<u>Biomass to lactic acid</u>: Microbial cellulases and their application in cellulosic lactic acid production

Thesis submitted to AcSIR for the award of
the degree of
DOCTOR OF PHILOSOPHY
in
Biological Sciences

By
MAMATA B KHIVASARA
(10BB13J26041)

Research Supervisor
Dr. D. V. GOKHALE

NCIM RESOURCE CENTRE,
NATIONAL CHEMICAL LABORATORY,
PUNE-411008,
INDIA

TABLE OF CONTENTS

CHAPTER 2: STRAIN IMPROVEMENT OF *LACTOBACILLUS LACTIS* NCIM 2368 FOR D-LACTIC ACID PRODUCTION

CHAPTER 3: CELLULASE PRODUCTION BY MUTANTS OF *PENICILLIUM JANTHINELLUM* NCIM 1171 AND ITS APPLICATION IN HYDROLYSIS OF AVICEL AND CELLULOSE

CHAPTER 4: D-(-)-LACTIC ACID PRODUCTION FROM CELLOBIOSE AND CELLULOSE BY *LACTOBACILLUS LACTIS* MUTANT RM2-24

CHAPTER 5: IMPROVEMENT OF ACID TOLERANCE IN *LACTOBACILLUS* THROUGH INTERGENERIC PROTOPLAST FUSION

DECLARATION

This is to certify that the work incorporated in the thesis ***entitled "Biomass to lactic acid: Microbial cellulases and their application in cellulosic lactic acid production",*** *submitted by* ***Ms. Mamata B. Khivasara*** *was carried out under my supervision at NCIM Resource Centre, National Chemical Laboratory, Pune, 411008, Maharashtra, India. Materials obtained from other sources have been duly acknowledged in the thesis.*

Dr. D. V. Gokhale — *(Research Guide)*

Dr. Vidya Gupta — *(Research Co-Guide)*

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the work of the thesis entitled ***"Biomass to lactic acid: Microbial cellulases and their application in cellulosic lactic acid production",*** *submitted for the Degree of Doctor of Philosophy to the AcSIR has been carried out by me at NCIM Resource Centre, National Chemical Laboratory, Pune, 411008, Maharashtra, India, under the supervision of Dr. D. V. Gokhale (Research Supervisor). This work is original and has not been submitted in part or full by me for any other degree or diploma to any other university.*

Ms. Mamata B. Khivasara

(Research Scholar)

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to those who have contributed to this thesis and supported me in one way or the other during this amazing journey.

First of all, I am extremely grateful to my research supervisor, ***Dr. D. V. Gokhale****, for his guidance, patience, motivation and all the useful discussions. His deep insights helped me at various stages of my research. I remain amazed that despite his busy schedule, he was able to go through the final draft of my thesis in very less span of time and commented and suggested on almost every page. He is an inspiration to me.*

I deeply appreciate my Co-guide Dr. Vidya Gupta for her guidance in Molecular characterization studies which has been performed in their laboratory. My special thanks to Dr. A.J. Varma for his support and guidance during the biomass conversion studies.

I am also grateful to Dr. J. M. Khire, Dr. K.B. Bastawde, Dr. U.R. Kalkote, Dr. Mahesh Dharne, Dr. Syed Dastager and Dr. Gayatri Gurjar for their timely help and suggestions.

I would like to convey my sincere thanks to Mr. S. K. Roychoudhuri and Dr. S. M. Kotwal for their constant encouragement throughout this work.

I admire the co-operation shown by lab members like, Mrs. Kavita Bhavsar, Mr. Mukund Adsul, Dr. Nutan, Mrs. Shivani Choudhari, Mrs. Sandhya Sudge, Mrs. Shalaka Gaikaiwari and Mr. Sathish Yadav, during the course of this work.

This acknowledgement would not be complete without thanking all my labmates, Anil, Priyanka, Kumar Raja, Parin, Alfina, Uma, Vidya, Meghana, Rahul, Gajanan, Anupama, Pradnya, Kapil for their co-operation.

I also thank the Director, NCL, for providing the necessary research facilities.

Finally, I would like to acknowledge the most important person in my life – my husband Mr. Sachin. He has been a constant source of strength and inspiration. There were times during the past two years when everything seemed hopeless and I didn't have any hope. I can honestly say that it was only his determination and constant encouragement that ultimately made it possible for me.

ABSTRACT

Rationale and significance

Lignocellulosic substances are abundantly available sources of renewable biopolymer (carbohydrates) for the production of biofuels or other bio-based products. Cellulases are the key enzymes required for the degradation of lignocellulosic polysaccharides into simple monomeric sugars, which are the main 'C' sources for the production of biofuels or other value added products through microbial fermentation. The production of cellulase is a major factor in the hydrolysis of cellulose materials. Unfortunately, high cost of cellulase production is one of the factors, which poses problems in the economics of biofuels and chemicals production from biomass. We have reported the isolation of mutants of *P janthinellum* NCIM 1171 capable of producing enhanced levels of cellulases. These improved strains could be exploited to convert biomass to monomers that can then be diverted to produce value added chemicals like ethanol or lactic acid using SSAF processes. Biomass to ethanol process loses carbon in the form of CO_2 yielding only 50% conversion of biomass to ethanol. On the other hand, production of lactic acid from biomass does not lose any carbon yielding almost 100% conversion of carbon to lactic acid.

Poly lactic acid (PLA) is one of the most promising biodegradable plastics. When PLA is produced from a pure isomer, the product is crystalline and has better stability than polymers obtained from racemic mixtures. Recently, the development of stereo-complex of poly (L-lactic acid) and poly (D-lactic acid) is one of the hot topics in the application of PLA because its melting point (225 ^{o}C) is higher than that of poly (L-lactic acid) (177 ^{o}C). There are many reports on L-lactic acid production by

fermentation with higher productivities and yields. We have already isolated *L. delbrueckii* mutant strain, Uc-3 producing L-lactic acid with high productivity. However, much less attention has been paid to microbial production of D-lactic acid and hence we proposed to screen the in-house microbial strains of *Lactobacillus* which produce D-lactic acid. We also proposed to improve the selected strain for D-lactic acid production with high productivity.

Chapter 1: General introduction and literature search.

This Chapter provides the information on biomass structure, conversion of biomass to sugar, lactic acid production using microbial fermentation, polylactate production by polymerization of lactic acid and biodegradation of polymer. In this chapter, we outline the process of exploiting biomass for the production of polylactic acid, a biodegradable polymer which is well-known as a sustainable bio-plastic material. Lignocellulosic biomass is renewable and cheap, and it has the potential to displace fossil fuels for the production of fuels and chemicals. Biomass derived lactic acid is an important compound that can be used as a chemical platform for the production of a variety of important chemicals on a large scale. The quality of the monomers of lactic acid and lactide, as well as the chemical changes induced during polymerization and processing, are crucial parameters for controlling the properties of the resulting polylactic acid (PLA) products.

<u>Chapter 2</u>: Strain improvement of *Lactobacillus lactis* and optimisation studies for D-lactic acid production.

During screening program, a strain i.e. *Lactobacillus lactis* NCIM 2368 was found to be producing pure D-lactic acid but with very less productivity. This strain was further improved using UV mutagenesis and the improved strain was named as *Lactobacillus lactis* RM2-24. An improved strain was compared with the wild type for D-lactic acid production using 100 g/l of hydrolyzed cane sugar in the fermentation medium. The improved strain RM2-24 produced 81 g/l lactic acid which was over three times that of the wild type. The highest D-lactic acid (110 g/l) in batch fermentation was obtained with 150 g/l of hydrolyzed cane sugar with a 73% lactic acid yield. The mutant utilized cellobiose very efficiently and converted it into D-lactic acid suggesting the presence of cellobiase in the mutant strain. Thus, this strain could be used to obtain D-lactic acid from cellulosic materials that are pre-hydrolyzed with cellulase.

The production of D-lactic acid by *Lactobacillus lactis* RM2-24 was investigated using modified media to increase the efficiency of the fermentation process. The results indicated that the addition of 5 g/l peptone and 1 g/l $(NH_4)_2HPO_4$ enhanced D-lactic acid production by 32%, as compared to that obtained from non-supplemented media, with a productivity of 3.0 g/l/h. We analyzed the fermentation broth for total lactic acid by HPLC, L-lactic acid by L-lactate oxidase. Surprisingly, we found that the broth contained approximately 5% of L-lactic acid. Therefore, we studied lactate dehydrogenase (LDH) expression profile in these different media which resulted in appearance of additional LDH isoform produced by cells when they were grown in

HSYE medium supplemented with $(NH_4)_2HPO_4$. The additional LDH band appeared to be L-LDH contributing to production of L-lactic acid in the fermented broth.

Chapter 3: Comparative production of cellulase by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose.

Mutants of *Penicillium janthinellum* NCIM 1171 were evaluated for cellulase production using both submerged fermentation (SmF) and solid state fermentation (SSF). Mutant EU2D-21 gave highest yields of cellulases in both SmF and SSF. Hydrolysis of Avicel and cellulose were performed using SmF and SSF derived enzyme preparations obtained from EU2D-21. Surprisingly, the use of SSF derived preparation gave less hydrolysis compared to SmF derived enzymes. We studied the thermostability profile of FPase, CMCase and β-glucosidase activites at 50 ^{o}C. We found that the β-glucosidase derived from SSF lost almost 80% of the original activity. Hence the less hydrolysis of the Avicel and cellulose was due to inactivation of inactivation of β-glucosidase derived from SSF. During activity staining, we found that SmF derived enzyme preparations contained both thermo-stable and thermo-sensitive β-glucosidases whereas SSF derived enzyme preparations contained predominantly thermo-sensitive β -glucosidase.

Chapter 4: D-(-)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-24.

Lactobacillus lactis mutant RM2-24 utilized cellobiose efficiently, converting it into D(-) lactic acid. Cellobiose-degrading enzyme activities were determined for whole cells, cell extracts and disrupted cells. Aryl-β-glucosidase activity was detected in

whole cells and disrupted cells, suggesting that these activities are confined to the cells. The mutant produced 80 g/l of lactic acid from100 g/l of cellobiose with 1.66 g/l/h productivity. Production of D-lactic acid from different cellulose samples was also studied. The cellulose samples at high concentration (10%) were hydrolyzed by cellulase enzyme preparation (10 FPU/g cellulose) derived from *Penicillium janthinellum* mutant EU1 generated in our own laboratory. We obtained a maximum 72% hydrolysis, yielding glucose and cellobiose as the main end products. Lactic acid was produced from these cellulose samples by simultaneous saccharification and fermentation (SSAF) in a media containing a cellulase enzyme preparation derived from *Penicillium janthinellum* mutant EU1 and cellobiose utilizing *Lactobacillus lactis* mutant RM2-24. A maximum lactic acid concentration of 73 g/l was produced from a concentration of 100 g/l of bagasse-derived cellulose, the highest productivity and yield being 1.52 g/l/h and 0.73 g/g, respectively. Considering that bagasse is a waste material available in abundance, we propose to use this biomass to produce cellulose and then sugars, which can be fermented to valuable products such as ethanol and lactic acid.

Chapter 5: Improvement of acid tolerance in *Lactobacillus* through inter-generic protoplast fusion.

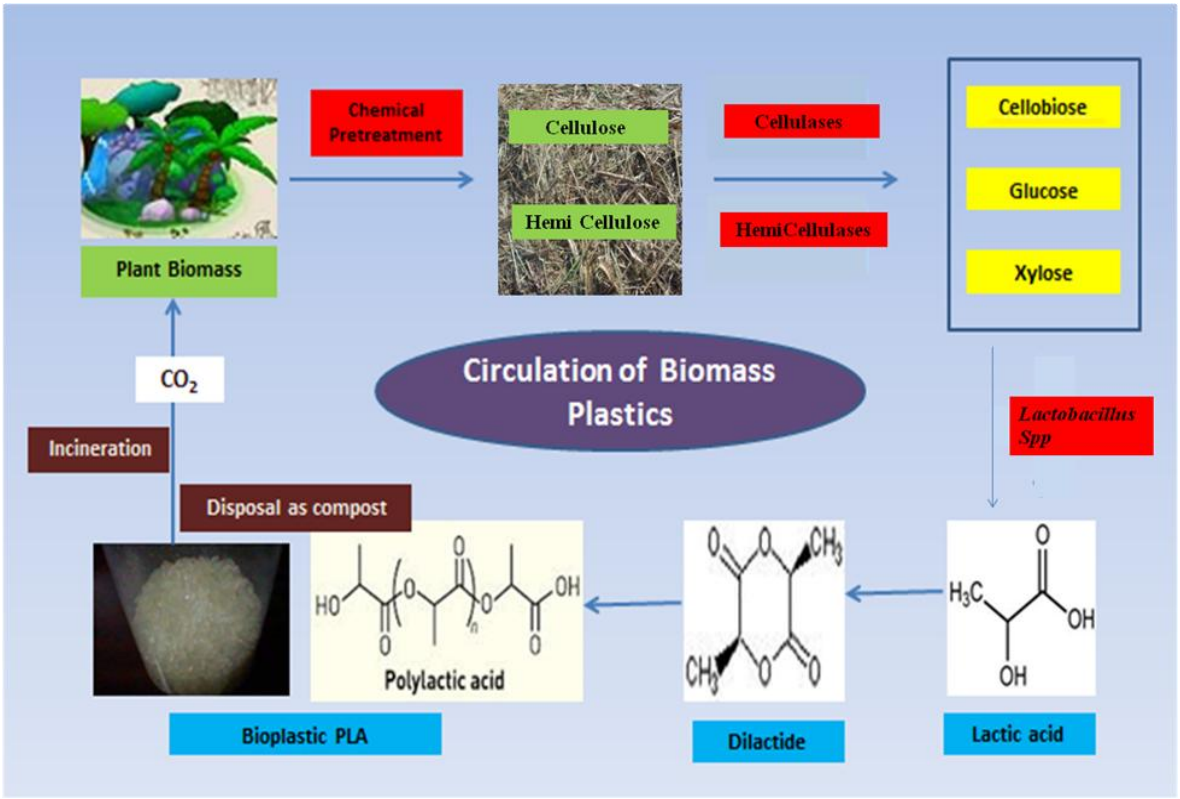
Global warming and environmental problems forced us to develop sustainable processes based on the use of catalysts/biocatalysts that are eco-friendly with least potential toxicity. Scientists all over the world are engaged in exploring/developing such novel, well defined biocatalysts. For rapid development of industrially important microbial biocatalysts (microbial strains), genome shuffling through protoplast fusion is a proficient approach. Fermentation based technologies depend comprehensively on

biocatalyst development for commercialization. Here we describe the application of protoplast fusion technology followed by UV mutagenesis to improve acid tolerance of *Lactobacillus delbrueckii* mut Uc3. Fusant F3 was generated using protoplast fusion between *Lactobacillus delbrueckii* mut Uc3 and *Acetobacter pasteurianus* NCIM 2314 on solid media at pH4.0. Fusant F3 was further treated by UV irradiation to generate a mutant, FM1, with improvements in pH tolerance. This mutant FM1 produced five-fold more lactic acid than that produced by the parent strain at acidic pH. Thus the use of such acid tolerant strains will offer an effective means for designing environmentally benign processes for lactic acid production.

CHAPTER 1

Introduction: Biomass to biodegradable polymer (poly lactic acid)

Graphical Abstract



- Singhvi M., et al (2014) *RSC Advances.* 4, 8271-8277.
- Singhvi M. and Gokhale D. V. (2013) *RSC Advances.* 3, 13558-13568.
- Adsul M. G, Singhvi M. S., et al (2011) *Bioresour. Technol.* 102, 4304-4312.

Biomass, being renewable, is the only sustainable source of energy and organic carbon for our industrial society (Chheda et al., 2007). Nature produces a vast amount of biomass by photosynthesis, 75% of which is assigned to the class of carbohydrates. Surprisingly, only 3–4% of these compounds are used by humans for food and non-food purposes (Roper et al., 2002). Biomass carbohydrates are the most abundant renewable resources available, and they are currently viewed as feedstock for the green chemistry of the future (Lichtenthaler and Peter, 2004). The production of fuels and chemicals from biomass is beneficial concerning an environment which is associated with the reduction of the net emissions of CO_2 (a greenhouse gas) into the atmosphere. In contrast to fossil fuels, biofuels are considered to be carbon neutral because any CO_2 produced during fuel combustion is consumed by a subsequent biomass re-growth (NSF, 2008). A biomass based energy system would improve the economy of those countries determined to accept the challenges. In addition, the use of the lignocellulosic biomass (LB) does not affect the food supplies, thereby permitting a sustainable production of fuels (so-called second-generation fuels) and chemicals. Additionally, the lower cost and faster growth of the LB compared with food crops (Klass, 2004) and its ample availability (Huber et al., 2006) makes this resource an attractive raw material suitable for the substitution of fossil fuels.

Lactic acid (2-hydroxypropionic acid), CH_3–CHOHCOOH, is the most widely occurring hydroxyl-carboxylic acid in nature. Lactic acid is frequently used in the food industry, especially in beverage production and in the pharmaceutical and chemical industry, or in medicine (Vick Roy, 1985). Because lactic acid has both carboxylic and hydroxyl groups, it can also be converted into different and potentially useful chemicals

such as pyruvic acid, acrylic acid, 1,2-propanediol and lactate esters (Fan et al., 2009). The recent growing interest for the manufacture of biodegradable plastic necessitates a high demand for lactic acid as the raw material for PLA production (Sodergard and Stolt, 2002). Another very promising lactic acid application is the production of environmentally friendly ‘‘green’’ solvents (lactate esters) which can replace traditional solvents made from petrochemical feedstock (Yang et al., 2012). Lactic acid exists as two optical isomers. One is known as L-(+)-lactic acid or (S)-lactic acid, and its mirror image is D-(-)-lactic acid or (R)-lactic acid. Among the two isomers, L-(+)-lactic acid is the biologically important isomer.

Poly (lactic acid) is one of the most promising biodegradable plastics (Sakai et al., 2004). Much research effort is currently focused on the modifications of polylactide to make it suitable for a wider range of applications. Optically pure lactic acid is necessary to obtain high crystalline poly (lactic acid) which leads to the high strength, chemical and heat resistance properties of the polymer (Lunt, 1998). In this chapter, we focus on the utilization of the LB for the production of PLA, a biodegradable polymer. Environmental, economic and safety challenges have pushed towards the partial replacement of petrochemical-based polymers with bio-based ones.

BIOMASS STRUCTURE

Lignocellulose, a carbohydrate source, is an interesting raw material for biotechnological processes, owing to its renewable character, widespread distribution, abundance and low price. LB (plant biomass) is a prodigious potential resource for the production of fuels and chemicals because it is abundant, inexpensive and the

production of such resources is environmentally sound. Agricultural residues are a great source of LB which is renewable, mainly unexploited and inexpensive. Such resources include: leaves, stems and stalks from corn fiber, corn stover, sugarcane bagasse, rice hulls, woody crops and forest residues. Also, there are multiple sources of lignocellulosic waste from industrial and agricultural processes, e.g. citrus peel waste, sawdust, paper pulp, industrial waste, municipal solid waste and paper mill sludge (Maki et al. 2009). The abundance of LB pinpoints to the need and potential for efficient, cost effective processes which convert it into those value added chemicals presently obtained from non-renewable resources such as fossil fuels.

Biomass is a mixture of carbohydrate polymers (cellulose, hemicellulose and pectin, to varying degrees) and the non-carbohydrate polymer lignin. Cellulose consists of long micro-fibrils containing repeating units of cellobiose, which is dimer of glucose molecules. These hydrogen-bonded micro-fibrils may be quite long, up to 14,000 glucose units as observed in Arabidopsis, corresponding to a fibril length of 7 mm (Sommerville et al., 2004). Cellulose has strong physico-chemical interaction with hemicelluloses and lignin. Cellulose, a linear glucose polymer, is highly ordered polymer of cellobiose (D-glucopyranosyl-β-1,4-D-glucopyranose) representing about 50% of the wood mass. Native cellulose has about 10,000 glycosyl units in the cellulose chain that form fibrils which are stabilized by strong intermolecular hydrogen bonds between hydroxyl groups of the adjacent molecules. Cellulosic materials have crystalline domains separated by less ordered, amorphous, regions. These amorphous regions are the potential points for chemical and enzymatic attacks. The crystalline cellulose is highly resistant to chemical and enzymatic hydrolysis due to its structure in

which chains of cellodextrins are precisely arranged. Cellulose is degraded by acid or enzymes known as cellulase to its monomer, glucose, that is fermented further to commodity chemicals such as ethanol, lactic acid and succinic acid (Figure 1.1).

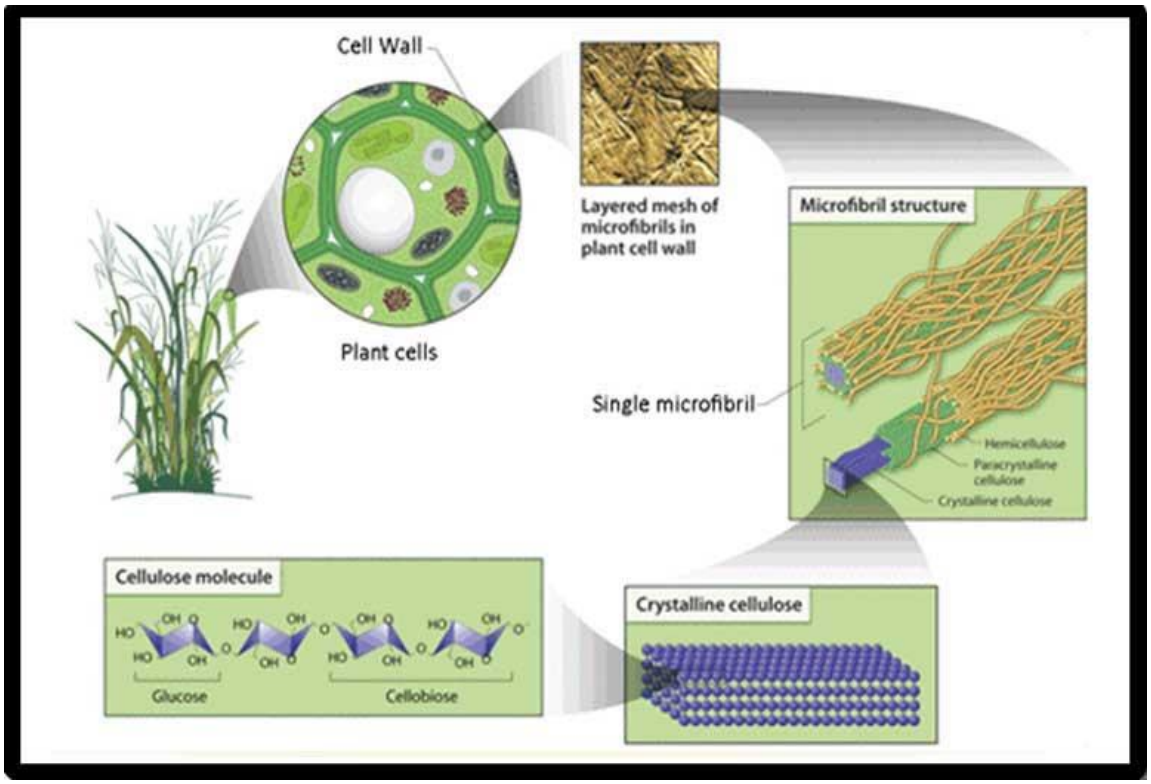


Figure 1.1: Structural organization of the plant cell wall

Hemicellulose is short, highly branched hetero-polymer of mainly xylose, plus glucose, mannose, galactose and arabinose and sometimes uronic acids. Hemicelluloses are referred to as mannans, xylans or galactans depending upon the predominance of type of sugars present in the polymer. The C5 and C6 sugars that are linked through 1,3; 1,6, and 1,4 glycosidic bonds and often acetylated, form a loose, very hydrophilic structure acting as a glue between cellulose and lignin. Hemicelluloses are branched

polysaccharides with backbones of neutral sugars hydrogen-bonded to cellulose. Hemicellulose is a heterogeneous polymer, which varies in composition from plant to plant and also within different parts of the same plant. In hardwoods, hemicellulose contains mainly xylans, while in softwood mainly glucomannans are present. The hydrolysis of hemicellulose requires various types of enzymes (such as xylanase, mannanase, etc.). Briefly, xylan degradation requires endo-1-4,-β-xylanase, β-xylosidase, α-glucuronidase, α -L-arabinofuranosidase, as well as acetyl xylan esterases. In glucomannan degradation, β –mannanase and β -mannosidase are required to cleave the polymer backbone. Pectins are the cement that hold the plant cell walls together and are defined by the presence of uronic acids.

Lignin is a polymer of three closely-related phenyl propane moieties (Mishra and Singh, 1993). It is a highly cross-linked polymer built up of substituted phenols and, together with cellulose and hemicellulose, it gives strength to plants. Lignin is made up of phenyl propanoid units derived from the corresponding *p*-hydroxycinnapyl alcohols. These phenylpropanoid units are made up of dimethoxylated (syringyl), monomethoxylated (guaiacyl) and nonmethoxylated (*p*-hydroxyphenyl) alcohols. Lignin is hydrophobic and highly resistant to chemical and biological degradation. It is present in the middle lamella and acts as cement between the plant cells. It is also located in the layers of the cell walls, forming, together with hemicelluloses, an amorphous matrix in which cellulose fibrils are embedded and protected against biodegradation. The processing of lignocellulosic biomass will make the lignin enormously available for conversion into value added products, rather than its fuel value. The partially hydrolyzed lignin has excellent properties for use as substitutes for

phenol-formaldehyde resins, polyurethane foams, adhesives, insulation materials, rubber processing, antioxidants, etc. It also provides a cheap source for vanillin and syringol for the flavor and fragrance industry. Plants are also able to store energy in products such as lipids, sugars and starch, as well as other products relatively rich in hydrogen and carbon (terpenes). Terpenes are found in essential oils that are components of resins, steroids, and rubber. This association between cellulose, hemicelluloses and lignin makes the plant cell wall resistant to mechanical and biological degradations (Figure 1.2). The processing of LB has to include the conversion of lignin to value added products in addition to its use as fuel in order to economically transform the biomass into useful chemicals.

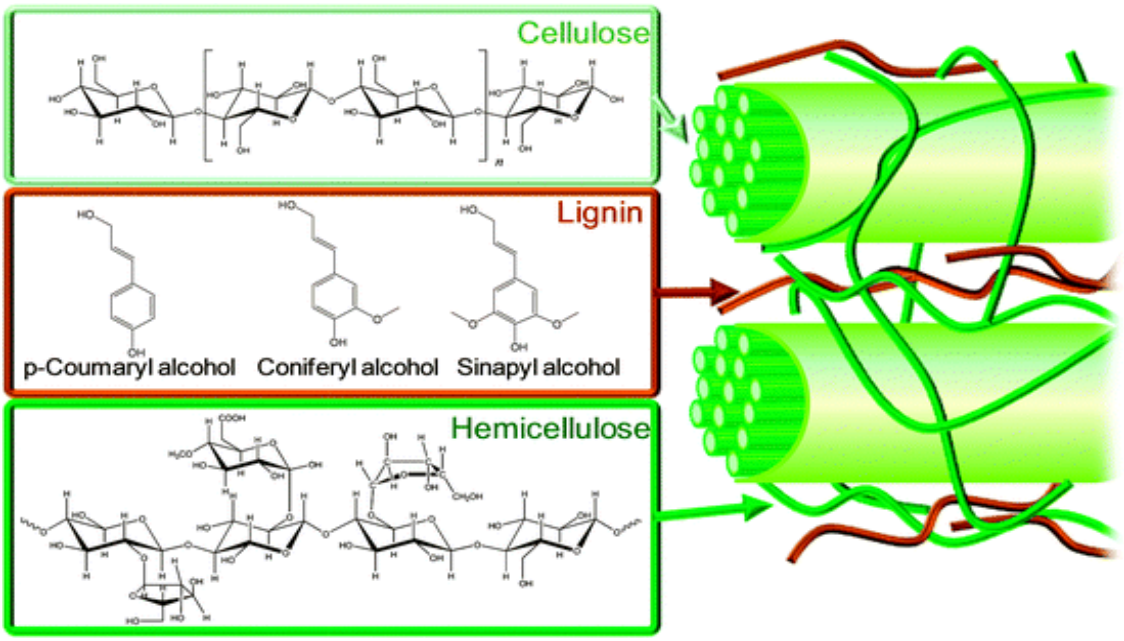


Figure 1.2: Structure of lignocellulosic biomass

CONVERSION OF BIOMASS TO SUGAR

The biotechnological conversion of LB is a potentially sustainable approach to develop novel bioprocesses and products. The complex structure of lignocellulose, with its highly crystalline structure protected by lignin, confers this material with a high degree of recalcitrance that makes its de-polymerization a difficult task (Mosier et al., 2005a). There are four main consecutive steps involved in production of chemicals from lignocellulosic substrates: pretreatment, hydrolysis, fermentation and separation. Due to its low bio-accessibility, a number of chemical and physical methods, such as acid or base treatments and steam explosion, have been developed and used to hydrolyze lignocellulosic materials to produce oligosaccharides, prior to fermentation by microorganisms. Biological and biochemical pretreatment methods for the conversion of cellulosic materials into sugars also appear to be attractive alternatives from both economic and environmental viewpoints. The major challenges in biomass conversion are: the relatively low rate of hydrolysis, the high cellulase costs and the little understanding of cellulase kinetics on lingo-cellulosic substrates. Two main approaches have been developed in parallel for the conversion of lignocellulosic materials to commodity chemicals- ‘‘acid based’’ and ‘‘enzyme based’’ (Hahn-Hagerdal et al., 2006). Consequently, the deconstruction of lignocellulose requires an effective pretreatment step to break the lignin protection which makes it more accessible to acids or enzymes resulting in a complete hydrolysis of cellulose and hemicellulose fractions of the biomass. The morphology of the lignin network is modified in aqueous solutions at mild temperatures, which allows the hydrolysis of hemicellulose to occur under the same conditions in the presence of acids. The crystalline structure of cellulose protects

its β-glycoside ether linkages from being accessed by the acid catalyst, so more severe conditions are required for a complete deconstruction of this polymer (Lange, 2007). Several pretreatments involving physical, chemical and biological methods have been developed to depolymerize lignocellulosic materials (Kumar et al., 2009a). Table 1.1 summarizes several advantages and disadvantages of the pretreatment processes involved in the conversion of LB. Recently, the effects of these pretreatments on the morphology and structure of the biomass have been studied (Kumar et al., 2009b). In the acid catalyzed pretreatment, the major part of the hemicellulose is degraded while in the alkali-catalyzed pretreatment part of the lignin is removed (Kovacs et al., 2009). This pretreated cellulose is further hydrolyzed using cellulase enzymes. Very recently, Ding et al. (2012) reported the use of correlative imaging in real time to assess the impact of pretreatment as well as the resulting nanometer scale changes in the cell wall structure upon digestion by the cellulase systems. Very recently, Sathitsuksanoh et al (2014) used non-toxic and recyclable ionic liquid, [C2mim][OAc], for pretreatment of wheat straw, Miscanthus, and Loblolly pine followed by enzymatic hydrolysis of the treated material. They found that ionic liquid pretreatment process conditions play a major role in obtaining lignin structures suitable for its valorization thereby improving the economics of bio-refinery. The developments in the use of solid acid (Rinaldi et al., 2010) in biomass hydrolysis may also establish the efficient and economically viable process for biomass hydrolysis in near future. From these studies, it was concluded that the complete/maximum removal of lignin from the biomass without modifying the native microfibrillar structure of carbohydrates could be the ideal pretreatment for

enhancing the digestibility of the biomass. The production of cellulase is another major factor in the hydrolysis of cellulose materials.

