to enzymatic hydrolysis of CFW.

Tailor made CCN matrix was found to be the best suitable support matrix for hydrolysis of cassava fibre waste. For instance, the reduction in contact angle is highly favourable this is because the gelatinization of CFW was carried out in an aqueous environment, the enzyme support material should preferably be hydrophilic as that may reduce fluid flow resistance and thereby resulting in better enzyme-substrate contact to give enhanced enzymatic hydrolysis. Recently, polymer nanocomposite with graphene nanoplatelets was developed to increase the hydrophilicity of the polymeric

nanocomposite and eventually to enhance the lipase-substrate contact [36]. Further, the increase in the chitosan coating concentration resulted in a sharp reduction in the percentage elongation (Figure 2.8b), which enumerates the suitability of the CCN matrix as packing material in the packed bed reactors dealing with the enzymatic hydrolysis process. In order to handle the high inlet flowrate and shock loads in the packed bed system a packing material with excellent properties like high tensile strength and low elongation properties are solicited [37]. On top of all the aforementioned advantages, it is generally claimed that almost all the conventional immobilization support prepared using alginate or any other polymer suffers reduced relative activity due to mass transfer limitation. However, such a mass transfer and other inherent limitations with support material were overcome by the tailor-made CCN matrix having very high surface area providing the intimacy between the enzyme and substrate complex [36, 37]. Also, enzymatic hydrolysis by immobilization outperformed the freely suspended system owing to the protection provided by the support material for α - amylase enzyme from harsh acidic environment. Recyclability analysis has shown a slight reduction in the relative activity, which can be attributed to leaching of enzymes from the immobilized support and adsorption of the substrate onto the active sites thereby making them inaccessible for the next cycle of hydrolysis [10]. Recycle efficiency observed in the present study was significantly higher in comparison similar research studies reported in literature. Recently, a maximum of 80% relative activity at the end of the 6th cycle for α-amylase immobilized on naringin functionalized magnetic nanoparticles was reported [10]. Finally, the packed bed studies established the scalability of the present system and its labile operation under both recycling and non-recycling mode.

Hence, the present study developed novel electrospun PLA and modified chitosan nanofiber immobilized with α - amylase for hydrolyzing highly viscous starchy solution

resulting from the CFW. As mentioned before, in section 3.2, the CCN matrix used in the present study was tailor-made to handle the viscous solution. For instance, the higher tensile strength needed to hold the huge pressure drop across the packed bed reactor was imparted by the addition of chitosan onto the PLA polymer. Further, the reduction in the elongation is an added advantage as it maintains the packing material to be intact throughout the operation of the packed bed reactor. Consecutively, reduced hydrophobicity and nano features associated with the packing material is an added advantage for the successful implementation of the present packing material in the pilot-scale packed bed reactor system. The abundance availability of the CFW and the ease associated with the automated production of CCN matrix through electrospinning process suggests the scalability of the present study for commercial production of fermentable sugars. Alternatively, the packed bed reactor system can be integrated as pretreatment hydrolysis unit in a biorefinery setup. Novel electrospun CCN matrix dealt in this present study as the support material could be extended for the immobilization of other industrially important enzymes like lipozyme, lipase and cellulase as these enzymes also handle viscous substrate such as cheese, vegetable oil and liquefied lignocellulose, respectively.

## 2.5. Conclusion

Suitability of the CFW as a potential feedstock for the production of fermentable sugars was established in the light of different results observed from the characterization techniques like FESEM, XRD, thermogravimetric analysis (TGA) and rheological analysis. Electrospun CCN matrix was tailor-made to overcome the bottlenecks associated with the processing of CFW, and the same is used as the support material for the immobilization of α - amylase enzyme. Hydrolysis performance of the immobilized enzyme was compared with the freely suspended enzyme. Immobilized system outperformed the freely suspended in terms of its robustness and capacity for continuous

production of fermentable sugars for several cycles. Packed bed studies carried out with electrospun CCN matrix as the packing material, and its simulation results revealed the scalability of the present system. Furthermore, the present chapter paves an avenue for application of novel support material for immobilization of different other commercially valuable enzymes on handling viscous feedstock in the near future.

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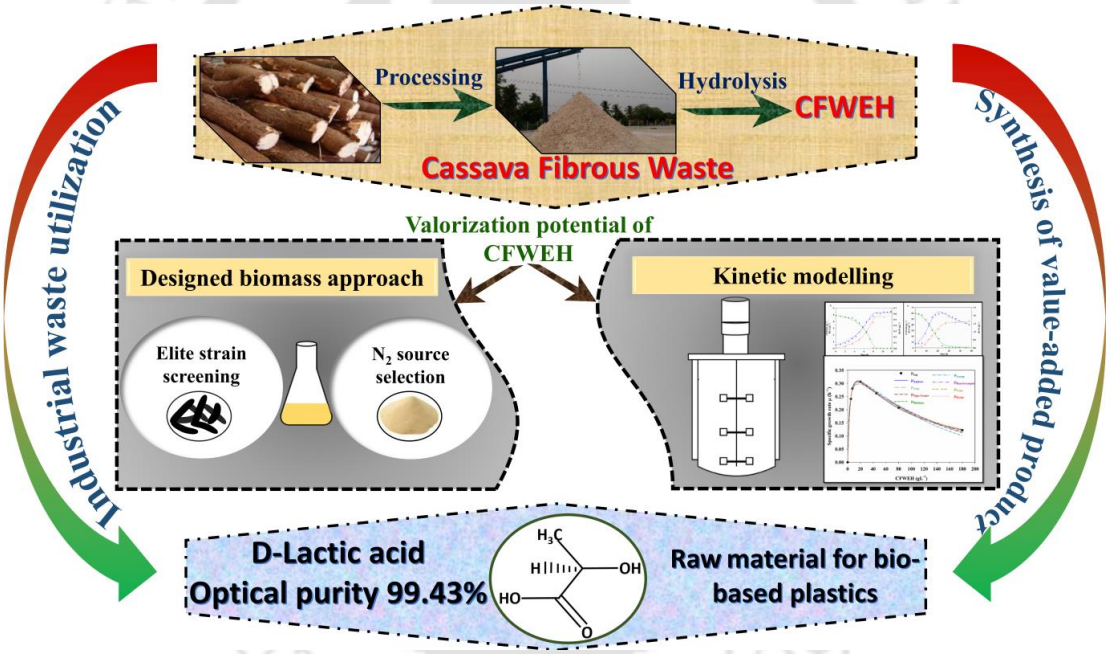
# Chapter 3

## Cost-effective waste valorization of cassava fibrous waste into enantiomerically pure D lactic acid : Process engineering and kinetic modeling approach



# Chapter 3

## Cost-effective waste valorization of cassava fibrous waste into enantiomerically pure D lactic acid: Process engineering and kinetic modeling approach

**Abstract**

Cassava fibrous waste (CFW) valorization on the synthesis of D (-) lactic acid (DLA) holds enormous importance, particularly for the production of thermostable biodegradable polymers. In this study, kinetic modelling was carried out to gain insight on the dynamics of cassava fibrous waste enzyme hydrolysate (CFWEH) utilization towards DLA production. Designed biomass approach was attempted to evaluate the potential of organism ability to metabolize CFWEH into high optically pure DLA. *Sporolactobacillus inulinus* (NBRC 13595) was found to be one of the elite strains for the production of DLA with an optical purity of 99.43 % on CFWEH. Yeast extract (2 $gL^{-1}$) was observed to be potential nitrogen source over peptone, tryptone and whey protein hydrolysate for kinetic modelling investigation. Kinetic parameters predicted from the proposed model for DLA production were found maximum specific growth rate, $\mu_{max}$ - 0.36 ($h^{-1}$); growth-associated product coefficient ($\alpha$ = 0.47 $gg^{-1}$) and specific productivity ($q_{P,max}$ = 1.12 $gg^{-1}h^{-1}$) respectively. Good synchronization between simulated model and experimental data was observed for biomass growth, substrate consumption and DLA production with initial sugar concentrations ranging from 20 – 180 $gL^{-1}$. Kinetic investigation reported in this study is a novice attempt enumerating the valorization potential of CFW for the synthesis of value-added products including DLA at commercial scale in near future.

### 3.1. Introduction

Economical production of value-added compounds by valorization of 2nd generation agricultural feedstocks say corn cobs, wheat straw, sugar cane waste, barley husk, etc advantageous not only for cost-effective production process but also benefits from environmental friendly perspective. Promoting agro-based feedstock's for the production of bulk chemicals would cut down the costs of carbonaceous raw materials drastically. Geographical nature of agro by-products and its characterization about the organization of sugar residues and other impurities would determine its sustainability; qualify for fermentation, followed by the synthesis of a bioproduct. Cassava fibrous waste (CFW) is a sago industrial waste, rich in starch content (40.1 – 75.1 % on dry weight basis) and generated from cassava processing industries during the peeling, crushing and sieving process[1]. Cassava, being one of the staple crop in the sub-tropical region of the world, produces 291,993 $\times 10^3$ tonnes as per FAO Statistics 2019 [2].The waste generated from cassava processing in both liquid and solid forms have been used for the production of bio-fuels and platform chemicals like ethanol, citric acid, lactic acid etc. [3]. D lactic acid (DLA) is an important platform chemical of potential commercial interest and can be obtained from microbial fermentation of organisms, which naturally synthesizes the product. This investigation aimed at valorizing the CFW peel wastes by microbial degeneration to reduce the overall organic loading in the natural environment. Commercial interests of D lactic acid (DLA) vest in the production of stereo-complexed poly D (-)/L (+) lactic acid , which is a bioplastic derivative.

Biodegradable polymers are obtained from starch/cellulose based agricultural feedstock [4-5] and food/beverage industry waste [6-7] through microbial fermentation directly or by polymerization of their monomers. Few examples include synthesis of Poly(Lactic Acid) (PLA) [8] Polyhydroxybutyrate (PHB) [1] and Poly(3-hydroxybutyrate-co-3-

hydroxyvalerate) (PHBV) [9] a co-polymer of PHB through metabolic routes of various organisms. PLA distinctly exhibits lesser structural disintegration under moisture conditions, which possesses good elastic and thermal properties at par with the petroleum driven plastic composites. Especially, PLA application in the package industry is driven by several factors viz. readily biodegradable, efficient municipal waste management and Food-grade package material. D lactic acid (DLA), being a monomer of PLA and remains an important platform chemical, its versatile applications suit well for chemical, pharmaceutical, agriculture, textile, and plastic industries. Synthesis of thermostable polymer, poly D (-)/L (+) lactic acid opens up new avenue for futuristic applications [8], which is obtained by blending controlled ratio of poly L (+) lactic acid (PLLA) with poly D lactic acid (PDLA). PDLLA has improved crystallinity and enhanced melting point from 180$^{o}$C to 230$^{o}$C compared to poly lactic acid (PLA) obtained from optically pure isomers (DLA or L (+) lactic acid (LLA)) alone or racemic mixture [10]. According to lactic acid market analysis report 2019-2025 (Grand view research, Inc., USA), the estimated global lactic acid market in 2019 was 3.1 billion USD and would reach 8.7 billion USD by 2025 [11]. The worldwide PLA production capacity was estimated to be 375,000 tons by 2020 whereas its market demand would be around 500,000 tons by 2021 [8]. Industrial production of DLA can be achieved either through conventional chemical route or microbial fermentation. The petrochemical based chemical process yields racemic mixture, whereas microbial fermentation by Lactic acid bacteria (LAB) synthesizes enantiomerically pure D(-)/L(-) lactic acid [12].

LAB preferentially adopt fermentative route than oxidative TCA cycle conserving more energy and almost all LAB species can be characterized based on its homofermentative nature [13]. Microbial production of DLA is significantly influenced by the cost of raw material. Renewable and low cost feedstock is the optimal choice for fermentative

production of DLA [14-16]. Published reports on successful utilization of readily available cheaper agricultural feedstocks substantiated agro-industrial waste as potential choice for optically pure DLA production[17-26]. Characterization of CFW in our preliminary investigation hints that the peel waste contains mainly sucrose, packed with lignocellulosic biomass and demands effective pretreatment strategy for the recovery of utilizable sugars. Hydrolysis was carried out to digest complex glycan polymer into simple saccharins. Optimization of CFW hydrolysis by both acidic and enzymatic treatment processwas reported earlier by our research group [27]. Previous reports highlight that the growth and product profile obtained for LAB strains in the MRS media substituted with the respective CFW hydrolysate were found to be highly promising for DLA production.

Gaining kinetic insight on DLA production from Cassava Fibrous Waste Hydrolysate (CFWH) would be immensely useful in understanding the process behaviour, crucial to optimize, design, control and improve the sustained production of DLA. Several research groups developed the kinetic models for the lactic acid production from renewable feedstocks [28-31]. Fermentative lactic acid production was found to be inhibited at high substrate and product concentrations, which lead to reduction of overall product yield and productivity.

In this chapter, a novice attempt involving screening the process factors (xxx) was dealt with Designed Biomass Approach (DBA) by Zhao et al [32]. Molecular cloning technique possesses advantages for the improvement of utilization of various substrates by manipulating the expression rates of appropriate genetic elements. In general, processed raw feedstock contains fibrous network harbouring significant quantities of pectin, lignin and other inhibitory sugar residues that may not find suitable for metabolically engineered strains. But DBA method simplifies the selection of existing wild type strains appropriate for the utilization of the processed raw feedstock than genetic manipulation. DBA is

practically simpler for selection, can be assessed based on higher product titer, yield and other quality attributes (Figure1.7). Following the organism selection, kinetic investigation of experimental data using broad range of unstructured models for DLA production from CFWH was dealt in this study. The kinetic model parameters reported in this study would be of great significance in addressing the technical bottlenecks for a sustained DLA production. Cassava based waste valorization possess greater impact in the reduction of higher carbonaceous Biological Oxygen Demand (BOD) in the water bodies. In a nutshell, the proposed study of waste valorization, followed by kinetic model and its impact can be explained further in section 3.3.2.

## 3.2. Materials and Methods

### 3.2.1. Raw material

CFW was procured from small scale sago industries located around Salem, India. Optimization of process conditions for acidic and enzymatic digestion of raw CFW improved the efficiency of its overall conversion into utilizable sugars by 2.47 % (w/v) and 7 % (w/v) respectively, which was already reported in our preliminary study [27]. Also, optimal concentration of HCl for CFW hydrolysis was determined to be 1.67 M. Enzyme hydrolysate was obtained using α- amylase (12 AGU, M/s Himedia Laboratories, Mumbai, India) for the hydrolysis of linear chain and amyloglucosidase (M/s Richcore Lifesciences Pvt. Ltd., Bengaluru, Karnataka, India) digesting the branched segment of the CFW biomass respectively. The temperature and pH were maintained at optimized conditions.

### 3.2.2. Organism and culture conditions

Homo-fermentative LAB (HFLAB) organisms used in this study are *Lactobacillus delbrueckii subsp. delbrueckii* (NBRC 3534 and 3202 strains), *Sporolactobacillus inulinus* (NBRC 13595), *Sporolactobacillus laevolacticus* (NBRC 102473) and *Sporolactobacillus terrae* (NBRC 101527). All organisms/strains were procured from NITE (National Institute of Technology and Evaluation) Biological Research Center, Japan. Glycerol stocks (30 % v/v) of the cultures were prepared and preserved at -80 ºC.

MRS (de Man, Rogosa and Sharpe) media ingredients in the preculture volume (100 mL) are as follows (in $gL^{-1}$): Glucose - 20; Yeast extract- 5; Beef extract - 10; Peptone - 10; Sodium acetate - 5; Di-potassium hydrogen phosphate - 2; Tri-ammonium citrate - 2; Magnesium sulphate heptahydrate - 0.2; Manganese sulphate tetrahydrate - 0.05 and Tween 80. Aseptic transfer of preserved stock (organism) into the autoclaved preculture media was carried outto initiate the culture growth at flask level. 250 mL closed screw cap bottles were used as culture flasks in a static mode for anaerobic growth and incubated overnight at 37 ºC. The pH was maintained in a range between 5 – 7 by the addition of $CaCO_3$ (60 w/w % of initial substrate concentration) as a neutralizing agent [33]. Growth was continued until the cells reach mid-exponential phases (optical density ($OD_{600}$) ≈ 1) and the cells were harvested by centrifuging the culture at 5000 RPM for 10 minutes. The pellet was wash with 0.9 % saline solution and used to inoculate production medium. All the experiments were repeated in duplicates and average of the estimated titer was reported.

### 3.2.3. Production medium

Optimal formulation of the production medium composition remains same composition to the preculture medium except two modifications:

(i) Glucose in standard MRS medium was replaced with the Acidic/Enzymatic hydrolysate of CFW (CFWH) as carbon source. Henceforth, the production medium (CFWH + MRS) can be termed as Cassava Fibrous waste substituted media (CFWSM); (ii) Concentration of selected nitrogen source in CFWSM was varied based on one factor at a time (OFAT) approach.

### 3.2.4. Screening of elite strain

The HFLAB strains were grown in CFWSM and the initial concentration of CFWH was maintained uniformly at 20 $gL^{-1}$. Inoculated with the glycerol stocks, the prepared static flasks were incubated overnight at 37 ºC. The sample collected at the end of the batch was analysed to estimate the DLA concentration and Optical Purity (OP) towards selection of elite strain. The OP computation establishes the relationship between the concentrations of DLA and LLA by Eq. (3.1.)

$$OP = \frac{DLA}{DLA+LLA} * 100 \qquad \text{(Eq.3.1)}$$

Elite DLA producer screened from the CFW hydrolysate (Acidic/Enzymatic) exhibiting optimal DLA productivity and higher OP was chosen for the subsequent nitrogen source screening experiment.

### 3.2.5. Screening of nitrogen source by OFAT approach

Yeast extract, Peptone, Tryptone, and Whey protein hydrolysate were used as nitrogen sources for the screening experiments. Selection process weighs on the higher DLA productivity and enantiomeric purity. The combined nitrogen sources (beef extract, peptone and yeast extract) originally present in the MRS medium was replaced by 25$gL^{-1}$ of sole nitrogen sources (Yeast extract, peptone, tryptone, and whey protein hydrolysate) in CFWSM. Static flask experiments for DLA production were carried out to elucidate the effects of sole nitrogen sources and identify the elite source. The selected nitrogen source

was further varied at different levels by OFAT approach to determine the optimal nitrogen concentration requirement by the elite strain i.e. (1, 2, 4, 12, 20 and 25 $gL^{-1}$). Thus, an elite nitrogen source and its optimized concentration chosen in CFWSM at later stages was employed for DLA production at bioreactor level.

### 3.2.6. Bioreactor experiments

Batch reactor experiments were conducted in a 3 Litre bioreactor (M/s Applikon Biotechnology, Netherlands) with the different initial CFWH concentration. Autoclaved MRS equivalent CFWSM medium substituted with different initial concentration of CFWH (5 – 180 $gL^{-1}$) served as production medium and elite producer strain was employed. Purging of nitrogen gas for 15 mins to remove the traces of oxygen is an important step before preceding the inoculation of anaerobic LAB (elite strain). Overnight grown preculture ($OD_{600} \approx 1.0$) was transferred aseptically into the reactor to initiate growth and samples were collected at regular intervals from therein. The process temperature and agitation rate were set to 37 ºC and 180 RPM respectively. The pH was maintained at 6.8 by addition of the minute pulses of 4 M NaOH and 4 M HCl.