Table 1.1: Various processes used for pretreatment of lignocellulosic biomass

Pretreatment process	Advantages	Limitations and Disadvantages	Reference
Mechanical comminution	• reduces both the degree of polymerization (DP) and cellulose crystallinity • increases the available specific surface area	• power consumption usually higher than inherent biomass energy	Zhua et al., 2009
Steam Explosion	• causes hemicellulose degradation and lignin transformation • makes limited use of chemicals • requires low energy	• destruction of a portion of the xylan fraction • incomplete disruption of the lignin-carbohydrate matrix	Chandra et al., 2007
AFEX	• Ammonia pretreatments have a high selectivity for reaction with lignin • does not produce inhibitors for downstream processes • the ability to reduce, recover and recycle the ammonia used in both AFEX/ARP makes the process economically viable	• not efficient for biomass with high lignin • the cost of ammonia basically drives the process and its application on large scale • environmental concerns with the stench of ammonia also have a negative impact on pilot as well as industrial scale applications.	Galbe and Zacchi, 2007
Ionic Liquids (ILs)	• environmentally friendly • nonderivatising, nonvolatile, thermostable. single component solvent for cellulose with potential applications in cellulose fractionation and dissolution in the IFB.	• High cost • Poor biodegradability • Toxic to microrganisms	Zhang and Lynd, 2006

Table 1.1 continues			
Pretreatment process	**Advantages**	**Limitations and Disadvantages**	**Reference**
CO2 explosion	• increases accessible surface area • cost-effective • does not cause formation of inhibitory compounds	• does not modify lignin or hemicelluloses	Hendricks and Zeeman, 2009
Ozonolysis	• reduces lignin content • does not produce toxic residues	• large amount of ozone required • expensive	Quesada et al., 1999
Acid hydrolysis	• hydrolyzes hemicellulose to xylose and other sugars • alters lignin structure	• high cost • equipment corrosion • formation of toxic substances	Mosier et al., 2005b
Alkaline hydrolysis	• removes hemicelluloses and lignin • increases accessible surface area	• long residence times required • irrecoverable salts formed and incorporated into biomass	Wyman et al., 2005
Organosolv	• organosolv lignin is sulfur free with high purity and low molecular weight. • can be used as fuel to power pretreatment plant or further purified to obtain high quality lignin which is used a substitute for polymeric materials • very effective for the pretreatment of high-lignin lignocellulose materials	• solvents need to be drained from the reactor, evaporated, condensed, and recycled • high cost • generation of compounds inhibitory to microorganisms	Zhang, 2008
Pyrolysis	• produces gas and liquid products	• high temperature • ash production	Zwart and Boerrigter, 2006
Hot water Treatment	• lower temperatures minimizing the formation of degradation products • eliminates the need for a neutralisation	• down-stream processing is more energy demanding because of the large volumes of water involved.	Mosier et al., 2005a
Biological	• degrades lignin and hemicelluloses • low energy requirements	• rate of hydrolysis is very low	Magnusson et al., 2008

CELLULASE PRODUCTION

Cellulase refers to the class of hydrolytic enzymes (O-glycoside hydrolases, EC.3.2.1-) which hydrolyze the β-1,4 linkage in the cellulose. Cellulases are mainly produced by Fungi and bacteria. Cellulase is a multi-enzyme system composed of several enzymes with numerous isozymes, which act in synergy. The basic enzymatic process for the de-polymerization of cellulose requires three types of enzymes.

i) Endoglucanase (endo-1,4-β-D-glucanases, EC 3.2.1.4): It hydrolyses the internal β-1,4-glucan chain of cellulose at random, primarily within the amorphous regions and displays a low hydrolytic activity towards crystalline cellulose.

ii) Exoglucanase (exo-1,4-β-D-glucanases, EC 3.2.1.74): It is exo-acting cellobiohydrolase (CBH), which removes cellobiose from the reducing (CBH1) and non-reducing end (CBHII) of cellooligosaccharide and crystalline, amorphous and acid or alkali treated cellulose.

iii) β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21): It hydrolyses cellobiose to yield two molecules of glucose which completes the de-polymerization of cellulose (Himmel et al., 1994).

Cellulases have been used for several years in food processing, feed preparation, wastewater treatment, detergent formulation, textile production and in many other areas. Nevertheless, the requirement of cellulase for such uses is small compared to cellulase requirements for the bioconversion of the LB to fuel ethanol. The insoluble recalcitrant nature of cellulose represents a challenge for cellulase systems. Cellulases are composed of an independent folding structure and functional discrete units called

domains or modules (Henrisaat et al., 1998). A general feature of most cellulases is a modular structure often including both catalytic and carbohydrate-binding modules (CBMs). The CBM effects the binding to the cellulose surface, presumably to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the insoluble cellulose substrate. The presence of CBMs is particularly important for the initiation and processivity of exoglucanases (Teeri, 1998). Cellulase systems exhibit a higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism. Structurally fungal cellulases are simpler compared to bacterial cellulase systems known as "cellulosomes" (Zhang et al., 2006). Improvement in the cellulase production as well as the hydrolysis of cellulosic substrates can make the process more cost effective (Himmel et al., 2007). Consequently it is necessary to understand the enzyme substrate interactions and both identify and quantify the contribution of various system properties to the hydrolysis process. The high cost of cellulase production is a major bottleneck in the conversion of biomass to any value added product. Hence, efforts are needed to produce cellulase at an affordable cost which can be used for hydrolyzing the biomass to monomers with high economical potential. Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation. The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa. However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrates in particular (Lynd et al., 2002). One of the most extensively studied fungus is *Trichoderma reesei*, which converts native as well as derived

cellulose to glucose. Besides *Trichoderma reesei*, other fungi, like *Humicola*, *Aspergillus* and *Penicillium,* have the ability to secrete extracellular cellulase. Fungal cellulases are being commercially produced for biomass saccharification. The economic viability of biomass conversion depends on the pretreatment of the substrates and the cost of the enzyme. The need for lower costs triggered a search for high cellulase producing organisms using classical mutagenesis, genetic engineering and enzyme engineering techniques that included advanced biotechnological procedures, such as directed evolution and rational design studies (Adsul et al., 2011). These improved enzyme preparations are expected to possess desirable properties such as higher catalytic efficiencies, increased stabilities at elevated temperatures and higher tolerance to end product inhibition. Improvement in cellulase activities by imparting desired features to enzymes by protein engineering is probably another area where cellulase research has to advance. Previously we reported cellulase production by *P. janthienllum* NCIM 1171 using bagasse as the carbon source and its application in bagasse hydrolysis (Adsul et al., 2004; Adsul et al., 2005). The mutants of *P. janthinellum* NCIM 1171 capable of producing enhanced levels of cellulase using submerged fermentation have been isolated (Adsul et al., 2007a), which also exhibited high levels of filter paper degrading activity in solid state fermentation (Adsul et al., 2009a). By using cellulase producing improved strains and suitable pretreatments, LB can be converted into sugars which would be diverted to produce value added products like ethanol, butanol succinic acid, lactic acid, etc.

In anaerobic microorganisms, the enzyme system involves the formation of a large, extracellular enzyme complex called the cellulosome. The cellulosome can be

described as one of the nature's most well-designed and highly structured machines. To overcome the recalcitrant nature of the plant cell wall, the cellulosome multicomponent system can serve as a key biological macromolecule. One of the major challenges of biomass degradation is that the required hydrolyzing enzymes have high costs, low hydrolysis rates and less efficient enzyme cocktails. The designer cellulosome approach would be beneficial for reduction of the cost and the efficient degradation of lignocellulose. Designer cellulosome technology can overcome the recalcitrant properties of lignocellulosic substrates and thus increase the level of enzymatic degradation of treated or native lignocellulose. Recently, Morais et al (2012) have integrated six dockerin-bearing cellulases and xylanases from the highly cellulolytic bacterium, *Thermobifida fusca*, into a chimeric scaffolding, engineered to bear a cellulose binding module and the appropriate matching cohesion modules. The resultant hexavalent designer cellulosome was fully functional and achieved enhanced levels (up to 1.6-fold) of untreated wheat straw degradation compared to those of the wild-type free enzymes. Unfortunately, the de-crystallization of biomass by enzymatic processes is very critical and not yet well understood. Considerable research and development inputs are necessary to look for untapped microbial sources for new enzymes with better performances. The knowledge-based protein engineering may also be a better approach to developing improved cellulase enzyme cocktails. *Fibrobacter succinogenes*, an anaerobe, and *Cytophaga hutchinsonii*, anaerobic soil bacterium, produce cellulases that are devoid of CBM and hence remain bound to cellulose during growth and allow their enzymes to act on it (Wilson, 2011). Another marine bacterium, *S. degradans* produces an unusual set of cellulases and processive endoglucanases, but

the exact mechanism for degradation of cellulose by this organism is not clear (Watson et al., 2009).

Hemicellulose conversion to fermentable sugars is mediated by hemicellulases containing a myriad of enzymes with different catalytic functions. These include endoxylanases, exoxylanases and β-xylosidases along with esterases releasing acetyl and ferulic acid groups. These enzymes show significant synergy between themselves as well as with other lignocellulose degrading enzymes (Gusakov, 2011). Some of the microorganisms possessing cellulases also have the ability to degrade hemicellulose. It is known that *Trichoderma* and *Penicillium* strains have efficient hemicellulolytic enzymes. In addition, *Aspergillus spp*. have also been identified for their capability to produce hemicellulose degrading enzymes. Specific xylanases capable of producing xylo-oligosaccharides (XOS) from xylan could add new dimensions to the economics of hemicellulose (biomass) conversion to value added products (Adsul et al., 2009b). XOS are most popular as dietary fibers or functional foods since they act as prebiotics which stimulate the growth and/or activity of one or a limited number of bacteria in the colon (*Bifidobacterium* and *Lactobacilli*) by suppressing the activity of entero-putrefactive and pathogenic organisms and also facilitate the absorption of nutrients. Apart from prebiotics and bulking agents, it is also employed in cosmetics as stabilizers, immune-stimulating agents and antioxidants and in pharmaceuticals. It is assumed that efficient extraction and conversion of hemicellulose sugars is required for developing economically viable biomass conversion technologies. The total processing of the individual components in biomass is shown in Figure 1.3.

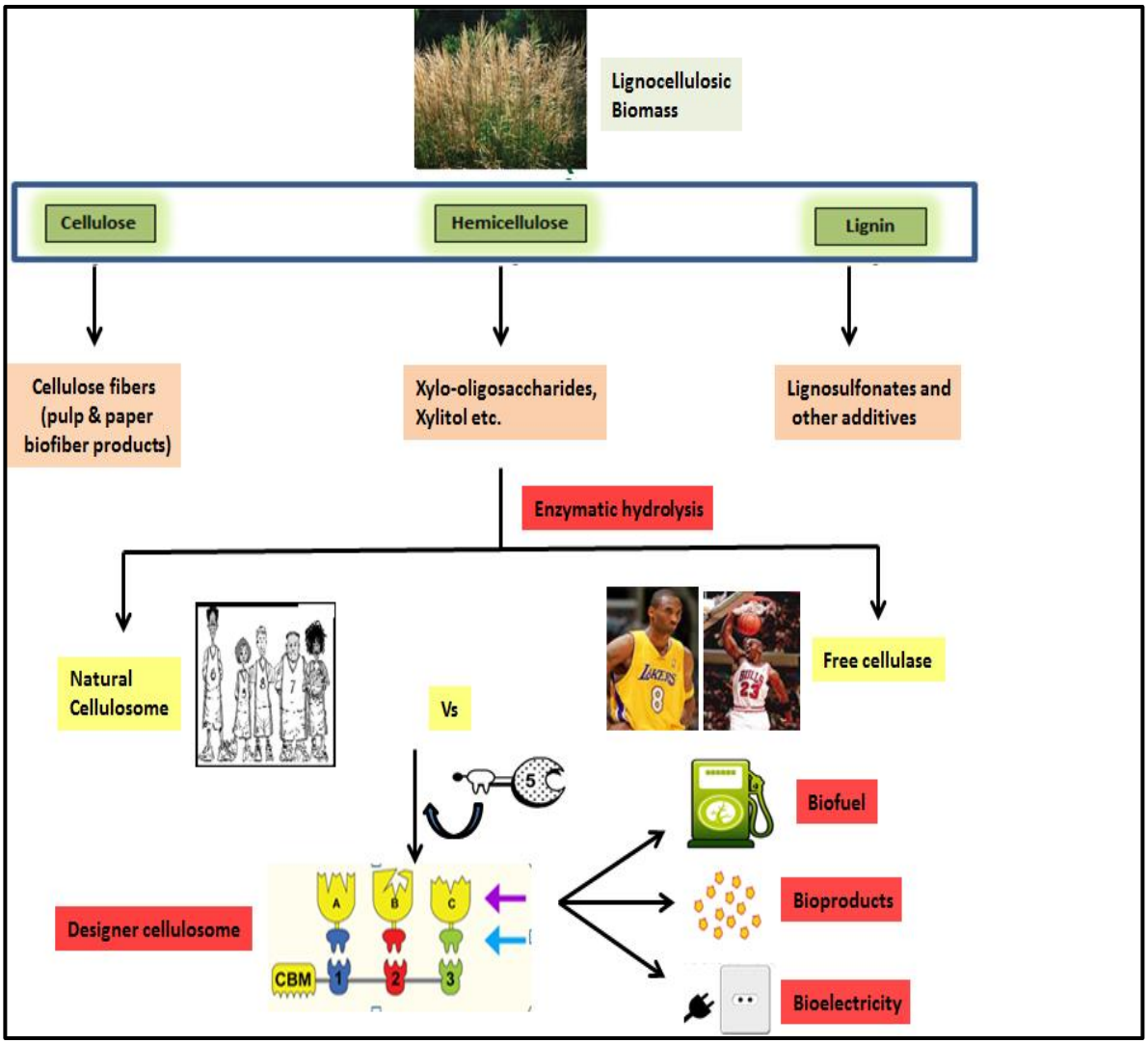


Figure 1.3: The total processing of the individual components of biomass

LACTIC ACID PRODUCTION

Lactic acid production by chemical reaction

Lactic acid can be manufactured by either chemical synthesis or fermentation. The commercial process for chemical synthesis is based on lactonitrile which is synthesized by addition of hydrogen cyanide to acetaldehyde in the presence of a base.

This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered and purified by distillation. It is then hydrolyzed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and lactic acid. Lactic acid is then esterified with methanol to produce methyl lactate before being purified by the means of distillation, and is hydrolyzed by water under acid catalyst to produce lactic acid and methanol. The chemical synthesis method produces a racemic mixture DL-lactic acid. This process of lactic acid synthesis is represented by the following reactions (Narayanan et al., 2004).

(a) Addition of Hydrogen Cyanide

$$CH_3CHO + HCN \xrightarrow{\text{Catalyst}} CH_3CHOHCN$$

Acetaldehyde Hydrogen cyanide Lactonitrile

(b) Hydrolysis by H_2SO_4

$$CH_3CHOHCN + H_2O + \tfrac{1}{2}H_2SO_4 \longrightarrow CH_3CHOHCOOH + \tfrac{1}{2}(NH_4)_2SO_4$$

Lactonitrile Sulphuric acid Lactic acid Ammonium salt

(c) Esterification

$$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$$

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H_2O

$$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$$

Methyl lactate Lactic acid Methanol

Lactic acid production by microbial fermentation

Compared to chemical synthesis, the biotechnological process for lactic acid production offers several advantages, such as low substrate costs, reduced production temperature and less energy consumption (Datta and Henry, 2006). Although DL-lactic acid is always produced by chemical synthesis, an optically pure L(+)- or D(-)-lactic acid can be obtained by microbial fermentation when the appropriate microorganism is selected (Hofvendahl and Hahn-Hagerdal, 2000). The majority of the world's commercially produced lactic acid is made by the bacterial fermentation of carbohydrates, using homo-lactic organisms belonging to the genus *Lactobacillus*, which exclusively produce lactic acid. The organisms that predominantly produce the L(+)-isomer are *Lactobacillus amylophilus, L. bavaricus, L. casei, L. maltaromicus* and *L. salivarius*. Strains such as *L. delbrueckii, L. jensenii* or *L.acidophilus* produce either the D-isomer or mixtures of both. These strains show high carbon conversions from feedstock under standard fermentation conditions, such as neutral pH, temperatures around 40 ^{o}C and low oxygen concentrations.

The optical purity of lactic acid is crucial to the physical properties of poly (lactic acid) (PLA). An optically pure L(+)- or D(-)-lactic acid can be polymerized to a highly crystalline PLA that is suitable for commercial use. Therefore, the biotechnological production of lactic acid has received a significant amount of importance recently, since it offers an alternative to environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources. Lactic acid producing organisms, most of which are anaerobic, utilize pyruvic acid, which is the end product of the Embden–Meyerhof pathway. The conversion of pyruvic acid to

lactate can be effected by either of the two enzymes, L-lactate dehydrogenase (LDH) or D-lactate dehydrogenase (LDH). Lactic acid is produced by oxidizing NADH (nicotinamide adenine dinucleotide) generated during glycolysis with pyruvate as the electron acceptor.

Lactic acid production using food carbohydrates

Raw material cost is one of the major factors in the economic production of lactic acid. Pure sugars or edible crops have been traditional substrates for lactic acid production. The use of pure sugars or edible crops results in obtaining pure lactic acid which lowers the costs of pretreatment and recovery. However, these substrates cannot be used since they impact on human food chain. Recently, lactic acid has been produced from a variety of carbohydrates, including starchy and lignocellulosic biomasses, depending on the substrate availability in the producing country (Vink et al., 2007). The major homo-fermentative LAB used in the lactic acid production from different carbon sources are *Lactobacillus delbrueckii* (Kadam et al., 2006), *L. helveticus* (Tango and Ghaly, 2002) and *L. casei* (Rojan et al., 2005). Some of the homo-fermentative bacteria, like *L. amylophilus*, *L. manihotivorans* can directly consume complex carbohydrates like starch (Naveena et al., 2005). Amylolytic bacteria *Lactobacillus amylovorus* ATCC 33622 is capable of converting liquefied corn starch to lactic acid with a productivity of 2.0 g/l/h (Zhang and Cheryan, 1991). Most studies on lactic acid fermentation have been focused on using refined carbohydrates such as glucose (Zhou et al. 2003a; Ishida et al. 2006), starch (Fukushima et al. 2004; Tanaka et al. 2006). Hence extensive studies are currently underway to search for inexpensive and sustainable substrates such as lignocellulosic biomass for lactic acid production. The pretreatment and saccharification

of raw materials by physicochemical and enzymatic treatment are one of the bottleneck processes for cost-effective lactic acid production (Abdel-Rahman et al., 2011). Direct fermentation of starchy biomass has been demonstrated to produce lactic acid; however, further development is still necessary before these processes are commercially feasible. Various materials have been considered as attractive alternative substrates and renewable resources, including byproducts of agricultural industries, food industries, and natural unutilized biomasses such as starchy biomass, lignocellulosic biomass, whey, yogurt, glycerol, and algal biomass.

Lactic acid production using biomass

The lactic acid market has been estimated to reach approximately 300,000 tons by 2015 showing that its demand is continuously increasing. Hence there is need to produce lactic acid from sustainable and plentiful renewable resources. Lignocellulosic biomass is abundant carbohydrate source that has recently drawn a lot of interest for lactic acid production. The use of agro-waste materials for lactic acid production appears to be more attractive because they do not impact on the food chain for humans. The direct transformation of cellulose component of lignocellulosic biomass into commodity chemicals is a key requirement to establish biomass-based sustainable chemical processes. However, only limited successes have been achieved for such transformations under mild conditions. Wang et al. (2013) have reported simple and efficient chemo-catalytic conversion of cellulose in water into lactic acid using dilute lead (II) ions. In addition, the lactic acid production from lignocellulosic biomass and its transformation to commodity chemicals is very well summarized in recent review (Maki-Arvela et al., 2014). Unfortunately, the bioprocess of converting cellulosic

material into lactic acid is not yet feasible due to the high cost of cellulase enzymes involved in cellulose hydrolysis (Yanez et al., 2003). In addition, cellulase inhibition by glucose and cellobiose during the hydrolysis of cellulosic material by cellulase is the main bottleneck, which remarkably slows down the rate of hydrolysis. Thus, it is advantageous to use a lactic acid producing strains that have the ability to utilize both glucose and cellobiose efficiently (Moldes et al., 2000). It is known that some *Lactobacillus* strains utilize cellobiose as a carbon source (Carr et al., 2002) but very little information is available about lactic acid production from cellobiose. We have reported the production of L-lactic acid (Adsul et al., 2007b) from cellobiose with the highest productivity and yields. The bioconversion of carbohydrate materials to lactic acid can be made much more effective by coupling the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of the derived sugars into a single step, known as the ‘‘Simultaneous Saccharification And Fermentation (SSAF)’. However, it is more difficult to ferment lignocellulosic biomass than starchy biomass to lactic acid. This is because lignocellulosic biomass contains cellulose as the main component. Cellulose is a persistent polymer, and its degradation requires physicochemical pretreatments and multi-enzymatic reactions (Okano et al., 2010). For example, corn stover or cobs, sugarcane bagasse, and wood processing waste are alternative substrates used for lactic acid production after pretreatment followed by chemical or enzymatic hydrolysis (Cui et al., 2011; Wang et al., 2010). Different cellulosic substrates have been used for lactic acid production by the SSAF process and the comparative results are given in Table 1.2. SSAF eliminates the need for a complete hydrolysis of the carbon substrates prior to the fermentation.

In the SSAF process, enzymatic hydrolysis, cell growth and microbial production occur simultaneously. A direct benefit of the SSAF is to decrease the inhibition caused by mono or disaccharide accumulation, leading to an increase in the saccharification rate, consequently increasing productivity and reducing reactor volume and capital costs. We have reported the L-lactic acid (Adsul et al., 2007c) production from sugarcane bagasse derived cellulose using SSAF process (Figure 1.4).

Table 1.2: Comparison of lactic acid production using different type of renewable raw materials by different organisms

Organism	Substrate	Lactic acid			References
		Concentration (g/l)	Yield (g/g)	Productivity (g/l/h)	
Lactobacillus delbrueckii Uc- 3	Molasses	166	0.87	4.2	Dumbrepatil et al., 2008
Lactobacillus delbrueckii Uc- 3	α-Cellulose	67	0.83	0.93	Adsul et al., 2007c
Lactobacillus delbrueckii IFO 3202	Defatted rice bran	28	0.28	0.77	Tanaka et al., 2006
Lactobacillus coryniformis ATCC 25600	Pretreated cardboard	23	0.56	0.49	Yanez et al., 2005
Lactobacillus delbrueckii HG 106	Unpolished rice	90	0.73	1.5	Lu et al., 2009
Bacillus sp. Strain 36D1	Solka Floc	40	0. 65	0.22	Patel et al., 2005
Lactococcus lactis IO-1	Sugar cane bagasse	10.9	0.36	0.17	Laopaibon et al.,2010
Sporolactobacillus sp. CASD	Peanut meal, glucose	207	0.93	3.8	Wang et al., 2011
Lactobacillus rhamnosus strain CASL	Cassava powder	175.4	0.71	1.8	Wang et al., 2010

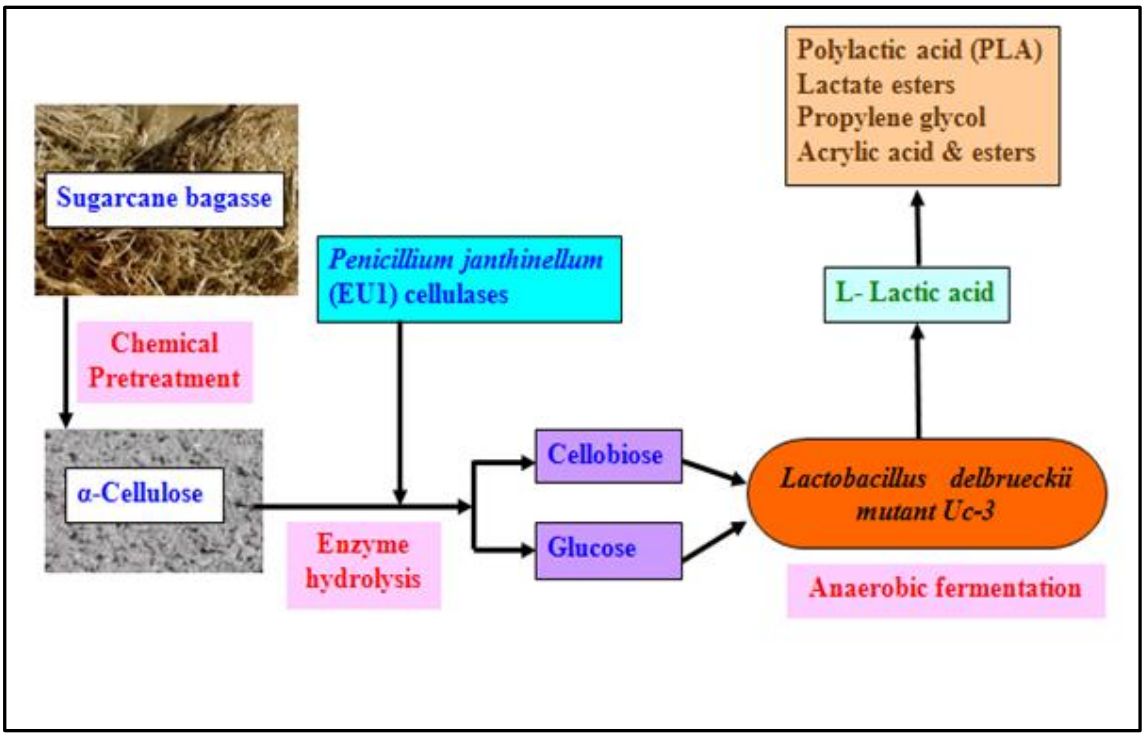


Figure 1.4: Biomass utilization through improved strains for lactic acid production using SSAF approach

Hemicelluloses are the second most abundant polysaccharide in nature due to their enormous availability, low cost and environmentally benign nature. The major fraction in hemicelluloses is pentosan and the conversion of pentose sugars is still challenging. Lactic acid bacteria are capable of fermenting glucose and other hexoses but lack the ability to ferment pentoses (Patel et al., 2006). For a complete conversion of biomass to lactic acid, lactic acid bacteria should have the capability to ferment pentoses. A genetically modified strain of *E. coli* containing the genes for pentose utilization and lignin degradation could be the suitable strain for the fermentation of hemicellulose derived pentose sugars (Graber et al., 2006). The lack of industrially suitable strains for the efficient conversion of xylose into lactate has been cited as a

major technical obstacle for the development of the poly(lactic acid) industry. *Enterococcus mundtii* QU 25, a newly isolated lactic acid bacterium, efficiently metabolized xylose into L-lactate. This strain may provide an ideal wild-type microorganism for an economical L-lactate production from renewable biomass substrates (Abdel-Rahman et al., 2011). The use of pure sugars as the carbon source for lactic acid production which leads to an increase in cost of the product is the major bottleneck in the biotechnological production of optically pure lactic acid. This problem can be resolved through the fermentative production of lactic acid from cheaper materials such as molasses, starch, lignocelluloses and wastes from agricultural and agro-industrial residues that can be used as substrates for lactic acid fermentation. However, most lignocellulose materials need to be pretreated by physicochemical and enzymatic methods because lactic acid fermenting microorganisms cannot directly use those materials due to their recalcitrance nature (Okano et al., 2010). Improvement of these microorganisms through gene modification is an essential and interesting method that has been extensively studied. The other hurdle in lactic acid production is the operating cost. For example, sterilization is necessary for the fermentative production of lactic acid. Therefore, it is difficult to avoid contamination if the medium is not sterilized. To avoid contamination, highly thermo-tolerant and acid tolerant strains may be useful in lactic acid production. The fermentative production of L-lactic acid by a newly isolated thermophilic strain, *Bacillus sp*. 2–6, has been recently reported (Qin et al., 2009).

The down streaming process after fermentation also elevates the cost of lactic acid production. Owing to the inhibitory effects of low pH on cell growth and lactic

acid production, $CaCO_3$ must be added to maintain a constant pH. Fermentation of sugars to lactic acid at low pH (below 4.5) is essential to avoid the use of calcium carbonate which generates high concentrations of calcium sulfate during acid hydrolysis to liberate free lactic acid. *Lactobacillus* strains capable of producing lactic acid at acidic conditions have not yet been developed. The use of such acid tolerant strains will change the entire scenario of downstream processes for lactic acid purification. Recently, lactic acid (77.0 g/l) production from 100 g/l cellulose equivalent of paper sludge was reported using *Bacillus coagulans* strains. The semi-continuous saccharification and fermentation was carried out without pH control since these strains are thermophilic and acid tolerant (Budhavaram and Fan, 2009). An important step in the lactic acid production is the recovery from the fermentation broth. The conventional process for the recovery of lactic acid is still far from ideal. Indeed, it involves the precipitation of calcium lactate after the separation of micro-organisms and the conversion of the salt to lactic acid by the addition of sulfuric acid. The dilute lactic acid produced is then submitted for purification. The separation and purification stages account for up to 50% of the production cost. Moreover, reactor productivities are low and the process is unfriendly to the environment since it consumes sulfuric acid and produces a large quantity of calcium sulfate (1 ton of calcium sulfate per ton of lactic acid). Recent advances in membrane-based separation and purification technologies, particularly in micro- and ultrafiltration and electrodialysis, have led to the inception of new processes which may lead to low-cost production without the environmental problems associated with the conventional process. Biotechnology is providing new, low-cost and highly efficient fermentation processes for the production of chemicals

from biomass resources (Varadarajan and Miller, 1999). However, the current economic impact of lactic acid fermentation is still limited, in large part owing to difficulties in product recovery. Thus, improvements in the existing recovery technologies are needed in order to allow the chemicals from fermentation to penetrate further in the organic chemical industry.

LACTIC ACID RECOVERY PROCESS

Several recovery techniques have been reported on the separation of lactic acid produced in the fermentation broth without precipitation such as solvent extraction (Hano et al., 1993), liquid surfactant membrane extraction (Sirman et al., 1991), adsorption (Kaufman et al., 1994), chromatographic methods (Hauer and Marr, 1994), ultrafiltration (Hauer and Marr, 1994), reverse osmosis (Hauer and Marr, 1994), conventional electrodialysis (Heriban et al., 1993; Kim and Moon, 2001; Thang et al., 2005) and bipolar membrane electrodialysis (Bailly, 2002). Among those, the electrodialysis technique has been found to be potentially effective in the recovery of lactic acid from fermentation broth due to its rapid treatment, effective removal of non-ionic molecules, concentration of lactic acid, and environmentally sustainable process (Boniardi et al., 1997). Additionally, in-situ recovery technique has the potential to not only recover lactic acid but also relieve product inhibition. Conventional electrodialysis generally uses ion-exchange membranes under the influence of direct current for separating and concentrating ions in solution. In-situ conventional electrodialysis with the cathode located in the compartment of the fermenter has been shown to damage the bacterial cells by direct contact with the electrodes. To prevent the cells from contacting with the electrodes, an ultrafiltration step has been introduced before the electrodialysis

process (Nomura et al., 1991). Furthermore, pH control is an important factor that should limit the technical and commercial viability in electrodialysis processes (Hongo et al., 1986; Vonktaveesuk et al., 1994). In order to maintain a pH at the suitable value, an additional pH control system with the addition of alkaline solution was incorporated into the electrodialysis process (Vonktaveesuk et al., 1994). Several recent studies have also applied electrodialysis to recover lactic acid from fermentation broth (Gao et al., 2005; Wee et al., 2005; Yi et al., 2008).

To date, various attempts have been made to develop the technology of electrodialysis by using bipolar membrane. This process is advantageous in the capability of separation, purification and concentration of salts, and in the conversion of these salts into acid and base without generating waste effluents containing high concentrations of salts. In addition, the generated base can be recycled for the neutralization of the fermentation process (Franken, 2000). Min-tian et al. (2005) achieved production of 2637 g lactic acid from 4000 g glucose at a productivity of 8.18 g/l/h and a yield of 0.69 g/g in continuous fermentation integrated with bipolar membrane electrodialysis using *L. rhamnosus* IFO 3863. However, there is still a compatibility issue between electro-dialysis and fermentation process that should be carefully arranged to achieve a synchronous operation between fermentation and electro-dialysis process.

POLYMERISATION OF LACTIC ACID

Lactic acid is a building block for the manufacture of poly (lactic acid) (PLA), a biodegradable polymer used as an environmentally friendly biodegradable plastic. PLA is the first commodity polymer produced annually from renewable resources. PLA is a

representative bio-based plastic that is used in packaging, stationery, containers, etc (Nampoothiri et al., 2010). In addition, the utilization of the polyester (polyhydroxyalkonates) has been expanded to the medical field for drug delivery, resorbable sutures and as a material for medical implants and other related applications (Auras et al., 2004). Among the biomaterials (biopolymers) used in the medical field, PLA has received significant attention. PLA and its copolymers are being used in the biomedical area in the form of implants or devices due to their biocompatibility and biodegradability. The rate of degradation of PLA depends on the degree of crystallinity. As the lactide content increases, the degradation of the graft polymer decreases (Luckachan and Pillai, 2006). The Poly (L-lactic acid) PLLA is a semi-crystalline polymer with a glass transition temperature between 55 ^{o}C and 59 ^{o}C and a melting point 170 ^{o}C–180 ^{o}C. It shows good mechanical stiffness, high Young's modulus, thermal plasticity and has good processability (Auras et al., 2010). It is relatively hydrophobic polyester, unstable in wet conditions, which can undergo chain disruptions in the human body and degrades into nontoxic byproducts, lactic acid, carbon dioxide and water, which are subsequently eliminated through the Krebs cycle and in the urine. The most widely used method for improving PLLA processability is based on the melting point depression by the random incorporation of small amounts of lactide enantiomers of opposite configuration into the polymer (i.e. by adding a small amount of D-lactide to L-lactide to obtain PDLLA). Unfortunately, the melting point depression is accompanied by a significant decrease in crystallinity and crystallization rates. Recently, lactic acid consumption has increased considerably because of its role as a monomer in the production of biodegradable PLA, which is well-known as a

sustainable bio-plastic material. However, the global consumption of lactic acid is expected to increase rapidly in the near future.

PLAs are basically synthesized via three processes: (i) production of lactic acid (LA) by microbial fermentation, (ii) purification of LA and preparation of its cyclic dimer (lactide) and (iii) poly-condensation of LA or ring-opening polymerization of lactides. Figure1.5 shows the reaction mechanism for both the poly-condensation of LA and the ring-opening polymerization of lactides. In direct condensation, a solvent is used and higher reaction times are required. The resulting polymer is a material with low to intermediate molecular weights. Lactide is obtained by the de-polymerization of low molecular-weight PLA under reduced pressure to give L-lactide, D-lactide or meso-lactide. The different percentages of the lactide isomers formed depend on the lactic acid isomer feedstock, temperature and catalyst. Poly (lactic acid) can undergo cationic ring-opening polymerization. It has been found that trifluoromethane sulfonic acid (triflic acid) and methyl trifluoromethane sulfonic acid (methyl triflate) are the only cationic initiators to polymerize lactide. The polymerization proceeds via triflate ester end-groups instead of free carbenium ions, which yield at low temperatures an optically active polymer without racemization. The chain growth proceeds by cleavage of the alkyloxygen bond. The propagation mechanism begins with positively charged lactide ring being cleaved at alkyl-oxygen bond by an SN2 attack by the triflate anion. The triflate end-group reacts with a second molecule of lactide again in an SN2 fashion to yield a positively charged lactide that is opened. Then the triflate anion again opens the charged lactide and polymerization proceeds. Anionic lactide polymerizations proceed by the nucleophilic reaction of the anion with the carbonyl and the subsequent acyl-

oxygen cleavage. This produces an alkoxide end-group, which continues to propagate (Garlotta, 2002). Ring-opening polymerization (ROP) of the lactide needs a catalyst but it results in PLA with a high molecular weight. Depending on the monomer used and the reaction conditions, it is possible to control the ratio and sequence of D- and L-lactic acid units in the final polymer. The ring-opening polymerization of lactide can be carried out in melt or solution by cationic, anionic and coordination mechanisms, depending on the initiator utilized. The most considered active initiator for the L-lactide ring-opening polymerization is stannous octoate (bis-2-ethyl hexanoate, SnOct2), which causes a low degree of racemization at high temperatures. It is catalyzed by transition metals such as tin, aluminum, lead, zinc, bismuth, ion and yttrium (Agrawal and Bhalla, 2003). PLA is chemically synthesized by the heavy metal-catalyzed ring-opening polymerization of lactide, which in turn is derived from fermentative lactate (LA). However, the trace residues of the heavy metal catalyst are unfavorable for certain applications, in particular, medical and food applications. Thus, the replacement of the heavy metal catalyst with a safe and environmentally acceptable alternative is a crucial issue. For this purpose, enzymes are attractive targets because they are natural and non-harmful catalysts that can drive the reactions under mild conditions (Matsumoto and Taguchi, 2009). In addition, highly specific enzymatic reactions and/or whole-cell systems bearing them may be capable of synthesizing polymers with a fine structure from inexpensive raw materials. In comparison, chemical processes require extremely pure monomers, anhydrous conditions and high temperatures in order to avoid side reactions and produce high quality polymers. Therefore, a complete biosynthesis of PLA may be advantageous over the chemical process provided this challenge can be

met. Thus, an ‘‘LA-polymerizing enzyme (LPE)’’, which can function as an alternative to a metal catalyst, would be desirable to establish the bioprocess. The appropriate strategy (albeit difficult) would be the discovery of a PLA-producing microorganism. There is no information available on PLA production using natural strains. However, engineered strains have been used extensively for producing LA based polyesters. Overall, the configuration of the enantiomers of the LA unit in the LA based polyester is mainly determined by the enantio-selectivity of LDH and LPE. In that case, the *Lactobacillus* strain could be useful in establishing the one step process for the synthesis of LA-based polyesters. We suggest that *Lactobacillus* could be the host organism for the cloning and expression of the LPE gene, which directly converts lactic acid into polylactate. In this case, lactic acid is directly diverted to the synthesis of PLA, thereby reducing the requirement of a neutralizing agent to maintain the pH during fermentation.

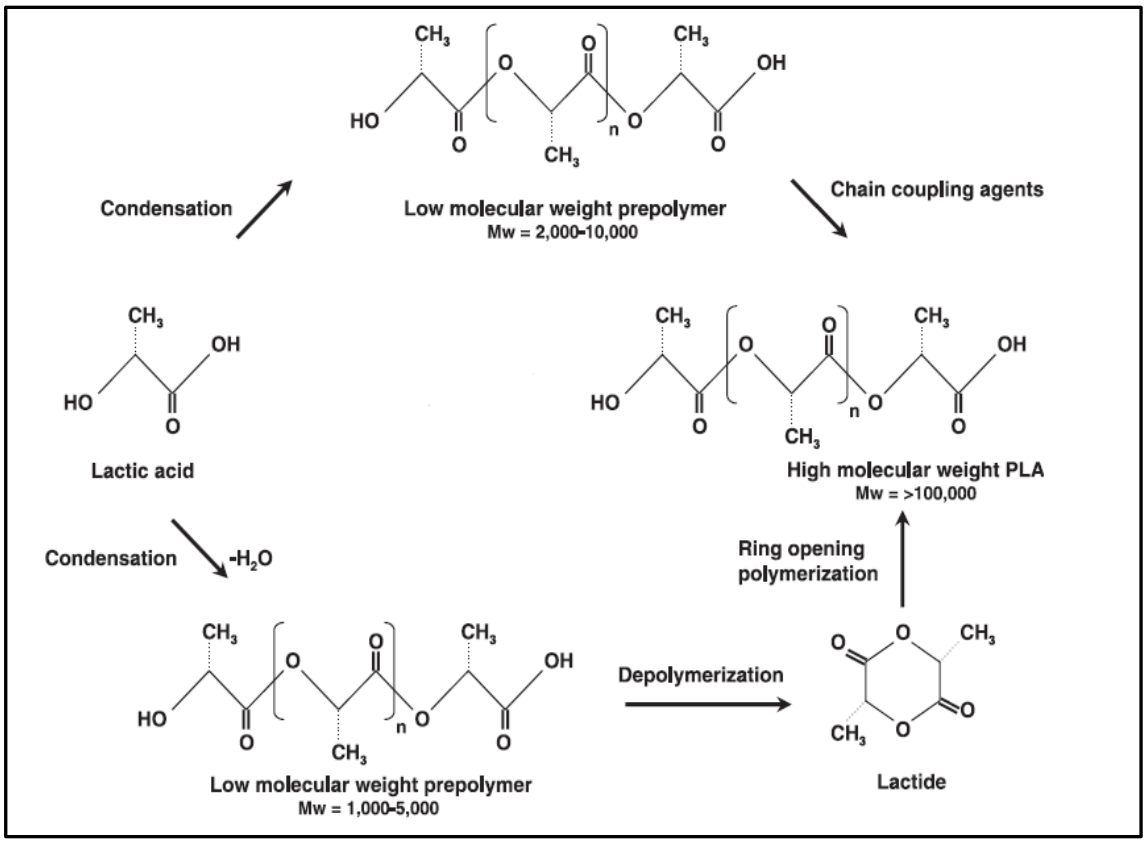


Figure 1.5: Schematic representation of PLA synthesis (Gupta et al 2007)

Bacterial polyhydroxyalkanoates (PHAs) are also a major class of bio-based plastics, which are intracellularly produced by the PHA synthase-catalyzed polymerization of hydroxyacyl-CoAs. Among them, poly(3-hydroxybutyrate) [P(3HB)] is the most common PHA, and is efficiently produced from renewable carbon sources. However, in considering practical uses, there is the obstacle that P(3HB) tends to be a stiff and brittle material due to crystallinity. In addition, because of such crystallization, P(3HB) becomes opaque. These properties have limited the range of applications of these materials. Recently, a whole-cell biosynthesis system for LA-based polyester production without heavy metal catalyst has been achieved using engineered *Escherichia coli* (Matsumoto and Taguchi, 2009; Taguchi et al., 2008). In this

biological system, LA synthesized in the cell is directly converted into the polymer without any extraction and purification processes. The discovery of LPE (Taguchi et al., 2008), an engineered polyhydroxyalkanoate (PHA) synthase (Taguchi and Doi, 2004), was a key to develop the first microbial system. To date, the *E. coli* platform has been used to produce various LA-based polymers incorporating 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) (Shozoui et al., 2009; Yamada et al., 2009). Most recently, the successful incorporation of new 2-hydroxy acids, such as 2- hydroxybutyrate and glycolate, using LPE has been reported (Han et al., 2011; Matsumoto et al., 2011). PLA homo-polymer and its copolymer, poly(3-hydroxybutyrate-co-lactate) have been produced by the direct fermentation by metabolically engineered *E. coli* (Jung et al., 2010). The engineered *E. coli* strain was constructed by introducing heterologous metabolic pathways involving engineered propionate CoA-transferase and polyhydroxyalkanoate (PHA) synthase. This resulted in the efficient generation of lactyl-CoA which is incorporated into the polymer. However, the PLA and poly(3-hydroxybutyrate-co-lactate) were synthesized with low frequency in engineered *E. coli*. This strategy of combined metabolic engineering and enzyme engineering could be useful for developing other engineered organisms capable of producing different unnatural polymers by direct fermentation from the biomass. Song et al (2012) designed metabolic pathways in *C. glutamicum* to generate monomer substrates, lactyl-CoA (LA-CoA) and 3-hydroxybutyryl-CoA (3HBCoA), for the copolymerization catalyzed by LPE. LA-CoA was synthesized by D-LDH and propionyl-CoA transferase, while 3HB-CoA was supplied by β-ketothiolase (PhaA) and NADPH (nicotinamide adenine dinucleotide phosphate)-dependent acetoacetyl-CoA

reductase (PhaB). The functional expression of these enzymes led to a production of P(LA-co-3HB) with high LA fractions (96.8 mol%). The newly engineered *C. glutamicum* potentially serves as a food-grade and bio-medically applicable platform for the production of a poly(lactic acid)-like polyester. Tajima et al.(2012) cloned a pha locus from a thermo-tolerant bacterium, *Pseudomonas sp.* SG4502, which is capable of accumulating polyhydroxyalkanoate (PHA) even at 55 ^{o}C, as a source of thermo-stable enzymes and identified two genes encoding PHA synthases (PhaC1SG and PhaC2SG). Two mutations, Ser324Thr and Gln480Lys, corresponding to those of a LPE from mesophilic *Pseudomonas sp*. 61-3, were introduced into PhaC1SG to evaluate the potential of the resulting protein as a ‘‘thermostable LPE’’. The mutated PhaC1SG [PhaC1SG(STQK)] showed a high thermal stability in synthesizing P(LA-co-3HB) in an in vitro reaction system under a range of high temperatures which could be useful in synthesizing LA based copolymers.

BIODEGRADATION OF POLYMER

Biodegradable polymers have received much more attention in the last decades due their potential applications in the fields related to environmental protection and the maintenance of physical health. Two classes of biodegradable polymers can be distinguished: synthetic or natural polymers. There are polymers produced from feedstock derived either from petroleum resources (non-renewable resources) or from biological resources (renewable resources). To improve the properties of biodegradable polymers, a lot of methods have been developed, such as random and block copolymerization or grafting. For example, THE anchoring of minute quantities of saccharide moieties onto polyolefins improved their rates of biodegradation (Galgali et

al., 2002; Galgali et al., 2004). These methods improved both the biodegradation rate and the mechanical properties of the final products. Physical blending is another route to prepare biodegradable materials with different morphologies and physical characteristics. Biodegradation takes place through the action of enzymes and/or chemical deterioration associated with living organisms. This event occurs in two steps. The first one is the fragmentation of the polymers into lower molecular mass species by means of either abiotic reactions, i.e. oxidation, photo-degradation or hydrolysis, or biotic reactions, i.e. degradation by microorganisms. This is followed by THE bio-assimilation of the polymer fragments by microorganisms and their mineralization. Biodegradability depends not only on the origin of the polymer but also on its chemical structure and the environmental degrading conditions (Figure 1.6). The degradation of PLA has been studied for several years, but understanding of the mechanisms involved in biodegradation is still incomplete. Several reports concluded that PLA degradation occurs strictly through hydrolysis with no enzymatic involvement (Li et al., 1990; Hakkarainen et al., 1996). Other reports suggested that enzymes have a significant role in PLA degradation (Hoshino and Isono, 2002). In the last years, a new type of material called nano-composites, have been developed (Sinha Ray and Bousmina, 2005). Fukushima et al. (2009) reported the degradation PLA and its nano-composites in composts under controlled conditions. Recently, Arena et al (2011) reported that the degradation of PLA and its composites by *Bacillus licheniformis* is due to the extracellular esterase activity.

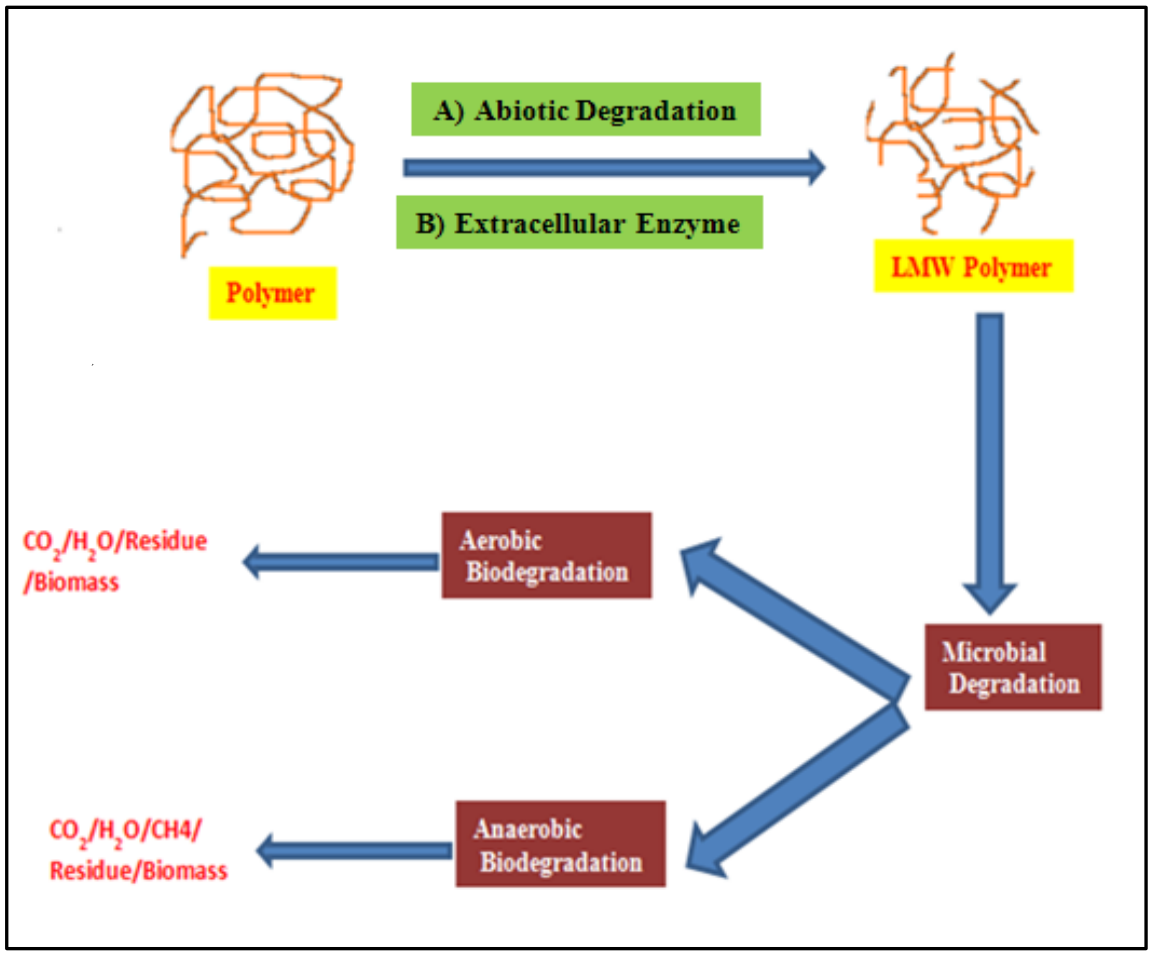


Figure 1.6: Schematic representation of chemistry of biodegradation of polymer

STRAIN IMPROVEMENT

The science and technology of manipulating and improving microbial strains, in order to enhance their metabolic capacities in biotechnological applications, are referred to as strain improvement. Strain improvement is usually aimed at increasing yields of the desired metabolites (Macdonald and Holt, 1976). However, other strain characteristics can also be improved such as the removal of unwanted co-metabolites. There are three methods of strain improvement available to increase the production of

desired metabolites: 1) Mutagenesis and selection 2) Genome shuffling and 3) Gene cloning

Mutagenesis and selection

The random mutagenesis followed by selection of the desired phenotype is also called as classical strain improvement. In this method, there is permanent alteration of one or more nucleotides at specific site along the DNA strand. Mutation may be associated with the change of the single nucleotide (point mutation) through substitution, deletion or rearrangement of one or more base pairs in chromosomes. In many cases mutations are harmful but sometimes it creates the strain more adaptable to environment and improves its bio-catalytic performance. This method involves three basic steps: 1) Mutagenesis of population to induce the genetic variability 2) random selection and primary screening of surviving population to find improved strain 3) actual checking of desired improvement in fermentation or by assay. The improved mutant may again act as a parent strain for further mutagenesis. This is continued till we get superior mutant. An improvement in the strain is achieved by subjecting the cells or spores of microbial strain to variety of chemicals or physical agents called mutagens. There are two types of mutagens namely physical mutagens and chemical mutagens.

***Physical mutagens*:** Mutations can be naturally or artificially induced by a variety of physical mutagens. The physical agents are broadly divided into two types:

Ionizing radiation: X-rays and gamma (Y) rays are ionizing radiations. They have short wavelength and high penetration power. They can penetrate into deeper tissues causing

ionization of the molecules along their way. When X-rays penetrate into cells, electrons are ejected from the atoms of molecules encountered by the radiation. As a result the stable molecules and atoms change into free radicals and reactive irons. The radicals and ions can initiate a variety of chemical reactions, which can affect the genetic material, resulting in point mutations affecting only one base pair in a given location. The rate of mutation increases with the increasing dose of X-rays administered.

Nonionizing radiation: Ultra Violet (UV) rays are nonionizing radiations. They have long wavelength and low penetration power. The purines and pyrimidines absorb UV radiation most intensely at about 260 nm. This property has been useful in the detection and analysis of nucleic acids. In 1934 it was discovered that UV radiation is mutagenic. The major effect of UV radiation is formation of pyrimidine dimmers, particularly between two thymines. Cytosine-cytosine and cytosine-thymine dimmers are less prevalent. The dimers damage the DNA structure and effects normal replication.

***Chemical mutagens*:** Chemical mutagens can remove, replace or modify DNA bases e.g.5-chlorouracil, 5-bromouracil, Nitrous acid, hydroxylamine. Base analogs are structurally similar to normal nitrogenous bases and can be incorporated into the growing polynucleotide chain during replication. These analogs will have base pairing properties different from the bases they replace. One of the first base analogs formed to induce mutations in phage T2 is 5 bromouracil (BU) an analog of thymine. In the normal keto form BU base pairs with adenine. But its tautomeric enol form pairs with guanine like cytosine.

*Alkylating agents***:** Alkylating of nitrogenous bases by the alkylating agent either removes the base or modifies it. Guanine residues can be alkylated by the methyl methane sulfonate and ethylmethanesulfonate.

*Intercalating agents***:** Intercalating agents produces frame shift mutation in bacteriophages like T4. For example acridines are mutagenic to bacteriophphages but not to bacteria. As the acridines are unable to enter bacterial cell.

Protoplast Fusion

Protoplast fusion is a method which leads to genome shuffling. It has been used to modify the phenotypic traits of a variety of prokaryotic and eukaryotic cells since the late 1970s (Petri and Schmidt-Dannert, 2004; Gong et al., 2009). Protoplast fusion was successfully achieved first in animals, and later in plants (Rai and Rai, 2006). Protoplast from different species, and even from different kingdoms, can be successfully fused. This indicates a very broad applicability of this technique in cell engineering (Verma et al., 1992; Rassoulzadegan et al., 1982; Petri and Schmidt-Dannert, 2004). Protoplasts are the cells of which cell walls are removed and cytoplasmic membrane is the outermost layer in such cells. Protoplast can be obtained by specific lytic enzymes to remove cell wall. Protoplast fusion is a physical phenomenon in which two or more protoplasts come in contact and adhere with one another either spontaneously or in presence of fusion inducing agents. By protoplast fusion it is possible to transfer some useful genes such as diesese resistance, nitrogen fixation, rapid growth rate, more product formation rate, protein quality, frost hardiness, drought resistance, herbicide resistance, heat and cold resistance from one species to another. Protoplast fusion an important tools in strain improvement for bringing genetic recombinations and

developing hybrid strains in filamentous fungi. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties. This is the powerful technique for engineering of microbial strains for desirable industrial properties. Protoplast fusion would continue to be an existing area of research in modern biotechnology. This technique in the future will be one of the most frequently used research tools for tissue culturists, molecular biologists, biochemical engineers and biotechnologists technology and its biotechnological applications.

Conditions for isolation of healthy and functional protoplasts from various bacteria, however are not identical and need to be established for individual organisms. Efficiency of fusion and appropriate phenotype transfer, therefore, is dependent on efficiency of protoplast formation and their regeneration. Specific conditions for protoplast isolation have been developed in plant cells (Vardi et al., 1989, Deng et al., 1992), fungi (Kim et al., 2000) and bacteria (Hopwood 1981). For protoplast fusion it is important that the cell wall of plant or microorganisms is degraded. Various enzymes are used for this process i.e. Cellulase and pectinase or macerozyme acting on plant cell wall, Bacterial cell wall are degraded by the action of lysozyme. Fungal wall are degraded by Novozyme -234 which includes glucanase and chitinase. Streptomyces cell wall degraded by action of lysozyme and achromopeptidase (Narayanswamy 1994, Jogdand 2001).

For any genetic work on protoplast fusion or protoplast transformation, successful regeneration of protoplasts into normal cell is essential in order to give progeny which could be genetically characterized. The minimum requirement for the regeneration of protoplasts is the presence of osmotic stabilizer in the solid media. For

regeneration of protoplasts of yeast and Gram positive bacteria, an osmotic stabilizers such as 0.5M Nacl (Okanishi et al., 1974), 20% sucrose (Akamatsu and Sekiguchi 1981) or 16% sorbitol (Van Solingen and vander Platt, 1977) have been used.

The generalized scheme for fusion of bacterial protoplasts has been shown in Figure 1.7. The procedure involves a use of two parental strains that are genetically marked with complementary auxotrophies or additional selectable markers unique to either parent or generated through prior mutagenesis. Protoplasts of both the strains are mixed in equal numbers and treated with fusogen. PEG induces the aggregation of protoplasts and fusion events occur after the fusogen is washed. The PEG treated mixed protoplasts are then plated onto suitable media and the fusion products are recovered by direct or indirect selection. It is considered as classical or fundamental method of gene transfer in which multiple genes can be simultaneously introduced in contrast to genetic engineering suitable for introducing only one or few genes. We have been successful in transferring multiple phenotype using protoplast fusion approach leading to changed properties of enzymes such as cellulase (Gokhale et., 1984; Gokhale and Deobagkar, 1989) and amylase (Gokhale and Deobagkar, 1990). Here we describe its application to improve acid tolerance in one of the strains of *Lactobacillus* with commercial potential. This approach has already been used to improve the acid tolerance in *Lactobacillus* (Patnaik et al., 2002; Wang et al., 2007) for L- lactic acid production. This was also used in *Streptomyces fradiae* to improve the production of the polyketide antibiotic, tylosin (Zhang et al., 2002) and in *Sphingobium* for the degradation of pentachlorophenol (Dai and Copley, 2004).