### 3.2.7. Analytical methods

Collected samples were stored at 4 ºC before estimating biomass and metabolite concentrations. Cells were separated from the broth by centrifuging samples at 10000 RPM for 10 min. Supernatant was retained separately and pellet was analyzed for biomass estimation ($OD_{600}$) by UV visible spectrophotometer (GeneQuant 1300, M/s GE Health care, NJ, USA). Measured OD values were converted into dry cell weight (DCW) values by using estimated relationship 1 OD = 0.49 DCW ($gL^{-1}$). Glucose consumption was estimated enzymatically by glucose oxidase – peroxidase method using GOD-POD kit (M/s Tulip Pharmaceuticals, Mumbai). DLA and LLA concentrations were assessed by

enzymatic method using D/L Lactic acid assay kit (NYZ Tech assay kit) and OP can be computed from the relationship described previously (Eq.3.1).

### 3.2.8. Kinetic modelling

Major metabolic activities can be grouped into either of growth, substrate utilization and product formation, which can be explained by the mathematical expressions appropriately. Differential equations for biomass growth $\left(dX/_{dt}\right)$, substrate consumption $\left(dS/_{dt}\right)$ and product formation $\left(dP/_{dt}\right)$ accounting the fermentation processes describes complex biological functions.

#### 3.2.8.1. Biomass growth kinetics

Eq.3.2 addresses biomass growth of a LAB, concerning a steady state balance between specific growth and death rates.

$$dX/_{dt} = (\mu - k_d)X \qquad \text{(Eq.3.2)}$$

Where μ is the specific growth rate ($h^{-1}$), can be calculated during the exponential growth phase and $k_d$ is cell death rate constant ($h^{-1}$). A well-illustrated monod model explains the relationship between the specific growth rate (μ) and substrate concentration (S). As represented in Eq. 3.3, the monod kinetics suits well for the substrate limited growth processes.

$$dX/_{dt} = \left(\frac{\mu_{max}S}{K_s+S} - K_d\right)X \qquad \text{(Eq.3.3)}$$

Where $\mu_{max}$ = maximum specific growth rate ($h^{-1}$), $k_s$ = Substrate limitation constant($gL^{-1}$). Higher substrate and product concentrations are the most important factors influencing microbial growth, especially in synthesizing organic acids. Different models were employed to study the proposed microbial growth with substrate limitation anD lactic acid

inhibition on various substrates. The model equations representing LAB growth reported by Boonmee *et al* 2003 was adopted for the present investigation on the assumption that non-competitive type of inhibition founds valid at extremely higher substrate concentrations [35]. At higher DLA concentration, the inhibition can be assumed to be in linear manner. Cell death rate isconsidered to be negligible and notation, $k_d$ finds no relevance to the proposed outcome. The modified equation governing the biomass growth can be represented as shown in Eq.3.4.

$$dX/dt = \mu_{max} * \left(\frac{S}{K_{sx}+S}\right) * \left(\frac{K_{ix}}{k_{ix}+S}\right) * \left(1 - \frac{P-P_{ix}}{P_{mx}-P_{ix}}\right) * X \qquad \text{(Eq.3.4)}$$

### 3.2.8.2. DLA production kinetics

Leudeking – Piret (LP) model suggests the DLA production rate dependent upon instantaneous biomass concentration (X) and the specific growth rate (μ) linearly as shown in Eq. 3.5 [34].

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \qquad \text{(Eq.3.5)}$$

Where α and β are the growth associated and non-growth associated constants. Modified LP modelequation contains substrate limitation, inhibition constants as well as DLA inhibitory terms. The model was represented in Eq. 3.6

$$\frac{dP}{dt} = \alpha\frac{dx}{dt} + q_{p,max} * \left(\frac{S}{K_{sx}+S}\right) * \left(\frac{K_{ip}}{k_{ip}+S}\right) * \left(1 - \frac{P-P_{ip}}{P_{mp}-P_{ip}}\right) * X \qquad \text{(Eq.3.6)}$$

### 3.2.8.3. Substrate consumption kinetics

In general, substrate consumption gets channelled towards biomass growth, product formation and for the maintenance of the cell. The mathematical form of substrate utilization kinetics is given by Eq. 3.7.

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - \frac{1}{Y_{P/S}}\frac{dP}{dt} - m_s X \qquad \text{(Eq.3.7)}$$

Where, $Y_{X/S}$ and $Y_{P/S}$ are respective yields of biomass and product per gram of substrate utilized, $m_s$ is the cell maintenance coefficient. Boonmee *et al* 2003 and Nandasana *et al* 2008 reported a variation in yield at different initial substrate concentrations in the batch production of LA [30] [35]. Under this condition, Eq. 3.7 is unlikely to be applied for studying substrate consumption kinetics. To describe the substrate utilization, the Eq. 3.8 can be employed.

$$\frac{dS}{dt} = q_{s,max} * \left(\frac{S}{K_{ss}+S}\right) * \left(\frac{K_{is}}{k_{is}+S}\right) * \left(1 - \frac{P-P_{is}}{P_{ms}-P_{is}}\right) * X \qquad \text{(Eq.3.8)}$$

### 3.2.8.4. Kinetic parameters estimation

Batch reactor runs operated at different initial substrate concentrations yielding offline biomass, substrate and product concentrations. The data thus obtained was subjected to determine kinetic parameters, in which minimization of residual sum of squared errors (RSS) between model predicted data and experimental data was obtained from the following equation (Eq. 3.9) [36].

$$RSS = \sum_{i=1}^{n}(X_{model} - X_{exp})^2 + \sum_{i=1}^{n}(S_{model} - S_{exp})^2 + \sum_{i=1}^{n}(P_{model} - P_{exp})^2 \text{ (Eq.3.9)}$$

Where, $X_{exp}$ = experimental value, $X_{model}$ = model predicted value and 'n' is the number of data points. All numerical calculations for modified Monod equations involving substrate inhibitory terms (Table for different substrate inhibition models) were performed in

Microsoft Excel (Ver. 14.7.7) solver add-in (Microsoft Inc, USA). The numerical calculations for solving the non-differential equations were performed using MATLAB R2014a version 8.3 (M/s Math Works Inc., Massachusetts, USA). Correlation (regression) coefficient was determined using the statistical analysis program, StatPlus: mac LE (Analyst Soft Inc.) for Macintosh Operating System. From MATLAB tools ODE 23S solver and 'fmincon' optimization tool has been utilized for resolving complex differential equations of the model. Simulated X, S, P values were derived by minimizing objective function and plotted against time and compared with their corresponding offline datasets. ODE 23S solver is more efficient to solve complex differential equations with appreciable error tolerance.

## 3.3. Results

### 3.3.1. Screening experiments

#### 3.3.1.1. Selection of elite strain and suitable carbon source

DBA was adopted in order to assess the performance of an elite strain utilizing CFWH based on final DLA titer, $Y_{P/S}$ and OP (Table 3.1). Outcomes were promising for the enzymatic hydrolysate (CFWEH) and LAB strains positively responded by complete utilization of the substituted carbon except for *S. terrae*. Growth and overall metabolic activities of LAB strains were found to be relatively inhibitory for Acid hydrolysate (CFWAH), illustrating lesser DLA productivity and yield. Both the strains of *L. delbreuckii* sub sp. *delbreuckii* (NBRC 3202 and NBRC 3534) exhibited absolutely zero growth in CFWAH, proving the presence of inhibitory by-products. DLA titer (19.13 $gL^{-1}$), $Y_{P/S}$ (0.96 $gg^{-1}$) and OP (99.43 %) were found to be significantly high for *S. inulinus* growth in CFWEH among other LAB strains. It is clearly evident from Table 3.1 that both the strains *S. inulinus* and *L. delbreuckii* are performing efficiently based on titre,

productivity and optical purity. Also from the literature these two strains are performing predominantly over other substrate too. In order to understand the starin behaviour for CFWEH feedstock inhibition, growth and fermentation kinetics detailed investigations are conducted at higher concentrations of CFWEH. The results are discussed for *S. inulinus* in this chapter where as for *L. delbreuckii* the results are discussed in detail in the next chapter i.e Chapter 4.

### 3.3.1.2. Selection of nitrogen source and optimal concentration

Supplementation of various nitrogen sources viz. YE, Tryptone, Peptone, and WPH for *S. inulinus* growth were proven to be effective for better DLA productivity (>18.89$gL^{-1}$) and OP (>99 %) (Table 3.2). The control run (without supplementing nitrogen source) yield low DLA titer (7.05 $gL^{-1}$) suggesting the significance of the role of nitrogen source selection in DLA production. All the nitrogen sources used in this study were proved to be promising but YE was chosen for subsequent investigation owing to its complex vitamin and mineral content. OFAT approach in determining appropriate YE concentration supporting DLA yield was represented in Figure 3.1. YE level above 2 $gL^{-1}$ (Optimal YE concentration ≥ 2 $gL^{-1}$) do not have pronouncing effect on the final DLA titer. Hence, *S. inulinus* grown in CFWEH as carbon source and YE (2 $gL^{-1}$) as nitrogen source was considered for lab-scale cultivation and microbial kinetic studies.

**Table 3.1** Screening of microbial strains on CFWEH and CFWAH for production of optically pure DLA

| Microorganism | Substrate | $Y^{a}_{P/S}$ ($gg^{-1}$) | DLA titer ($gL^{-1}$) | $r^{b}_{p}$($gL^{-1}h^{-1}$) | Optical purity (%) |
|---|---|---|---|---|---|
| *Lactobacillus delbrueckii delbrueckii* NBRC 3202 | CFWEH[c] | 0.93 ± 0.02 | 16.22 ± 0.61 | 0.91 ± 0.01 | 98.24 ± 0.32 |
| | CFWAH[d] | NG | NG | NG | NG |
| *Lactobacillus delbrueckii delbrueckii* NBRC 3534 | CFWEH | 0.76 ± 0.05 | 15.62 ± 0.54 | 0.67 ± 0.03 | 93.22 ± 0.27 |
| | CFWAH | NG | NG | NG | NG |
| *Sporolactobacillus inulinus* | CFWEH | 0.96 ± 0.02 | 19.13 ± 0.43 | 0.89 ±0.03 | 99.43 ± 0.13 |
| NBRC 13595 | CFWAH | 0.97 ± 0.01 | 15.41 ± 0.71 | 0.59 ±0.02 | 98.95 ± 0.51 |
| *Sporolactobacillus terrae* | CFWEH | 0.80 ±0.04 | 14.10 ± 0.47 | 0.59 ± 0.01 | 75.61 ± 0.34 |
| NBRC 101527 | CFWAH | 0.64 ± 0.03 | 8.25 ± 0.22 | 0.34 ±0.03 | 56.64 ± 0.62 |

[a]DLA Yield ($Y_{P/S}$, $gg^{-1}$) was calculated as a ratio of DLA produced (g) to substrate consumed (g). [b]Volumetric productivity ($r_p$, $gL^{-1}h^{-1}$) was calculated as a ratio of concentration of DLA produced ($gL^{-1}$) to fermentation time (h). [c]CFWEH – Cassava fibrous waste enzyme hydrolysate. [d]CFWAH – Cassava fibrous waste acid hydrolysate. All the cultures were grown in static condition at 37°C, NG- No growth.

**Table 3.2** Screening of nitrogen source for production of optically pure DLA from CFWEH by *S. inulinus* NBRC 13595

| Nitrogen source | $Y_{P/S}$ ($gg^{-1}$) | DLA titer ($gL^{-1}$) | $r_P$ ($gL^{-1}h^{-1}$) | Optical purity (%) |
|---|---|---|---|---|
| Yeast extract | 0.96 ± 0.01 | 19.16 ± 0.53 | 0.48 ± 0.02 | 99.57 ± 0.17 |
| Peptone | 0.95 ± 0.02 | 19.11 ± 0.21 | 0.39 ± 0.02 | 99.75 ± 0.11 |
| Tryptone | 0.94 ± 0.02 | 18.93 ± 0.64 | 0.41 ± 0.03 | 99.50 ± 0.25 |
| Whey Protein hydrolysate | 0.95 ± 0.02 | 18.89 ± 1.01 | 0.41 ± 0.02 | 99.64 ± 0.21 |
| Control | 0.92 ± 0.01 | 7.05 ± 0.77 | 0.13 ± 0.01 | 99.01± 0.13 |

Experiments were performed in static condition at 37 ºC

### 3.3.1.2. Selection of nitrogen source and optimal concentration

Supplementation of various nitrogen sources viz. YE, Tryptone, Peptone, and WPH for *S. inulinus* growth were proven to be effective for better DLA productivity (>18.89$gL^{-1}$) and OP (>99 %) (Table 3.2). The control run (without supplementing nitrogen source) yield low DLA titer (7.05 $gL^{-1}$) suggesting the significance of the role of nitrogen source selection in DLA production. All the nitrogen sources used in this study were proved to be promising but YE was chosen for subsequent investigation owing to its complex vitamin and mineral content. OFAT approach in determining appropriate YE concentration supporting DLA yield was represented in Figure 3.1. YE level above 2 $gL^{-1}$ (Optimal YE concentration ≥ 2 $gL^{-1}$) do not have pronouncing effect on the final DLA titer. Hence, *S. inulinus* grown in CFWEH as carbon source and YE (2 $gL^{-1}$) as nitrogen source was considered for lab-scale cultivation and microbial kinetic studies.

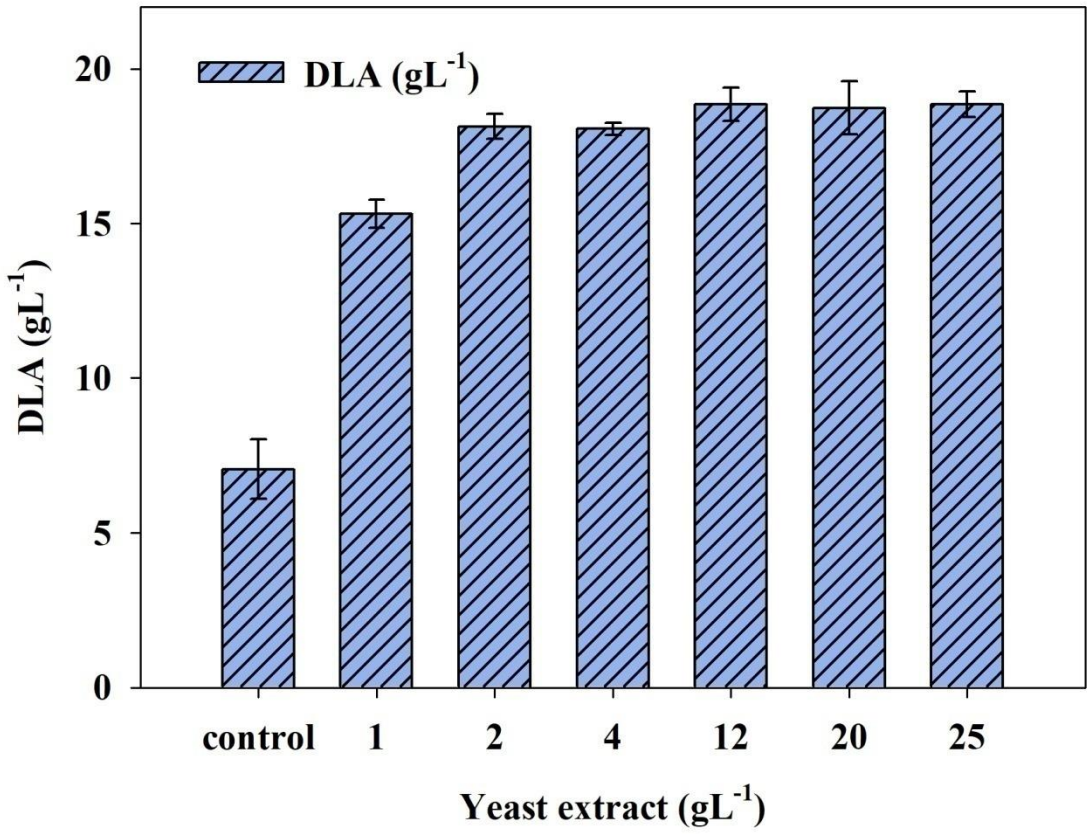



**Figure 3.1** Final DLA titer of the shake flask assessment carried out on varying initial YE concentration using OFAT approach

### 3.3.2. Kinetic modelling

Simple unstructured and non-segregated models were investigated to explain DLA production by microbial fermentation. Monod model serve to be universally applicable to all genera of bacteria and yeast except fewer microbial systems. It reliably explains microbial kinetics of growth, substrate utilization and product formation with suitable modifications interpreting substrate and product inhibition terms. The resolved differential equations 3.4, 3.6 & 3.8 representing growth, DLA formation and CFWEH utilization were plotted with their respective offline values at different substrate concentrations of growth-promoting (Figure 3.2) and inhibitory concentrations (Figure 3.3). The kinetic equations enlisted in Table 3.3 were evaluated with the offline estimated μ values against

their respective CFWEH concentrations to explain respective models and other kinetic parameters.

**Table 3.3** Substrate inhibition kinetic models used in this study

| **Name of the model** | **Model** | **Reference**[41-46] |
|---|---|---|
| Andrews | $\mu = \frac{\mu_{max} * S}{\left(K_S + S + \frac{S^2}{K_I}\right)}$ | [Andrews, 1968] |
| Aiba | $\mu = \frac{(\mu_{max} * S)}{(K_S + S)} * exp^{\left[\frac{-S}{K_I}\right]}$ | [Aiba et al., 1968] |
| Edward (Tipo Teisser type) | $\mu = \mu_{max} * \left[exp^{\left[\frac{-S}{K_I}\right]} - exp^{\left[\frac{-S}{K_S}\right]}\right]$ | [Edwards, 1970] |
| Yano | $\mu = \frac{(\mu_{max} * S)}{S + K_S + \left[\left(1 + \frac{S}{K}\right) * \left(\frac{S^2}{K_I}\right)\right]}$ | [Yano et al., 1966] |
| Webb | $\mu = \frac{\mu_{max} * S * \left(1 + \frac{S}{K}\right)}{S + K_S + \left(\frac{S^2}{K_I}\right)}$ | [Webb, 1963] |
| Luong | $\mu = \frac{(\mu_{max} * S)}{(K_S + S)} * \left[1 - \frac{S}{S_m}\right]^n$ | [Luong, 1987] |
| Han-Levenspiel | $\mu = \mu_{max}\left(1 - \frac{S}{S^*}\right)^n * \frac{S}{\left(S + K_S\left(1 - \frac{S}{S^*}\right)^m\right)}$ | [Han and Levenspiel, 1998] |
| Haldane | $\mu = \frac{\mu_{max} * S}{\left[(S + K_S) * \left(1 + \frac{S}{K_I}\right)\right]}$ | [Armstrong, 1930] |

All the models could convincingly explain the overall growth and resolve kinetic parameters ($\mu_m$, $K_S$, $K_I$) were tabulated (Table 3.4). The model parameters are highly likely reliable from operational perspective as the regression coefficient, $R^2$ was above 0.99.

Relatively higher $R^2$ value (0.9994) and lower RMSE value (0.000712) corresponds to Edward (Tipo-Teisser) model indicates closeness to the estimated parameters.