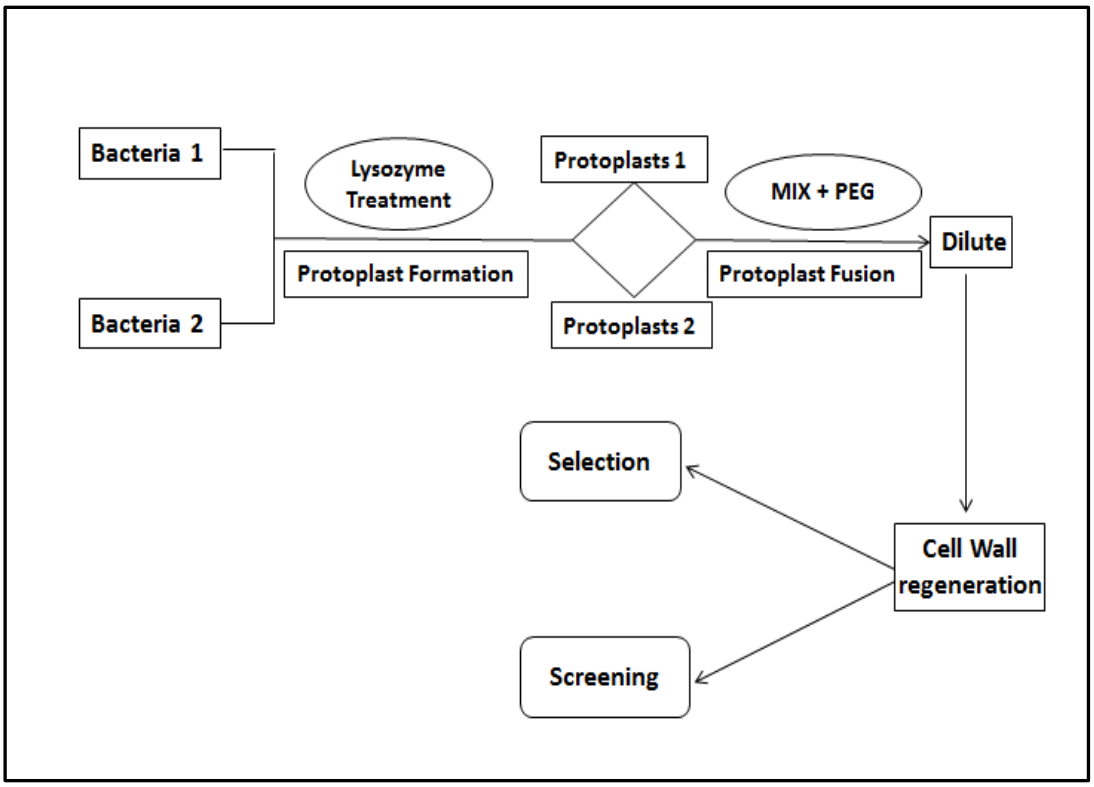


Figure 1.7: Schematic diagram of protoplast fusion in Bacteria

Gene cloning/rDNA technology

With increasing demand for lactic acid and increasing concern over environmental impact of gypsum accumulation as a by-product of traditional fermentation, enhanced efforts for development of alternative technologies/methods are being made. The advent of biotechnology led to the efforts being focused on use of biotechnological tools to engineer for the production of lactic acid. The use of insertion sequence elements for deletion of *Lac Z* gene of *Lactobacillus bulgaricus* resulted in its limited fermentation of lactose. Hence, this strain could be used in yoghurt fermentation and limited lactose utilization prevents further souring of yoghurt (Mollet et al., 1993). The gene inactivation method based upon integration of insertion sequence (IS) elements into chromosome leads to altered gene expression pattern, which may give rise

to a strain with enhanced qualities (Maguin et al., 1996). The pyruvate decarboxylase (*PDC*) deficient *Saccharomyces cerevisiae* strain used for expression of lactate dehydrogenase showed increased lactate yield with suppressed ethanol production. The findings also suggested that out of three genes (*PDC1*, *PDC5* and *PDC6*) responsible for pyruvate decarboxylase, *PDC1* plays a key role (Adachi et al., 1998). An ethanol-genic *Escherichia coli* strain, SZ470, was reengineered for homo-fermentative production of L-lactic acid from xylose by deleting the alcohol dehydrogenase gene (adhE) and integrating the L-lactate dehydrogenase gene (ldhL) of *Pediococcus acidilactic*i (Zhao et al., 2013). *T. aotearoense* SCUT27 was engineered to block the acetic acid formation pathway to improve the lactic acid production. The genetic manipulation resulted in 1.8 and 2.1 fold increase of the lactic acid yield using 10 g/l of glucose or 10 g/l of xylose as substrate, respectively (Yang et al., 2013). To improve lactic acid production, *E. coli* strains have been engineered in the field of metabolic engineering (Zhou et al., 2003a). Several studies reported the use of engineered *E. coli* strains for lactic acid production from glucose (Dien et al., 2001; Zhou et al., 2003a,b, Zhu, 2007), xylose (Dien et al., 2001), sucrose (Wang et al., 2012; Zhou et al., 2005),and glycerol (Mazumdar et al., 2010).

APPLICATIONS OF LACTIC ACID

Lactic acid has been receiving a great attention due to their variety of applications in various industries, some of the applications are reported here (Figure 1.8).

Figure 1.8: Applications of lactic acid in different fields

Food industry

The major use of lactic acid is in food and food-related applications, which, in the U.S., accounts for approximately 85 % of the demand. The rest (~ 15 %) of the uses are for non-food industrial applications. As a food acidulant, lactic acid has a mild acidic taste, in contrast to other food acids. It is nonvolatile, odorless and is classified GRAS for general-purpose food additives by the FDA in the USA and by other regulatory agencies elsewhere. It is a good preservative and pickling agent for sauerkraut, olives and pickled vegetables. It is used as an acidulant, flavoring, pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods Another potential application of lactic acid in the food industry is the mineral fortification of food products.

Bakery products

A large fraction (>50 %) of the lactic acid for food-related uses goes to produce emulsifying agents used in foods, particularly for bakery goods. These emulsifying agents are esters of lactate salts with longer chain fatty acids, and the four important products are calcium and sodium, stearoyl-2-1actylate, glyceryl lactostearate, and glyceryl lactopalmirate. Of the stearoyl lactylates, the calcium salt is a very good dough conditioner, and the sodium salt is both a conditioner and an emulsifier for yeast leavened bakery products. The glycerates and palmitates are used in cake mixes and other bakery products.

Pharmaceutical industry

Lactic acid has long been used in pharmaceutical applications and formulations, particularly in topical ointments, lotions, parenteral solutions and biodegradable polymers for medical applications such as surgical sutures, controlled-release drugs and prostheses. A substantial part of pharmaceutical lactic acid is used as the sodium salt for parenteral and dialysis applications. The calcium salt is widely used for calcium deficiency therapy and as an effective anti-caries agent. Lactic acid based formulations find use for their antitumor activity

Cosmetic industry

Lactic acid offers natural ingredients for cosmetic applications. Although primarily used as moisturizers and pH regulators, they possess multiple other properties such as antimicrobial activity, skin lightening, and skin hydration. The moisturizing effect is related directly to lactate's water retaining capacity, and the skin-lightening

action of lactic acid is produced by the suppression of the formation of tyrosinase. As humectants in cosmetic applications, the lactates are often superior as natural products and more effective than polyols. Ethyl lactate is the active ingredient in many anti acne preparations. they produce such effects as skin lightening and rejuvenation which makes them very useful as active ingredients in cosmetics. It is primarily used as an anti-aging chemical claimed to soften lines, reduce photo damage from the sun, improve skin texture and tone and improve overall appearance.

Chemical industry

Currently, lactic acid is considered the most potential feedstock monomer for chemical conversions, because it contains two reactive functional groups, a carboxylic group and a hydroxyl group. Lactic acid can undergo a variety of chemical conversions into potentially useful chemicals, such as propylene oxide (*via* hydrogenation), acetaldehyde (*via* decarboxylation), acrylic acid (*via* dehydration), propanoic acid (*via* reduction), 2,3-pentanedione (*via* condensation), and dilactide (*via* self-esterification). In the chemical industries, lactic acid is used in the dyeing of silks and other textile goods, as a mordant in the printing of woolens, in the bating and plumping of Due to the high solvency power and solubility of lactic acid, it is an excellent remover of polymer and resins.

Polymer industry

Lactic acid has recently received a great deal of attention as a feedstock monomer for the production of polylactic acid (PLA), which serves as a biodegradable commodity plastic. The optically pure lactic acid can be polymerized into a high

molecular mass PLA through the serial reactions of polycondensation, depolymerization, and ring opening polymerization. The resultant polymer, PLA, has numerous uses in a wide range of applications, such as protective clothing, food packaging, mulch film, trash bags, rigid containers, shrink wrap, and short shelf-life trays.

Other applications

Technical grade lactic acid is used as an acidulant in vegetable and leather tanning industries. Lactic acid is being used in many small scale applications like pH adjustment hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyl resin modifier, solder flux, lithographic and textile printing developers, adhesive formulations, electroplating and electro-polishing baths, detergent builders. It is also used for the extraction of fish skin gelatin. In recent days it is used in the field of soft tissue augmentation and also used as adhesive in lamination industries.

Lactate esters like ethyl, methyl lactate etc. are used for degreasing since they have excellent action for oils, oligomeric and polymeric stains. Lactic acid is used in Ni – plating process because of its unique complexing constant for Ni. Lactic acid is used as a pH regulator and complexing agent in various binder systems for water–based coatings such as electro-deposition coatings. Lactates find use as neutralizers in the production of certain types of surfactants, used in special detergents and personal care products.

REFERENCES

Abdel-Rahman M. A., Tashiro Y., Sonomoto K. (2011) Lactic acid production from lignocellulose derived sugars using lactic acid bacteria: overview and limits. *Journal of Biotechnology*, 156, 286-301.

Adachi E., Torigoe M., Sugiyama M., Nikawa J. I., Shimizu K. (1998) Modification of metabolic pathways of *Saccharomyces cerevisiae* by the expression of lactate dehydrogenase and deletion of pyruvate decarboxylase genes for the lactic acid fermentation at low pH value. *Journal of Fermentation and Bioengineering*, 86(3), 284-289.

Adsul M. G., Ghule J. E., Singh R., Shaikh H., Bastawde K. B., Gokhale D. V., Varma A.J, (2004) Polysaccharides from bagasse: applications in cellulase and xylanase production. Carbohydrate Polymers, 57, 67–72.

Adsul M. G., Ghule J. E., Shaikh H., Singh R., Bastawde K. B., Gokhale D. V., Varma A.J, (2005) Enzymatic hydrolysis of delignified bagasse polysaccharides. *Carbohydrate Polymers*, 62, 6–10.

Adsul M. G., Bastawade K. B., Varma A. J., Gokhale D. V. (2007a) Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. *Bioresource Technology*, 98, 1467-1473.

Adsul M. G., Khire J. M., Bastawade K. B., Gokhale D. V. (2007b) Lactic acid production from cellobiose and cellotriose by *Lactobacillus delbrueckii* mutant Uc-3. *Applied and Environmental Microbiology*, 73, 5055–5057.

Adsul M. G., Varma A. J., Gokhale D. V. (2007c) Lactic acid production from waste sugar cane bagasse derived cellulose. *Green Chemistry*, 9, 58–62.

Adsul M. G., Terwadkar A. P., Varma A. J., Gokhale D. V. (2009a) Cellulases from *Penicillium janthinellum* mutants: solid state production and their stability in ionic liquids. *Bioresources*, 4 (4), 1670–1681.

Adsul M. G., Bastawde K. B., Gokhale D. V. (2009b) Biochemical characterization of two xylanases from yeast *Pseudozyma hubeiensis* producing only xylooligosaccharides. *Bioresource technology*, 100, 6488-6495.

Adsul M. G., Singhvi M., Gaikaiwari S., Gokhale D. V. (2011) Development of biocatalyst for production of commodity chemicals from lignocellulosic biomass. *Bioresource Technology*, 102, 4304-4312.

Agrawal A. and Bhalla R. (2003) Advances in the Production of Poly(Lactic Acid) Fibers: A Review. *Macromolecular Sciences and Polymer Review*, 43, 479-503.

Akamatsu T. and Sekiguchi J. (1981) Studies on regeneration media for *Bacillus subtilis* protoplasts. *Agricultural and Biological Chemistry*, 45, 2887-2894.

Arena M., Abbate C., Fukushima K., Gennari M. (2011) Degradation of poly (lactic acid) and nanocomposites by *Bacillus licheniformis*. *Environmental Science and Pollution Research*, 18, 865-870.

Auras R., Harte B., Selke S. (2004) An overview of polylactides as packaging materials. *Macromolecular Biosciences*, 4, 835–864.

Auras R., Selke S., Tsuji H. (2010) Poly(Lactic Acid): Synthesis,Structures, Properties, Processing, and Applications, Wiley & Sons, Inc.

Bailly M. (2002) Production of organic acids by bipolar electrodialysis: realizations and perspectives. *Desalination*, 144, 157–162.

Boniardi N., Rota R., Nano G., Mazza B. (1997) Lactic acid production by electrodialysis part I:experimental tests. *Journal of Applied Electrochemistry*, 27, 125–133.

Budhavaram N. K. and Fan Z. (2009) Production of lactic acid from paper sludge using acid-tolerant, thermophilic *Bacillus coagulans* strains. *Bioresource Technology*, 100, 5966–5972.

Carr F.J., Chinn D., Maida N. (2002) The lactic acid bacteria: a Literature survey. *Critical Reviews in Microbiology*, 28, 281-370.

Chandra R. P., Bura R., Mabee W. E., Berlin D. A., Pan X., Saddler J. N. (2007) Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics?. In *Biofuels*, . 67-93.

Chheda J., Huber G., Dumesic J. (2007) Liquid-phase catalytic processing of biomass-derived oxygenated hydrocarbon to fuels and chemicals. *Angewandte Chemie International Edition*, 46, 7164–7183.

Cui F., Li Y., Wan C. (2011) Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*. *Bioresource Technology*, 102, 1831-1836.

Dai M. H. and Copley S.D. (2004) Genome Shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. *Applied and Environmental Microbiology*, 70, 2391–2397.

Datta R. and Henry M. (2006) Lactic acid: recent advances in products, processes and technologies—a review. *Journal of Chemical Technology and Biotechnology*, 81, 1119-1129.

Deng X. W., Matsui M., Wei N., Wagner D., Chu A. M., Feldmann, K. A., Quail, P. H. (1992) An arabidopsis regulatory gene encodes a protein with both a zinc-binding motif and a homologous domain. *Cell*, 71, 791-801.

Dien B. S., Nichols N. N., Bothast, R. J. (2001) Recombinant *Escherichia coli* engineered for production of L-lactic acid from hexose and pentose sugars. *Journal of Industrial Microbiology and Biotechnology*, 27, 259-264.

Ding S., Lui Y., Zeng Y., Himmel M., Baker J., Bayer E. (2012) How does plant cell wall nanoscale architecture correlate with enzymatic digestibility? *Science.* 338, 1055-1060.

Dumbrepatil A., Adsul M., Chaudhari S., Khire J., Gokhale D. (2008) Utilization of molasses sugar for lactic acid production by *Lactobacillus delbrueckii* mutant Uc-3 in batch fermentation. *Applied and Environmental Microbiology*, 74, 333-335.

Fan Y., Zhou C, Zhu X. (2009) Selective catalysis of lactic acid to produce commodity chemicals. *Catalysis Reviews*, 51, 293–324.

Franken T. (2000) Bipolar membrane technology and its applications. *Membrane Technology*, 2000, 8-11.

Fukushima K., Sogo K., Miura S., Kimura Y. (2004) Production of D-lactic acid by bacterial fermentation of rice starch. *Macromolecular Bioscience*, 4, 1021-1027.

Fukushima K., Abbate C., Tabuani D., Gennari M., Camino G. (2009) Biodegradation of poly (lactic acid) and its nanocomposites. *Polymer Degradation and Stability*, 94, 1646-1655.

Galbe M. and Zacchi G. (2007) Pretreatment of lignocellulosic materials for efficient bioethanol production. In *Biofuels*, 41-65.

Galgali P., Puntambekar U., Gokhale D.V., Varma A. J. (2004) Fungal Degradation of Carbohydrate-Linked Polystyrenes. *Carbohydrate Polymer*, 55, 393-399.

Galgali P., Varma A., Puntambekar U., Gokhale D.V. (2002) Towards biodegradable polyolefins: strategy of anchoring minute quantities of monosaccharides and disaccharides onto functionalized polystyrene, and their effect on facilitating polymer biodegradation. *Chemical Communications*, 23, 2884-2885.

Gao M. T., Michiteru K., Rie G., Hirokazu T., Makoto H., Tadashi H. (2005) Development of a continuous electrodialysis fermentation system for production of lactic acid by *Lactobacillus rhamnosus*. *Process Biochemistry*, 40, 1033–1036.

Garlotta D. (2002) A literature review of poly(lactic acid). *Journal of Polymers and the Environment*, 9, 63–84.

Gokhale D. V. and Deobagkar D. N. (1989) Different expression of xylanases and endoglucanases in the hybrid derived from intergeneric protoplast fusion between *Cellulomonas sp.*and *Bacillus subtilis. Applied Environmental Microbiology*, 55, 2675-2680 .

Gokhale D. V. and Deobagkar D. N. (1990) Secretion of thermostable β-glucosidase by an intergeneric bacterial hybrid between *Cellulomonas sp*. and *Bacillus subtilis. Applied Biochemistry and Biotechnology*, 26, 207-216 .

Gokhale D. V., H. Eun Soo-Han Srinivasan S. R., Deobagkar D. N. (1984) Transfer of DNA coding for cellulase from *Cellulomonas species* to *Bacillus subtilis* by protoplast fusion. *Biotechnology Letters*, 6, 627-632.

Gong J., Zheng H., Wu Z., Chen T., Zhao X. (2009) Genome shuffling: Progress and applications for phenotype improvement. *Biotechnology Advances*, 27, 996-1005.

Grabar T. B., Zhou S., Shanmugam K., Yomano L., Ingram L. (2006) Methyl glyoxal bypass identified as source of chiral contamination in L(+) and D(-)-lactate fermentations by recombinant Escherichia coli. Biotechnology Letters, 28, 1527–1535.

Gusakov A. V. (2011) Alternatives to *Trichoderma reesei* in biofuel production. *Trends in biotechnology*, 29, 419-425.

Hahn-Hagerdal B., Galbe M., Gorwa-Grauslund M., Liden G., Zacchi G. (2006) Bio-ethanol – The fuel of tomorrow from the residues of today. *Trends in Biotechnology*, 24, 549–556.

Hakkarainen M, Albertsson A-C, Karlsson S. (1996) Weight losses and molecular weight changes correlated with the evolution of hydroxyacids in simulated *in vivo* degradation of homo- and copolymers of PLA and PGA. *Polymer Degradation and Stability*, 52, 283–291.

Han X., Satoh Y., Satoh T., Matsumoto K., Kakuchi T., Taguchi S., Dairi T., Munekata M. and Tajima K.(2011) Chemo-enzymatic synthesis of polyhydroxyalkanoate (PHA) incorporating 2-hydroxybutyrate by wild-type class-I PHA synthase from *Ralstonia eutropha*. *Applied Microbiology and Biotechnology*, 92, 509–517.

Hano T., Matsumoto M., Uenoyama S., Ohtake T., Kawano Y., Miura S. (1993) Separation of lactic acid from fermented broth by solvent extraction. *Bioseparation*, 3, 321–326.

Hauer E. and Marr R. (1994) Liquid extraction in biotechnology. *International Chemical Engineering*, 34, 178–187.

Hendriks A. T., and Zeeman G. (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource technology*, 100, 10-18.

Henrissat B., Terri T., Warren R. (1998) A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants. *Federation of European Biochemical Societies*, 425, 352–354.

Heriban V., Skara J., Sturdik E., Ilavsky J. (1993) Isolation of free lactic acid using electrodialysis. *Biotechnology Techniques*, 7, 63–68.

Himmel M., Baker J., Overend R. (1994) Approaches to cellulase purification. In:Enzymatic Conversion of Biomass for Fuel Production. ACS symposium series 566.

Himmel M., Ding S., Johnson D., Adney W., Nimlos M., Brady J., Foust T. (2007) Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. *Science*. 2207, 804-807.

Hofvendahl K. and Hahn-Hägerdal B.(2000) Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme and Microbial Technology*, 26, 87–107.

Hongo M., Nomura Y., Iwahara M. (1986) Novel method of lactic acid production by electrodialysis fermentation. *Applied and Environmental Microbiology*, 52, 314–319.

Hopwood A. (1981). Uses of protoplasts in the genetic manipulation of *streptomycetes. Zentrulblatt fur Bakteriologie* , 11, 523-531.

Hoshino A. and Isono Y. (2002) Degradation of aliphatic polyester films by commercially available lipases with special reference to rapid and complete degradation of poly(L-lactide) film by lipase PL derived from Alcaligenes sp. Biodegradation, 13, 141-147.

Huber G., Iborra S., Corma A. (2006) Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering. *Chemical Reviews*, 106, 4044–4098.

Ishida N., Suzuki T., Tokohiro K., Nagamori E., Onishi T., Saitoh S., Takahashi H. (2006) D-lactic acid production by metabolically engineered *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering*, 101, 172-177.

Jogdand S.N. (2001) Protoplast Technology, Gene Biotechnology,Himalaya Publishing house 3rd ed 171-186.

Jung Y., Kim T., Park S., Lee S. (2010) Metabolic engineering of *Escherichia coli* for the production of polylactic acid and its copolymers. *Biotechnology Bioengineering*, 105, 161–171.

Kadam S. R. Patil S. S., Bastawde K.B., Khire J. M., Gokhale D. V. (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. *Process Biochemistry*, 41, 120-126.

Kaufman E. N., Cooper S. P., Davison B. H. (1994) Screening of resins for use in a biparticle fluidized-bed bioreactor for the continuous fermentation and separation of lactic acid. Applied Biochemistry and Biotechnology, 46, 545–554.

Kim C. Y., Lee S. H., Park H. C., Bae, C. G., Cheong Y. H., Choi Y. J., Cho, M. J. (2000) Identification of rice blast fungal elicitor-responsive genes by differential display analysis. *Molecular plant-microbe interactions*, 13, 470-474.

Kim Y. H. and Moon S. H. (2001) Lactic acid recovery from fermentation broth using one-stage electrodialysis. *Journal of Chemical Technology and Biotechnology*, 76, 169–178.

Klass D. (2004) Biomass for renewable energy and fuels. In Encyclopedia of Energy, ed. CJ Cleveland London: Elsevier 1,193–212.

Kovacs K., Macrelli S., Szakacs G., Zacchi G. (2009) Enzymatic hydrolysis of steam-pretreated lignocellulosic materials with *Trichoderma atroviride* enzymes produced in-house. *Biotechnology for Biofuels*, 14, 1-11.

Kumar P., Barrete D., Delwiche M., Stroeve P. (2009a) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial and Engineering Chemistry Research*, 48, 3713–3729.

Kumar R., Gaurav Mago B., Venkatesh Balan C., Charles E., Wyman D. (2009b) Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource Technology*, 100, 3948–3962.

Lange J. (2007) Lignocellulose conversion: an introduction to chemistry, process and economics. *Biofuels Bioproducts and Biorefining*, 1, 39–48.

Laopaiboon P., Thani A., Leelavatcharamas V., Laopaiboon L.(2010) Acid hydrolysis of sugarcane bagasse for lactic acid production. *Bioresource Technology*, 101, 1036–1043.

Li S. M., Garreau M., Vert H. (1990) Structure-property relationships in the case of the degradation of massive aliphatic poly-(a-hydroxy acids) in aqueous media, part 1: poly(DL-lactic acid) *Journal of Material Science and Material Medicine*, 1, 123–130.

Lichtenthaler F. and Peter S. (2004) Carbohydrates as green raw materials for the chemical industry. *Comptes Rendus* Chimie, 7, 65-90.

Lu Z., Lu M., He F., Yu L. (2009) An economical approach for D-lactic acid production utilizing unpolished rice from paddy as major nutrient source. *Bioresource Technology*, 100, 2026-2031

Luckachan G. and Pillai C. (2006) Chitosan/oligo L-lactide graft copolymers: effect of hydrophobic side chains on the physico-chemical properties and biodegradability. *Carbohydrate Polymers*, 24, 254-266.

Lunt J. (1998) Large-scale production, properties and commercial applications of polylactic acid polymers .*Polymer Degradation and Stabilility*, 9, 145–152

Lynd L., Weimer P., Van Zyl W., Pretorious I. (2002) Microbial Cellulose Utilization: Fundamentals and Biotechnology .*Microbiology and Molecular Biology Review*, 66, 506-577.

Macdonald K. D. and Holt G. (1976) Genetics of biosynthesis and overproduction of penicillin. *Science progress*, 63, 547.

Magnusson L., Islam R., Sparling R., Levin D., Cicek N. (2008) Direct hydrogen production from cellulosic waste materials with a single-step dark fermentation process. *International Journal of Hydrogen Energy*, 33, 5398-5403.

Maguin E., Prevost H., Ehrlich S. D., Gruss A. (1996) Efficient insertional mutagenesis in *lactococci* and other gram-positive bacteria. *Journal of Bacteriology*, 178, 931-935.

Maki M., Leung K., Qin W. (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences*, 5, 500-516.

Maki-Arvela P., Simakova I.L., Salmi T., Murzin D.U. (2014) Production of Lactic Acid / Lactates from Biomass and Their Catalytic Transformations to Commodities. *Chemical Reviews* 114, 1909-1971.

Matsumoto K. and Taguchi S.(2009) Enzymatic and whole-cell synthesis of lactate-containing polyesters: toward the complete biological production of polylactate. *Applied Microbiology and Biotechnology*, 85, 921–932.

Matsumoto K., Ishiyama A., Sakai K., Shiba T., Taguchi S. (2011) Biosynthesis of glycolate-based polyesters containing mediumchain-length 3-hydroxyalkanoates in recombinant *Escherichia coli* expressing engineered polyhydroxyalkanoate synthase. *Journal of Biotechnology*, 156, 214–217.

Mazumdar S., Clomburg J. M., Gonzalez R. (2010) *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. *Applied and Environmental Microbiology*, 76, 4327-4336.

Min-tian G., Koide M., Gotou R., Takanashi H., Hirata M., Hano T. (2005) Development of a continuous electrodialysis fermentation system for production of lactic acid by *Lactobacillus rhamnosus. Process Biochemistry*, 40, 1033–1036.

Mishra P. and Singh P. (1993) Microbial pentose utilization, in: S. Neidlemen, A.I. Laskin (Eds.), *Adv. App. Microbiol.* 39, 351 Academic press, California.

Moldes A. B., Alonso J. L., Parajo J. C. (2000) Multi-step feeding systems forlactic acid production by simultaneous saccharification and fermentation of processed wood. *Bioprocess Engineering,* 22, 175-180.

Mollet B., Knol J., Poolman B., Marciset O., Delley M. (1993) Directed genomic integration, gene replacement, and integrative gene expression in *Streptococcus thermophilus*. *Journal of bacteriology,* 175, 4315-4324.

Moraïs S., Morag E., Barak Y., Goldman D., Hadar Y., Lamed R., Bayer E. A. (2012) Deconstruction of lignocellulose into soluble sugars by native and designer cellulosomes. *MBio*, 3(6), e00508-12.

Mosier N., Wyman C., Dale B., Elander R., Lee Y. Y., Holtzapple M., Ladisch M. (2005a) Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource technology*, 96, 673-686.

Mosier N. S., Hendrickson R., Brewer M., Ho N., Sedlak M., Dreshel R., Ladisch M. R. (2005b) Industrial scale-up of pH-controlled liquid hot water pretreatment of corn fiber for fuel ethanol production. *Applied Biochemistry and Biotechnology*, 125, 77-97.

Nampoothiri M., Nair R., John P. (2010) An overview of the recent developments in polylactide (PLA) research. *Bioresouce Technology*, 101, 8493–8501.

Narayanswamy S. (1994) Plant cells and tissue cultures. Plant Protoplast: Isolation,Culture and Fusion ,391-469.TATA MCGraw Hill Publishing Company, New Delhi,India.

Narayanan N., Roychoudhury P.K., Srivastava A. (2004) L (+) lactic acid fermentation and its product polymerization. *Electronic Journal of Biotechnology*, 7, 167-179.

National Science Foundation (NSF) (2008) Breaking the Chemical and Engineering Barriers to Lignocellulosic Biofuels: Next Generation Hydrocarbon Biorefineries, ed. GW Huber, Workshop.

Naveena B. J., Altaf M., Bhadriah K., Reddy G. (2005) Selection of medium components by Placket Burman design for the production of L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. *Bioresource Technology*, 96, 485–490.

Nomura Y., Yamamoto K., Ishizaki A. (1991) Factors affecting lactic acid production rate in the built-in electrodialysis fermentation, an approach to high speed batch culture. *Journal of Fermentation and Bioengineering*, 71, 450–452.

Okanishi M., Suzuki K., Umezawa H. (1974) Formation and reversion of *Streptomycete* protoplasts: cultural condition and morphological study. *Journal of general microbiology*, 80, 389-400.

Okano K., Tanaka T., Ogino C., Fukuda H., Kondo A. (2010) Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. *Applied Microbiology and Biotechnology*, 85, 413–423.

Patel M. A., Ou M. S., Ingram L. O., Shanmugam K. T. (2005) Simultaneous Saccharification and Co-Fermentation of Crystalline Cellulose and Sugar Cane Bagasse Hemicellulose Hydrolysate to Lactate by a Thermotolerant Acidophilic *Bacillus sp. Biotechnology progress*, 21, 1453-1460.

Patel M., Ou M., Harbrucker R., Aldrich H., Buszko M., Ingram L., Shanmugam K. (2006) Isolation and characterization of acid tolerant, thermophilic bacterial biocatalysts for effective fermentation of biomass-derived sugars to lactic acid. *Applied and Environmental Microbiology*, 72, 3228-3235.

Patnaik R., Louie S., Gavrilovic V., Perry K., Willem P. C., Stemmer M., del Cardayré S. (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nature Biotechnology*, 20, 707–712 .

Petri R. and Schmidt-Dannert C. (2004) Dealing with complexity: evolutionary engineering and genome shuffling. *Current Opinion in Biotechnology*, 15, 298-304.

Qin J., Zhao B., Wang X., Wang L., Yu B., Ma Y. (2009) Non-sterilized fermentative production of polymer-grade L-lactic acid by a newly isolated thermophilic strain *Bacillus sp.* 2–6. *PLoS One,* 4, 43-59.

Quesada J., Rubio M., Gómez D. (1999) Ozonation of lignin rich solid fractions from corn stalks. *Journal of wood chemistry and technology*, 19, 115-137.