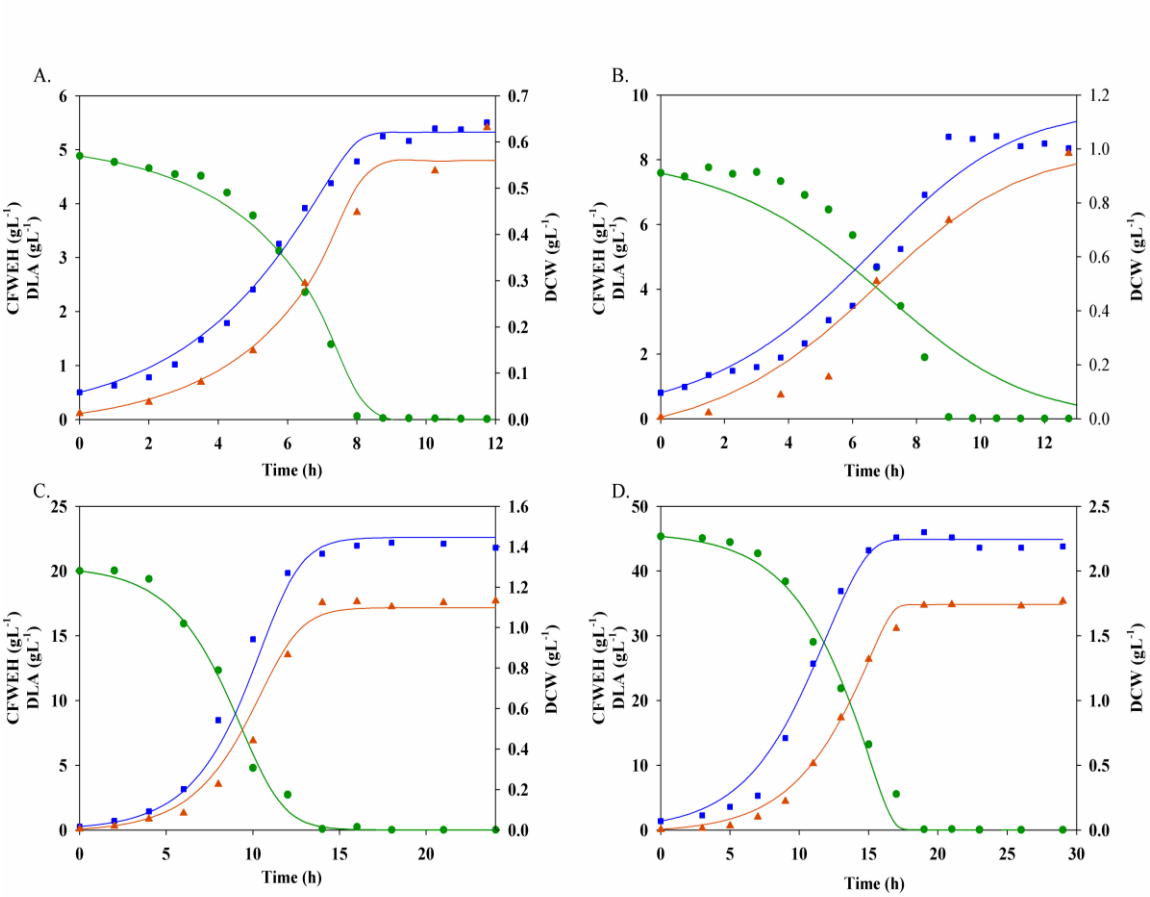



**Figure 3.2** Comparison of experimental profiles of biomass (filled squares), CFWEH (filled triangles) and DLA (filled circles) to the respective simulated curves of monod type growth viz. biomass (continuous), CFWEH (continuous) and DLA (continuous) carried out by least square minimization method for different initial CFWEH concentrations A. 5 $gL^{-1}$; B. 8 $gL^{-1}$; C. 20 $gL^{-1}$ D. 45 $gL^{-1}$;

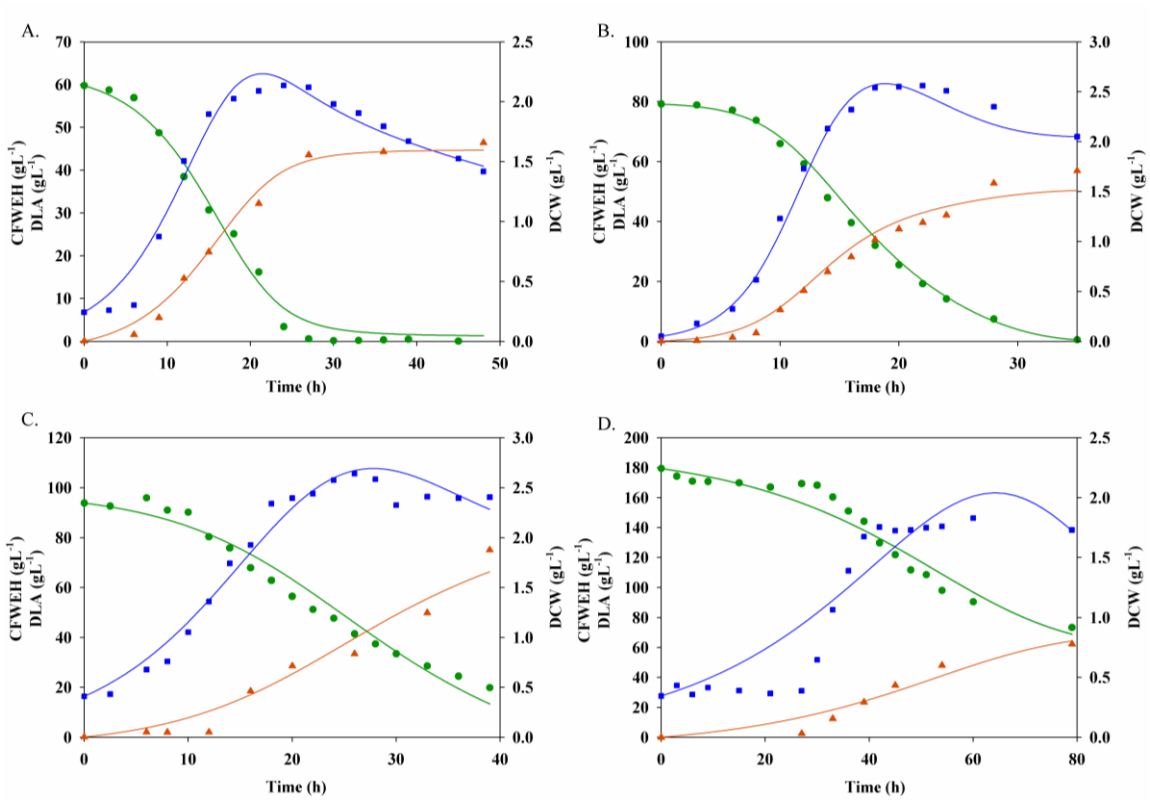



**Figure 3.3** Comparison of experimental profiles of biomass (filled squares), CFWEH (filled triangles) and DLA (filled circles) to the respective simulated curves of inhibitory monod type growth viz. biomass (continuous), CFWEH (continuous) and DLA (continuous) carried out by least square minimization method for different initial CFWEH concentrations A. 60 $gL^{-1}$; B. 80 $gL^{-1}$; C. 95 $gL^{-1}$; D. 180 $gL^{-1}$

### 3.4 Discussion

Several reports are available for L-lactic acid production from renewable resources at pilot scale throughput, but similar investigations related to DLA fermentation are in scarce. Metabolic engineering of *Bacillus subtilis* by incorporating thermo-tolerant *ldh* gene derived from *L. delbrueckii* yielded OP of 98 % [37]. But, DBA was found to be simpler strategy and successful in case of LAB species for identifying suitable microbial host to valorize CFW-based substrate by its natural selection. Yield of DLA (0.96 $gg^{-1}$) and the promising OP (99.5 %) were accomplished under controlled operating conditions at a laboratory scale. Pre-treatment by mineral acid finds more suitable for any lignocellulosic

biomass to achieve better recovery of utilizable sugars. In the present study, acidic hydrolysate did not work for any LAB strains employed for both the strains as discussed in section 3.3.1.1 where as for *S. inulinus*, showing moderate DLA productivity (15.41 $gL^{-1}$) and better OP (98.95 %). Significant DLA synthesis in CFWAH containing medium for *S. inulinus* can be attributed for its higher survival rate in acidic and bile salts medium, also determined to be highly stable than other LAB organism [38]. Also presence of inhibitory compounds like furfurals and its derivatives in CFW were determined from preliminary investigation [27]. Drastic improvement in DLA productivity when compared to other LAB strains proves that *S. inulinus* growth invariably metabolizes in the presence of inhibitory compounds. Although organism appears stable in CFWSM containing acidic hydrolysate, but DLA titer was significantly lesser than CFWEH containing medium. Owing to the enzyme specificity (α-amylase and amyloglucosidase) and hydrolysis at optimized conditions, major fraction of complex carbon in CFW was channelized towards product formation and CFWEH was found to be a better substitute for reducing sugar in production medium. *S. inulinus* was also observed to be highly stable for most of the nitrogen sources and obtained results were found to be reproducible at different instances. No drastic change in final DLA titers (18.89 – 19.16 $gL^{-1}$) for various nitrogen sources exhibiting the capability of organism in metabolizing the carbon and nitrogen substrates. YE as a nitrogen source would be highly conducible for reactor studies owing to its rich amino acid and mineral salt content. A significant drop in DLA titer (7.05 $gL^{-1}$) for control run exemplifies nitrogen source supplementation is vital for the fastidious LAB growth. Kinetics of LAB strains are reported and entailed elaborately with different carbon sources and compared in the Table 3.2.

**Table 3.4** Estimated kinetic parameter values for the selected kinetic models

| Name of the Kinetic model | $\mu_{max}$ ($h^{-1}$) | $K_S$ ($gL^{-1}$) | $K_I$ ($gL^{-1}$) | n | M | $S^*/S_m$ ($gL^{-1}$) | K | $R^2$ | SSE | RMSE |
|---|---|---|---|---|---|---|---|---|---|---|
| Andrews | 0.46 | 4.13 | 67.45 | - | - | - | - | 0.9973 | 1.14E-05 | 0.001511 |
| Aiba | 0.42 | 3.20 | 117.59 | - | - | - | - | 0.9838 | 6.98E-05 | 0.003736 |
| Edward (Tipo-Teisser) | 0.35 | 3.99 | 150.93 | - | - | - | - | 0.9994 | 2.59E-06 | 0.000712 |
| Haldane | 0.50 | 4.46 | 62.46 | - | - | - | - | 0.9973 | 1.15E-05 | 0.001512 |
| Luong | 0.41 | 3.13 | - | 12.24 | - | 1499.0 | - | 0.9821 | 7.77E-05 | 0.003926 |
| Han - Levenspiel | 0.35 | 4.09 | - | 2.273 | 54.89 | 389.8 | - | 0.9994 | 2.99E-06 | 0.000705 |
| Yano | 0.40 | 3.04 | 134.01 | - | - | - | 138.89 | 0.9834 | 7.15E-05 | 0.003781 |
| Webb | 0.46 | 4.18 | 64.89 | - | - | - | 4945.44 | 0.9975 | 1.05E-05 | 0.001447 |

**Table 3.5** Optimum parameter values for the kinetic model of *S. inulinus* NBRC 13595 and their comparison with reported values for lactic acid using similar models

| Kinetic parameter | This work | *Lactococcus lactis* NZ133 grown on lactose[35] | *Enterococcus faecalis* RKY1 grown on molasses[30] |
|---|---|---|---|
| Biomass formation model | | | |
| $\mu_{max}$ ($h^{-1}$) | 0.36 | 1.1 | 1.6 |
| $K_{sx}$ ($gL^{-1}$) | 0.85 | 1.32 | 0.89 |
| $K_{ix}$ ($gL^{-1}$) | 193.94 | 304 | 167.46 |
| $P_{ix}$ ($gL^{-1}$) | 1.26 | 1.39 | - |
| $P_{mx}$ ($gL^{-1}$) | 27.51 | 49.9 | - |
| $K_d$ ($h^{-1}$) | 0.01 | - | 0.00318 |
| CFWEH consumption model | | | |
| $q_{s,\,max}$ ($gg^{-1}h^{-1}$) | 1.54 | 3.42 | 3.33 |
| $K_{ss}$ ($gL^{-1}$) | 0.56 | 2.05 | 0.1 |
| $K_{is}$ ($gL^{-1}$) | 99.59 | 140 | 303.17 |
| $P_{is}$ ($gL^{-1}$) | 39.74 | 47.1 | - |
| $P_{ms}$($gL^{-1}$) | 66.71 | 95.5 | - |
| DLA production model | | | |
| $\alpha$ ($gg^{-1}$) | 0.47 | 0.39 | 0.26 |
| $q_{p,\,max}$ ($gg^{-1}h^{-1}$) | 1.12 | 3.02 | 3.0 |
| $K_{sp}$ ($gL^{-1}$) | 0.56 | 2.05 | 0.1 |
| $K_{ip}$ ($gL^{-1}$) | 99.59 | 140 | 303.17 |
| $P_{ip}$ ($gL^{-1}$) | 39.74 | 47.1 | - |
| $P_{mp}$($gL^{-1}$) | 66.71 | 95.5 | - |

### 3.4.1. Growth kinetics

Applying simpler monod growth model to account LAB growth can hardly differentiate the exponential and stationary phases. Therefore, many reports suggest the adequacy of mathematical modelling can be fulfilled by incorporating logistic equation, explaining various phases of growth more reliably [7]. Invariably the depiction of simulated biomass represented in Figure 3.2 & 3.3 showed a good overlapping with the estimated offline values. The regression coefficient ($R^2 > 0.99$) for the proposed logistic equation could be highly reproducible for different initial CFWEH concentrations. Table 3.4 illustrates the application of different model equations previously developed kinetic models were fitted with the respective offline μ and $S_0$ to obtain saturation, $k_s$ and inhibitory concentrations, $k_I$. The obtained values were found to be more realistic and comparable with the previous kinetic investigations of LAB organism as shown in Table 3.5. Estimated specific growth rate is an important indicator and relates the affinity of an organism to the alternate carbon source formulated in this study. Maximum specific growth rate, ($\mu_m$) determined to be 0.31 $h^{-1}$ approximately by offline and could be convincingly predicted by the deployed models (0.36 $h^{-1}$) between 20 – 45 $gL^{-1}$. Biomass limitation rate, $k_{sx}$ was found to be 0.85 $gL^{-1}$ and in most of the reactor runs yielded a consistent biomass concentration above 1.7 $gL^{-1}$. Death constant, $k_d$ determined by the simulation was found to be 0.01 $h^{-1}$ and insignificant compared to the higher growth rate. From Figure3.4 the effect of inhibition can be understood by a steady decline in μ above 20 $gL^{-1}$. Another important indicator of inhibition is the extended lag phase at higher $S_0$ and much smaller lag phases at lower concentrations, as it can be observed from Figure 3.2 & 3.3. Biomass concentration was much reduced to 1.8 $gL^{-1}$ upon a highest substituted CFWEH concentration of 180 $gL^{-1}$. Unutilized residual substrate concentration was found to be significantly higher at 95 $gL^{-1}$ and 180 $gL^{-1}$ and also rate of substrate utilization and biomass

growth was significantly reduced (Figure 3.3). These observations confirm that inhibitory substrate concentration was exceedingly higher at higher substrate concentrations above 80 $gL^{-1}$. From the predicted inhibitory concentration of biomass i.e. $k_{ix}$ at 193.94 $gL^{-1}$ may unlikely to occur for lab scale fermentations. Therefore, the inhibitory effects dealt in this study may be confined to CFWEH and DLA concentrations.

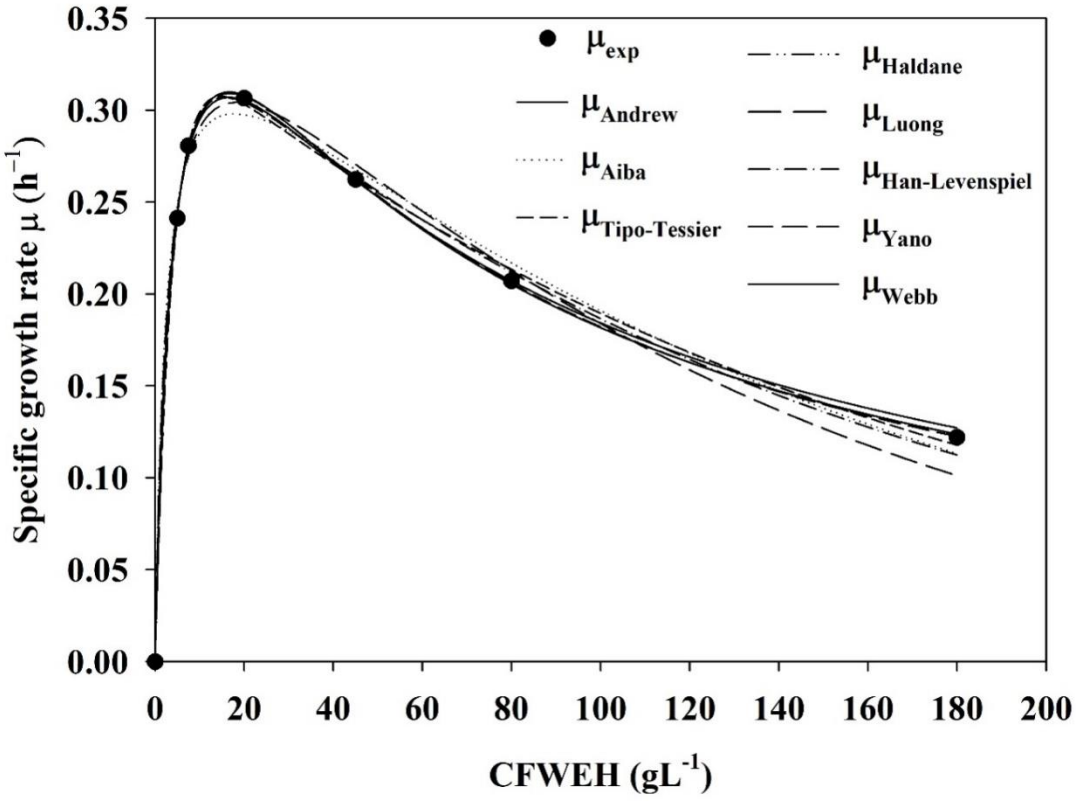



**Figure 3.4** Comparison of the experimental data and simulations for different substrate inhibition models at different initial sugar concentrations

### 3.4.2 Utilization of CFWEH

Unlike acid hydrolysate (CFWAH), enzymatic digests remain free from additional inhibitory compounds like furfurals [27]. The determination of CFWH inhibitory concentration i.e. $k_I$ and its

relevance to $\mu_{max}$ value of *S. inulinus* could be helpful for the determination of stability of continuous operation of the process. Results of the model points at 99.59 $gL^{-1}$ as a threshold limit until which the microbial system suits well for the Monod type growth. Substrate inhibition was imminent beyond the critical concentration and proceeds at destabilizing DLA fermentation process. Based on our previous study [27], pretreated/processed feedstock biomass with sugar residue composition was determined to be lesser than the inhibitory levels. It would remain non-problematic even if feeding can be carried out continuously to meet inherent metabolic requirements more dynamically. The specific substrate utilization rate (1.54 $gg^{-1}h^{-1}$) was found to be lesser when compared to other LAB species viz. *Lactobacillus helveticus* (4.8 $gg^{-1}h^{-1}$) [39] and *Lactococcus lactis* (3.42 $gg^{-1}h^{-1}$) [35]. Also comparison with other bacterial genera, say *Enterococcus faecalis* (3.33 $gg^{-1}h^{-1}$) [30] was also found to be lower for sucrose based feedstock. But *S. inulinus* seamlessly engaged to build upsignificant biomass, higher DLA productivity and yield (> 0.99 g. DLA g. $DCW^{-1}$) at par with other high yielding LAB strains, amidst retarded utilization rate. This can be confirmed from higher affinity constant, $k_s$ (0.85 $gL^{-1}$) reported for *S. inulinus* in utilizing CFWEH shown previously represents the ability of organism in metabolizing the substrate with more ease. Further it was observed that offline DLA titer showed gradual increment upon increasing CFWEH concentrations and reached maximum to 75 $gL^{-1}$ for the substrate loading at 95 $gL^{-1}$, also achieved product yield of 1.01 g.DLA. g $DCW^{-1}$. Inhibition due to higher substrate concentration was visible beyond this point and as a result product titer was significantly reduced (62.27 $gL^{-1}$) at 180 $gL^{-1}$. The impact of CFWH utilization on the DLA productivity would have pronouncing effects on the kinetic parameters of product formation as established by Boonmee et.al 2003 [35]. These findings and its justifications are crucial for the interpretation of the subsequent DLA production kinetics.

### 3.4.3. DLA product kinetics

Leudeking-Piret model postulated based on the experimental outcome of recurrent lactic acid production processes [34]. The segregation of growth and non-growth-associated product coefficient was already proven to be a best fit for DLA production. From the growth and product profiles (Figure 3.2), it can be well discerned that DLA production is predominantly a growth associated. Evolution of DLA creates acidic efflux in the reaction broth, inhibiting the organism growth beyond reaching threshold concentration. The modified model predicts that the coefficient of growth-assisted DLA production (α) found to be 0.47 $gg^{-1}$, while non-growth term (β) was negligible. The lowered production coefficient concomitant with the utilization profile proves that proposed kinetic model was stoichiometrically more appropriate. From the model, *S. inulinus* projects that the maximum overall product coefficient ($q_{P,max}$) was 1.12 $gg^{-1}h^{-1}$. The product coefficient of a raw feedstock at this level is of greater significance in terms of efficient product conversion. Yield coefficient ($Y_{P/S}$) of most reactor runs accounts 75 – 99 % of the total carbon input and acidic product competes with regular metabolic machinery at higher product concentrations. An extreme proton gradient generated across the cell membrane necessitates spending of higher maintenance energy drawn from the substituted carbon [40]. The practical difficulties in carrying out fermentations at higher substrate concentrations can be eased at the incorporation of adding neutralizing agents/buffers. Our previous investigation on *S. inulinus* yielded DLA titer of above 200 $gL^{-1}$ metabolizing whey protein hydrolysed medium by incorporating $CaCO_3$ as a neutralizing agent [17]. This investigation exclusively overweighs the sustainable technologies of the future to treat industrial wastes of higher carbon loading. Unstructured, non-segregated model elucidated the dynamic process more reliably to account process level intricacies.

### 3.5. Conclusion

This study emphasizes the suitability of DBA on valorizing various industrial wastes into a value-added product. LAB strains were successful in utilizing the enzymatic hydrolysate of CFWH and achieving 99 % optical purity and the obtained DLA yield coefficients showed promising outcome. *S. inulinus* exhibited a good track record in adopting to the supplied CFWEH (20 $gL^{-1}$) and YE (2 $gL^{-1}$) as limiting nutrients. Application of the kinetic model encumbered monod/inhibition ranges defined for the synthesis of optimal DLA production. The model simulation data showed very good correlation with the experimental data at different initial substrate concentrations with negligible standard errors. In future, proposed model would be helpful in designing and development of bioprocesses for the sustainable production of optically pure DLA from renewable agricultural waste feed stocks.

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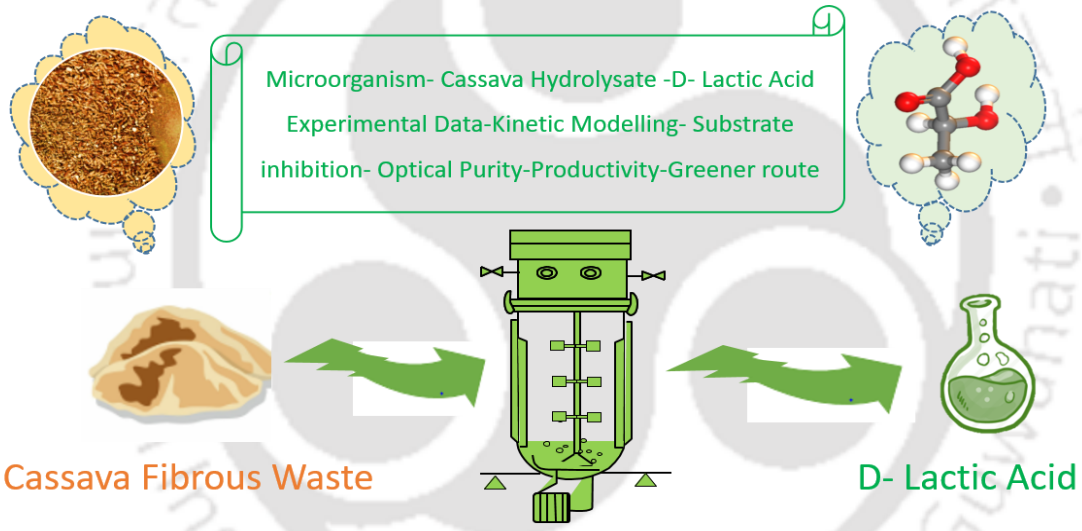
# Chapter 4

## Bioprospecting of cassava fibrous waste as a precursor for stereospecific lactic acid production: Inhibition insights for value addition and sustainable utilization



# Chapter 4

## Bioprospecting of cassava fibrous waste as a precursor for stereospecific lactic acid production: Inhibition insights for value addition and sustainable utilization

**Abstract**

A novice attempt on valorizing Cassava Fibrous Waste by *Lactobacillus delbreuckii* into D lactic acid (DLA) may assuage the massive supply chain for the synthesis of alternative bioplastics i.e. PLA. Enzymatic hydrolysate of cassava fibrous waste (CFWEH) contains free glucose, which was siphoned off to the fermentation as potential substrate. Among other complex nitrogen sources, Yeast Extract (YE) based medium yielded 17.75 $gL^{-1}$of DLA. Further optimization based on one factor at a time approach (OFAT), YE at 5 $gL^{-1}$ was found to be optimal. At different initial CFWEH concentrations from 20 – 120 $gL^{-1}$, kinetic modeling of biomass and DLA formation and CFWEH consumption was carried out by weighted-average least square method. Predicted parameters show that the inhibitory concentration for substrate was above 99 $gL^{-1}$, also inhibition due to DLA synthesis occurred as high as 59 $gL^{-1}$ for 120 $gL^{-1}$ substrate loading. This research finding offers the knowledge of kinetic parameters, its transformation into operational parameters, which would be helpful for sustainable synthesis of DLA.

### 4.1. Introduction

Synthetic plastics are family of polyalkanoates, containing aliphatic carbon chains and gets disposed into the environment to about 8 million tonnes every year as reported by International Union for Conservation of Nature (IUCN). Also in United States, the plastic industries consume 4.6 % of petroleum products every year and approximately 50 % of the polymer output is employed towards packaging applications [1]. The impact of the plastic sequestration into oceans and landfill amounts tremendous pressure on the respective ecosystems due to the secretion of toxic chemicals like phthalates and bisphenol A, which was known to degenerate endocrine system of animals and humans [2]. The challenges associated with plastic recycling and inherent demerits of incineration usher the focus of plastic industries towards the production of biodegradable plastics. Synthesis of bioplastics through microbial fermentation is the only viable alternative and has been successfully adopted for several decades. Utilization of renewable biomass such as cellulosic agricultural feedstock's and food/beverage industry wastes were employed for the synthesis of cost-effective bioplastics such as Poly hydroxyl alkanoates (PHA) [3-4] and D lactic acid (DLA) [5] monomer for Polylactic acid (PLA) production.

DLA is an important commodity chemical and eco-friendly product, finds its application in the food, pharmaceutical, polymer, plastics and composite industries. Global demand forecasted for DLA was found to be 8.77 $ billion by 2025 [6]. Stereo complexing of DLA with the conventional PLA (polymerized solely with L-lactic acid monomers) increase the melting point of the resultant composite, thus advantageous in terms of its thermal stability. Enantiomeric D/L isomers of lactic acid form the backbone of the PDLLA chain, a bioplastic derivative with superior mechano-elastic properties. Thus the contribution of DLA in the PLA and other composites is credited with the structural

stabilization and mechanical strength improvement. In addition to the polymer industry, DLA possess much wider applications in the other sectors of moderate economy like probiotics [7] animal nutrition [8], food additives [9] etc. Traditional chemical methods like stronger oxidation of petrochemical hydrocarbons into lactic acid yields racemic mixture of both D and L isomers in equal proportion at a cost of greater energy demand for fossil fuels. But microbial fermentation inherently possesses 2 advantages: Feasibility of utilizing cheaper renewable feedstocks and likelihood of achieving highest optical purity of the product. Corbion (Amsterdam), NatureWorks (United States), and Henan Jindan Lactic acid Technology Co. Ltd. (China) are the major supply chains of DLA to meet global demand of PLA for packaging, plastic wraps, biodegradable single use trays, etc.

Selection of alternative 2$^{nd}$ generation agro-based feedstock with promising sugar content is the first step in the development of cost-effective DLA production process. Food and agricultural wastes are potential sources, which contributes highly carbonaceous Biological Oxygen Demand (BOD) in the water streams. Organic carbon loading in the waste feed stream requires appropriate treatment facilities before disposing into external environment as directed by Global Wastewater Initiative (GW$^2$I) guidelines. It proposes in establishing the measures enabling nutrients to be removed from wastewater to ensure controlled levels of BOD in the waste stream. This present study highlights the utilization of starch rich Cassava Fibrous Waste (CFW), an agricultural field residue for the synthesis of DLA production. Cassava (*Manihot esculenta*) is the third largest source of food carbohydrates in the tropical region after rice and maize, which is grown across a broad range of agro-climatic conditions [10]. It is a biannual tuber crop with worldwide cultivability and sustainability with a production of over 250 million tonnes as per FAO

statistics. Different processing stages of cassava generate huge amounts of rich organic biomass waste which is cheaper and holds saccharin to about 40 – 45 % of its total weight [11]. CFW has been used as a raw material for the production of various value added bio-products viz. glutamic acid, ethanol, pullulan, L-lactic acid. Pretreatment of agro feedstock breaks down the fabric entangling sugar residues, easing out microbial population to utilize for DLA production. The assessment of acidic/enzymatic hydrolysis of CFW and fraction of sugars released was addressed in our previous published literature [11]. This present study focuses to improve the bioprocessing of DLA production by Lactic acid bacteria (LAB), utilizing hydrolysate of CFW at lab scale fermentation process and subsequent kinetic modeling.

Homo-fermentative Lactic acid bacteria (HFLAB) are specialized genera, characterized by strong D/L-lactate dehydrogenase activity and capable of metabolizing any hexoses/pentoses into lactic acid more efficiently. Unlike hetero-fermentative species, it sustains at lowest terminal pH value owing to lactate efflux into the reaction medium. The organisms are generally regarded as safe (GRAS) for the probiotic application and also serves as a host for the production of various recombinant therapeutic proteins and biopharmaceuticals. Relative expression rates of D/L-lactate dehydrogenases determine the optical purity of the lactic acid. Few strains of HFLAB secrete DLA inherently were already reported [5] [12]. Screening of natural HFLAB strains by Designer Biomass Approach (DBA) possesses a distinct advantage over metabolic engineering strategies. It attempts to adapt the metabolic requirements of an organism through the supplied pretreated feedstock containing C-source. This methodology was proven to be successful for cheese whey-based valorization in synthesizing DLA. As per the result obtained in section 3.3.1.1 the preliminary screening of HFLAB organisms identified *Lactobacillus*

*delbreuckii* to be an elite strain capable of synthesizing DLA at optimal concentration and higher optical purity. Further exploring the kinetics of growth and other metabolic activities can be studied to engineer the DLA production process at the reactor level amidst inhibitory concentrations of substrate and product.

Differential equations deciphering metabolic processes can be broadly segregated into growth and other metabolic processes for model development purposes. Majority of the research work discussing kinetics of DLA production deals with Unstructured & Non-segregated models, which were simple and precise for accounting LAB growth. Model accuracy can be accrued to its minimized residual sum of squares of errors experimental and simulated profiles. This present chapter remains to be first exploratory work incorporating *L. delbreuckii* growth on cassava fibrous waste enzymatic hydrolysate (CFWEH) and subsequent valorization into DLA. This present investigation comprehensively addresses the DLA product kinetics over a range of CFWEH concentrations in addition to the cell growth process.

## 4.2. Materials and Methods

### 4.2.1. Raw Materials and Enzymes

The preprocessed organic cassava peel was dried and finely powdered to generate CFW. It was procured from the processing unit of a sago industry in Salem, India. Enzymes (α-Amylase & Glucoamylase) involved in the digestion of starch residues were procured from M/s Richcore Enzymes, India. The activities of both the enzymes were estimated by the relative product formation rates per unit time, i.e. synthesis of free glucose and were used for hydrolysis of CFW. The optimal process condition such as pH, temperature range for enzymatic hydrolysis was adopted from our previously published research study [11]. All

the chemicals of media constituents were procured from M/s Himedia Laboratories, Bangalore, India.

### 4.2.2. Organism, storage and culture methods

*Lactobacillus delbrueckii subsp. delbrueckii* (NBRC 3202) was chosen for shake flask and lab-scale bioreactors for the synthesis of DLA based on the results of screening studies reported in our previous chapter 3.3.1.1. The cryopreserved *L. delbreuckii* culture was maintained in de Man, Rogosa and Sharpe (MRS) broth with 30 % (v/v) glycerol at -80˚C.

### 4.2.3. Process strategies

#### 4.2.3.1. Nitrogen source screening and optimization (Screw cap culture)

Based on the improved DLA productivity in *L. delbreuckii* compared with other natural DLA producers, the effect of various nitrogen sources was assessed in this strategy. Screening of elite nitrogen source for DLA production was accomplished in autoclaved screw cap tubes comprising medium (100 mL) with CFWEH as chief carbon source (20 $gL^{-1}$ conc.) supplemented with different nitrogen sources (25 $gL^{-1}$ conc.) as shown in Table 4.1. One factor at a time approach (OFAT) was deployed to elucidate the optimal concentration of the selected nitrogen source. OFAT experiments were carried out in autoclaved screw cap tubes comprising MRS medium with CFWEH as chief carbon source (20 $gL^{-1}$ conc.) and the nitrogen concentration was varied in the range between 2-20 $gL^{-1}$ (Table 4.1). Optimal concentration of nitrogen source is obtained by comparative analysis of its influence on DLA titer, Yield, productivity and optical purity.

#### 4.2.3.2. Kinetic modeling of *L. delbreuckii* (Bioreactor operation)

Autoclaved CFWEH at different initial concentrations (20, 40, 60, 80 & 120 g/L) were substituted before the inoculating preculture of *L. debreuckii*. The concentration of

substrate was carefully chosen such that *L. debreuckii* follow Monod's kinetics for both cell growth and its inhibition. Specific growth rate as a function of initial substrate concentration can be plotted and resolved for other kinetic parameters. Subsequent sections detail more elaborately about the formulation of kinetic equations of growth, substrate utilization and product formation.

#### 4.2.3.3. Static flask culture

Transfer of *L. delbreuckii* from glycerol stock was carried out aseptically into shake flasks. Composition of preculture medium remains same as MRS constituents except carbon source was replaced with the enzymatic hydrolysate of CFW (CFWEH). A detailed description of media composition were presented in Table 4.1. The experiments were carried out in screw cap tubes for 48 h at 37°C, 160 RPM and at controlled pH (5-7), by addition of neutralizing agent (Sodium bi carbonate, 100 g/L) at regular time interval.

#### 4.2.3.4. Bioreactor cultivation of *L. debreuckii* for DLA production

Preculture of *L. delbreuckii* was grown in modified MRS medium replacing glucose by CFWEH (similar composition to production medium) in order to minimize the lag time during DLA production at bioreactor level. The elite nitrogen source obtained from nitrogen source screening studies was supplemented as nitrogen substrate in the modified MRS medium (Table 4.1). All the bioreactor runs with the production media intended for the study of microbial kinetics of *L. delbreuckii* for the production of DLA utilizing CFWEH was carried out under anaerobic condition. Fermentation of CFWEH at different initial concentrations was carried out in a lab scale bioreactor (Biojenik engineering, Chennai) at 1.7 L working volume ($V_R$). The reactor was sterilized *in-situ* prior to its operation at 121ºC for 20 min by maintaining 15 lbs pressure. Freshly prepared hydrolysate of CFW containing micronutrients were autoclaved separately before charging

into reactor. Similarly, for nitrogen source and other mineral salts were autoclaved separately following by which charged into the reactor. $N_2$ gas was sparged into reactor system to scrub even minute traces of $O_2$ present in the medium. DLA fermentation was initiated by the transfer of inoculum (10% v/v of $V_R$) aseptically into the reactor. Temperature was controlled at 37ºC, while pH got regulated at 6.8 by the addition of suitable neutralizing agents (4 M NaOH & 4 M HCl) and agitation rate was maintained constantly at 160 RPM. All the experiments were carried out for 48 h and samples were collected for offline estimation at regular intervals.

**Table 4.1** MRS media constituents at different stages of the investigation

| **Culture methods/Strategies** | **Carbon substrate ($gL^{-1}$)** | **Nitrogen source ($gL^{-1}$)** | **Mineral salts ($gL^{-1}$)** |
|---|---|---|---|
| Preculture/Test tube culture | Pure glucose (20) | Yeast extract (5); Beef extract (10); Peptone (10); | Sodium acetate (5); Di-potassium hydrogen phosphate (2); Tri-ammonium citrate (2); Magnesium sulphate heptahydrate (0.2); Manganese sulphate tetrahydrate (0.05); Tween 80. |
| Selection of nitrogen source/Static flask culture | CFWEH (20) | Single nitrogen source under investigation (25 g/L)* | |
| Optimization of Nitrogen source | CFWEH (20) | Different levels of elite nitrogen substitution (2 to 20 g/L) | |
| Kinetic modelling/Reactor operation | CFWEH (20 to 120 ) | Elite nitrogen source at an optimized concentration | |

*25 $gL^{-1}$ is cumulative concentration of all nitrogen sources in MRS medium

### 4.2.4. Analytical Methods

The batch growth process continued for 48 h uniformly for both static flask and bioreactor operations. The samples collected at regular intervals were subjected to offline assessment for the estimation of biomass, substrate and DLA. Insoluble phase containing biomass as a solid pellet was derived after centrifugation of broth samples at 8000 RPM for 10 min. The biomass pellet was repeatedly washed with saline (9% v/v of NaCl), followed measurement of optical density ($OD_{600}$) at 600 nm. Biomass concentration was reported as dry cell weight (DCW) based on the calibration plot correlation between OD and DCW ($OD_{600}$ = 4.558 DCW in g/L). Concentration of glucose was estimated by glucose oxidase-peroxidase (GOD-POD) method. D lactic acid Concentration was measured using the enzymatic assay [13]. The D/L-lactic acid concentrations were estimated by enzymatic analysis using D/L lactic acid assay kit, hence the optical purity was calculated as percent of D lactic acid to racemic mixture of lactic acid (both D/L forms). The optical purity of the DLA was calculated (Eq. 4.1) as described elsewhere [14].

$$DLA\ Optical\ Purity\ (\%) = \frac{DLA}{DLA+LLA} \qquad \text{(Eq.4.1)}$$

### 4.2.5. Microbial model elucidating *L. delbreuckii* growth

Mathematical expression formulated for growth, nutrient utilization and product formation represents the core metabolic activities of an organism. Unstructured and Non-segregated models are a straight-forward and better platform to illustrate black-box nature of simpler microbial systems without accounting the intracellular activities (Table 4.2). Representation by differential equations for biomass growth $\left({}^{dX}/_{dt}\right)$, substrate consumption $\left({}^{dS}/_{dt}\right)$ and product formation $\left({}^{dP}/_{dt}\right)$ was reported for various bacterial

systems and dynamics of metabolic activities could be predicted [15]. The reliability of the prediction can be assessed by the residual errors between predicted and true values.