Rai N. and Rai M. (2006) Reproduction, Pollen Biology, and Pollen Biotechnology. In: Heterosis breeding in vegetable crops, ed. Kalloo G., New India Publishing Agency 184-185.

Rassoulzadegan M., Binetruy B., Cuzin F. (1982) High frequency of gene transfer after fusion between bacteria and eukaryotic cells. *Nature*, 295, 257-259.

Rinaldi R., Meine N., vom Stein J., Palkovits R., Schüth; F. (2010) Which controls the depolymerization of cellulose in ionic liquids: The solid acid catalyst or cellulose?; *Chem Sus Chem*, 3(2), 266-276.

Rojan P., Noomphhothiri K., Nair A., Pandey A. (2005) L(+)-Lactic acid production using *Lactobacillus casei* in solid-state fermentation. *Biotechnology Letters*, 27, 1685–1688.

Roper H. (2002) Renewable Raw Materials in Europe — Industrial utilisation of starch and sugar. *Starch-Starke.* 54, 89-99.

Sakai K. M., Taniguchi S., Miura H., Ohara T., Matsumoto M., Shirai Y. (2004) Making plastics from garbage—a novel process for poly-L-lactate production from municipal food waste. *Journal of Industrial Ecology*, 7, 63–73.

Sathitsuksanoh N., Holtman K. M., Yelle, D. J., Morgan, T., Stavila, V., Pelton, J., Blanch, H., Simmons B. A. George, A. (2014) Lignin fate and characterization during ionic liquid biomass pretreatment for renewable chemicals and fuels production. *Green Chemistry*, 16(3), 1236-1247.

Shozui F., Matsumoto K., Nakai T., Yamada M., Taguchi S. (2009) Biosynthesis of novel terpolymers poly-(lactate-co-3- hydroxybutyrateco-3-hydroxyvalerate) in lactate-overproducing mutant *Escherichia coli* JW0885 by feeding propionate as a precursor of 3-hydroxyvalerate. *Applied Microbiology and Biotechnology*, 85, 949–954.

Sinha Ray S. and Bousmina M. (2005). Biodegradable polymers and their layered silicate nanocomposites: in greening the 21st century materials world. *Progress in Materials Science*, 50(8), 962-1079.

Sirman T., Pyle D. L., Grandison A. S. (1991) Extraction of organic acids using a supported liquid membrane. *Biochemical Society Transactions*, 19, 274–279.

Södergård A. and Stolt M. (2002) Properties of lactic acid based polymers and their correlation with composition. *Progress in Polymer Science*, 27, 1123–1163.

Sommerville D., Bradley R., Mailley D. (2004) Leaf litter quality and decomposition rates of yellow birch and sugar maple seedlings grown in mono-culture and mixed-culture pots at three soil fertility levels. *Trees*, 18, 608–613.

Song Y., Matsumoto K. I., Yamada M., Gohda A., Brigham C. J., Sinskey A. J., Taguchi S. (2012). Engineered *Corynebacterium glutamicum* as an endotoxin-free platform strain for lactate-based polyester production. *Applied microbiology and biotechnology*, 93(5), 1917-1925.

Taguchi S. and Doi Y. (2004) Evolution of polyhydroxyalkanoate (PHA) production system by "enzyme evolution": successful case studies of directed evolution. *Macromolecular Biosciences*, 4, 145–156.

Taguchi S., Yamada M., Matsumoto K., Tajima K., Satoh Y., Munekata M. (2008) A microbial factory for lactate-based polyesters using a lactate polymerizing enzyme. *Proceedings of the National Academy of Sciences*, 105, 17323–17327.

Tajima K., Han X., Satoh Y., Ishii A., Araki Y., Munekata M., Taguchi S.(2012) In vitro synthesis of polyhydroxyalkanoate (PHA) incorporating lactate (LA) with a block sequence by using a newly engineered thermostable PHA synthase from *Pseudomonas sp*. SG4502 with acquired LA-polymerizing activity. *Applied Microbiology and Biotechnology*, 94, 365-376.

Tanaka T., Hoshina M., Tanabe S., Sakai K., Ohtsubo S., Taniguchi M. (2006) Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation. *Bioresource Technology*, 97, 211–217.

Tango M. and Ghaly A. (2002) A continuous lactic acid production system using an immobilized packed bed of *Lactobacillus helveticus*. *Applied Microbioliology and Biotechnology*, 58, 712–720.

Teeri T. (1998) *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochemical Society Transactions*, 26, 173–178.

Thang V.H., Koschuh W., Kulbe K. D., Novalin S. (2005) Detailed investigation of an electrodialytic process during the separation of lactic acid from a complex mixture. *Journal of Membrane Science*, 249, 173–182.

Van Solingen, P. and Van Der Plaat J. B. (1977) Fusion of yeast spheroplasts. *Journal of bacteriology*, 130, 946-947.

Varadarajan S. and Miller D. (1999) Catalytic Upgrading of Fermentation-Derived Organic Acids. *Biotechnology Progress*, 15, 845-854.

Vardi A., Arzee-Gonen P., Frydman-Shani A., Bleichman S., Galun, E. (1989) Protoplast-fusion-mediated transfer of organelles from Microcitrus into Citrus and regeneration of novel alloplasmic trees. *Theoretical and applied genetics*, 78, 741-747.

Verma V., Qazi G.N., Parshad R. (1992) Intergeneric protoplast fusion between *Gluconobacter oxydans* and *Corynebacterium species*. *Journal of Biotechnology*, 26, 327-330.

Vick Roy T. (1985) Lactic acid. In: Young M.M. (ed.) Comprehensive Biotechnology. Pergamon Press, New York 3, 761–775 .

Vink E. T. H., Glassner D. A, Kolstad J. J., Wooley R. J, O'Connor R. P. (2007) The eco-profiles for current and near-future NatureWorks polylactide (PLA) production. *Industrial Biotechnology*, 3, 58–81.

Vonktaveesuk P., Tonokawa M., Ishizaki A. (1994) Stimulation of the rate of L-lactate fermentation using *Lactococcus lactis* IO-1 by periodic electrodialysis. *Journal of Fermentation and Bioengineering*, 77, 508–512.

Wang Y. H., Li Y., Pei X. L., Yu L., Feng Y. (2007) Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity in *Lactobacillus rhamnosus. Journal of Biotechnology*, 129, 510–515.

Wang L., Zhao B., Liu C., Yang B., Yu S., Li Q. (2010) Efficient production of L-lactic acid from cassava powder by Lactobacillus rhamnosus. Bioresour Technol, 101, 7895–7901.

Wang L., Zhao B., Li F., Xu K., Ma C., Tao F. (2011) Highly efficient production of D-lactate by *Sporolactobacillus sp*. CASD with simultaneous enzymatic hydrolysis of peanut meal. *Appl Microbiol Biotechnol*, 89, 1009–1017.

Wang Y., Tian T., Zhao J., Wang J., Yan T., Xu L., Zhou S. (2012) Homofermentative production of d-lactic acid from sucrose by a metabolically engineered *Escherichia coli*. *Biotechnology letters*, 34, 2069-2075.

Wang Y., Deng W., Wang B., Zhang Q., Wan X., Tang Z., Wang Y., Zhu C., Cao Z., Wang G., Wan H. (2013) *Nature Communications*, 4, Article No. 2141 (doi:10/ncomms3141).

Watson B. J., Zhang H., Longmire A. G., Moon Y. H., Hutcheson S. W. (2009) Processive endoglucanases mediate degradation of cellulose by *Saccharophagus degradans*. *Journal of bacteriology*, 191, 5697-5705.

Wee Y. J., Yun J. S., Lee Y. Y., Zeng A. P., Ryu H. W. (2005) Recovery of lactic acid by repeated batch electrodialysis and lactic acid production using electrodialysis wastewater. *Journal of Biosciences and Bioengineering*, 99, 104–108.

Wilson D. B. (2011) Microbial diversity of cellulose hydrolysis. *Current Opinion in Microbiology*, 14, 259-263.

Wyman C. E., Dale B. E., Elander R.T., Holtzapple M., Ladish M. R., Lee Y.Y. (2005) Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*, 96, 1959-1966.

Yamada M, Matsumoto K., Nakai T., Taguchi S. (2009) Microbial production of lactate-enriched poly[(R)-lactate-co-(R)-3-hydroxybutyrate] with novel thermal properties. *Biomacromolecules*, 10, 677–681.

Yanez R., Moldes A. B., Alonso J. L., Parajo J. C. (2003) Production of D (-)-lactic acid from cellulose by simultaneous saccharification and fermentation using *Lactobacillus coryniformis subsp. torquens*. *Biotechnology Letters*, 25, 1161-1164.

Yanez R., Alonso J. L., Parajo J. C. (2005) D-Lactic acid production from waste cardboard. *Journal of Chemical Technology and Biotechnology*, 80, 76–84.

Yang J., Neng Tan J., Gu Y. (2012) Lactic acid as an invaluable bio-based solvent for organic reactions. *Green Chemistry*, 14, 3304-3317.

Yang X., Lai Z., Lai C., Zhu M., Li S., Wang J., Wang, X. (2013) Efficient production of L-lactic acid by an engineered *Thermoanaerobacterium aotearoense* with broad substrate specificity. *Biotechnology for Biofuels*, 6, 124.

Yi S. S., Lu Y. C., Luo G. S. (2008) Separation and concentration of lactic acid by electro-electrodialysis. *Separation and Purification Technology*, 60, 308–314.

Zhang D. and Cheryan M. (1991) Direct fermentation of starch to lactic acid by *Lactobacillus amylovorus*. *Biotechnology Letters*, 10, 733–738.

Zhang Y. and Lynd L. R. (2006) A functionally based model for hydrolysis of cellulose by fungal cellulase. *Biotechnololy and Bioengineering*, 694, 888–898.

Zhang Y. H. P. (2008) Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. *Journal of Industrial Microbiology and Biotechnology*, 35, 367-375.

Zhang Y., Himmel M., Mielenz J. (2006) Outlook for cellulase improvement: screening and selection strategies. *Biotechnology Advances*, 24, 452–481.

Zhang Y., Perry K., Powell K., Stemmer P. C., del Cardayré S. (2002) Evolution of *Streptomyces fradiae* by whole genome shuffling. *Nature*, 415, 644–646.

Zhao J., Xu L., Wang Y., Zhao X., Wang J., Garza E., Zhou, S. (2013) Homofermentative production of optically pure L-lactic acid from xylose by genetically engineered *Escherichia coli Microbial Cell Factories*, 12, 57.

Zhou S. D., Causey T. B, Hasona A., Shanmugam K. T., Ingram L. O. (2003a) Production of pure D-lactic acid in mineral salts medim by metabolically engineered *Escherichia coli* W3110. *Applied and Environmental Microbiology*, 69, 399-407.

Zhou S. D ., Shanmugam K. T., Ingram L. O. (2003b) Functional replacement of the *Escherichia coli* D-(-)-lactate dehydrogenase gene (ldhA) with the L-(+)-lactate dehydrogenase gene (ldhL) from *Pediococcus acidilactici Applied and Environmental Microbiology*, 69, 2237-2244.

Zhou S., Yomano L. P., Shanmugam K. T., Ingram, L. O. (2005) Fermentation of 10%(w/v) sugar to D (−)-lactate by engineered *Escherichia coli*. *Biotechnology letters*, 27, 1891-1896.

Zhu S. D. (2007) Exp-function method for the Hybrid-Lattice system. *International Journal of Nonlinear Sciences and Numerical Simulation*, 8, 461-464.

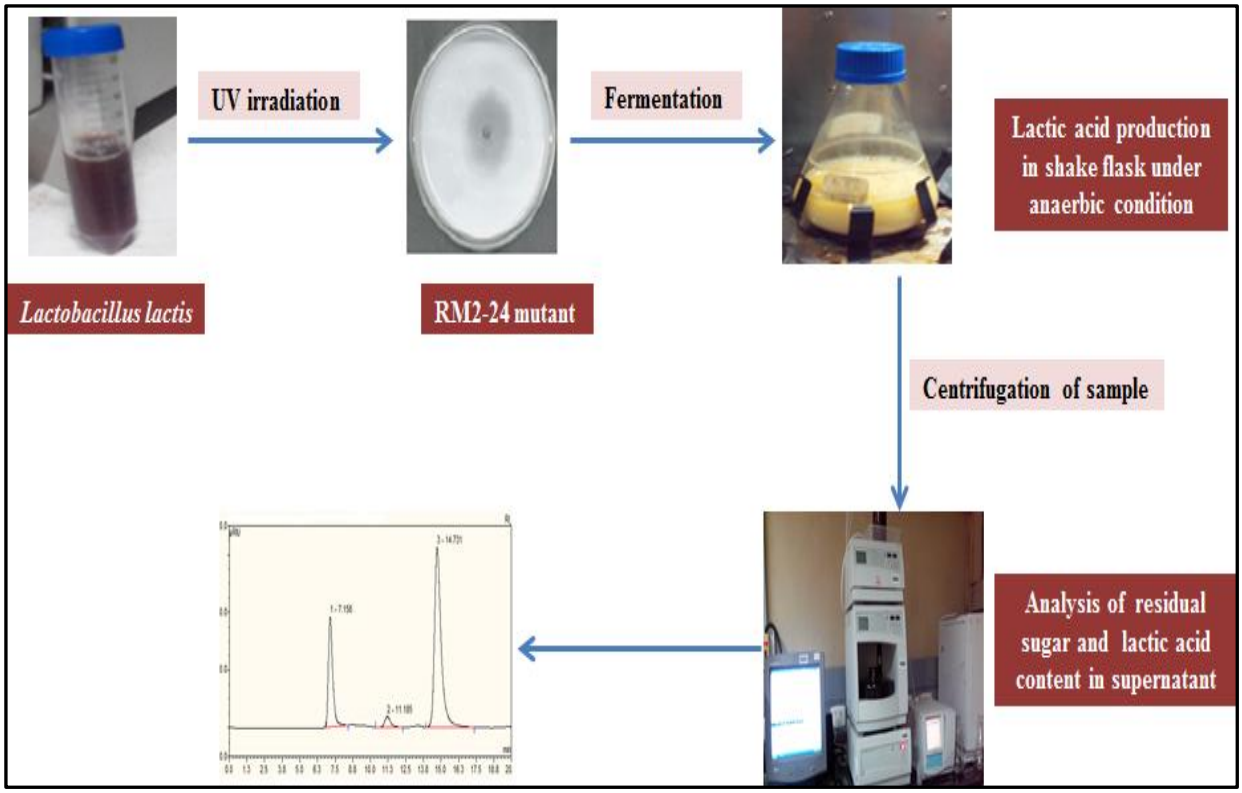
Zhua J. Y., Wang G. S., Pan X. J., Gleisner, R. (2009) Specific surface to evaluate the efficiencies of milling and pretreatment of wood for enzymatic saccharification. *Chemical Engineering Science*, 64, 474-485.

Zwart R. W., Boerrigter H., van der Drift A. (2006) The impact of biomass pretreatment on the feasibility of overseas biomass conversion to Fischer-Tropsch products. *Energy and Fuels*, 20, 2192-2197.

CHAPTER 2

Strain improvement of *Lactobacillus lactis* NCIM 2368 for D-lactic acid production

Graphical Abstract



- **Singhvi M. et al (2013) *Bioresource Technol.* 146, 536-739.**
- **Joshi D.S., Singhvi,M.S. et al (2010) *Biotechnol. Lett*. 32, 517-520.**

SUMMARY

Approximately, 90 lactic acid producing bacterial strains were screened for the production of D-lactic acid and *Lactobacillus lactis* NCIM 2368 was selected which produced pure D-lactic acid but with very less productivity. This strain was further improved using UV mutagenesis and the improved strain was named as *Lactobacillus lactis* RM2-24. *Lactobacillus lactis* RM2-24 was compared with the wild type for D-lactic acid production using 100 g/l of hydrolyzed cane sugar in the fermentation medium. The improved strain RM2-24 produced 81 g/l lactic acid which was over three times that of the wild type. The highest D-lactic acid (110 g/l) in batch fermentation was obtained with 150 g/l of hydrolyzed cane sugar with a 73% lactic acid yield. The mutant also utilized cellobiose very efficiently and converted it into D-lactic acid suggesting the presence of cellobiase. Thus, this strain could be used to obtain D-lactic acid from cellulosic materials that are pre-hydrolyzed with cellulase.

The production of D-lactic acid by *Lactobacillus lactis* RM2-24 was investigated using modified media with a view to increasing the efficiency of the fermentation process. The results indicated that the addition of 5 g/l of peptone and 1 g/l of $(NH_4)_2HPO_4$ enhanced D-lactic acid production by 32%, as compared to that obtained from non-supplemented media, with a productivity of 3.0 g/l/h. The fermented broth was checked for the purity of D-lactic acid which surprisingly showed the presence of small amount (5%) of L-lactic acid. Therefore, lactate dehydrogenase (LDH) expression profiles in these different media was studied which resulted in appearance of additional LDH isoform produced by cells when they were grown in

HSYE medium supplemented with $(NH_4)_2HPO_4$. The additional LDH band appeared to be L-LDH contributing to production of L-lactic acid in the fermented broth.

INTRODUCTION

In recent years, demand for lactic acid in production of biodegradable plastics and coating for application in biocompatible materials used in medical implants, has increased (Cheng et al., 1991; Goncalves et al., 1992). It is a raw material for the production of polylactic acid (PLA) that is biodegradable and eco-friendly polymer which could be the substitute for synthetic plastics derived from petroleum feedstock. It is also a product that has found immense applications in diverse fields as an acidulant in the food and pharmaceutical industries (Schepers et al., 2002). The chemical synthesis of lactic acid leads to racemic mixture, which is a major disadvantage. Fermentative production of lactic acid offers a great advantage since D- or L-lactic acid is produced depending upon the strains selected for fermentation. The optical purity of the lactic acid is crucial factor for the physical properties of PLA. Though L-lactic acid can be polymerized to get crystalline poly L-lactic acid (PLLA), its application is limited due to its low melting point (Sodegard and Stolt, 2002). The attractive solution to increase the heat stability of PLLA is the complexing of PLLA with poly D-lactic acid (PDLA) (Tsuji et al., 1991; Suji and Fukui, 2003). This finding has generated a great interest in the production of D-lactic acid. L-Lactic acid production by fermentation is well established (Yu and Hang, 1989). However, fermentation of sugars to D-lactic acid is not well studied and its microbial productivity is not well known (Zhou et al., 2003). Therefore, we decided to investigate D-lactic acid fermentation with a view to obtaining the improved strains capable of producing D-lactic acid with enhanced productivities.

India is one of the largest producers of sugar cane (approximately 300 million tons) from which about 20 million tons of cane sugar is produced. During this process, large amounts of molasses are generated as a byproduct which contains 40–60% (w/v) sucrose. All these byproducts can be converted to value-added products such as lactic acid. Most of the lactic acid bacteria generally have complex nutrient requirements and very low growth rates, which are the main drawbacks of industrial lactic acid fermentation processes. Therefore, there is a need for developing lactic acid bacteria, which require minimum amount of nutrients in a medium and convert carbohydrates in to optically pure lactic acid through homo-fermentative pathway.

Lactobacillus lactis NCIM 2368 is a homo-fermentative D(-)- lactic acid producing strain selected through screening protocol. This chapter deals with the isolation of mutants of *Lactobacillus lactis* capable of producing enhanced levels of D-lactic acid. The isolated mutants showed greater acid formation zones on the selected medium. We also describe the effect of addition of various carbon and inorganic and organic nitrogen sources in the fermentation medium on lactic acid production. It was found that the addition of peptone and $(NH_4)_2HPO_4$ increased the growth of *L. lactis* RM 2–24 remarkably with maximum D-lactic acid production.

MATERIALS AND METHODS

Chemicals

Glucose, cellobiose, yeast extract and MRS liquid medium were obtained from Hi-Media (India). Cane sugar was obtained from local market. All other chemicals were of analytical grade and procured from Hi-Media or from B.D.H. UK. Molasses was a kind gift from local sugar factory.

Microorganisms and growth media

Lactobacillus lactis NCIM 2368 and its mutants were maintained in liquid MRS medium (Hi-Media, Mumbai, India) supplemented with 0.1% $CaCO_3$ which was used as stock culture for preparation of inoculum. L-lactic acid producing mutant strain, *Lactobacillus delbrueckii* Uc-3 was isolated in our laboratory using UV mutagenesis (Kadam et al., 2006). The stock cultures maintained in lyophilized vials at -50^{o}C were used for experiments. These cultures were grown on growth medium consisting of 100 g hydrolyzed cane sugar/l; 10 g yeast extract/l; and 45 g $CaCO_3$/l (HSYE media). The basic fermentation medium was the same as the growth medium. Molasses and cellobiose were also used as carbon sources at 10% (w/v) for D-lactic acid production. The cane sugar and molasses were hydrolyzed by adding 1-ml of 20% H_2SO_4 in 100-ml of sugar solution. The acidified sugar solution was heated in a boiling water bath for 20 min. The pH of the medium was adjusted to 6.8 with 4.0 M KOH prior to sterilization. The different nitrogen sources were added separately to the medium after sterilization at 121 ^{o}C for 20 min.

Inoculum preparation

Cells from stock cultures were transferred to 30 ml sterile growth medium in 50 ml screw cap tubes and incubated at 42 ^{o}C for 24 h under stationary conditions. This culture (2 ml) was then transferred to 100 ml of the growth medium in 250 ml screw cap conical flask. The flask was incubated at 42 ^{o}C with shaking (150 rpm) for 24 or 48 h till the optical density (growth) reached approximately 10. This culture was used as an inoculum to be transferred to fermentation medium.

Mutagenesis and mutant selection

Lactobacillus lactis was grown in 30 ml MRS liquid medium with 0.1% $CaCO_3$ at 37 ^{o}C under stationary conditions for 24 h. The culture (10 ml) was centrifuged at 10,000 rpm for 10 min, the supernatant was decanted and the cell pellet was washed three times with saline. The cells were re-suspended in 10 ml of saline and the total viable count was adjusted to 10^7/ml. Ten ml of the diluted sample in a 9 cm Petri dish was irradiated with a UV lamp at 254 nm at 6 cm for 20 min. The samples were serially diluted in sterile saline and survivors determined by spreading 0.1 ml of diluted samples on a agar medium containing 100 g cane sugar/l; 10 g yeast extract/l; 10 g $CaCO_3$/l and 20 g agar/l. The viable counts were determined after anaerobic incubation at 42 ^{o}C until the distinct colonies appeared with zones of acid production. The scheme of obtaining mutants is shown in Figure 2.1.

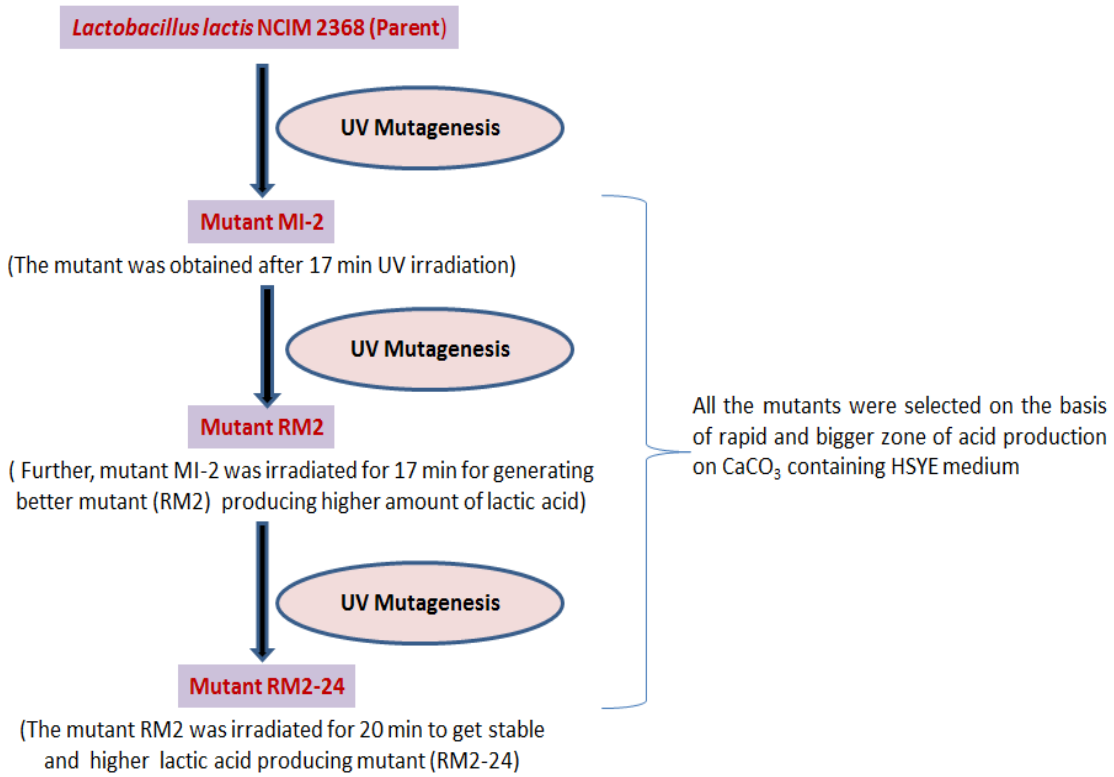


Figure 2.1: Schematic representation of Mutant's isolation

Lactic acid production in shake-flasks

An overnight culture (~5 ml) was transferred to 100 ml growth medium in 250 ml screw-cap conical flasks and then shaken (150 rpm) at 42 ^{o}C for 24 or 48 h. This culture was inoculated at 5% (v/v) into HSYE fermentation media. Fermentation experiments were carried out in screw-cap conical flasks containing 100 ml fermentation medium and also shaken at 150 rpm at 42 ^{o}C. Culture samples removed after certain time intervals were centrifuged at 10,000 rpm for 10 min to separate the cells. The supernatant was analyzed for sugar and lactic acid and pH measurement. The supernatant was acidified with equal volume of 1 M HCl where free acid is liberated. The acidified supernatant was further used for determination of total sugar and lactic acid. The cells recovered after centrifugation, were further processed for determination of biomass and intracellular lactate dehydrogenase (LDH) activity.

Sonication of cells

The cells (1.0 g) were washed three times with Tris–HCl buffer (50 mM, pH 8.0) and re-suspended in the same buffer (5 ml) containing 10% (v/v) glycerol, 0.1% Triton X-100, lysozyme (100 µg/ml), 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 5 mM β-mercaptoethanol. The cell suspension was incubated at 37 ^{o}C for 15 min and subjected to sonication (SONICS Vibra cell; model VC 130). The sonication was performed at 60% amplitude (125 µm) for 10 min by using 2 mm probe under cold conditions. Almost 90% of the cells were disrupted by this method. The supernatant after centrifugation was used for analyzing LDH activity and zymogram staining.

Determination of LDH activity

In order to determine the LDH activity, a continuous assay using UV absorbance at 280 nm was employed to monitor the NADH concentration during the oxidation catalyzed by LDH. The assay mixture contained 2.7-ml Tris buffer (0.2 M, pH 7.3), 0.1-ml substrate solution (30 mM sodium pyruvate in 0.2 M Tris buffer, pH 7.3), 0.1-ml NADH (6.6 mM in distilled water) and 0.1-ml supernatant. The reaction was started by addition of NADH solution to the mixture and measured over a period of 1 min. The enzyme activity was expressed as the initial rate [U /min/ (mg protein)] of substrate oxidation calculated from $\Delta A\ d^{-1}\ e^{-1}$, where ΔA is the change in absorbance at 340 nm, a measure of the oxidation of NADH; d is the light path; and e is the molar absorption coefficient of NADH at 340 nm.

Zymogram staining of lactate dehydrogenase (LDH)

Native polyacrylamide gel electrophoresis (10%) was performed by a modification of the method of Laemmli (1970) and gels were stained for LDH activity according to direct method described by Garvie (1969). Native PAGE was performed as described by Laemmli (1970) at pH 8.3 using 10% acrylamide as resolving gel with 5% stacking gel. Aliquots of 15µl were loaded into sample wells and electrophoresed at a constant voltage of 150 volt for 3h. After electrophoresis at pH 8.3 the lactate dehydrogenase (LDH) enzymes were located by direct staining by immersing the gel in a solution consisting of 50.0 mg of NAD, 10.0 mg of nitro-blue tetrazolium, 2.0 mg of phenazine methosulphate, 1mM sodium DL-lactate in 100 ml. 0.1M Tris-HCl buffer (pH 8.3). The gels were left for 30 min at room temperature in the dark and then examined.

Analytical methods

Lactic acid was analyzed by high-performance liquid chromatography with UV or refractive index detectors using Aminex HPX-87H column. The presence of L-lactic acid was analyzed by L-lactate oxidase enzyme kit (Randox Laboratories, UK) and the D-lactic acid content was calculated by subtracting L-lactic acid values from total lactic acid estimated by HPLC. The total lactic acid estimated by HPLC equipped with UV or RI detectors. An ion exclusion column (Aminex HPX-87H) was used with 4 mM H_2SO_4 as a mobile phase at 0.6 ml/min. An injection volume of the sample was 50 µl. The temperature of the column was maintained at 30 ^oC (Kadam et al., 2006). The sugar was analyzed by the dinitrosalicylic acid method (Fischer and Stein, 1961). The protein in the sample was determined by Bradford method (Bradford, 1976). The cell growth (optical density) was measured spectrophotometrically using Spectrophotometer-117 (Systronics, Mumbai, India) at a wavelength of 660 nm. The biomass was determined by washing the cells with saline several times and kept for drying at 80 ^oC till we obtained constant weight.

L-lactate oxidase enzyme assay

The L-lactic acid content in the fermentation broth was analyzed by L-lactate oxidase enzyme kit (Randox Laboratories, UK). The 6.0 ml of buffer was added from the kit into one bottle of dry reagent powder and dissolved by gentle shaking and inversion which is called as "Reagent Solution".1.0 ml reagent solution was distributed in tubes and thermostated at 25 ^oC and then 10µl sample was added in each tube. The mixture was incubated for 10 min at 25 ^oC and mixed gently. The absorbance of the sample was measured at wavelength of 550 nm in 1cm path length cuvette.