### 4.2.6. Kinetic modeling – Procedure

Batch DLA fermentation experiments were carried out at varied initial CFWEH concentration levels and offline biomass, substrate and DLA concentrations were obtained. The offline data was subjected MATLAB (Mathworks, Massachusetts, United States) based ODE 23S solver to determine kinetic parameters employing minimization of least squares algorithm. Residual sum of squared errors (RSS) between model predicted and experimental data was obtained from Eq.4.8 [16]..

$$RSS = \sum_{i=1}^{n}(X_{model} - X_{exp})^2 + \sum_{i=1}^{n}(S_{model} - S_{exp})^2 + \sum_{i=1}^{n}(P_{model} - P_{exp})^2 \quad \text{(Eq.4.8)}$$

Where, $X_{exp}$ = experimental value, $X_{model}$ = model predicted value and 'n' is the number of data points. The optimization tool 'fmincon' (ODE 23S solver, MATLAB) was used for resolving complex differential equations of the model. Simulated X, S, P values were derived by minimizing objective function and plotted against time and compared with their corresponding offline datasets. ODE 23S solver is more efficient to solve complex differential equations with appreciable error tolerance.

**Table 4.2** Kinetic model equations representing biomass, product formations and substrate utilization

| **Biomass growth model** |
| --- |
| Monod model illustrates the relationship between the specific growth rate (μ) and substrate concentration (S). This differential equation tries to resolve the biomass concentration with respect to substrate concentration and other kinetic parameters. |

$$dX/dt = \left(\frac{\mu_{max} S}{K_s + S} - K_d\right) X \quad \text{(Eq.4.2)}$$

Kinetic constants are strongly influenced by initial substrate and inhibitory DLA concentrations. Kinetic equation of biomass growth can be devised based on the assumption that growth rate of an organism in parallel affected by the inhibitory activity of initial CFWEH and DLA concentrations as adopted in Boonmee *et al.*, 2003.

$$dX/dt = \mu_{max} * \left(\frac{S}{K_{sx}+S}\right) * \left(\frac{K_{ix}}{k_{ix}+S}\right) * \left(1 - \frac{P - P_{ix}}{P_{mx} - P_{ix}}\right) * X \quad \text{(Eq.4.3)}$$

**Product formation model**

Leudeking – Piret (LP) model proposed for DLA production rate takes the following form as shown below (Eq. 4.4). The former term with 'α' in Eqn. 4.4 becomes more predominant and varies linearly with respect to specific growth rate (μ).

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad \text{(Eq.4.4)}$$

Where α and β are the growth-associated and non-growth-associated constants. Incorporating saturation and inhibitory terms with respect to the growth can be applied for DLA production rate and the Eq. 4.4 gets modified into Eq. 4.5.

$$\frac{dP}{dt} = \alpha \frac{dx}{dt} + q_{p,max} * \left(\frac{S}{K_{sx} + S}\right) * \left(\frac{K_{ip}}{k_{ip} + S}\right) * \left(1 - \frac{P - P_{ip}}{P_{mp} - P_{ip}}\right) * X \quad \text{(Eq. 4.5)}$$

**Substrate consumption model**

The terms growth, DLA production and maintenance coefficient of a cell forms core of the entire metabolism, especially for LAB. Therefore dynamics of CFWEH utilization can be represented in differential equation format as follows (Eq. 4.6).

$$\frac{dS}{dt} = -\frac{1}{Y_{\frac{X}{S}}}\frac{dX}{dt} - \frac{1}{Y_{\frac{P}{S}}}\frac{dP}{dt} - m_s X \qquad \text{(Eq. 4.6)}$$

$Y_{X/S}$ and $Y_{P/S}$ represents the fractional yields of biomass and product per gram of CFWEH utilized, $m_s$ is the cell maintenance coefficient. From earlier research works it can be well understood that both biomass and product yields do vary significantly at different substrate concentrations. Also the constraints with respect to the growth and DLA production are also applicable to CFWEH utilization kinetics. For describing CFWEH utilization, Eq.4.7 can be employed.

$$\frac{dS}{dt} = q_{s,max} * \left(\frac{S}{K_{ss} + S}\right) * \left(\frac{K_{is}}{k_{is} + S}\right) * \left(1 - \frac{P - P_{is}}{P_{ms} - P_{is}}\right) * X \qquad \text{(Eq. 4.7)}$$

## 4.3. Results

### 4.3.1. Preference of *L. delbreuckii* on different nitrogen sources

Nitrogen source screening experimental results presented in Table 4.3 shows the comparative productivity and optical purity (OP) of DLA influenced by different type of nitrogen sources combined with CFWEH as carbon source. Anaerobic cultures showed final DLA titers of several fold higher than that of aerobic growth in the same media composition at a comparative process conditions.

**Table 4.3** Screening of different nitrogen sources and its effect on Biomass productivity, Final DLA concentration, Optical Purity, DLA specific productivity and Yields of biomass and DLA

| Cultivation mode/Nitrogen Source | DLA titer ($gL^{-1}$) | Optical purity (%) | Biomass productivity, $r_P$ ($gL^{-1}.h^{-1}$) | Biomass yield, $Y_{X/S}$ ($gg^{-1}$) | DLA yield, $Y_{P/S}$ ($gg^{-1}$) | DLA specific productivity, $q_P$ ($gL^{-1}.h^{-1}$) |
|---|---|---|---|---|---|---|
| Aerobic | 2.14 | 94.55 | 0.03 | 0.15 | 0.51 | 0.015 |
| Anaerobic | 17.8 | 95.76 | 0.37 | 0.2 | 0.85 | 0.08 |
| Yeast extract | 17.75 | 96.5 | 0.37 | 0.115 | 1.01 | 0.053 |
| Beef extract | 17.26 | 96.2 | 0.36 | 0.134 | 0.895 | 0.042 |
| Peptone | 13.27 | 99.18 | 0.27 | 0.084 | 0.936 | 0.07 |
| Tryptone | 9.88 | 99.03 | 0.2 | 0.081 | 0.788 | 0.084 |
| Whey Protein Hydrolysate | 11.80 | 96.95 | 0.24 | 0.155 | 1.00 | 0.057 |
| Brain heart infusion | 16.13 | 97.70 | 0.33 | 0.135 | 0.92 | 0.044 |
| Ammonia | 0.81 | 89.33 | 0.016 | 0.021 | 0.14 | 0.083 |
| Soy protein | 16.32 | 96.83 | 0.34 | 0.156 | 1.00 | 0.05 |

Therefore, anaerobic cultivation mode was selected henceforth for subsequent experiments. The outcome of nitrogen source screening experiments was interesting to notice that DLA titers spanning 9.88 – 17.75 $gL^{-1}$ was found and OP was consistently above 95 % in all the cases. Therefore, selection of nitrogen source based on DLA titer would be a good choice as the OP being sub-optimal for the kinetic investigation at reactor scale. Table 4.3 concludes Yeast extract substituted media yielded highest DLA titer of

17.75 $gL^{-1}$ and 96.5 % OP. Also, similar observation for Beef extract (DLA titer of 17.26 $gL^{-1}$ and OP 96.2 %) could be noticed. Pure ammonia substitution as a nitrogen source was inhibitory to biomass growth and DLA productivity. Subsequently a stepwise increment of YE concentration at different levels proved that 5 $gL^{-1}$ of nitrogen source was found to be optimal for organism growth and DLA production (Figure 4.1).

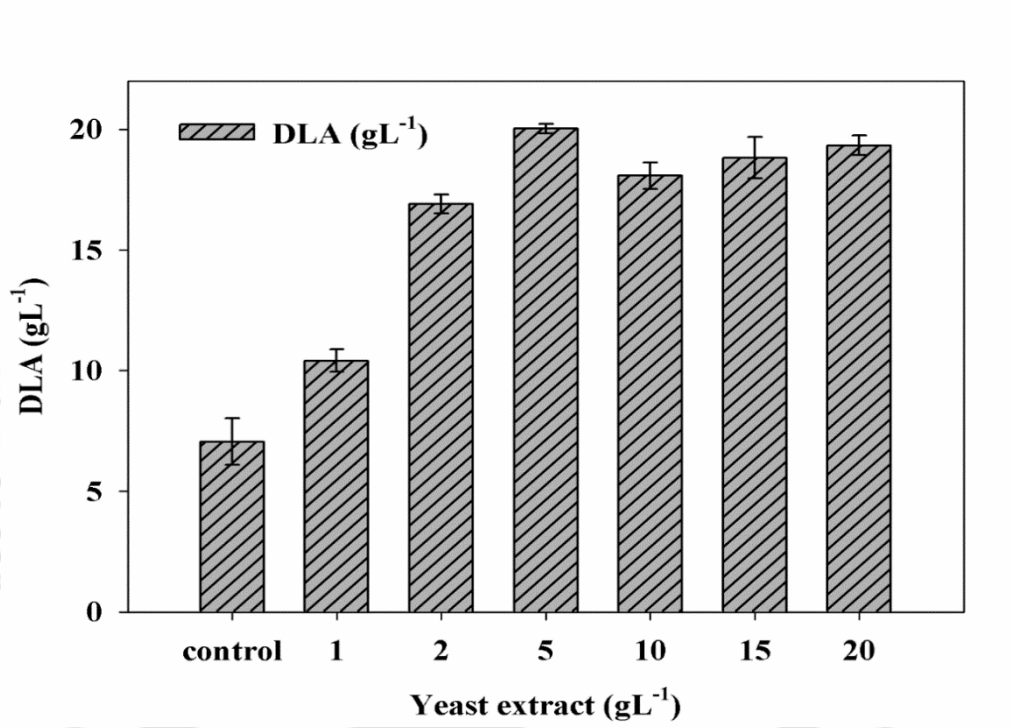



**Figure 4.1** DLA productivities reported for a stepwise increment of YE at different initial concentrations

### 4.3.2. Microbial growth and DLA production kinetics

Resolving experimental data containing biomass, CFWEH and DLA concentrations for different initial substrate concentrations, Table 4.4 demonstrates the predicted kinetic parameters. Advantageously weighted-average constraint method yields more reliable kinetic data than conventional mathematical ODE solvers (Data not shown). Regression coefficients and standard errors of the model derived from modified Monod equation seems to be showing good accuracy with the ongoing microbial activity. The derived

kinetic constants serve as a good indicator of the relative ease/difficulty in establishing homeostatis in its internal cellular metabolism. On this context, unstructured and non-segregated model dealt in this study entails a good insight on LAB fermentation with the available fewer offline data (CFEWH and DLA concentration) and deduced kinetic parameters. Moreover, the synchronized profile of offline and simulated biomass, DLA and CFWEH (Figure 4.2) characterizes the model of highly fiddle in nature.

For instance, the influence of initial substrate concentrations on specific growth rate (Figure 4.3) depicts an upright hyperbolic pattern, in which a continuous decline can be noticed at the onset of $\mu_m$, maximum specific growth rate, $h^{-1}$ (Figure 4.3). Moreover, the effect of inhibition can be implied from a continuous drop in μ value beyond 60 $gL^{-1}$ (Table 4.4). Models enlisted in Table 4.5 were tested for fitting with the offline estimated μ values and inhibitory trend was found (Figure 4.3) due to excess substrate and product inhibition. The kinetic constants $q_{s,\,max}$, $q_{p,\,max}$, α were highly conserved and reproducible with the offline estimation.

**Table 4.4** Predicted kinetic parameters of Biomass formation, CFWEH consumption and DLA productivity obtained at different Initial CFWEH concentrations

| Kinetic parameter | Initial substrate concentration ($gL^{-1}$) | | | | |
|---|---|---|---|---|---|
| | **20** | **40** | **60** | **80** | **120** |
| **Biomass formation** | | | | | |
| $\mu_{max}$ ($h^{-1}$) | 0.338 | 0.398 | 0.423 | 0.407 | 0.370 |
| $K_{sx}$ ($gL^{-1}$) | 1.999 | 2.000 | 0.502 | 1.089 | 2.000 |
| $K_{ix}$ ($gL^{-1}$) | 199.706 | 199.991 | 199.844 | 183.068 | 199.997 |
| $P_{ix}$ ($gL^{-1}$) | 6.503 | 6.622 | 6.435 | 6.364 | 5.801 |
| $P_{mx}$ ($gL^{-1}$) | 15.762 | 30.691 | 49.999 | 48.577 | 50.000 |
| $R^2$ | 0.99 | 0.98 | 0.99 | 0.96 | 0.97 |
| SSE | 0.07 | 0.14 | 0.15 | 0.56 | 0.34 |
| **Glucose consumption** | | | | | |
| $q_{s,\,max}$ ($gg^{-1}h^{-1}$) | 2.632 | 3.226 | 3.514 | 3.467 | 3.767 |
| $K_{ss}$ ($gL^{-1}$) | 4.998 | 5.000 | 4.995 | 2.862 | 4.997 |
| $K_{is}$ ($gL^{-1}$) | 99.895 | 99.997 | 99.937 | 78.687 | 99.989 |
| $P_{is}$ ($gL^{-1}$) | 13.838 | 23.117 | 39.078 | 39.998 | 40.270 |
| $P_{ms}$($gL^{-1}$) | 30.004 | 31.186 | 49.984 | 47.462 | 59.002 |
| $R^2$ | 0.996 | 0.997 | 0.986 | 0.996 | 0.92 |
| SSE | 3.07 | 11.37 | 111.31 | 62.02 | 562.86 |
| **DLA production** | | | | | |
| $\alpha$ ($gg^{-1}$) | 0.983 | 0.999 | 0.993 | 0.573 | 0.100 |
| $q_{p,\,max}$ ($gg^{-1}h^{-1}$) | 2.082 | 2.282 | 2.761 | 2.020 | 3.145 |
| $R^2$ | 0.99 | 0.96 | 0.99 | 0.93 | 0.98 |
| SSE | 4.51 | 101.11 | 67.86 | 950.70 | 170.54 |

**Table 4.5** Kinetic parameters estimated from different model equations

| Name of the model | Model equation | $\mu_{max}$ ($h^{-1}$) | $K_S$ ($gL^{-1}$) | $K_I$ ($gL^{-1}$) | $R^2$ | SSE | RMSE |
|---|---|---|---|---|---|---|---|
| Andrews | $\mu = \frac{\mu_{max} S}{\left(K_S + S + \frac{S^2}{K_I}\right)}$ | 0.393 | 6.490 | 82.07 | 0.99 | $1.004\times 10^{-5}$ | $1.29\times 10^{-3}$ |
| Aiba | $\mu = \frac{\mu_{max} S}{K_S + S} \exp^{\left(\frac{-S}{K_I}\right)}$ | 0.314 | 2.349 | 173.95 | 0.99 | $2.053\times 10^{-5}$ | $1.85\times 10^{-3}$ |
| Edward (Tipo-Tessier type) | $\mu = \mu_{max}\left(\exp^{\left(\frac{-S}{K_I}\right)} - \exp^{\left(\frac{-S}{K_S}\right)}\right)$ | 0.294 | 6.590 | 187.47 | 0.99 | $1.496\times 10^{-5}$ | $1.58\times 10^{-3}$ |
| Yano* | $\mu = \frac{\mu_{max} S}{S + K_S + \left[\left(1+\frac{S}{K}\right)\left(\frac{S^2}{K_I}\right)\right]}$ | 0.381 | 5.939 | 88.88 | 0.99 | $1.01\times 10^{-5}$ | $1.3\times 10^{-3}$ |

*Yano eq 'K'=3405.96

**Table 4.5** Kinetic parameters estimated from different model equations

| Name of the model | Model equation | $\mu_{max}$ ($h^{-1}$) | $K_S$ ($gL^{-1}$) | $K_I$ ($gL^{-1}$) | N | M | S*/$S_m$ ($gL^{-1}$) | $R^2$ | SSE | RMSE |
|---|---|---|---|---|---|---|---|---|---|---|
| Webb# | $\mu = \frac{\mu_{max} S\left(1+\frac{S}{K}\right)}{S + K_S + \left(\frac{S^2}{K_I}\right)}$ | 0.393 | 6.509 | 81.80 | - | - | - | 0.99 | $1.005\times 10^{-5}$ | $1.29\times 10^{-3}$ |
| Luong | $\mu = \frac{\mu_{max} S}{(K_S + S)}\left(1-\frac{S}{S_m}\right)^n$ | 0.312 | 2.253 | - | 28.17 | - | 4965 | 0.99 | $2.267\times 10^{-5}$ | $1.94\times 10^{-3}$ |
| Han–Levenspiel | $\mu = \mu_{max}\left(1-\frac{S}{S^*}\right)^n \frac{S}{\left(S + K_S\left(1-\frac{S}{S^*}\right)^m\right)}$ | 0.294 | 7.151 | - | 43.23 | 751.58 | 8147 | 0.99 | $1.548\times 10^{-5}$ | $1.61\times 10^{-3}$ |
| Haldane | $\mu = \frac{\mu_{max} S}{\left[(S + K_S)\left(1+\frac{S}{K_I}\right)\right]}$ | 0.430 | 7.106 | 74.96 | - | - | - | 0.99 | $1.004\times 10^{-5}$ | $1.29\times 10^{-3}$ |

#Webb eq 'K'=112346

**Table 4.6** Analytical estimation of kinetic parameters viz. Specific growth rate, Biomass yield, DLA yield, Maximum biomass concentration, Specific DLA productivity and DLA yield per unit biomass obtained at different initial CFWEH concentrations

| Initial CFWEH concentration ($gL^{-1}$) | Specific growth rate($h^{-1}$) | Biomass yield coefficient, $Y_{X/S}$ ($gg^{-1}$) | DLA yield coefficient, $Y_{P/S}$ ($gg^{-1}$) | Maximum biomass comcentration, $X_{max}$, ($gL^{-1}$) | Specific DLA productivity, $q_P$, ($gg^{-1}h^{-1}$) | DLA yield per unit of biomass, $Y_{P/X}$,($gg^{-1}$) |
|---|---|---|---|---|---|---|
| 20 | 0.25 | 0.084 | 0.946 | 1.69 | 0.460 | 11.059 |
| 40 | 0.237 | 0.051 | 0.951 | 2.09 | 0.647 | 18.129 |
| 60 | 0.216 | 0.044 | 0.949 | 2.692 | 0.703 | 21.114 |
| 80 | 0.189 | 0.031 | 0.965 | 2.55 | 0.995 | 29.872 |
| 120 | 0.156 | 0.027 | 0.850 | 2.096 | 1.000 | 30.018 |

## 4.4. Discussion

### 4.4.1. Implications of different nitrogen sources in CFWEH fermentation

From the cheaper to expensive range of nitrogen sources incorporated in this study supports anaerobic fermentation in the static flasks. Organism being a facultative anaerobe, fermentative type of metabolism found to be favored due to its lesser energy cost [17]. Energy intermediates are highly conserved and stored in the form of intermediary by products like organic acids unlike $CO_2$ in the aerobic growth. Under rotary shaking condition, flask cultivation lags both biomass and DLA productivity owing to its aerobic nature. Further experiments concerning nitrogen screening were carried out under anaerobic conditions (Table 3). DLA productivity and OP as seen before were promising for complex nitrogen sources than a simpler ammonia substitution. The organism was found non-prototrophic, which exhibits inability to utilize the inorganic nitrogen sources like ammonia for the synthesis of amino acids. Other complex nitrogen sources were rich in amino acids, vitamins, co-factors and mineral salts [18]. Drop in pH during static flask cultures could inhibit the growth of *L. debreuckii*, was controlled by 4 M NaOH in the production medium, which also helps the organism to utilize the sugar completely.

Substitution of nitrogen substrates are actively utilized for the building of structural compounds, proteins, nucleotide backbones. Out of the prominent nitrogen sources employed in this study, YE and BE were exceptionally better than other form of nitrogen constituents (Table 4.3). Relatively higher protein content (50 % of w/w) and rich source of other trace nutrients in YE support the organism in achieving higher growth rate and DLA productivity [19]. In order to promote process economy, nitrogen substrates of animal origin are discouraged for its usage towards commercial production of biochemical and YE could be an ideal choice for DLA fermentation.