RESULTS

Selection of mutants

Mutants were isolated by exposing the 10 ml cell suspension (10^7 cells) to UV irradiation for 17 min which gave approx. 99% killing. Selection was based on rapid growth as well as a wider zone of acid production. About 10 colonies showing greater acid production zones were tested for lactic acid production in shake-flask. One mutant, MI-2, with higher lactic acid production capacity was further treated with UV irradiation which resulted in seven mutants showing larger acid production zones on agar plates. Among the seven mutants, RM-2 was selected based on higher production of lactic acid in shake flask. RM2 was further mutated by exposing it to UV-irradiation for 20 min which resulted in selection of mutant RM2-24 showing enhanced D-lactic acid production in shake flasks and was therefore selected for further studies. The lactic acid production by selected mutants in shake flask is shown in Table 2.1.

Table 2.1: Comparison of D-lactic acid production by parent (NCIM 2368) and mutant strains of *L. lactis* in production medium containing hydrolyzed cane sugar (10%), yeast extract (1%) and CaCO3 (4.5%)

Organism	Cell mass (g/l)		D-Lactic acid (g/l)	
	24h	48h	24h	48h
NCIM 2368	2.6	3.2	16.3 (0.68)	26.0 (0.54)
MI-2	4.4	4.3	25.0 (1.04)	47.5 (0.98)
RM2	4.4	5.0	34.5 (1.43)	49.0 (1.02)
RM2-24	4.5	5.6	52.0 (2.17)	81.0 (1.68)

The cultures were grown anaerobically for 48 h at 42 ^oC with shaking (150 rpm). The values given are the average of three independent experiments. The numbers in the parenthesis show lactic acid productivities (g/l /h).

Lactic acid fermentation using different carbon sources

Lactic acid production from different substrates using RM2-24 was performed in fermentation medium with 10% (w/v) substrate and the results are given in Table 2.2. Since the *Lactobacillus* strains did not degrade sucrose, we hydrolyzed the cane sugar and molasses and then used in the fermentation medium. The mutant RM2- 24 produced lactic acid more efficiently than the parent strain. Lactic acid production using molasses was comparatively less probably due to the presence of non-fermentable sugars. The mutant RM2-24 was also found to produce lactic acid from cellobiose suggesting the presence of high cellobiase activity in the mutant strain. These are the highest productivity and efficiency values reported so far for lactic acid production from cellobiose. The optical purity of D-lactic acid derived from all substrates was 98%.

Table 2.2: Effect of different substrates on cell growth and D-lactic acid production by *L. lactis* RM2-24

Carbon sources	Cell mass (g/l)		D-Lactic acid (g/l)	
	24h	48h	24h	48h
Glucose	4.0	4.5	51.5 (2.12)	78 (1.61)
Hydrolyzed cane sugar	4.4	4.7	52 (2.17)	81 (1.68)
Molasses	5.1	5.5	50 (2.08)	70 (1.45)
Cellobiose	3.7	5.0	50 (2.08)	80 (1.66)

The cultures were grown anaerobically for 48 h at 42 ^{o}C with shaking (150 rpm). The numbers in the parenthesis show lactic acid productivities (g/l h). The values given are the average of three independent experiments.

Effect of initial cane sugar concentration on D- lactic acid production

Our main aim was to use cane sugar for lactic acid production. Hence we studied the effect of various concentrations of hydrolyzed cane sugar on lactic acid production by mutant RM2-24. As shown in Table 2.3, the mutant produced increasing amounts of lactic acid with increasing sugar concentrations up to 200 g/l. The profile of lactic acid production from different hydrolyzed cane sugar concentrations is given in Figure 2.2. The results revealed that higher sugar concentrations affected both the yield and productivity of lactic acid indicating the sugar inhibition at higher sugar concentrations (>100g/l). Maximum lactic acid production (120 g/l) was with 200 g cane sugar/l with a productivity of 1 g/l/ h.

Table 2.3: Effect of initial hydrolyzed cane sugar concentration on fermentation time required, lactic acid produced, and D-lactic acid productivity

Sucrose (g/l)	Cell mass(g/l)		D-Lactic acid (g/l)	
	48h	96h	48h	96h
50	4.1	4.0	40.5 (0.85)	40.0 (0.41)
100	4.7	4.5	81.0 (1.68)	80.0 (0.83)
150	3.5	4.1	65.5 (1.36)	110.0 (1.15)
200	3.6	4.2	60.0 (1.25)	108.0 (1.13)

The cultures were grown anaerobically for 48 h at 42 ^{o}C with shaking (150 rpm). The numbers in the parenthesis show lactic acid productivities (g/l/h).

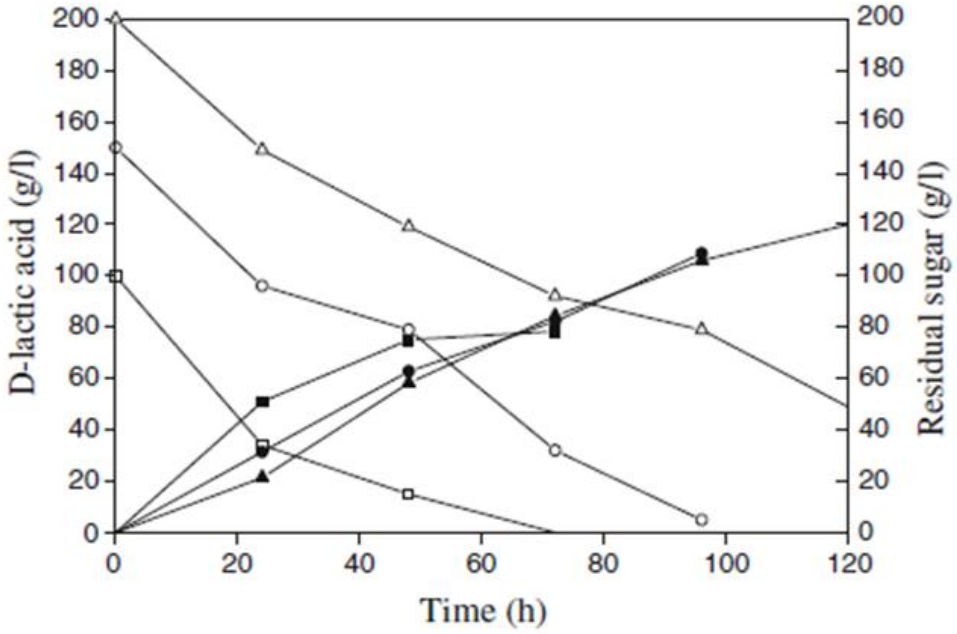


Figure 2.2: Effect of different initial concentrations of hydrolyzed cane sugar on D-lactic acid production by *Lactobacillus lactis* RM2-24. Closed symbols: L-lactic acid production and open symbols: residual sugar (open square, closed square) 100 g/l, (open circle, closed circle) 150 g/l (open triangle, closed triangle) 200 g/l.

Effect of various nitrogen sources on D-lactic acid production

The effect of different nitrogen sources on lactic acid production by RM2-24 was evaluated. Table 2.4 summarizes the profile of growth and lactic acid production in media containing $(NH_4)_2HPO_4$ or peptone. It was found that the supplementation of HSYE medium with only $(NH_4)_2HPO_4$ alone enhanced the lactic acid production (69 g/l) with 2.3 g/l/h productivity. No other inorganic nitrogen source (1 g/l) such as ammonium citrate, ammonium chloride, ammonium acetate or ammonium nitrate showed enhancement in lactic acid production (data not shown). Supplementation of HSYE with peptone increased the growth substantially with marginal increase in lactic acid productivity. The maximum lactic acid was produced within 30 h of fermentation

when the mutant was grown in media containing both $(NH_4)_2HPO_4$ and peptone. There was no further increase in lactic acid production after 30 h of incubation. This combination also resulted in maximum growth which may be contributing to the higher lactic acid productivity. The maximum amount of lactic acid (84 g/l) was produced with a productivity of 2.8 g/l/h and the 0.84 g/g yield.

LDH activity profiles of cells grown in $(NH_4)_2HPO_4$ and peptone containing media

We observed that $(NH_4)_2HPO_4$ alone and in combination with peptone produced higher levels of D-lactic acid. Therefore, we attempted to detect the levels of lactate dehydrogenase enzyme in the cells grown in media with and without $(NH_4)_2HPO_4$ or/and peptone. As LDH plays a key role in lactic acid production, its level in the cells grown in different fermentation media was studied and the data compared with lactic acid productivity is given in Table 2.5.

The specific activity of enzyme (U/mg of protein) was found to vary with the medium. The media showing high lactic acid productivity exhibit a corresponding high specific activity of LDH in cells grown in that medium. The LDH activity was highest in the media containing both $(NH_4)_2HPO_4$ and peptone. Increased LDH activity was observed in the cells in medium containing $(NH_4)_2HPO_4$ alone but peptone alone did not increase the LDH activity. Thus the increase in LDH levels in the cells grown in $(NH_4)_2HPO_4$ could be attributed to increased production of lactic acid in that respective media. To get a better understanding of LDH pattern in different media, we performed the LDH zymogram staining using native polyacrylamide gel electrophoresis.

Table 2.4: Effect of diammonium hydrogen phosphate and peptone (individually and in combination) on lactic acid production

Media	Time (h)	Growth (OD)	Lactic acid (g/l)	Productivity (g/l/h)
HSYE	0	0.7 ± 0.1	2.5 ± 0.2	--
	18	7.0 ± 0.5	35.3 ± 3.0	1.96
	24	9.6 ± 0.7	52.1 ± 3.4	2.17
	30	9.8 ± 0.7	57.7 ± 4.5	1.92
HSYE + $(NH_4)_2HPO_4$	0	0.6 ± 0.1	2.6 ± 0.2	--
	18	11.5 ± 0.9	42.3 ± 3.2	2.35
	24	12.7 ± 1.0	61.0 ± 4.6	2.54
	30	12.7 ± 1.2	69.1 ± 5.0	2.30
HSYE + Peptone	0	0.5 ± 0.1	2.4 ± 0.2	--
	18	8.9 ± 0.7	40.8 ± 3.1	2.26
	24	10.1 ± 0.9	55.0 ± 3.5	2.29
	30	10.4 ± 0.8	58.2 ± 3.6	1.94
HSYE + Peptone +$(NH_4)_2HPO_4$	0	0.6 ± 0.1	2.5 ± 0.2	--
	18	14.0 ± 1.2	53.1 ± 4.0	2.95
	24	15.1 ± 1.1	76.3 ± 4.7	3.18
	30	14.6 ± 1.0	84.7 ± 5.2	2.82

The cultures were grown anaerobically for 30 h at 42 ^oC with shaking at 150 rpm as described in section 2. The fermented broth analyzed for total lactic acid by HPLC. All the values are the average of three independent experiments.

Table 2.5: Comparison of D-lactic acid productivity and specific activity of LDH in different media

Medium	Lactic acid productivity (g/l/h)	Specific activity of LDH (U/mg of protein)
HSYE	2.19	0.15
HSYE + $(NH_4)_2HPO_4$	2.58	0.21
HSYE + Peptone	2.35	0.16
HSYE + Peptone +$(NH_4)_2HPO_4$	3.12	0.25

The cells were harvested at 24 h for the determination of enzyme activity. Values are the averages of three independent experiments

The activity (broadness of band) on the gel and the spectrophotometric measurements of LDH activity matched well with corresponding production levels in the media. In the electrophoregram, one band corresponding to D-LDH was observed when the cells were grown in un-supplemented media. However, $(NH_4)_2HPO_4$ grown cells produced an additional LDH band with less electrophoretic mobility. We also performed LDH activity staining of cell extract of *L. delbrueckii* Uc-3 (producing only L-lactic acid) and observed single band of L-LDH with electrophoretic mobility comparable to that observed with additional LDH band in case of *L. lactis* RM2-24 (Figure 2.3). These results suggest that the additional LDH band in cell extract of *L. lactis* RM2-24 appears to be corresponding to L-LDH. To substantiate this finding, we analyzed the content of D- and L-lactic acid in the fermentation broths obtained by growing the mutant RM2-24 in different media. Surprisingly, we found that the fermentation broths derived from $(NH_4)_2HPO_4$ supplemented media contained some amount of L-lactic acid also (Table 2.6). This L-lactic acid could be the result of L-

LDH produced by RM2-24. This is very valuable information to the industries who are engaged in D-lactic acid production.

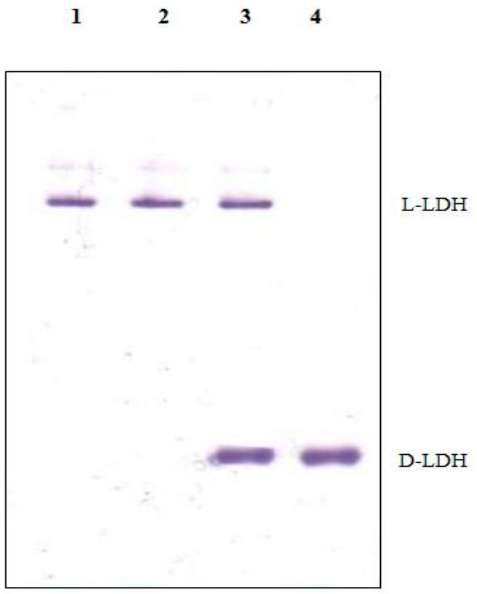


Figure 2.3: Activity staining of lactate dehydrogenase (LDH) from the cells grown in different media.

Lane 1 : Extract of *L. delbrueckii* Uc-3 cells grown in HSYE
Lane 2 : Extract of *L. delbrueckii* Uc-3 cells grown in HSYE + [$(NH_4)_2HPO_4$]
Lane 3 : Extract of *L. lactis* RM2-24 cells grown in HSYE + [$(NH_4)_2HPO_4$]
Lane 4 : Extract of *L. lactis* RM2-24 cells grown in HSYE

Table 2.6: D- and L- lactic acid content in total lactic acid produced by *Lactobacillus lactis*, RM2-24

Medium	Total Lactic acid (g/l)	L-lactic acid (g/l)	D-lactic acid (g/l)
HSYE	57.7	--	57.7
HSYE+ $(NH_4)_2HPO_4$	69.1	5.70	63.3
HSYE+ $(NH_4)_2HPO_4$ + Peptone	84.7	7.90	76.8

The fermented broth obtained after 30 h of fermentation was analyzed for total lactic acid by HPLC and L-lactic acid by L-lactate oxidase kit

DISCUSSION

The optical purity of D-lactic acid is vital to the physical properties of PDLA and hence homo-D-lactic acid-producing microorganisms are required for the production of D-lactic acid (Tsuji, 2002). To date, a few wild-type stains such as *Lactobacillus delbrueckii* (Fukushima et al.,2004) and *Lactobacillus coryniformis subsp. torquens* (Yáñez et al., 2003) have been identified as D-lactic acid-producing lactic acid bacteria (D-LAB) which produce little or no L-lactic acid. In addition, several studies have successfully the production of D-lactic acid by metabolically engineered *Lactobacillus plantarum* (Okano et al., 2009a, b, c), *Escherichia coli* (Shukla et al., 2004; Zhou et al., 2003), *Corynebacterium glutaminum* (Okino et al., 2008). Despite the increased focus on microbial D-lactic acid production, its information is limited compared to the microbes producing L-lactic acid. In this chapter, we have described the D-lactic acid production using *Lactobacillus lactis* NCIM 2368 and its mutants.

During screening programme, *Lactobacillus lactis* NCIM 2368 was found to be producing pure D-lactic acid but with very less productivities. The wild type, *Lactobacillus lactis* NCIM 2368 strain, has been improved using UV mutagenesis so as to get the higher lactic acid producing strain. The mutant strain, *Lactobacillus lactis* RM2-24 produced almost three times more D-lactic acid than the wild type strain. The mutant RM2-24 produced increasing amounts of lactic acid with increasing sugar concentrations up to 200 g/l. The highest D-lactic acid (110 g/l) in batch fermentation was produced from 150 g/l with a 73% lactic acid yield. The mutant also utilized cellobiose very efficiently and converted it into D-lactic acid proving its potentiality in

converting biomass into lactic acid. There are few reports on production of D-lactic acid from unpolished rice (Lu et al., 2009), defatted rice bran (Tanaka et al., 2006) and from waste cardboard (Yanez et al., 2005). A recombinant strain of *Corynebacterium glutamicum* capable of producing lactic acid from the mixture containing cellobiose, glucose and xylose was reported (Sasaki et al., 2008). Okino et al. (2008) constructed a genetically modified strain of *Corynebacterium glutamicum* which produced 120 g/l D-lactic acid with higher productivity. However, this organism produced succinic and acetic acid along with D-lactic acid. Calabia and Tokiwa (2007) reported 104 g/l D-lactic acid from molasses with productivity of 1.48 g/l/ h. Our strain, RM2-24, produced only D-lactic acid with comparable yields without the formation of succinic and acetic acids, as confirmed by HPLC. Previously, we reported the L-lactic acid production from bagasse derived cellulose using another mutant *L. delbrueckii* Uc-3 (Adsul et al., 2007a) which showed the presence of a cell-bound cellobiase (Adsul et al., 2007b).

Media optimization studies have been carried out using different carbon and nitrogen sources for enhanced lactic acid production by *Lactobacillus lactis* RM2-24. The mutant strain produced almost similar amount of lactic acid with the productivity of about 1.6g/l/h using carbon sources like glucose, cellobiose and cane sugar. In addition, it also uses molasses as substrate and converts it in to pure D-lactic acid with comparable productivity indicating its capability to use robust substrate like molasses, a major byproduct of sugar industries. For further improvement in lactic acid productivities, different organic and inorganic nitrogen sources were evaluated. The control medium i.e. HSYE medium supplemented with $(NH_4)_2HPO_4$ alone enhanced the lactic acid production. The combination of $(NH_4)_2HPO_4$ and peptone produced

maximum amount of lactic acid (84 g/l) with a productivity of 2.8 g/l/h and the 0.84 g/g yield. These are the highest productivity and efficiency values reported so far for D-lactic acid production. Thus the combination of $(NH_4)_2HPO_4$ and peptone proved to be efficient for D-lactic acid production. The similar experiments were performed using *L. delbrueckii* Uc-3 which produces L-lactic acid (Kadam et al., 2006). However, there was a marginal increase in levels and productivity of lactic acid suggesting that the incremental effect was selective for D-lactic acid production. The fermentation broth was analyzed for L and D-lactic acid content and surprisingly we detected small amount of L-lactic acid along with D-lactic acid. It is well known that bacterial LDH exhibits isozyme forms (Garvie, 1980) and hence LDH expression profiles were studied for both the supplemented and non-supplemented media. The appearance of additional band corresponding to L-LDH in case of supplemented media suggested that L-LDH is expressed when the cells were grown only in presence of $(NH_4)_2HPO_4$. This is very valuable information to the industries who are engaged in D-lactic acid production. The studies on induction of specific L-LDH in presence of $(NH_4)_2HPO_4$ during D-lactic acid production by RM2-24 needs further investigation. This observation helps create awareness in lactic acid industries in the blind use of any nitrogen sources to get enhancement in lactic acid production without analyzing the final product for its enantiomeric purity.

The results in this chapter suggest that the *Lactobacillus lactis* RM2-24 has the ability to produce D-lactic acid from different carbon sources such as glucose, sucrose, molasses and cellobiose. This strain has the potential for commercial production of D-

lactic acid using different carbohydrates including lignocellulosic biomass materials which is a present need for producing pure D-lactic acid to be used for PLA production.

CONCLUSION

Lactobacillus lactis RM2-24 is a promising strain for the production of D-lactic acid from molasses and hydrolyzed cane sugar. It also utilizes cellobiose efficiently and converts it into lactic acid. Thus bottlenecks, like feed-back inhibition by glucose and cellobiose, are removed leading to complete conversion of cellulosic substrates to D-lactic acid. This study shows the potentiality of such a strain for producing commodity chemicals from renewable resources. The strain produced pure D-lactic acid in a homo-fermentative manner. Supplementation of medium with alternate nitrogen sources is necessary to produce D-lactic acid with high productivity. The mutant produced lactic acid with productivity (2.8 g/l/h) in presence of both $(NH_4)_2HPO_4$ and peptone in the production medium. LDH activity of the cells grown in the modified medium increased which can be correlated to enhanced lactic acid productivity. The cells grown in $(NH_4)_2HPO_4$ produced one additional band corresponding to L-LDH which contributed to increased levels of L-lactic acid in the fermented broth. To our knowledge, such novel observation of appearance of L-LDH in presence of $(NH_4)_2HPO_4$ in D-lactic acid producing *Lactobacillus* strain is not reported so far. This strain utilized cellobiose also very efficiently, thus the strain could be exploited for the conversion biomass into lactic acid. For the conversion of biomass to lactic acid, cellulases are needed for biomass hydrolysis. Hence the next chapter deals with the production of cellulase under both submerged and solid state fermentation conditions using in house developed fungal strain, *Penicillium janthinellum* EU2D-21.

REFERENCES

Adsul M. G., Varma A. J., Gokhale D. V. (2007a) Lactic acid production from waste sugar cane bagasse derived cellulose. *Green Chemistry*, 9, 58–62.

Adsul M. G., Khire J. M., Bastawade K. B., Gokhale D. V. (2007b) Lactic acid production from cellobiose and cellotriose by *Lactobacillus delbrueckii* mutant Uc-3. *Applied and Environmental Microbiology*, 73, 5055–5057.

Bradford M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, 72, 227-254.

Calabia B. P., Tokiwa Y. (2007) Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by *Lactobacillus delbrueckii*. *Biotechnology Letters*, 29, 1329–1332.

Cheng P., Mueller R. E., Jaeger S., Bajpai R., Iannotti E. L. (1991) Lactic-acid production from enzyme-thinned corn starch using *Lactobacillus amylovorus*. *Journal of Industrial Microbiology*, 7, 27–34.

Fischer E. A. and Stein E. A. (1961) Amylase from human saliva. *Biochemical. Preparations*, 8, 27–33.

Fukushima K., Sogo K., Miura S., Kimura Y. (2004) Production of D-lactic acid by bacterial fermentation of rice starch. *Macromolecular Biosciences*, 4, 1021–1026.

Garvie E. I. (1969) Lactic dehydrogenases of Strains of the Genus *Leuconostoc*. *Journal of General Microbiology*, 58, 85-94.

Garvie E. I. (1980) Bacterial lactate dehydrogenase. *Microbiology Review*, 44, 106-139.

Goncalves L.D., Barreto M. O., Xavier A. B., Carrondo M. T., Klein J. (1992) Inert supports for lactic acid fermentation a technological assessment. *Applied Microbiology and Biotechnology*, 38, 305–311.

Kadam S. R., Patil S. S., Bastawade K. B., Khire J. M., Gokhale D. V. (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. *Process Biochemistry*, 41, 120–126.

Laemmli U. K. (1970) Cleavage of structural protein during assembly of the head of bacteriophage-T4. *Nature*, 227, 680-685.

Lu Z., Lu M., He F., Yu L. (2009) An economical approach for D lactic-acid production utilizing unpolished rice from aging paddy as major nutrient source. *Bioresource Technology*, 100, 2026–2031.

Okano K., Zhang Q., Shinkawa S., Yoshida S., Tanaka T., Fukuda H., Kondo A. (2009a) Efficient production of optically pure D-lactic acid from raw corn starch by using a genetically modified L-lactate dehydrogenase gene-deficient and α-amylase-secreting *Lactobacillus plantarum* strain. *Applied and Environmental Microbiology*, 75, 462–467.

Okano K., Yoshida S., Tanaka T., Ogino C., Fukuda H., Kondo A. (2009b) Homo-D-lactic acid fermentation from arabinose by redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, 75, 5175–5178.

Okano K., Yoshida S., Yamada R., Tanaka T., Ogino C., Fukuda H., Kondo A. (2009c) Improved production of homo-D-lactic acid via xylose fermentation by introduction of xylose assimilation genes and redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, 75, 7858–7861.

Okino S., Suda M., Fujikura K., Inui M., Yukawa H. (2008) Production of D-lactic acid by *Corynebacterium glutamicum* under oxygen deprivation. *Applied and Environmental Microbiology*, 78, 449–454.

Sasaki M., Jojima T., Inui M., Yukawa H. (2008) Simultaneous utilization of D-cellobiose, D-glucose and D-xylose by recombinant *Corynebacterium glutamicum* under oxygen deprived conditions. *Applied Microbiology and Biotechnology*, 81, 691–699

Schepers A. W., Thibault J., Lacroix C. (2002) *Lactobacillus helveticus* growth and lactic acid production during pH-controlled batch cultures in whey permeate/yeast extract medium. Part I. Multiple factor kinetic analysis. *Enzyme Microbial Technology*, 30, 176-186.

Shukla V. B., Zhou S., Yomano L. P., Shanmugam K. T., Preston J. F., Ingram L. O. (2004) Production of D(−)-lactate from sucrose and molasses. *Biotechnology Letters*, 26, 689–693.

Sodegard A. and Stolt M. (2002) Properties of lactic acid based polymers and their correlation with composition. *Progress in Polymer Science*, 27, 1123–1163.

Tanaka T., Hoshina M., Tanabe S., Sakai K., Ohtsubo S., Taniguchi M. (2006) Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation. *Bioresource Technology*, 97, 211–217.

Tsuji F.(2002) Autocatalytic hydrolysis of amorphous-made polylactides: effects of L-lactide content, tacticity, and enantiomeric polymer blending. *Polymer*, 43, 1789–1796.

Tsuji H. and Fukui I. (2003) Enhanced thermal stability of poly (lactide)s in the melt by enantiomeric polymer blending. *Polymer*, 44, 2891–2896.

Tsuji H., Hyon S. H., Ikada Y. (1991) Stereocomplex formation between enantiomeric poly (lactides). Calorimetric studies on blend films cast from dilute solution. *Macromolecules*, 24, 5651-5656.

Yanez R., Alonso J. L., Parajo J. C. (2005) D-Lactic acid production from waste cardboard. *Journal of Chemical Technology and Biotechnology*, 80, 76–84.

Yanez R., Moldes A. B., Alonso J. L., Parajo J. C. (2003) Production of D(−)-lactic acid from cellulose by simultaneous saccharification and fermentation using *Lactobacillus coryniformis* subsp. torquens. *Biotechnology Letters*, 25, 1161–1164.

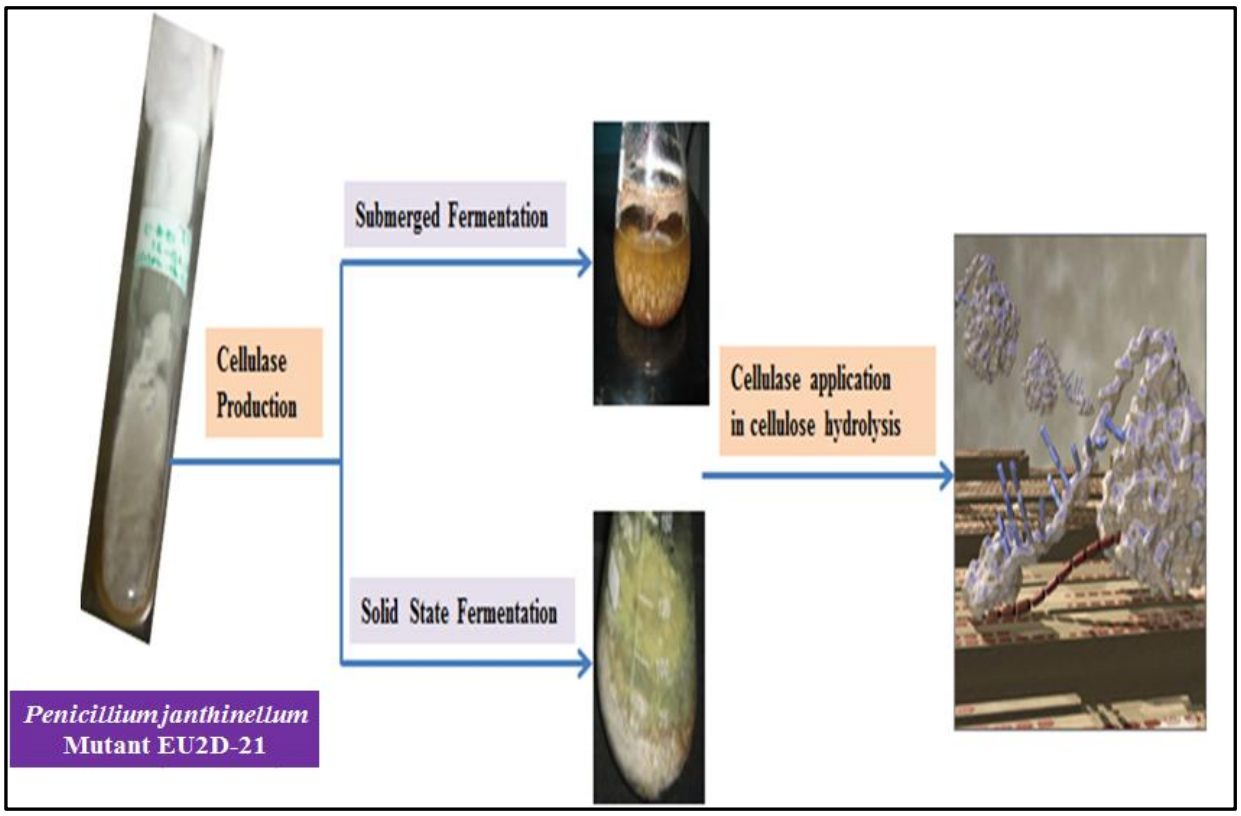
Yu R. and Hang Y.D. (1989) Kinetics of direct fermentation of agricultural commodities to L(+) lactic acid by *Rhizopus oryzae*. *Biotechnology Letters*, 11, 597-600.

Zhou S., Causey T. B., Hasona A., Shanmugam K. T., Ingram L. O. (2003) Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *Escherichia coli* W3110. *Applied and Environmental Microbiology*, 69, 399–407.

CHAPTER 3

Cellulase production by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose

Graphical Abstract



- **Singhvi M. et al (2011) *Bioresour. Technol.* 102, 6569-572.**

SUMMARY

Mutants of *Penicillium janthinellum* NCIM 1171 were evaluated for cellulase production using both submerged fermentation (SmF) and solid state fermentation (SSF). Mutant EU2D-21 gave highest yields of cellulases in both SmF and SSF. Hydrolysis of Avicel and cellulose were performed using SmF and SSF derived enzyme preparations obtained from EU2D-21. Surprisingly, the use of SSF derived enzyme preparation gave less hydrolysis compared to SmF derived enzymes. We studied the thermo-stability profile of FPase, CMCase and β-glucosidase activites at 50 ^{o}C. We found that the β-glucosidase derived from SSF lost almost 80% of the original activity. Hence the less hydrolysis of the Avicel and cellulose could be due to inactivation of β-glucosidase present in SSF derived enzyme preparation. The studies of activity staining of β-glucosidase demonstrated that SmF derived enzyme preparations contained both thermo-stable and thermo-sensitive β-glucosidases whereas SSF derived enzyme preparations contained predominantly thermo-sensitive β-glucosidase. This is the first report on less thermostability of SSF derived β-glucosidase which is the main reason for getting less hydrolysis of cellulose substrates.

INTRODUCTION

Lignocellulosic substances are abundantly available sources of renewable biopolymer for the production of biofuels or other bio-based products. The bioconversion of cellulosic materials has been receiving a great attention in recent years. Hydrolysis of lignocellulosic materials by cellulases and hemicellulases could be the most efficient method for the release of fermentable sugars (Lynd et al., 2002). Cellulases are the key enzymes required for the degradation of lignocellulosic

polysaccharides into the simple monomeric sugars that are converted through microbial fermentation processes to biofuels or other value added products. Cellulase enzymes have been reported for bioconversion of cellulosic biomass to useful products (Kanosh et al., 1999). Development of large scale bioconversion process would alleviate shortages of food and animal feeds, solve modern waste disposal problems and also diminish the dependence on fossil fuels by providing an energy source in the form of glucose. Much work has been done on the production of cellulases from different microorganisms (Depaula et al., 1999; Solomon et al., 2000). The application of these enzymes for biofuel production is hindered by the high cost of enzyme production and the suitability of pretreatment of biomass which makes it amenable to cellulase attack.

Cellulose is degraded by synergistic action of three types of enzymes in the cellulase complex: exo-1,4-β-D-glucanase (EC 3.2.1.91), endo-1,4- β-D -glucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). Among fungi, *Trichoderma* and *Aspergillus* have been extensively studied particularly due to their ability to secrete cellulose degrading enzymes. We have reported the hyper-production of β-glucosidase (Gokhale et al., 1984) and β-xylosidase (Gokhale et al., 1986) by *Aspergillus niger* NCIM 1207 isolated in our laboratory. However the search for an efficient and possibly better source of cellulase continues due to the low activity of β-glucosidase in *Trichoderma* which limits the rate and extent of hydrolysis. Thus the conversion of waste cellulose to glucose is not yet commercially feasible. Hence, efforts are needed to produce cellulases at affordable cost which can be used for hydrolyzing biomass to monomers with high economical potential. *Penicillium janthinellum* NCIM 1171 was identified as cellulase producer which produces cellulases using bagasse as carbon source (Adsul et

al., 2004) and for its application in bagasse hydrolysis (Adsul et al., 2005). Mutants of *P. janthinellum* NCIM 1171 were isolated that are capable of producing enhanced level of cellulases (Adsul et al., 2007).

This chapter describes the comparative enzyme production by the wild type *P. janthinellum* NCIM 1171 and its mutants, EMS-UV-8 and EU2D-21 using different substrates like Avicel, cellulose powder, solka floc and tissue paper under submerged and solid state fermentation conditions. The hydrolysis of Avicel and cellulose powder using mutant EU2D-21 enzyme preparations produced by solid state and submerged fermentation conditions is also described.

MATERIALS AND METHODS

Chemicals

Avicel PH 101 was obtained from Fluka AG, Switzerland. Solka Floc SW44 was purchased from Brown Co., Berlin, NH. Tissue paper was purchased locally. Cellulose powder, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), 4-methylumbelliferyl-β-D-glucoside, carboxymethylcellulose (CMC), xylan (oat spelts), 3,5-dinitrosalysilic acid were obtained from Sigma–Aldrich Co. st. Louis, USA. Phenyl methane sulfonyl flouride (PMSF), yeast extract and peptone were obtained from Hi-Media Laboratories Pvt. limited Mumbai, India. All the other chemicals were of analytical grade and were obtained locally.

Microorganisms and culture media

P. janthinellum NCIM 1171 and *A. niger* NCIM 1207 were obtained from NCIM Resource Centre, National Chemical laboratory, Pune, India. Mutants of *P.*

janthinellum (EMS-UV-8, EU-1, EU2D-21) were generated by exposing the spores of parent strain to ethyl methyl sulfonate followed by UV irradiation (Adsul et al 2007). All the fungal strains were maintained on Potato Dextrose Agar (PDA) and sub-cultured once in every 3 months. PDA contained (g/l): extract from 200 g of potatoes; glucose 20; yeast extract 1; and agar 20. Basal medium (BM) described by Mandels and Weber (1969) was used as a fermentation medium for the enzyme production. The submerged fermentation medium contained (g/l): KH_2PO_4 2.0, $CaCl_2.2H_2O$ 0.3, Urea 0.3, $MgSO_47H_2O$ 0.3, $(NH_4)_2SO_4$ 1.4, Peptone 0.25, Yeast extract 0.1, Tween 80 0.1% $FeSO_4$ $7H_2O$ 0.005, $MnSO_4$ $7H_2O$ 0.0016 , $ZnSO_4$ $7H_2O$ 0.0014, $COCl_2$ $6H_2O$ 0.002. The pH of the medium was adjusted to 5.2 before addition of cellulose powder and /or wheat bran. Cellulose powder and wheat bran were added separately. This fermentation medium containing 1% (w/v) Cellulose powder and 2.5% (w/v) wheat bran was used for enzyme production under submerged fermentation. The components of the fermentation medium used in solid state fermentation are same as that of submerged fermentation medium except that the concentration of the components is ten times more than submerged media components. The Erlenmeyer flask containing 4 g of wheat bran, 1 g of different cellulosic substrates and fermentation medium (8ml) was used for cellulase production under solid state fermentation conditions. The *Aspergillus* minimal medium (AMM) for β-glucosidase production by *Aspergillus niger* NCIM 1207 contained (g/l) $NaNO_3$ 0.5; $MgSO_4.7H_2O$ 0.5; KCl 0.5; KH_2PO_4 2.0; yeast extract 1.0, and bacto-peptone 5.0; xylan 3.0; urea 5; glycerol 25.

Cellulase production

Submerged fermentation (SmF)

Submerged fermentation (SmF) was carried out in 250 ml Erlenmeyer flask with 70 ml of fermentation medium containing 1% (w/ v) different cellulosic substrates and 2.5% (w/v) wheat bran. The flasks were inoculated with spores (approximately 10^7) from 15-days old culture grown on PDA slant and incubated at 30 ^oC with shaking at 180 rpm. The samples were removed at various time intervals and centrifuged at 3000 rpm for 10 min. The supernatant was analyzed for extracellular enzyme activities and soluble protein.

Solid state fermentation (SSF)

Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flask containing 4 g of wheat bran, 1 g of different cellulosic substrates and 8 ml of fermentation medium to moistened the substrate. The flasks were inoculated with spore suspension (1 ml) containing approximately 10^7 spores from 15-day sold culture grown on PDA slant. The contents of each flask were mixed thoroughly for uniform distribution of spores in the medium. The flasks were incubated at 30 ^oC in a humidified chamber with 90% humidity maintained throughout the incubation. The culture in the flasks was harvested and the enzyme was extracted in citrate buffer (50 mM, pH4.5). Citrate buffer (50 ml) was added in each flask and the mixture shaken at 180 rpm on rotary shaker for 2 h at room temperature. The mixture was squeezed through a double layer muslin cloth and centrifuged at 5000g for 20 min at 4 ^oC. The supernatant obtained was used as an extracellular enzyme and analyzed for extracellular enzyme activities and soluble protein.

β-Glucosidase production by *A. niger* NCIM 1207

The β-glucosidase of *A. niger* NCIM 1207 was produced as reported earlier (Khisti et al. 2013). The enzyme was produced in 100 mL Erlenmeyer flasks with 75 ml of the Aspergillus minimal medium (AMM) with xylan as carbon source. The medium was inoculated with the spore suspension (1 ml) containing 10^7 spores from 7 day old culture grown on PDA slope and incubated at 30 ^oC on a rotary shaker (150 rpm) for 14 days. The mycelium was separated by filtration, and the culture filtrate was used as a source of β-glucosidase enzyme.

Enzyme assays

Filter paper cellulase (FPase), endoglucanase, xylanase and β-glucosidase activities were determined as reported by Gokhale et al (1988). Filter paper activity was assayed by incubating the suitably diluted enzyme (0.1 ml) with 1.9 ml citrate buffer (50 mM, pH 4.5) containing filter paper, Whatman no. 1 (50 mg, 1.6 cm). The reaction mixture was incubated at 50 ^oC for 60 min. Endoglucanase (CMCase, Endo-1, 4- β -D-glucanase; EC 3.2.1.4) activity was carried out in the total reaction mixture of 1 ml containing 0.5 ml of suitably diluted enzyme and 0.5 ml of 1% (w/v) CMC solution in citrate buffer (50 mM, pH 4.5). This mixture was incubated at 50 ^oC for 30 min. Xylanase (1,4- β -D-xylan xylanohydrolase, EC (3.2.1.8) activity was determined under similar conditions as described above, except that 1% xylan solution was used as substrate in place of CMC. The reducing sugar liberated was analyzed by DNSA method as glucose and xylose equivalents (Fischer and Stein, 1961). The β-Glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) activity was estimated using *p*NPG as substrate. The total of assay mixture (1 ml) consisting of 0.9 ml of pNPG (1 mg/ml) and

0.1 ml of suitably diluted enzyme was incubated at 50 ^{o}C for 30 min. The reaction was stopped by the addition of 2 ml of sodium carbonate (2%) and the *p*-nitrophenol liberated was measured at 410 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of glucose, xylose or *p*-nitrophenol produced from the appropriate substrates/ min of crude filtrate under the assay conditions.

Enzymatic hydrolysis

The Enzyme preparations for hydrolysis of Avicel and cellulose powder were derived from the growth of mutants in shake flask culture containing basal medium with cellulose (1%) and wheat bran (2.5%) as mentioned above. The hydrolysis of both Avicel and cellulose powder were carried out in 100 ml conical flask containing 50 ml citrate buffer (50 mM, pH 4.5), 2.5 g Avicel or cellulose powder, 5 mg sodium azide and 10 filter paper units (FPU) per gram of substrate. In case of hydrolysis using solid state enzyme preparations, we added same units of *A. niger*, β-glucosidase for hydrolysis. This mixture was incubated at 50 ^{o}C with shaking at 150 rpm. The samples removed at different time intervals were analyzed for the reducing sugars. Residual activities of all enzymes were estimated in the hydrolysis mixtures during hydrolysis to determine inactivation of the enzymes at 50 ^{o}C.

Thermo-stability studies for FPase, endoglucanase and β-glucosidase

The thermo-stability experiments were performed for both Smf and SSF derived enzyme preparations. The residual activity of FPase, endoglucanase and β-glucosidase enzymes were determined under standard assay conditions. The enzyme assays were

carried out at different temperatures (50, 60 and 70 ^{o}C) and the residual activity was assayed after every hour of incubation up to 24 h.

Native polyacrylamide gel electrophoresis and zymogram of β-glucosidase

Crude enzyme preparations (protein 100 µg) were fractionated by native polyacrylamide gel electrophoresis (PAGE) using 10% acrylamide as resolving gel and 4% stacking gel (Laemmli, 1970). The β-glucosidase activity in the gel was detected by developing zymogram against 10 mM 4-methylumbelliferyl-β-D-glucoside (Sigma) as substrate prepared in sodium citrate buffer (50 mM, pH 4.5). Upon completion of electrophoresis, the gel was immersed in substrate solution for 45 min at 50 ^{o}C in the dark. The β-glucosidase bands in the gel were visualized under UV light using Gel Documentation system (Syngene). The enzymes from the gel corresponding to zymogram bands were eluted using 50 mM citrate buffer, pH 4.5 and thermo-stability was determined by incubating the enzymes at 50 ^{o}C. The residual activity was estimated under standard assay conditions.

RESULTS

Production of enzyme in SmF & SSF by *P. janthinellum* strains

The wild strain and selected mutants were evaluated for extracellular production of cellulases and xylanases in shake flask containing optimized basal medium with different substrates such as Avicel, cellulose powder, solka floc and tissue paper (1%) and wheat bran (2.5%) as substrates. The cellulase and xylanase activities of the wild and mutant strains using different substrates under submerged fermentation are as shown in Table 3.1. Both the mutants, EMS-UV-8 and EU2D-21, showed highest activities of FPase and CMCase. FPase activity of mutant strain was two times higher than the parent strain. No mutant produced as high β-glucosidase activity as that of wild strain. The mutant EU2D-21 produced highest FPase (3.49 IU/ml), CMCase (94.4 IU/ml) when it was grown in Avicel containing media.

The wild and mutant strains were evaluated for cellulase production in SSF and the various enzyme activities using different substrates under SSF conditions are shown in Table 3.2. It was found that both mutants produced enhanced levels of FPase, CMCase and xylanase and β-glucosidase in comparison to the wild strain during growth on wheat bran and different substrates. Tissue paper and solka floc also proved to be the suitable substrates for cellulase production by mutant strains. Mutant EU2D-21 produced the highest FPase (67.8 IU/g), CMCase (3558 IU/g), and β-glucosidase (149 IU/g) when grown on 1 g of cellulose and 4 g of wheat bran. We used tissue paper as the cheap source for the production of cellulases which yielded cellulase activities comparable to the values obtained for cellulose or Avicel. The comparison of cellulase activities produced by mutant EU2D-21 in SSF with those reported in the literature

demonstrated the superiority of the mutant to the other fungal strains with respect to CMCase and β -glucosidase production.

Table 3.1: Comparison of enzyme activities of *P. janthinellum* NCIM 1171 and its mutant using different substrates under submerged fermentation

Medium + carbon source	Strain	Enzyme activities (IU/ml)			
		FPase	CMCase	Xylanase	β-Glucosidase
BM+WB(2.5%)+ Avicel(1%)	Parent	1.21±0.10	37.2±2.99	160±10.5	6.8±0.4
	EMS-UV-8	3.24±0.21	95.3±6.20	129±11.2	4.8±0.3
	EU-2D-21	3.49±0.20	94.4±6.78	155±14.2	3.2±0.2
BM+WB(2.5%)+ Cellulose(1%)	Parent	1.10±0.11	33.2±2.55	135±11.5	6.8±0.4
	EMS-UV-8	3.13±0.21	81.3±5.82	120±10.2	5.1±0.4
	EU-2D-21	3.44±0.22	82.8±6.60	188±8.20	3.1±0.2
BM+WB(2.5%)+ Solka Floc(1%)	Parent	1.00±0.06	24.2±2.00	121±10.0	7.0±0.5
	EMS-UV-8	2.23±0.11	76.9±3.72	70.0±7.20	5.2±0.3
	EU-2D-21	2.99±0.20	76.6±3.60	116±9.10	3.6±0.2
BM+WB(2.5%)+ Tissue paper (1%)	Parent	0.40±0.02	24.2±2.00	122±10.0	5.2±0.5
	EMS-UV-8	2.19±0.10	76.0±3.70	114.0±9.0	3.4±0.2
	EU-2D-21	2.89±0.20	79.6±3.66	115±9.1	2.6±0.2

The cultures were grown in basal medium containing different cellulosic substrates (1%) and wheat bran (2.5%) as described above. The enzyme activities were estimated after 8 days of incubation. The values given are the average of three independent experiments.

Table 3.2: Comparison of enzyme activities of *P. janthinellum* NCIM 1171 and its mutant using different substrates under solid state fermentation

Medium + carbon source	Strain	Enzyme activities (IU/g)			
		FPase	CMCase	Xylanase	β-Glucosidase
BM+WB(4g)+ Avicel(1g)	Parent	11.1±2.10	688±2.99	221±10.0	88.9±9.9
	EMS-UV-8	54.4±.5.21	3255±34.2	3000±33.2	141±11.1
	EU-2D-21	59.9±5.20	3421±36.7	3250±34.2	143.2±12.2
BM+WB(4g)+ Cellulose(1g)	Parent	16.7±1.10	710±25.5	231±15.5	98.8±10.4
	EMS-UV-8	65.1±.7.11	3300±38.8	2990±40.2	144±8.88
	EU-2D-21	67.8±3.50	3558±36.6	3100±42.2	149±9.81
BM+WB(4g)+ Solka Floc(1g)	Parent	8.90±0.06	591±16.0	185±10.0	81±8.05
	EMS-UV-8	40.0±4.50	2562±28.2	2875±36.2	151±9.20
	EU-2D-21	42.2±4.20	2775±30.0	3000±40.1	139±8.80
BM+WB(4g)+ Tissue Paper(1g)	Parent	7.40±1.02	510±12.0	120±10.0	49.2±5.10
	EMS-UV-8	36.9±4.50	2000±22.5	2331±30.5	111±7.51
	EU-2D-21	38.0±4.80	2131±24.5	2451±32.1	100±7.00

The cultures were grown in basal medium containing different cellulosic substrates (1 g) and wheat bran (4 g) as described above. The enzyme activities were estimated after 4 days of incubation. The values given are the average of three independent experiments.

Production of β-glucosidase by *A. niger* NCIM 1207

A. niger NCIM 1207 was grown in AMM containing xylan as carbon source. The supplementation of urea was shown to maintain the pH of the fermentation medium between 3.0 and 4.0 and to protect β-glucosidase from pH inactivation (Gokhale et al. 1992). To investigate whether the addition of urea to glycerol containing medium helps to maintain the pH of the medium, the culture was grown in a medium containing xylan (3%) in combination with glycerol (2.5%) and urea (0.5%). High levels of both β-

glucosidase (14.0 IU/ml) were produced under these conditions. This enzyme preparation was further used to supplement cellulase enzyme preparations from *P. janthinellum* mutant for hydrolysis of cellulose substrates.

Hydrolysis of Avicel and cellulose powder

Among the wild and mutant strains, mutant EU2D-21 exhibited higher cellulase and xylanase activities under both submerged and solid state fermentation conditions. Hence, the enzyme preparations of mutant, EU2D-21, produced both under submerged and solid state fermentation, have been used to hydrolyze Avicel and cellulose powder at 5% concentration. Yield of hydrolysis was higher (60–62%) with submerged enzyme preparation as shown in Table 3.3. Enzyme preparation produced by solid state fermentation gave comparatively less (20–25%) hydrolysis yield. The addition of PMSF along with solid state enzyme preparation during hydrolysis did not give much difference in hydrolysis indicating no role of proteases in inactivation of β-glucosidase. However, the addition of β-glucosidase from *A. niger* NCIM 1207 gave hydrolysis equal to that of submerged enzyme.

Table 3.3: Comparison of hydrolysis between Avicel (5%) and cellulose (5%) using enzyme preparations of mutant EU2D-21 (10 FPU/g of substrate) at 50 ^{o}C for 60 h

Enzymes	Avicel		Cellulose	
	Residual Sugar (mg/ml)	Hydrolysis (%)	Residual Sugar (mg/ml)	Hydrolysis (%)
SSF Enzyme (10U/g Fpase + 21 U/g β-Glucosidase)	600±12	24±0.50	535±11	21.4±0.44
SmF Enzyme (10U/g Fpase + 12.5 U/g β- Glucosidase)	1506±20	60.2±0.81	1533±22	62.3±0.91
SSF(10U/g Fpase+ 21 U/g β-Glucosidase) + β-glucosidase (12.5 U/g) from *A.niger* NCIM 1207	1500±18	60±0.73	1458±16	58.32±0.62
SSF Enzyme (10U/g Fpase + 21 U/g β-Glucosidase) +PMSF	675±16	27±0.81	688±18	27.5±0.80

The values of hydrolysis given in table are the average of three independent experiments.

Thermostability studies for FPase, endoglucanase and β-glucosidase

Figures 3.1a and 3.1b showed the thermo-stability profiles of FPase, CMCase and β-glucosidase produced under submerged and solid state fermentation conditions at different temperatures. FPase and CMCase produced under both solid state and submerged fermentation exhibited good thermo-stability retaining 60-80% of its activity at 50 ^{o}C for 48 h. The β-glucosidase derived from submerged fermentation showed 90% of its original activity when incubated at 50 ^{o}C. It also did not show much inactivation even at 60 ^{o}C retaining almost 75% of its activity. On the contrary, the solid state

fermentation derived β-glucosidase lost its activity rapidly with the retention of only 25% activity after incubation at 50 ^{o}C.

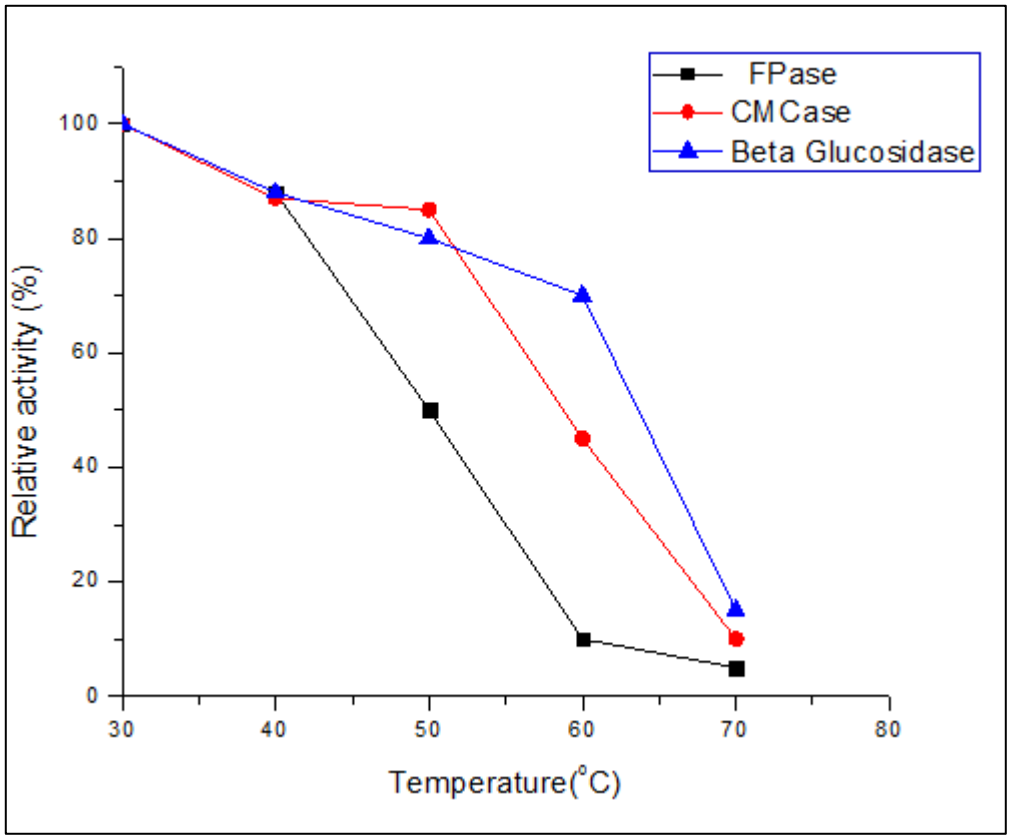


Figure 3.1a: Effect of temperature on stability of FPase (■) CMCase (●) and β-glucosidase(▲) produced under submerged fermentation conditions. The stability was checked at 50, 60,70 ^{o}C for 48 h.

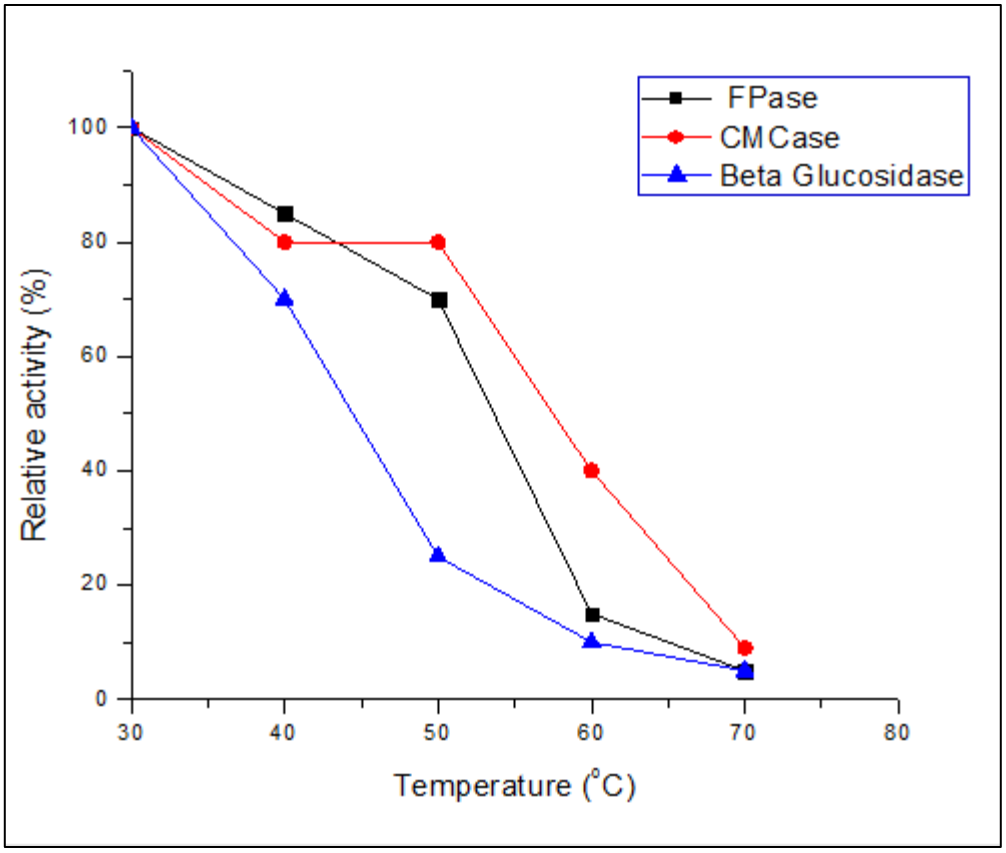


Figure 3.1b: Effect of temperature on FPase (□), CMCase (○) and β-glucosidase (Δ) stability produced under solid state fermentation conditions. The stability was checked at 50,60,70 °C for 48 h.

Inactivation profile of all enzymes during hydrolysis demonstrated that FPase and CMCase of both solid state and submerged enzyme preparations exhibited good thermo-stability retaining more than 80% of their activity at 50 °C even after 60 h (data not shown since these enzymes were not much inactivated at 50 °C even after 48 h as shown in Figures 3.1a, 3.1b). However, β-glucosidase of solid state enzyme preparation lost its 80% of its original activity within 8 h at 50 °C. In comparison, β-glucosidase in submerged enzyme preparation retained about 80% of its activity at 50 °C even after 60 h. The submerged derived β–glucosidase showed more thermo-stability than the SSF derived β –glucosidase at 50 °C as shown in Figure 3.2.

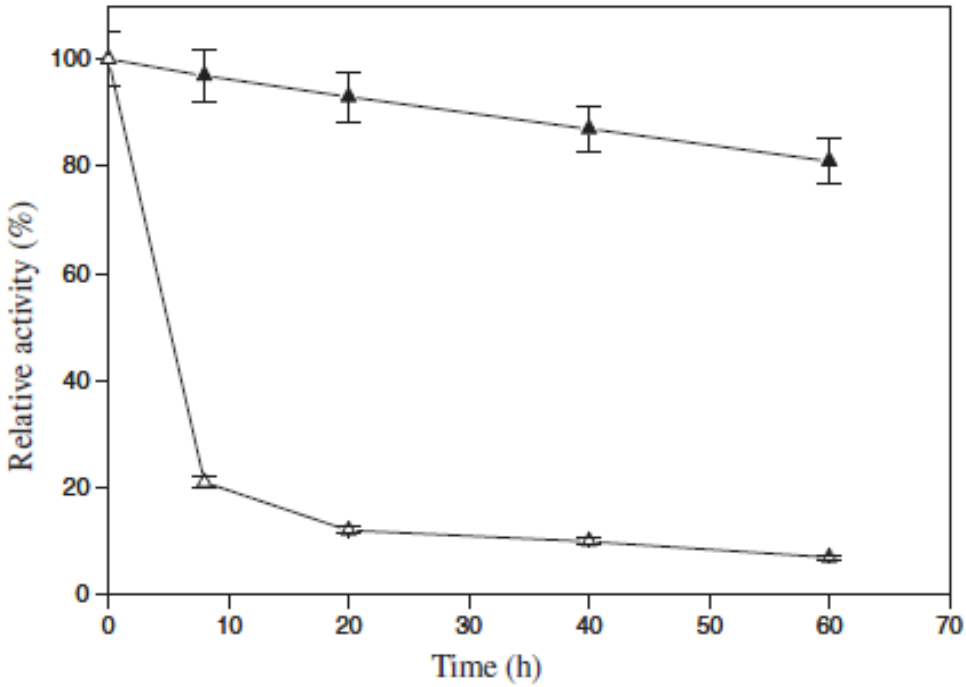


Figure 3.2: Effect of temperature on stability of β-glucosidase produced by submerged fermentation (▲) and solid state fermentation (Δ) at 50 ^{o}C for 60 h.

Zymogram staining and thermo-stability of β-glucosidase

The enzyme preparations derived from submerged fermentation showed two β - glucosidases, BGL1 and BGL2. While SSF derived preparations contained predominantly BGL2 and significantly less amount of BGL1 (Figure 3.3). The thermo-stability studies revealed that BGL1 is more thermo-stable than BGL2 (Figure 3.4). The less hydrolysis of Avicel and cellulose by SSF derived enzyme preparations could be attributed to the significantly low levels of thermo-stable BGL1. Production of such β - glucosidases exhibiting differential thermo-stability is not reported so far.