### 4.4.2. *L. delbreuckii* growth-related kinetic parameters

The offline data (biomass, CFEWH and DLA concentration) were subjected to simulation as discussed in Table 4.2. Implementation of Monod model to distinguish various phases of microbial growth was successfully carried out in this present investigation Resolved differential equation (Eq. 4.3) by weighted-average square method represents the simulated profile of biomass. The curves of different CFWEH concentrations illustrate good overlapping with the plotted values of biomass, CFWEH and DLA as shown in Figure 4.2. Goodness of fit i.e. Regression coefficient, $R^2$ was determined to be 0.999 for the all the batch runs with different initial CFWEH concentrations. Different model equations given in Table 4.5 were attempted to fit *L. delbreuckii* growth by an ordinary ODE solver. Resultant kinetic parameters were shown in Table 4.5. Interestingly the obtained profiles (Figure 4.3) suggest that the LAB microbial system is highly adaptable for different constraints/conditions governing the metabolic activities.

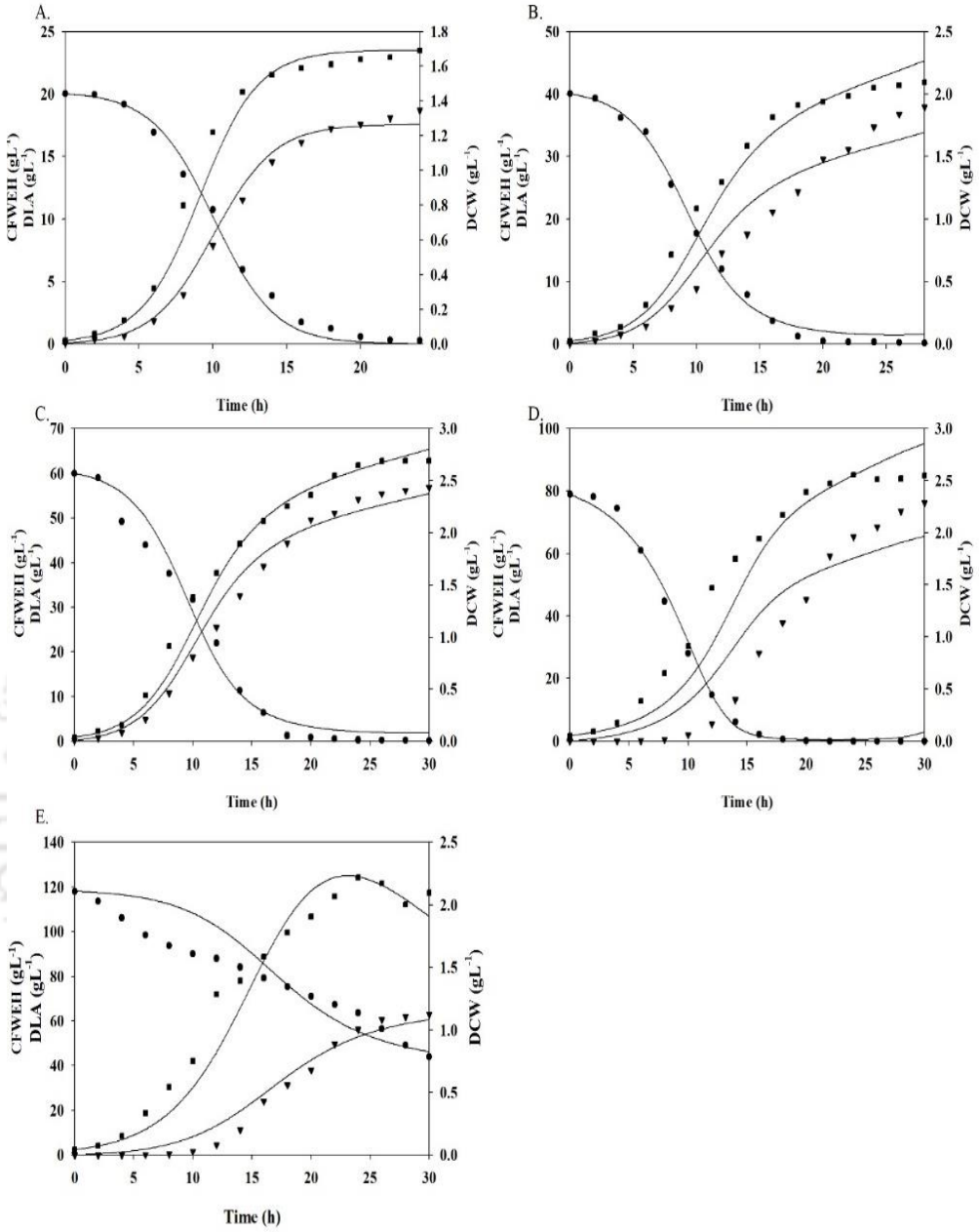



**Figure 4.2** Experimental plot representing biomass (filled squares), CFWEH (filled triangles) and DLA (filled circles) compared to the simulated curves (continuous) of Monod type growth viz. biomass, CFWEH and DLA were carried out by weighted-average least square minimization method at different initial CFWEH concentrations A. 20 $gL^{-1}$; B. 40 $gL^{-1}$; C. 60 $gL^{-1}$; D. 80 $gL^{-1}$; D. 120 $gL^{-1}$.

Relative comparison of μ values of various substrates shows their affinity over the utilized substrate. From the mechanistic nature of the model, the concluded μ values (ranging 0.338 – 0.423 $h^{-1}$) were slightly higher than the realistic determination i.e. estimation by offline (ranging 0.25 – 0.156 $h^{-1}$). Table 4.6 elucidates the estimated values of significant kinetic parameters of all the reactor runs. Saturation constant of biomass in all the cases were approximately equal to 2 $gL^{-1}$ and practical experimental biomass estimated were slightly higher than 2 $gL^{-1}$. This observation offers a clue that the organism in real can achieve closer to theoretical minimum value only, even at optimal conditions. Inhibitory nature of the product and excess substrate may limit the growth of an organism. The developed model was adopted from the previous study conducted in *Lactococcus lactis* [15]. Their reported kinetic constants for the said study and its comparison with the present investigation showed that DLA related saturation and inhibitory concentrations were similar. The cumulative effect of inhibition by substrate and DLA can be remarked from the drop in μ above 60 $gL^{-1}$, can be inferred from both predicted (Table 4.4) and experimental values (Table 4.6). A significant drop in the biomass concentration was observed at CFWEH at 120 $gL^{-1}$, also accumulation of available substrate is a strong indication that the organism is highly unlikely to undergo with its house-keeping activities. The inhibitory contribution is predominantly by DLA and CFWEH, the organisms tend to undergo metabolic activities with much difficulty and a sharp slump in biomass and DLA can be generally observed at higher CFWEH concentrations.

### 4.4.3. Inhibitory kinetics due to CFWEH supply and DLA productivity

Utilization of CFWEH was rapid as being a complex carbon substrate, which can be attributed to the absence of inhibitory compounds like furfurals [11]. *L. debreuckii* was found to be inhibited at CFWEH above 99 $gL^{-1}$ in most of the batch experiments. Also the

lower saturation constant, $K_S$ at 5 $gL^{-1}$ of substrate in all the cases indicates that CFWEH is preferred substrate for *L. debreuckii*. The upper and lower bounds of the kinetic constants for the operative specific growth rate determines stability of the process and would be useful in case of continuous production of DLA by tightly regulating the substrate concentration of feed. Specific CFWEH utilization rate (2.63 – 3.76 $gg^{-1}h^{-1}$) predicted in this study were in the same range reported for other LAB species viz. *Lactobacillus helveticus* (4.8 $gg^{-1}h^{-1}$) [20] and *Lactococcus lactis* (3.42 $gg^{-1}h^{-1}$) [15].

An increasing trend in final DLA titer was from 18.69 to 76.15 gL-1corresponding to 20 – 80 $gL^{-1}$ of CFWEH substitution. Growth inhibition was evident at excess CFWEH addition of 120 $gL^{-1}$, where DLA concentration got dropped to 62.94 $gL^{-1}$ and also DLA yield coefficient dropped to 0.85 g DLA. g CFWEH $^{-1}$ (Table 4.6). The reported values of kinetic parameters for *L. debreuckii* utilizing CFWEH as chief carbon source are comparable to experimental results report for pure reducing sugars [21], which substantiates the potential application of CFWEH at large scale DLA fermentation. DLA production in *L. delbreuckii* was highly robust and consistent till 80 $gL^{-1}$ amidst excessive acid flux developed in the broth. The DLA yield per gram of biomass harvested showed an increasing trend and reached as high as 30.1 g DLA. g Biomass$^{-1}$ (Table 4.6). Meanwhile the biomass yield decreased from 0.084 to 0.027 g Biomass. g CFWEH $^{-1}$ as the substrate concentration (Table 4.6). These observations infer that increase in biomass beyond certain threshold limit was not possible even under favorable conditions, but that does not limit the DLA synthesis.

Growth-associated product formation can be understood from the dynamic plots shown in Figure 4.3. All the plots (Figure 4.3) look that the DLA production follows similar trend of increase as is as biomass profile. Adoption of Leudeking-Piret model [22] to explain

the DLA formation kinetics would be more suitable for *L. delbreuckii*. Non growth-associated term was neglected in the simulation due to its insignificant index. Predicted α values obtained from simulation were tabulated in Table 4.4. The model predicts α value to be in the range 0.1 to 0.99 and the lowermost α value was recorded at highest initial CFWEH concentration (120 $gL^{-1}$). On the other hand specific DLA production coefficient was found to be at higher most (3.145 $gg^{-1}h^{-1}$) for 120 $gL^{-1}$ of CFWEH (Table 4.4). This determination proves that the severity in the inhibition at higher substrate concentration is due to excessive DLA efflux into the broth.

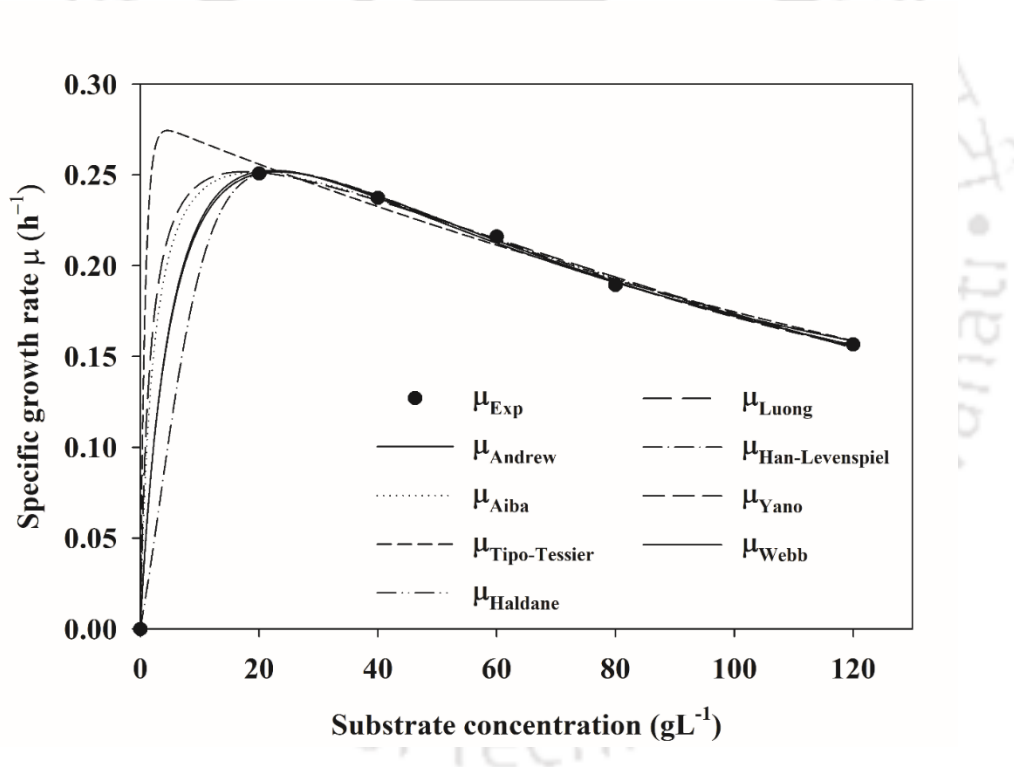



**Figure 4.3** Comparison of the experimental data and simulations for different substrate inhibition models at different initial CFWEH concentrations

Yield coefficient ($Y_{P/S}$) of every reactor run (Table 4.6) contributes 85 – 95 % of the CFWEH and the synthesis of organic acid is likely to disturb other house-keeping activities. Also it draws impact on the ability of the formation of biomass/cellular

components, as the cost of maintenance energy spend to pump proton flux into the cell to counter-balance osmotic shock due to excess acid (proton) in the broth[21]. Operational limitations of this kind can be reduced by the incorporation of suitable buffering agents in the medium. A previous report on *Sporolactobacillus inulinus* [5] found optimal with the addition of $CaCO_3$ which improved DLA titer up to 200 $gL^{-1}$ utilizing hydrolysed whey protein and palm sugar. Execution of process flow based on the kinetic models at lab-scale offers the knowledge on the design of the production process at larger scale.

### 4.5. Conclusion

DLA production by valorizing the processed CFW from sago industry by *Lactobacillus delbrueckii subsp. delbrueckii* is a first-step of producing cheaper bulk chemicals from industrial waste. DLA yield upto 95% of the totally substituted carbon and isomerically pure form of chemical was a valuable achievement of the study. Further on devicing input methods by sensing the acidic product would provide DLA concentration in real-time. Such methodologies may be important for industrial production/continuous synthesis of DLA from a relatively simpler source. The model simulation data showed very good correlation with the experimental data at different initial substrate concentrations with negligible standard errors. In future, proposed model would be helpful in designing and development of bioprocesses for the sustainable production of optically pure DLA from renewable agricultural waste feed stocks.

### 4.6. Comparitive assessment from Chapter 3 and Chapter 4: An observatory and concluding remarks

Designed biomass approach was advantageous over metabolic engineering method in identifying potential natural DLA producers of LAB family. Selection based on DLA productivity combined with optical purity has identified *S. inulinus* and *L. delbreuckii*

strains found to be elite strains of HFLAB family. The capability of strains at higher substrate and product concentrations which may turn inhibitory to the organism's metabolic activities and its preference to the complex carbon substrate should be investigated prior to its sustained synthesis. Plethora of articles already reported entailing the kinetic modeling of different LAB species by unstructured models. For this investigation, the model reliably predicts every parameter of the system and presents a comparison between both the organisms. Biomass growth-related parameters say specific growth rate prediction was found to be in the same range for *S. inulinus* and *L. delbreuckii.* The former one i.e. *S. inulinus* shows good affinity ($K_{sx}$ = 0.85 $gL^{-1}$) for biomass growth and can be corroborated for its easeness in metabolizing CFWEH ($K_{ss}$ = 4.998 $gL^{-1}$) compared to *L. delbreuckii*. Slightly a better CFWEH utilization can be noticed in *L. delbreuckii* than the other one.

Both the cases showed growth-associated product formation and the optimal coefficient of productivity ($\alpha \approx 1$) were almost in the same range. Selected strains were highly robust in acidic conditions and showed inhibitory due to excess substrate and DLA accumulation at later phases of the growth. Effectiveness in synthesizing DLA can be analysed from its optical purity, which showed 99% for *S. inulinus* and 95% for *L. delbreuckii.* From the recognizable kinetic parameters the former strain which had superior properties than the other one. The model based parameters are highly significant to their operational bounds and thus by assisting the continuous synthesis of the product.

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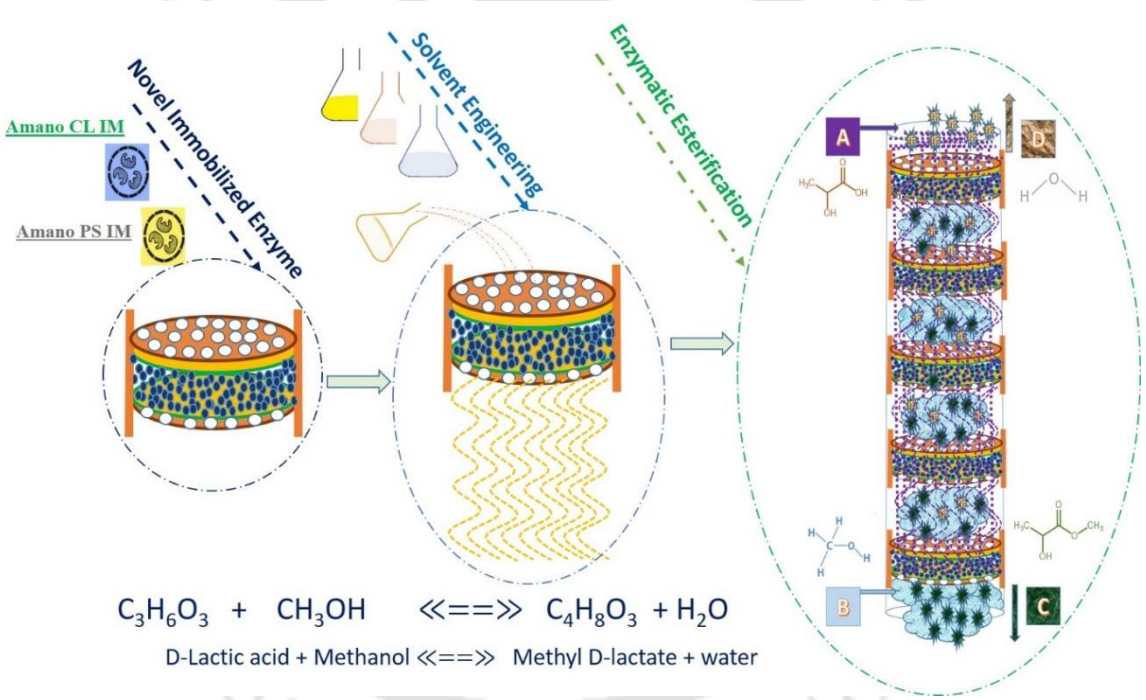
# Chapter 5

# Process Intensification strategy for D lactic acid purification: A novel biocatalytic enzyme coupled with solvent engineering and esterification.



# Chapter 5

## Process Intensification strategy for D lactic acid purification: A novel biocatalytic enzyme coupled with solvent engineering and esterification.

**Abstract**

Process Intensification strategies are necessary to achieve the desired economic and environmental aspects in any process. The design and operation of such strategies will enhance the productivity and sustainability of the process especially in integrated mode. Production of D lactic acid (DLA) by fermentation and reactive distillation is a typical example. An efficient method needed as purification is one of the costliest step in the entire DLA production process. Esterification is the only economical downstream process, which separates DLA from other components.Two different enzymes, *Candida antarctica* ( namely Amano CL IM) and *Pseudomonas cepacia* ( namely Amano PS IM) , gifted by Industrail enzyme division of Amano enzymes, Japan were screened for its efficiency in Methyl D-Lactate (MDL) production. Solvent engineering approach was used for the pre-treatment of both the enzymes and its consequent effect on ML production was analysed. Amano CL IM out performed Amano PS IM by yielding a maximum MDL conversion efficiency of 22.1% and 67.6% conversion efficiency at the end of $18^{th}$ h and $100^{th}$ h, respectively. Methanol to DLA ratio of 0.6 was found to be optimal for the screened Amano CL IM enzyme pre-treated with methanol. Esterification studies exhibit promising conversions on aqueous based fermentation broth.The investigation will address the above issues which will be suitable for future continuous fermentation coupled with modified reactive chambers of reactive distillation.

## 5.1. Introduction

The stereospecific nature and economic viability of Lactic acid monomer is very crucial for the sustainability of Poly(lactic acid) (PLA). It is a thermoplastic aliphatic polyester produced by mostly ring opening polymerization of lactide, which is a cyclic di-ester of lactic acid and is one of the biodegradable polymer obtained from renewable resources [1, 2]. The lactic acid is having two optical isomeric forms known as 'Dextro (D) rotatory and 'Levo (L)' rotatory. Enantiomeric purity is important for industrial uses and the greatest demand is for the pure isomers. Deliberate blending of the enantiomers provide an effective method to control both the physical properties of PLA and the rate of biodegradation. The subtle difference in stereochemistry has a drastic impact on mechanical properties and degradation which favours the production of lactic acid by biological processes rather than chemical processes which yield racemic mixtures (Figure 1.4). To have a carbon-neutral scheme, it is highly essential to substitute conventional petroleum-based polymers with the biopolymer [3]. In spite of its incitement to be a commercial alternative for the conventional polymer, the cost incurred during the D lactic acid (DLA) production keeps it back from realizing the actual commercial potential of PLA and its properties. Further, a recent study reported by López-Gómez et al., 2020 [4] quoted that the downstream processing of lactic acid production is even more challenging as the loss in this step amounts to 55% of the total lactic acid produced during the fermentation processes (Figure 1.8) [5]. Also, purification of Lactic acid is one of the crucial and costliest step in the entire production process. Lactic acid is the thermally unstable molecule and has a strong affinity to water and low volatility, which makes its separation by solvent extraction or distillation difficult [6]. The lactic acid separated at high temperatures can undergo thermal decomposition and forms dimers and oligomer. An efficient and alternative method for the purification is to esterify the acid and then

hydrolyse the ester to have the purified product. Chemically catalysed reactions are operated under energy consuming conditions with equilibrium issues along with by-product or unwanted product possibilities [7]. The design and operation of such strategies will enhance the productivity and sustainability of the process. Reactive distillation(RD) is one among such intensified and integrated designs in which reaction and separation is carried out simultaneously [8]. RD is an attractive alternative to the conventional method interms of enegy, capital savings, conversion of reactants, improved selectivity of desired products, reduced catalyst requirement, reduced by-product formation, and heat integration. It offers many advantages for equilibrium type reactions where separation is needed by carrying the reaction in the desired pathway. But at the same time it is not an eco friendly and cost effective one in terms of Greener process [9].

Enzymatically catalytic reactive processes are preferred industrially due to its specificity in preventing side reactions. Process Intensification strategies are also necessary to achieve the desired economic and environmental aspects in any commercial viable designs. In order to achieve the purity and stereospecificity of Lactic acid, enzymatically catalysed esterification and hydrolysis along with separation provision is the best possible way. Enzymatic Reactive Distillation will address all these issues successfully in one integrated design if equipped with a robust thermally active immobilized lipolytic enzyme which makes it a greener intensified process [10-12].

The aim of this chapter is to identify a suitable industrial enzyme which suits to DLA esterification as it is the typical equilibrium reaction which need to be carried in forward direction only. The lipolytic enzymes behave differently in aqueous as well as organic solvents [13-15]. In aqueous media they catalyse the breaking of ester bonds whereas in organic media they catalyse the formation of ester bonds. The enzymes such as esterages,

lipases and cutinases have the wide range of process capability in synthesis and hydrolysis reactions. They also can shift the equilibrium in the desired path of synthesis based on quantity of water and the nature of organic solvent. Most of the lipolytic enzymes perform these in an effective manner. The hindering aspect in this is the inactivation of enzyme due to its reaction conditions, solvent choice as well as concentrations of reactants which inhibits the desired reaction pathway in a progressive manner. To minimize the inactivation, deeper insights are needed in the structural aspects of the enzyme, surface modifications, thermal stability and a suitable inert matrix [13,16,17].

Immobilization of these enzymes with enhanced thermal stability may offer vast industrial scope for process intensification and to overcome phase equilibrium limitations. Lipases are widely used in transesterification and esterification reactions which are among the key players for industrial suitability. The key modification is to use different lipases derived from various organisms showing tolerance to organic solvents were used in the esterification reaction [18]. The use of solvent-stable lipase is inevitable as a high concentration of organic solvents was used in the esterification reaction. A high concentration of solvent was used to overcome the thermodynamic equilibrium by propelling reaction on the forward direction to produce Methyl D-Lactate (MDL) and simultaneously forbidding the backward response resulting in the hydrolysis of the formed esters. Few lipases as derived from *Bacillus subtilis*, *Acinetobacter* sp., *Bacillus thermoleovorans*, *Pseudomonas* sp., *Staphylococcus hominis* , *Streptomyces* sp. and especially *Candida antarctica* lipase species were already used for esterification reaction, and several commercially available lipases from Lipozyme® and Novozyme® were also used for the esterification of lactic acid [18] .

However, researches are still ongoing with a search for different lipases showing better performance. Surprisingly, the use of commercially available Amano lipase for the production of MDL was not reported so far for this DLA esterification. Investigation on the use of Amano lipase for MDL production is highly intriguing as these Amano® enzymes revolutionized the performance of halohydrins synthesis, epoxidation reaction and biodiesel production [19-21]. While the Amano enzymes used for the reactions mentioned above being derived from various microbes, till date Amano lipase derived from *Candida antarctica* (Amano CL IM) was not tested for its efficacy for any reactions. Thus, the present study is the first report on analyzing the performance Amano CL IM enzyme for methyl D-Lactate production. The objectives of the current works are as follows: (i)comparison of novel Amano lipase derived from *Candida Antarctica* (Amano CL IM) with that of the Amano lipase derived from *Pseudomonas cepacia* (Amano PS IM). (ii) To adopt solvent engineering approach to screen the best performing solvent for the methyl D-Lactate synthesis reaction. (iii) Finally, the concentration of the best performing solvent is varied and optimized to get a maximum MDL production by esterification reaction..

## 5.2. Materials and Methods

### 5.2.1. Chemicals

Both the lipases used in the present study was kindly gifted by the Industrial Enzyme division of Amano® enzymes (Japan) to Indian Institute of Technology Guwahati (IITG)-Amano®-Gifu University industry-academia consortium for academic research purpose. Lipase PS “Amano” IM is the commercial one in existence and Lipase CL “Amano” IM, which is *Aspergillus*(GMO) derived from *Candida antarctica*, currently under development stage at Amano enzyme research and development laboratory. The enzyme

was supplied in the form of Immobilized beads which was preserved at 4 $^0$C. D lactic acid (Source- TCI chemicals with 90% purity, density 1.2 g.cc$^{-1}$, liquid form, store at 4 $^0$C), The All the solvents used in the present study, like methanol and tetrahydrofuran was purchased from Wako® chemicals (Japan). All the chemicals were of analytical grade and used without any further purification. Wherever demanded double distilled water was used throughout the present study.

### 5.2.2. Enzyme catalyzed esterification by solvent engineering strategy for Methyl D-Lactate production

All enzymatic reactions were carried out with an immobilized enzyme dosage of 50 mg, unless and until stated all the experiments were carried out with a working volume of 1 ml. The first sets of investigation on screening of best-performing enzymes and best-suited solvents for enzyme pre-treatment were performed with similar reactants, i.e. DLA and methanol concentration of 2.7 of 3.2 M, respectively. Effect of enzymes on MDL production was studied using Amano CL IM and Amano PS IM enzyme. Effect of different solvents used for the pre-treatment of lipase was screened by using three different solvents viz. water, tetrahydrofuran and methanol. For the pre-treatment of lipase, 50 mg of respective lipase enzymes were incubated in 100 µL of different solvents as mentioned before. All the reactions were carried out with lipase pre-treatment solvent: DLA: methanol ratio of 0.8:0.1:0.1. The detailed strategy was provided in Table 5.1 for further assessment.

The second set of investigation on optimizing the concentration of the best performing solvent were studied by varying the methanol concentration from 50 to 90% (v.v$^{-1}$). While in both first and second sets of investigation, both the reactants DLA and methanol were diluted in tetrahydrofuran. However, for the final sets of experiments, the best performing

strain and the solvent at its suitable concentration was chosen, and the esterification reactions were carried out with reactants being diluted in the water. This final sets of experiments were carried out to mimic the real fermentation broth, which comprises 20% of DLA diluted in the water.

On the first investigation, to inspect the efficacy of enzymes over prolonged time, samples were collected at the end of 18th and 100th h. While on the other two investigations samples were collected only at the end of 18th. Enzyme esterification activity was assessed and compared by spectrophotometric protocol by examining p-nitrophenylbutyrate (pNPB) reaction at 405 nm as described elsewhere [22]. All the samples were analysed for the reaction products using 500 MHz proton NMR spectroscopy. These products estimation protocol was reported elsewhere in the literature[23]. Finally, the intact nature of the enzymes was analysed by using field emission scanning electron microscope (FESEM) analysis at 2.0 kV.

## 5.3. Results

### 5.3.1. Comparative performance of Immobilized enzyme and its activity

Separation of D lactic acid from the fermentation broth was envisaged through an esterification reaction catalysed in the presence of lipase enzyme. This process intensification strategy on the separation of D lactic acid and its consecutive production of methyl ester was investigated using both Amano CL IM and Amano PS IM enzymes. The enzyme esterification activities were examined by the release of 1 µmol of pNPB which was released in one minute under the specified conditions is counted as one unit (1U) of activity. The reaction was carried for 15 minutes in comparision with blank solution and in duplicate examined independently. The activities of both enzymes are on par with the reported values as 767 $U.g^{-1}$ and 877 $U.g^{-1}$ for Amano CL IM and Amano PS IM

respectively. This is evident from that of the information provided by Amano in Table 5.2 to proceed for further step on solvent engineering.

### 5.3.2. Solvent engineering approach for enzymatic esterification

The production of MDL ester was investigated using both Amano CL IM and Amano PS IM enzyme pre-treated with hydrophobic and hydrophilic solvents. Indeed, it is highly essential to choose the suitable pre-treatment solvent as it is a double-edged sword. If carefully selected, the solvent can promote the enzymatic activity or else it can be detrimental to the enzyme by changing the conformation of the enzyme (Koutinas et al., 2018). Further, as the reactants for the present esterification comprises of methanol, both conventional Amano PS IM enzyme and the novel solvent tolerant Amano CL IM enzyme were applied in the present system and analysed for its performance. Figure 5.1 shows the MDL conversion efficiency noticed in the presence of Amano CL IM and Amano PS IM enzyme pre-treated with tetrahydrofuran, methanol and water. In addition, an experiment was carried out in the absence of either of the lipases to determine MDL conversion arose due to the self-esterification reaction and the same is portrayed in Figure 5.1.

Figure 5.2 shows the entire reaction scheme and output in depicting the transformation of D lactic acid to MDL along with the DLA monomer, dimers and lactide formation in presence of Amano CL IM and Amano PS IM enzyme pre-treated with tetrahydrofuran, methanol and water detected at the end of 18th h. It can be noticed from Figure 5.1 that Amano PS IM has forbidden the esterification reaction, as the high concentration of the methanol (a reactant) acted as a catalytic poison resulting in no MDL formation. This can be evident from Figure 5.2, the formation of 20 – 27.5% of dimers on all the three different solvents pre-treated Amano PS IM clarifies that the Amano PS IM was not poisoned during the pre-treatment stage but only after introducing it into the esterification reaction.

Amano CL IM enzyme used in the present study was derived from a genetically modified organism, which was custom-designed to exhibit tolerance towards the organic solvents. As this being the case, the Amano CL IM enzyme showed remarkable performance in esterifying D lactic acid with the highly concentrated methanol for producing methyl D-Lactate. At the end of 18th h tetrahydrofuran and methanol pre-treated Amano CL IM enzyme resulted in a methyl D-Lactate conversion efficiency of 24.4% and 22.1%, respectively (Figure 5.1). And this conversion efficiency value was further enhanced to 24.8% and 67.6%, respectively at the end of 100th h.

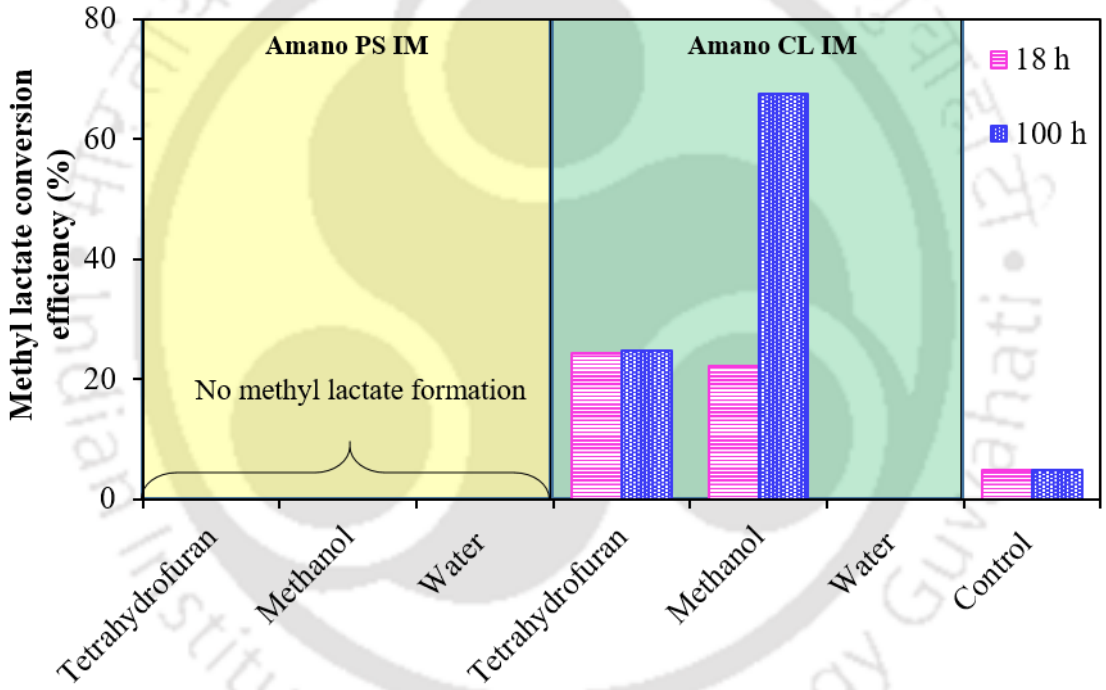



**Figure 5.1** Methyl D-Lactate conversion efficiency of esterification catalysed using Amano PS IM and Amano CL IM enzyme pre-treated with various solvents

Solvent tolerance of the Amano CL IM enzyme before and after pre-treatment can be verified by the intact nature of the enzyme as visualized from the FESEM images in Figure 5.3. Solvent tolerance of the present Amano CL IM enzyme and its consecutive performance in MDL conversion also answers its failure to catalyse esterification reaction after its pre-treatment with water. Control experiments revealed a negligible MDL

conversion efficiency of 4.8% this can be attributed to the equilibrating nature of the present esterification and the same is referred as self esterification reaction [24]

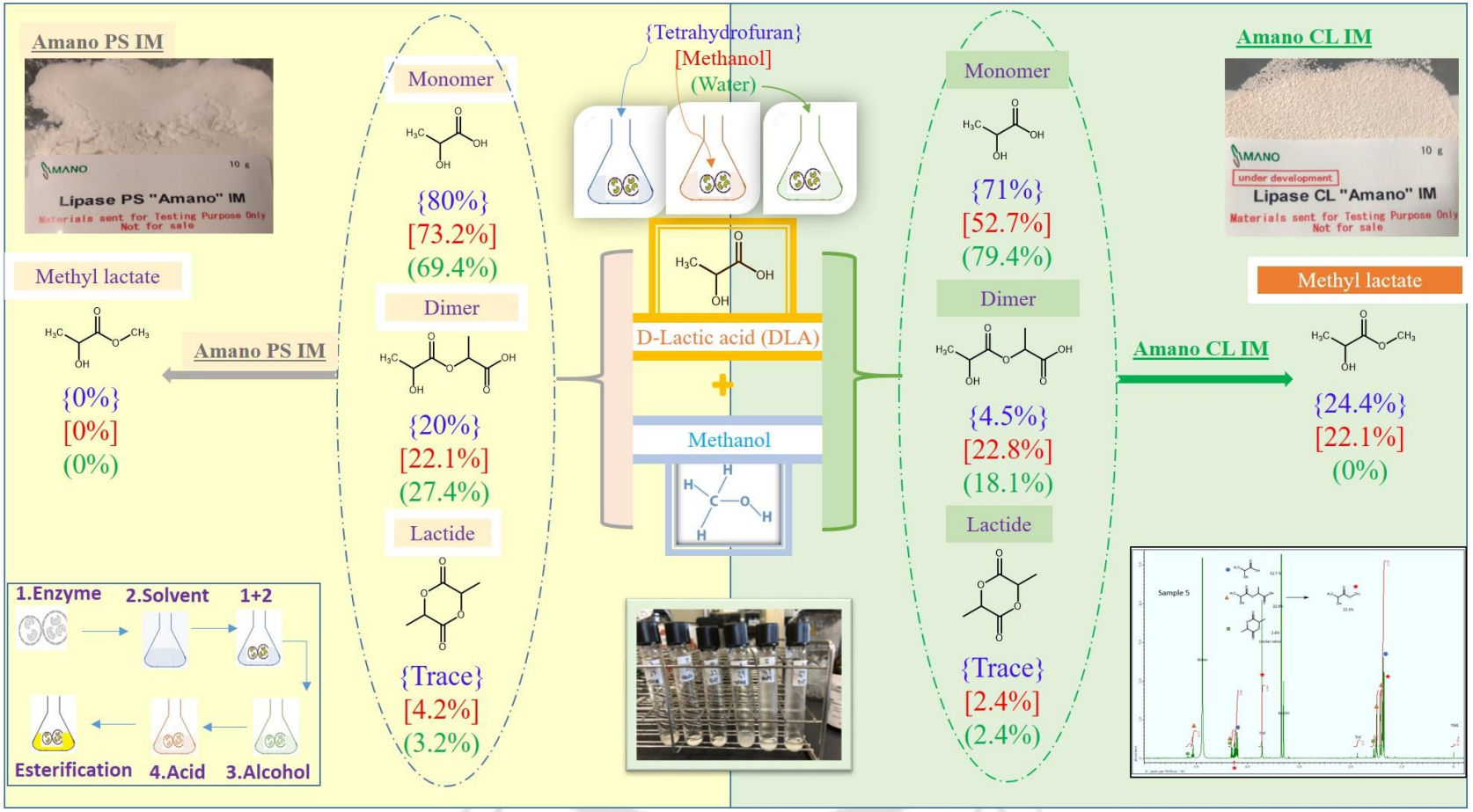



**Figure 5.2** Reaction scheme portraying the conversion of D lactic acid to MDL ester along with monomer, dimer, lactide form by Amano PS IM and Amano CL IM enzyme pre-treated with tetrahydrofuran, methanol and water for the samples drawn at the end of 18$^{th}$ h. { - denotes tetrahydrofuran pre-treatment, [ - denotes methanol pre-treatment and ( - denotes water pre-treatment.

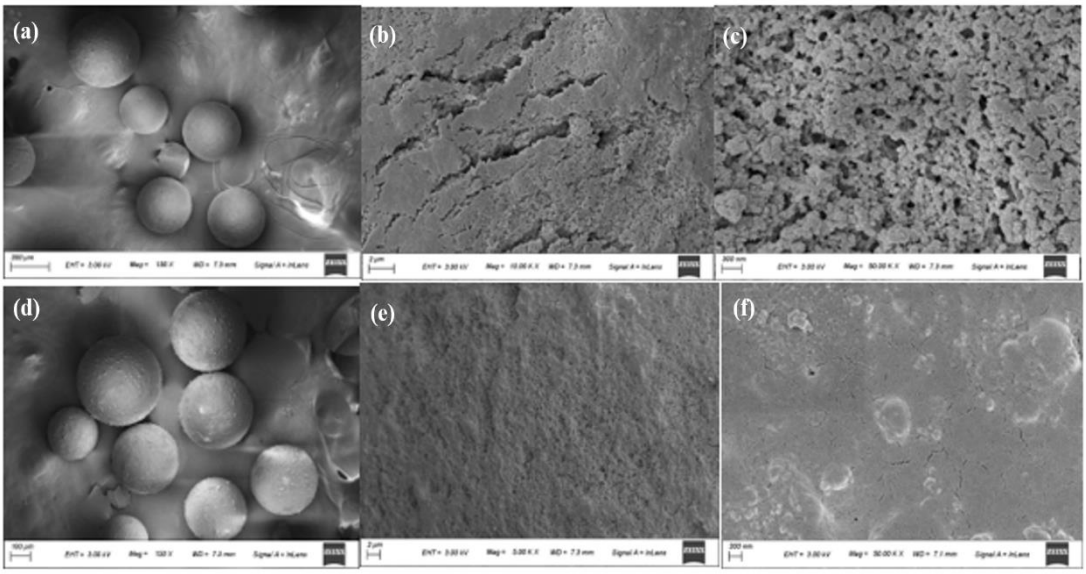



**Figure 5.3** Morphology of Immobilized enzyme (a) (b) (c) are without sovent at start , 18h, 100 h respectively. (d) (e) (f) are with sovent at start , 18h, 100 h respectively

Thus the screening experiments revealed that the Amano CL IM enzyme pre-treated with tetrahydrofuran gave a maximum MDL conversion efficiency.Swiftly after screening the enzyme and suitable solvent for its pre-treatment, MDL conversion efficiency was further enhanced by varying the concentration of methanol - a limiting reactant in the present esterification process. For this optimization, methanol to DLA ratio was varied from 0.5 to 0.9. Figure 5.4 revealed that the enhancement in methanol to DLA ratio from 0.5 to 0.6 resulted in an increase in the MDL conversion efficiency from 21 to 50%. However further increase in the methanol to DLA ratio promoted not the MDL conversion reaction, on the contradictory resulted in a reduced MDL conversion efficiency. Thus, the methanol to DLA of 0.6 was found to be optimal, and the same was considered for further studies. All the investigations carried out previously were conducted by diluting the reactants like methanol and DLA in the tetrahydrofuran. But on a real scenario, DLA is produced in the fermentation broth made of an aqueous solution. Thus to mimic the real scenario, 20% (v. $v^{-1}$) of DLA was diluted in aqueous solution, and this was compared with the previous tetrahydrofuran diluted DLA samples in Figure 5.4.

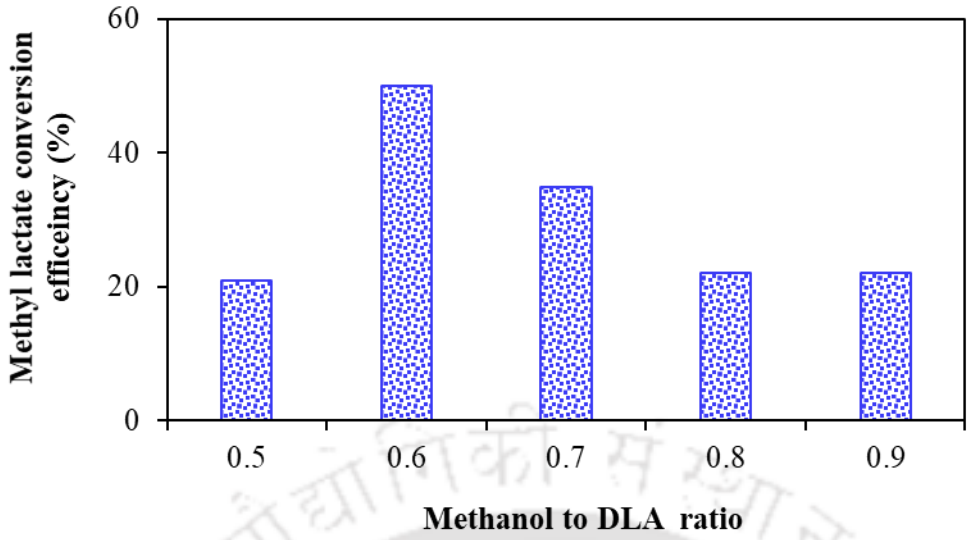



**Figure 5.4** Effect of methanol to DLA ratio on the MDL conversion efficiency

**Table 5.1** MDL Esterification conditions identified by Solvent Engineering approach

| S.No | Enzyme IMM - 50mg | Solvent(100%) | DLA (2.7M) (dil.in THF) | MeOH (3.2M) (dil. in THF) | Result* After 18 h/ 100 h |
|---|---|---|---|---|---|
| 1 | Amano PS IM | 800 µl THF | 100 µl | 100 µl | No Reaction |
| 2 | Amano PS IM | 800 µl MeOH | 100 µl | 100 µl | No Reaction |
| 3 | Amano PS IM | 800 µl Water | 100 µl | 100 µl | No Reaction |
| 4 | Amano CL IM | 800 µl THF | 100 µl | 100 µl | 24.4% / 24.8 % |
| 5 | Amano CL IM | 800 µl MeOH | 100 µl | 100 µl | 22.1% / 67.6% |
| 6 | Amano CL IM | 800 µl Water | 100 µl | 100 µl | No Reaction |
| 7 | -- | 800 µl MeOH | 100 µl | 100 µl | 4.8 % |

*All samples are reacted at 37 $^0$C and 146±2 RPM

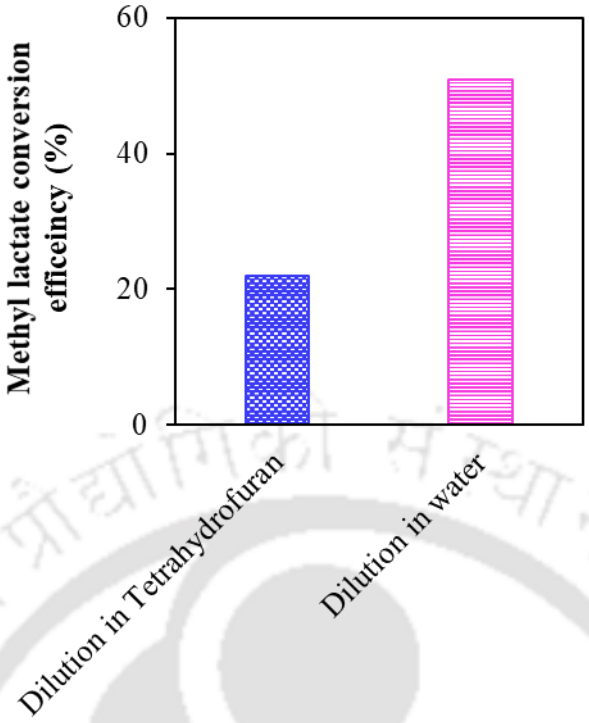



**Figure 5.5** Comparison on the MDL ester conversion efficiency of reactants diluted in tetrahydrofuran and water – mimicking the fermentation broth

MDL conversion reaction for the reactants diluted in aqueous solution was found to be highly favourable in giving more than a double-fold increase in the MDL conversion efficiency. For instance, the reactants diluted in tetrahydrofuran resulted in a MDL conversion efficiency of 22%, but this efficiency was significantly increased to 51% upon diluting the reactants in an aqueous solution. Thus, the present study documented the enormous potential offered by the Amano CL IM enzyme for the MDL using DLA produced in the fermentation broth.

**Table 5.2** Information provided by Amano enzymes about the two enzymes

| Lipase PS "Amano" IM | Lipase CL "Amano" IM |
|---|---|
| Burkholderia cepacia(GMO) | Aspergillus(GMO) derived from Candida antarctica |
| Immobilized on Diatomaceous earth | Immobilized on Diatomaceous earth |
| Optimum pH 7.0 | -- |
| Optimum Temp 50 0C | -- |
| Esterification activity 877 U/g | Esterification Activity 767 U/g |
| Lot No:LPP10511702 IM | Lot No:LCLQ1152102 IMR |
| High reactivity in enantioselective and regioselective synthesis/ Suitable for transesterification and esterification in organic solvents | -- |
| Loss on drying 10.0% , 1 g 105 0C, 4 h | -- |

### 5.4. Discussion

Screening of Amano enzymes and its pre-treatment with various organic solvents was investigated. Optimization of methanol to DLA ratio while mimicking the DLA from fermentation broth was examined for the potential application of the methyl ester production. Preliminary screening experiments revealed the suitability of the novel Amano CL IM enzyme rather than the conventional Amano PS IM enzyme, and this extraordinary performance is obvious because of the solvent tolerance nature of Amano CL IM. Unlike the polar and non-polar solvent that is water and methanol used in the present study, tetrahydrofuran being a neutral solvent played a vital role in pre-treatment of Amano CL IM enzyme. Tetrahydrofuran encapsulating the Amano CL IM enzyme facilitated the transport of D lactic acid, whereby enhancing the enzyme-substrate for better conversion. Methanol being a limiting reactant, retaining its concentration on a higher range than the

concentration of DLA resulted in better conversion efficiency. Higher process efficiency with a slightly higher methanol concentration is regularly noticed in various literature dealing with biodiesel production . Increase in methanol concentration beyond an optimal level reduced the process efficiency [25]. This can be attributed to the obvious reason of reduction in the DLA content – a key reactant in the reaction or its highlights the threshold level of methanol tolerance to Amano CL IM enzyme used in the present study. A double fold enhancement in the process efficiency with reactant diluted in an aqueous in comparison with the reactant diluted in water portrays the extension of the current strategy to the actual fermentation broth containing DLA monomers. Thus, further studies are envisaged in the mere future by applying the present Amano CL IM enzyme for the esterification of real fermentation broth on a simultaneous reactive distillation setup.

### 5.5. Conclusion

A potential process intensification strategy for enhanced production of methyl D-Lactate via a novel Amano CL IM enzyme catalysed esterification process was demonstrated. Due to a high concentration of methanol used in the present process, conventional Amano PS IM enzyme failed and the custom designed solvent tolerant Amano CL IM enzyme performed well. Pre-treatment of Amano CL IM facilitated the enzyme-substrate complex and eventually resulted in a higher methyl D-Lactate conversion. Methanol to DLA ratio played a decisive role in enhancing the methyl D-Lactate conversion efficiency. Final investigation on reactants diluted in aqueous solution disclosed the application potential of the present study.

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Chapter 6

# Conclusion and Future Perspective

# Chapter 6

## Conclusions and Future Perspective

Immense potential exists for cassava cultivation and its waste valorization in all developing countries including India. Cassava being a carbohydrate rich crop has a huge potential to improve the economic structure of a nation if properly executed. With more efforts in R&D, improvements can be made in agricultural and food sector. DLA being one of the versatile organic acids needs more attention from research point of view due to its emerging applications in the polymer industry. The demand for DLA production is rising globally and at the same time we also need to save nonrenewable resources.There is an urgent need to discover more viable feedstock's for the production of DLA by biological means at low price.

The thesis chapters in a nutshell conclude about outcome of imparting process intensification at the different stages of integrated bioprocessing (upstream and downstream processing) of DLA production. At pretreatment stage, the recalcitrant structure of starchy lignocelluloses and its processability is investigated effectively and intensification of continuous enzymatic hydrolysis of CFW was achieved by integration of a novel polymer matrix for enzyme immobilization. The output of this concept provided a ray of hope for largescale processability of bulk hydrolysate catering the need of continuous DLA fermentation. Although there are a number of host organisms available for the production of DLA through fermentation route, Lactic acid bacteria (LAB) can be prioritized for producing lactic acid naturally. The designed biomass approach (DBA) along with inhibition kinetic modelling (process intensification step) resulted in elucidation of robust LAB exhibiting minimal inhibition to feedstock and supporting

enhanced DLA production. DBA studies outcome proved that Lactobacillus delbrueckii subsp. delbrueckii and Sporolactobacillus inulinus were very competent strains and L. delbrueckii was finally selected as elite strain based on kinetic parameters. The solvent engineering approach with biocatalytic esterification strategy for process intensification of DLA purification will be a future game changer if it is adopted to the enzymatic reactive distillation as it will reduce the cost and energy aspects at least by 30% .Overall the strategies can be clubbed in a process flow diagram with economic assessment will ultimately improve the cassava based agro food industries economy. Moreover, the proposed strategies in the thesis work were ecofriendly, economically cheaper and technically viable thereby creating sustainable bioplastic for future generation.

The concept of bio refinery also need to be highlighted for a bright future. DLA production technology need to be developed along with modernization of traditional equipment's being used in the agro food industries. These industries are still emerging and the processing of the huge amount of waste generated is not a viable approach . To improvise this scenario continuous fermentative strategies of DLA which will minimize the salt formation and simplify the purification step. Continuous membrane recycle reactor for high cell throughput, better DLA productivity, in situ recovery DLA to avoid product inhibition. There exists a scope to have real time monitoring strategies for DLA production by using Process Analytical Techniques by Dielectric spectroscopy which monitor viable cells, Exhaust gas analyzer. Development of advanced control algorithms for effective monitoring and control of adopted fermentation process make the process more robust. The future of biobased plastics from second generation feedstock's is inevitable in which integrated approaches play a key role.New government policies have to be made which will promote cassava based industries and will lead to the foundation of more number of bio refineries. Bioprocessing equipment's have to progress for the efficient downstream

processing and marketing of optically pure DLA at much cheaper price. Once the whole manufacturing plan is established and optimized, biorefineries will definitely serve as a stepping stone for commercial scale production.

# List of Publications

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1. **Kiran Kumar Gali**, Narendren Soundararajan, Vimal Katiyar* and Senthilkumar Sivaprakasam*, "Electrospun chitosan coated polylactic acid nanofiber: A novel immobilization matrix for α – amylase and its application in hydrolysis of cassava fibrous waste" **Journal of Materials Research and Technology** - https://doi.org/10.1016/j.jmrt.2021.05.001 **[IF 5.289]**

2. **Kiran Kumar Gali**, Arun EVR, Subbi Rami Reddy Tadi Naresh Mohan, Nivedhitha Swaminathan, Vimal Katiyar* and Senthilkumar Sivaprakasam*, **"**Cost-effective waste valorization of cassava fibrous waste into enantiomerically pure D-lactic acid: Process engineering and Kinetic modeling approach" **Journal of Environment Technology and Innovation.** Vol.22, May **2021,** 101519**.** https://doi.org/10.1016/j.eti.2021.101519 **[IF 3.356]**

3. **Kiran Kumar Gali**, Manickavasagam Murugesan, Subbi Rami Reddy Tadi, Naresh Mohan, Nivedhitha Swaminathan, Vimal Katiyar* and Senthilkumar Sivaprakasam*, "Bioprospecting of cassava fibrous waste as a precursor for stereospecific lactic acid production: Inhibition insights for value addition and sustainable utilization" **Biomass conversion and Biorefinery (2021)** - https://doi.org/10.1007/s13399-020-01272-1 **[IF 2.602]**

4. **Kiran Kumar Gali**, Senthilkumar Sivaprakasam*, Vimal Katiyar*, "Enzyme catalyzed esterification of Lactic acid: Strategic insights to enhance Reaction kinetics in Reactive Distillation" (Ready for submission)

5. **Kiran Kumar Gali,** Aswathy Ramesh, Senthilkumar Sivaprakasam* and Vimal Katiyar*, "Sustainable utilization of North-eastern based Giant Taro flour in Polycaprolactone/Starch composite for biomedical applications". (Ready for submission)

6. Rajulapati, Vikky, Arun Dhillon, **Kiran Kumar Gali**, Vimal Katiyar* and Arun Goyal*. "Green bioprocess of degumming of jute fibers and bioscouring of cotton fabric by recombinant pectin methyl esterase and pectate lyases from Clostridium thermocellum." **Process Biochemistry,** Vol.92, May. **2020**.page:93-104. https://doi.org/10.1016/j.procbio.2020.02.024 **[IF 2.952]**

**Book Chapter (Published/communicated):**

1. **Kiran Kumar Gali**, Payal Mukherjee, Vimal Katiyar and Senthilkumar Sivaprakasam "Process efficacy in Cassava based Biorefinery: Scalable enzymatic process technology for the development of Green Monomer D-lactic acid" in Vol III "Enzymes in Valorization of Waste-CRC Press.

2. **Kiran Kumar Gali,** Purabi Bhagabati, Senthilkumar Sivaprakasam and Vimal Katiyar. "Sustainable Polymers for food Packaging: An Introduction" De Gruyter publishers. https://doi.org/10.1515/9783110648034-001

# List of Conferences

1. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "Integrated bioprocessing strategies for greener bio-economy: A cassava based Agri food Industry waste to sustainable monomer" for poster presentation in Bioprocessing India International symposium on Agri Food resources, BPI-19, during Dec 14-17, 2019 at Central Food Technological Research Institute (CSIR-CFTRI), Mysuru, India **(Bioprocessing India- Best poster award).**
2. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "Process Intensification strategies for optically pure D lactic acid: A Sustainable bio-based monomer production process" for poster presentation in International symposium on Sustainable Polymers, SPSI-19, during Aug 23-25, 2019 at IIT Guwahati, India **(ACS-Best poster award).**
3. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "Sustainable utilization of Cassava based Industry waste for Stereospecific Lactic Acid precursors and its characterization" for oral presentation in National conference for **Technologies to eradicate and control industrial Pollution (TECIP-2016**) during 21-22nd October,2016 organized at Annamalai University, India **(First prize for oral Presentation)**
4. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "The Fermentative Production of Stereospecific Lactic Acid Precursors for Poly (Lactic Acid): A Sustainable Approach to Utilize Cassava based Sago Industry Waste" for oral presentation in Polymers for Sustainable Global Development, POLY-CHAR 2019 during May 19-23,2019 at **Research Centre for Applied Science and Technology, Kathmandu, NEPAL.**
5. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "Sustainable utilization of north-eastern based Giant Taro flour in Polycaprolactone/starch composite" for oral presentation in **Indo Japan Bilateral Symposium (IJBS-17) during Feb, 1-4,2018 at IIT Guwahati, India.**
6. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "Reactive distillation for D-Lactic Acid Purification: A Process intensifying tool for Biorefineries" for poster presentation in Advances in Sustainable Polymers, ASP-17, 4th International conference during Jan 8-11, 2018 at Indian Institute of Technology Guwahati jointly organized by Department of Chemical Engineering of **IIT Guwahati and Polymer Processing Academy, India.**
7. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "Cassava fibrous waste enzymatic hydrolysis and fermentation: A systematic monitoring and control by LabVIEW based interface" for oral presentation in **Starch update-Agbio-2017**, 9th International conference on starch Technology during 27-28th Feb,2017 at Bangkok International trade and Exhibition **Centre organized by BIOTEC and NSTDA of Thailand**.

8. **Kiran Kumar Gali**, Vimal Katiyar and Senthilkumar Sivaprakasam, "Cassava fibrous waste as a sustainable feedstock for fermentable sugars: Characterization and emergy analysis" at 252nd **ACS National Meeting in Philadelphia, USA, Aug 21- 25, 2016**.

9. **Kiran Kumar Gali**, Aswathy Ramesh and Vimal Katiyar, "Extraction of starch from non-edible Taro tuber and its performance evaluation in poly(caprolactone) nanocomposites" for poster presentation in 3rd International Conference on **Khatmandu Symposia on Advanced Materials (KASAM-2016)** during **Oct 17-20, 2016 held in Pokhara, Nepal.**

10. **Kiran Kumar Gali**, Kaushik Bhowmick, Vimal Katiyar and Senthilkumar Sivaprakasam, "Immobilized enzymatic hydrolysis of Cassava fibrous waste for the fermentative production of optically pure D-Lactic acid" for poster presentation in **International Symposium on Advances in Sustainable Polymers held at Kyoto Institute of Technology, Japan during 4-6 Aug, 2016.**