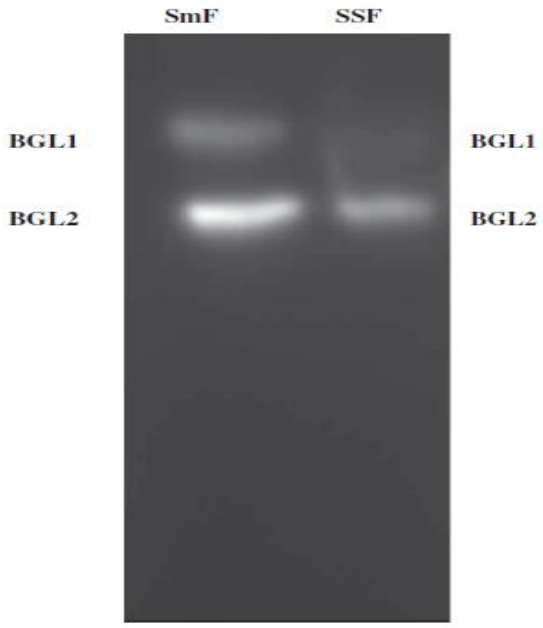


Figure 3.3: Zymogram staining of β-glucosidase(BGL) produced by EU2D- 21 in submerged fermentation (SmF) and solid state fermentation (SSF).

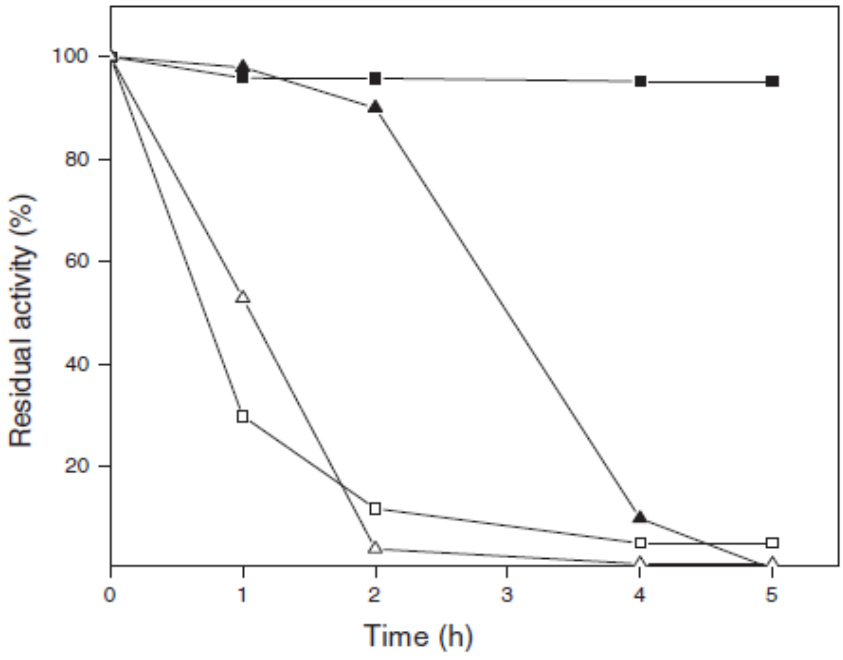


Figure 3.4: Effect of temperature on stability of *P. janthinellum* mutant EU2D-21 β-glucosidases (■, BGL1-SmF; □, BGL2- SmF; ▲, BGL1-SSF; Δ, BGL2-SSF).

DISCUSSION

The enzymatic degradation of cellulosic materials by fungal systems has been suggested as feasible alternative to produce fermentable sugars and fuel ethanol from lignocellulosic materials (Oksanen et al., 2000; Shin et al., 2000). One of the most extensively studied fungi is *Trichoderma reesei* which converts native as well as derived cellulose to glucose. Besides *Trichoderma reesei*, other fungi like *Humicola*, *Aspergillus, Penicillium* have the ability to secrete extracellular cellulases. Fungal cellulases are being commercially produced for biomass saccharification. The economic viability of biomass conversion depends on the pretreatment of substrates and the cost of the enzyme. The need for lower cost triggered a search for high cellulase producing organisms using classical mutagenesis, genetic engineering and enzyme engineering techniques that included advanced biotechnological procedures such as directed evolution and rational design studies (Adsul et al., 2011). These strains have been mutated and also genetically modified to obtain improved strains which have been used for the commercial production of cellulases (Szengyel et al., 2000). However attempts to use these enzymatic systems from *Trichoderma* mutants for the degradation of cellulosic waste have not been successful for several reasons such as low enzymatic yields, low specific activities and end product inhibition. We have reported earlier the production of enhanced level of cellulase by mutants of *P. janthinellum* in submerged fermentation (Adsul et al., 2007) and in solid state fermentation (Adsul et al., 2009) using only pure cellulose as substrate. Solid state fermentation (SSF) is advantageous over submerged fermentation because of superior enzyme yields in shorter time period, better oxygen circulation, less efforts in downstream processing. It resembles the

natural habitat for the filamentous fungi to grow and produce the fermented products. For cellulase production, SSF is most preferred due to its lower capital investment and lower operating cost (Rodriguez and Sanroman, 2005; Pandey et al., 2003). Additionally, SSF finds greater applications in solid waste management, biomass energy conservation and in the production of secondary metabolites. SSF also allows the use of low cost agricultural substrates for production of enzymes which helps in reducing the cost of production. Previously, agricultural residues such as wheat bran, corn stover, wheat straw, rice straw, bagasse, etc. were used in cellulase production. Among the mutants of *P. janthinellum*, mutant EU2D-21 was found to be producing highest amount of cellulases under both the submerged (Table 3.1) and solid state fermentation conditions (Table 3.2). These mutants produced enhanced level of cellulase using Avicel as well as cellulose powder. Tissue paper and solka-floc also gave good cellulase production. This work demonstrated that we can also use Avicel, solka-floc and especially tissue paper as substrates for cellulase production. Thus, tissue paper as cheap carbon source can also be used for effective cellulase production.

Among the mutant strains which have been evaluated for cellulase production, EU2D-21 showed higher production of cellulase under both submerged and solid state fermentation. Hence for further cellulose hydrolysis experiments, cellulase produced by the mutant EU2D-21 under submerged and solid state fermentations have been used. We used Avicel and Sigma cellulose as substrates for hydrolysis. The submerged enzyme preparation gave higher yield of hydrolysis (60–62%) than solid state enzyme preparations (20–25%). These results concluded that the decreased yield of hydrolysis by SSF enzyme preparations could be because of less thermo-stability of β-glucosidase

at 50 ^{o}C. Usually SSF derived enzymes are more thermo-stable than SmF derived enzymes (Archana and Satyanarayana, 1997). The β-glucosidases are considered as key enzymes for efficient cellulose saccharification, releasing glucanases from product inhibition. Moreover, since different applications are performed at elevated temperatures, higher thermal stability is desirable. The β-glucosidase from *Colletotrichum graminicola* maintained about 100% of the initial activity up to 72 h at 50 °C. Moreover, the enzyme was stable for 120 min at 55–60 °C (Zimbardi et al., 2013). Gao et al (2008) reported the thermo-stability of solid state derived β-glucosidase of *A. terreus* at 70 °C which maintained about 53% of their original activities even after incubation at 70 °C for 6 h. However, our results showed that the β-glucosidase in SSF derived cellulase preparation of mutant EU2D-21 exhibited less thermo-stability than that of β-glucosidase produced by submerged fermentation. This study also suggests that SSF derived β-glucosidase could be different from SmF derived β-glucosidase. Such reports on inactivation of β-glucosidase present in SSF derived cellulase preparations are not available so far.

CONCLUSION

Cellulose degrading fungal strains have attracted a great deal of interest as biomass degraders since they produce large amounts of extracellular ligno-cellulolytic enzymes. Attempts have been made by many researchers to improve the cellulolytic activity of fungal strains using traditional methods such as mutagenesis and also by recombinant techniques. In our laboratory, mutants have been isolated by subjecting *P. janthinellum* NCIM 1171 to mutation involving treatment of EMS followed by UV-irradiation. Among these strains, mutant EU2D-21 exhibited higher cellulase production

under both submerged and solid state fermentation conditions. The mutant EU2D-21 enzymes obtained from submerged and solid state fermentation have been used for hydrolysis of cellulosic substrates. Submerged fermentation derived enzymes hydrolyzed cellulosic substrates at greater extent than the solid state fermentation derived enzymes. *P. janthinellum* mutant EU2D-21 produced two β-glucosidases under submerged fermentation conditions. One of the β-glucosidase (BGL1) is more thermo-stable than other one (BGL2). SSF derived cellulase preparation contained predominantly less thermo-stable species of β-glucosidase which could be the cause of less hydrolysis of Avicel and cellulose by SSF derived cellulase preparation. Production of such β-glucosidases exhibiting differential thermo-stability is not reported so far. The cellulase produced under submerged fermentation conditions are more thermo-stable in this case hence this enzyme preparation can be used for hydrolysis of biomass material. This mutant could be the potential source of cellulase to be used for hydrolysis of lignocellulosic materials to produce monomeric sugars which can be converted to commodity chemicals such as ethanol or lactic acid. The next chapter deals with the hydrolysis of different cellulosic substrates using the cellulase preparation produced under submerged condition by *P. janthinellum* EU2D-21 and D-lactic acid production from these hydrolyzed substrates by an improved strain, *L. lactis* RM2-24 described in the 2nd chapter.

REFERENCES

Adsul M. G., Bastawde K. B., Varma A. J., Gokhale D. V. (2007) Strain improvement of *Penicilium janthinellum* NCIM 1171 for increased cellulose production. *Bioresource Technology*, 98, 1467–1473.

Adsul M. G., Ghule J. E., Shaikh H., Singh R., Bastawde K. B., Gokhale D. V., Varma A.J, (2005) Enzymatic hydrolysis of delignified bagasse polysaccharides. *Carbohydrate Polymers*, 62, 6–10.

Adsul M. G., Ghule J. E., Singh R., Shaikh H., Bastawde K. B., Gokhale D. V., Varma A.J, (2004) Polysaccharides from bagasse: applications in cellulase and xylanase production. *Carbohydrate Polymers*, 57, 67–72.

Adsul M. G., Terwadkar A. P., Varma A. J., Gokhale D. V. (2009) Cellulases *from Penicillium janthinellum* mutants: solid state production and their stability in ionic liquids. *Bioresources*, 4 (4), 1670–1681.

Adsul M.G., Singhvi M.S., Gaikaiwari S.A. Gokhale D.V. (2011) Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass. *Bioresource Technology*, 102, 4304-4312.

Archana A. and Satyanarayana T. (1997) Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid state fermentation. *Enzyme Microbial Technology*, 21, 12–17.

Depaula E. H., Ramos L. P., Azevedo M. D. (1999) The potential of *Humicola grisea* var. Thermoidea for bioconversion of sugarcane bagasse. *Bioresource Technology*, 68, 35–41.

Fischer E. A. and Stein E. A. (1961) Amylase from human saliva. *Biochemical Preparations* , 8, 27–33.

Gao J., Weng H., Zhu D., Yuan M., Guan F., Xi Y. (2008) Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. *Bioresource Technology*, 99, 7623–7629

Gokhale D. V., Puntambekar U. S., Deobagkar D. N. (1986) Xylanase and β – xylosidase production by *Aspergillus niger* NCIM 1207. *Biotechnology Letters*, 8, 137–138.

Gokhale D. V., Puntambekar U. S., Vyas A. K., Patil S. G., Deobagkar D. N. (1984) Hyper-production of β-glucosidase by *Aspergillus sp Biotechnology Letters*, 6, 719–722.

Gokhale D.V., Patil S.G., Bastawde K.B. (1992) Protection of *Aspergillus niger* cellulases by urea during growth on glucose or glycerol supplemented media. *Applied Biochemistry and Biotechnology,* 37, 11-17.

Kansoh A. L., Essam S. A., Zeinat A. N. (1999) Biodegradation and utilization of bagasse with *Trichoderma reesei. Polymer Degradation and Stability,* 62, 273–278.

Khisti U., Bastawde K., Gokhale D.V. (2011). Hyperpdrouction of β-glucosidases and β–xylosidase by *Aspergilus niger* NCIM 1207 in xylan containing media *BioResources*, 6(2), 2066-2076.

Laemmli U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage-T4. *Nature,* 227, 680–685.

Lynd L. R., Weimer P. J., van Zyn W. H., Pretorious I. S. (2002) Microbial cellulose utilization: fundamentals and bio-technology. *Microbiology and Molecular Biology Review,* 66, 506–577.

Oksanen T., Pere J., Paavilainen L., Buchert J., Viikari L. (2000) Treatment of recycled kraft pulps with *Trichoderma reesei* hemicellulases and cellulases. *Journal of Biotechnology*, 78, 39–48.

Pandey A. (2003) Solid state fermentation. *Biochemical Engineering Journal,* 13(2), 81-84.

Rodriguez S. and Sanroman M. A. (2005) Application of solid-state fermentation to ligninolytic enzyme production. *Biochemical Engineering Journal,* 22, 211-219.

Shin C. S., Lee J. P., Lee J. S., Park, S. C. (2000) Enzyme production of*Trichoderma reseei* Rut C-30 on various lignocellulosic substrates. *Applied Biochemistry and Biotechnology*, 86, 237–245.

Solomon B. O., Amigun B., Betiku E., Ojumu T.V., Layokun S. K. (2000) Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on bagasse. *Journal of Nigerian society of Chemical Engineers,* 16, 61–68.

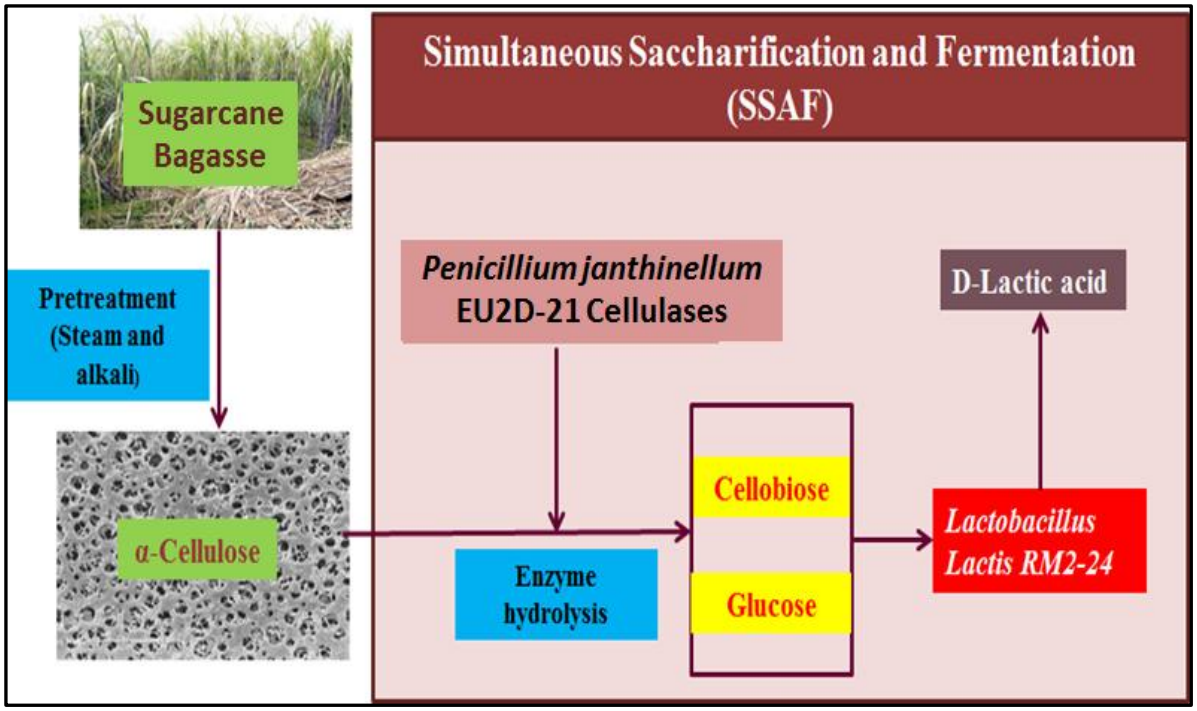
Szengyel Z., Zacchi G., Varga A., Reczey K. (2000) Cellulase production of *Trichoderma reseei* Rut C-30 using steam-pretreated spruce. Hydrolytic potential of cellulases on different substrate. *Applied Biochemistry and Biotechnology*, 86, 679–691.

Zimbardi A. L., Sehn, C., Meleiro L. P., Souza F. H., Masui D. C., Nozawa M. S., Furriel, R. P. (2013). Optimization of β-glucosidase, β-xylosidase and xylanase production by *Colletotrichum graminicola* under solid-state fermentation and application in raw sugarcane trash saccharification. *International journal of molecular sciences*, 14(2), 2875-2902.

CHAPTER 4

D-(-)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-24

Graphical Abstract



Singhvi M. S., et al (2010) *Green Chem.* 12, 1106-1109.

SUMMARY

Lactobacillus lactis mutant RM2-24 utilizes cellobiose efficiently, converting it into D-(-)-lactic acid. Cellobiose-degrading enzyme activities were determined for whole cells, cell extracts and disrupted cells. Aryl-β-glucosidase activity was detected in whole cells and disrupted cells, suggesting that these activities are confined to the cells. The mutant produced 80 g/l of lactic acid from100 g/l of cellobiose with 1.66 g/l /h productivity. Production of D-lactic acid from different cellulose samples was also studied. The cellulose samples at high concentration (10%) were hydrolyzed by cellulase enzyme preparation (10 FPU/g of cellulose) derived from *Penicillium janthinellum* mutant EU2D-21 generated in our own laboratory. We obtained a maximum 72% hydrolysis of bagasse derived cellulose, yielding glucose and cellobiose as the main end products. Lactic acid was produced from these cellulose samples by simultaneous saccharification and fermentation (SSAF) in a media containing a cellulase enzyme preparation derived from *Penicillium janthinellum* mutant EU2D-21 and cellobiose utilizing *Lactobacillus lactis* mutant RM2-24. A maximum lactic acid concentration of 73 g/l was produced from a concentration of 100 g/l of bagasse-derived cellulose, the highest productivity and yield being 1.52 g/l/h and 0.73 g/g, respectively. Considering that bagasse is a waste material available in abundance, we propose to use this biomass to produce cellulose and then sugars, which can be fermented to valuable products such as ethanol and lactic acid.

INTRODUCTION

Lactic acid and its derivatives have been widely used in food, pharmaceutical, cosmetic and industrial applications (Vickroy, 1985). It has a potential to become a commodity chemical for production of biodegradable polymers, oxygenated chemicals, plant growth regulators and special chemical intermediates. It has been receiving great attention as a feedstock for manufacture of polylactic acid (PLA), a biodegradable polymer used as environmentally friendly biodegradable plastic. Lactic acid is manufactured either chemically or by microbial fermentation. Chemical synthesis always results in racemic mixture of lactic acid, which is a major disadvantage. Microbial fermentation offers the advantage in both utilization of renewable carbohydrates and production of pure L- or D-lactic acid depending on the strain selected. The physical properties of PLA depend on the isomeric composition of lactic acid. Poly (L-lactide) (PLLA) obtained by polymerization of L-lactic acid or L-lactide has a melting temperature of 175 ^{o}C. The melting point of this polymer can be increased by blending with poly (D-lactide) (PDLA) in a solvent. Recently, it was found that the polymer blend of PLLA and PDLA produces a stereo-complex with melting temperature around 230 ^{o}C (Tsuji and Fukui, 2003). This finding has attracted more attention to the production of D-lactic acid.

Cellulosic substances are abundantly available resources of renewable biopolymer which can be utilized as a feedstock for producing a number of bulk chemicals like ethanol or lactic acid through fermentation processes. The production of such value-added products from renewable feedstock is a present need, and there is a demand to make it an economically and environmentally feasible process. Such

bioprocesses involving cellulosic residues not only provide alternative substrates but also help solve their disposal problems. With the advent of biotechnological innovations, mainly in the area of enzyme technology and fermentation technology, many new avenues have opened up for their exploitation as value-added products. Currently, optically pure lactic acid is produced mainly from corn starch. However, the use of agro-waste materials for lactic acid production appears to be more attractive because they do not then have any impact on the human food chain.

Unfortunately, the process for converting cellulosic material into lactic acid is not yet feasible due to the high cost of cellulase enzymes involved in cellulose hydrolysis (Wyman et al., 2005; Yanez et al., 2003) and also to the use of fastidious organisms (Moldes et al., 2000). For the utilization of cellulosic wastes such as cardboard and corn cobs as substrates for lactic acid fermentation, simultaneous saccharification and fermentation (SSAF) has been considered to be a promising approach (Rivas et al., 2004). However, there have been many technical problems – for instance, the enzymes of cellulose hydrolysis are inhibited by the intermediate products such as cellobiose, and lactic acid biosynthesis is inhibited by lactic acid (Lu et al., 2009). Many investigations have been carried out to relieve such inhibitions, for example, *in situ* product removal technology has been applied to the SSAF process, but this needs large electric energy or high-level equipment (Tanaka et al., 2006; Romani et al., 2008). Cellulase inhibition by glucose and cellobiose during the hydrolysis of cellulosic material by cellulases is the main bottleneck, which significantly slows down the rate of hydrolysis. The addition of β-glucosidase at the beginning of SSAF is recommended for the removal of cellobiose inhibition, but sometimes it does not work

because of rapid deactivation of the enzyme (Nakasaki and Adachi, 2003). Therefore, it is advantageous to use a lactic acid producing strain that has the ability to utilize both glucose and cellobiose efficiently (Moldes et al., 2000). It is known that some *Lactobacillus* strains utilize cellobiose as a carbon source, (Carr et al., 2002) but very little information is available about lactic acid production from cellobiose.

Lactic acid is produced by oxidizing NADH generated during glycolysis with pyruvate as the electron acceptor. Two separate genes encoding two lactate dehydrogenase enzymes are present in lactic acid bacteria each producing one or other enantiomer (L+ or D-) of lactic acid. *Escherichia coli* produces D(-) lactic acid with the help of D-lactate dehydrogenase present in it (Zhou et al., 2003a). A strain of *Escherichia coli* was constructed by transferring L-lactate dehydrogenase gene from *Pediococcus*, which produced L-lactic acid (Zhou et al., 2003b). Lactic acid bacteria are capable of fermenting glucose and other hexoses but lack the ability to ferment pentoses (Patel et al., 2006). For complete conversion of biomass to lactic acid, Lactic acid bacteria should have the capability to ferment pentoses. *E. coli* strains have been constructed for the production of optically pure lactic acid by deleting the competing pathways (Zhu and Shimizu, 2004).

Previously Adsul et al (2007) reported the production of L-lactic acid from sugarcane bagasse cellulose with high productivity and yield using a *Lactobacillus* mutant created in our laboratory (Adsul et al., 2007a). In this chapter, we describe the efficient utilization of cellobiose and cellulosic materials by *Lactobacillus lactis* mutant RM2-24 for lactic acid production. We also report the aryl-β-glucosidase activity (involved in cellobiose utilization) that this mutant displays.

MATERIALS AND METHODS

Chemicals

p-Nitrophenyl- β -D-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-D-cellobioside (*p*NPC), *p*-nitrophenyl-β-D-galactopyranoside (*p*NPgal), 3,5-dinitrosalicylic acid and Sigma cellulose were obtained from Sigma-Aldrich Co., St Louis, MO, USA. Avicel PH-101 was obtained from Fluka Chemie GmbH. Solka Floc SW44 was purchased from Brown Co., Berlin, NH. The α-cellulose with 0.18% lignin and 98% cellulose was prepared from sugarcane bagasse in our laboratory (Adsul et al., 2007a).

Preparation of sugarcane bagasse cellulose

Sugarcane bagasse was obtained from Tamil Nadu Pulp and Paper Mills, Chennai, India. This bagasse contains about 43% cellulose, 30% xylan, and 20% lignin, in addition to some silica and other constituents. It was cut into small shreds of 1–3 mm size and then pre-treated with steam and alkali by a proprietary process (under patenting) to remove the xylan, lignin, and other impurities. The final product consisted of 93.5% a-cellulose, 5.3% b-cellulose (low molecular weight cellulose and traces of hemicellulose), 1.02% c-cellulose, and 0.18% lignin.

Strain information, growth media and cellulase production

Mutant EU2D-21 was isolated by exposing conidia of *Penicillium janthinellum* NCIM 1171 to UV irradiation. The procedure of generation of mutant EU2D-21 and its crude enzyme preparation have already been reported earlier (Adsul et al., 2007b). The mutant was maintained on potato dextrose agar (PDA) and sub-cultured once every three months. PDA contained (per liter) extract from 200 g potatoes, glucose (20.0 g),

yeast extract (1.0 g), and agar (20.0 g). *Lactobacillus lactis* mutant RM2-24, producing lactic acid with high productivity, was isolated by UV mutagenesis. The procedure for generation of *Lactobacillus lactis* mutant RM2-24 is described in Chapter 2. The strain was maintained in liquid MRS medium supplemented with 0.1% $CaCO_3$. Enzyme production was carried out in a 250 ml Erlenmeyer flask, with 70 ml of production medium containing 1% (w/v) cellulose-123 powder and 2.5% wheat bran as described in Chapter 3.

Lactic acid production in shake-flasks using cellobiose

For the evaluation of lactic acid production from cellobiose, experiments were carried out in screw-cap conical flasks containing 100 ml fermentation medium and also shaken at 150 rpm at 42 ^{o}C. The flask contained 100 ml production medium consisting of 100 g/l cellobiose, 10 g/l yeast extract and 45 g/l $CaCO_3$. The flasks were inoculated (5% inoculum) with *Lactobacillus lactis* mutant RM2-24 grown in hydrolyzed sucrose medium (Kadam et al., 2006). The culture samples removed after certain time intervals were centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed for sugar and lactic acid and pH measurement. The supernatant was acidified with equal volume of 1 M HCl where free acid is liberated. The supernatant was further used for determination of total sugar and lactic acid.

Enzyme assay

The β-glucosidase (β-D-glucosideglucohydrolase; E.C. 3.2.1.21) activity was estimated according to the method described in Chapter 3 using *p*NPG as substrate. The total of assay mixture (1 ml) consisting of 0.9 ml of substrate and 0.1 ml of suitably

diluted enzyme was incubated at 50 ^{o}C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm after developing the color with 2 ml of sodium carbonate (2%). One unit of enzyme activity is equivalent to one µmole of *p*-nitrophenol liberated per minute. The other substrates used were *p*NPC and *p*NPgal to determine aryl-β-cellobiosidase and ayl-β-galactosidase activities.

To detect enzyme activities, the cells were grown in different sugars (glucose, lactose, hydrolyzed sucrose and cellobiose) at 42 ^{o}C and harvested at the late-exponential phase by centrifugation. After centrifugation, the supernatant and cells were used for an analysis of aryl-β-glucosidase and aryl-β-galactosidase activities. The cells were washed three times with phosphate buffer (50 mM, pH 7.0) and re-suspended in the same buffer. The suspension was used for analyzing cell-bound β-glucosidase and β-galactosidase activities.

To check the intracellular location of these enzymes, cells were subjected to sonication (SONICS Vibra cell; model VC 130) in phosphate buffer (pH 7.0, 50 mM) containing 0.5 mM EDTA and 1 mM dithiothreitol. The sonication was performed at 60% amplitude (125 mm) for 3 min by using a 2 mm probe under cold conditions. Almost 90% of the cells were disrupted by this method. The supernatant and sonicated cell debris were analyzed for all above-mentioned enzyme activities.

Enzymatic hydrolysis of different cellulosic substrates

The saccharification experiments were carried out in a 250 ml conical flask with 100 ml citrate buffer (pH 4.5, 50 mM), 10 g of Avicel, bagasse derived cellulose, sigma cellulose and solka-floc and crude enzyme preparations from *P. janthinellum* NCIM

1171 mutant, EU2D21. This mixture was incubated at 50 ^{o}C with shaking at 150 rpm. The samples were analyzed for the reducing sugars after suitable time intervals.

Simultaneous Saccharification and Fermentation (SSAF)

SSAF was carried out in a 250 ml screw cap conical flask with the production medium consisting of different cellulosic substrates (10.0 g), CaCO3 (5.0 g), yeast extract (1.0 g) in 100 ml citrate buffer (pH 4.5, 50 mM). The production medium was sterilized at 121 ^{o}C for 20 min, the crude enzyme preparation was added, and *Lactobacillus lactis* RM2-24 mutant cells (5%) grown in sucrose based medium were inoculated. The flasks were incubated at 42 ^{o}C with shaking at 150 rpm. All the SSAF experiments were performed for 72 h in media containing 10 filter paper units (FPU)/ g of substrate. The initial pH of the fermentation medium was 6.5. The samples harvested at various time intervals were centrifuged at 5000 rpm for 20 min to separate the cells. The supernatant was acidified by adding an equal volume of 1 N HCl where free acid is liberated and analyzed by HPLC for lactic acid.

Analytical methods

Cell growth was measured by spectrophotometrically using Spectrometer-117 (Systronics, Mumbai, India) at a wavelength of 660 nm. Reducing sugar concentration was determined by DNS method. The amounts of glucose and lactic acid were determined using a high-performance liquid chromatography (HPLC) system (Dionex India Ltd.) equipped with UV- or RI-detectors (Kadam et al., 2006). An ion exclusion column (Aminex, HPX-87H,Biorad, Hercules, CA) was used at a temperature of 30 ^{o}C with 0.008 N H_2SO_4 as a mobile phase at flow rate of 0.6 ml/min. An injection volume

of the sample was 50 µl. The presence of L-(+)-lactic acid was analyzed by an L-lactate oxidase enzyme kit (Randox Laboratories, UK), and the D-lactic acid content was calculated by subtracting L-lactic acid values from total lactic acid estimated by HPLC as described in Chapter 2.

RESULTS

Lactic acid production using cellobiose

The profile of growth (optical density) and lactic acid production from cellobiose is shown in Figure 4.1. The maximum amount of lactic acid was produced within 48 h of fermentation, with an increase in optical density from 0.6 to 11 and a decrease in pH from 6.5 to 4.8. The maximum amount of lactic acid (80 g/l) was produced from 100 g/l of cellobiose with 1.66 g/l productivity and 0.8 g/g yield. These are the highest productivity and efficiency values reported so far for the production of lactic acid from cellobiose.

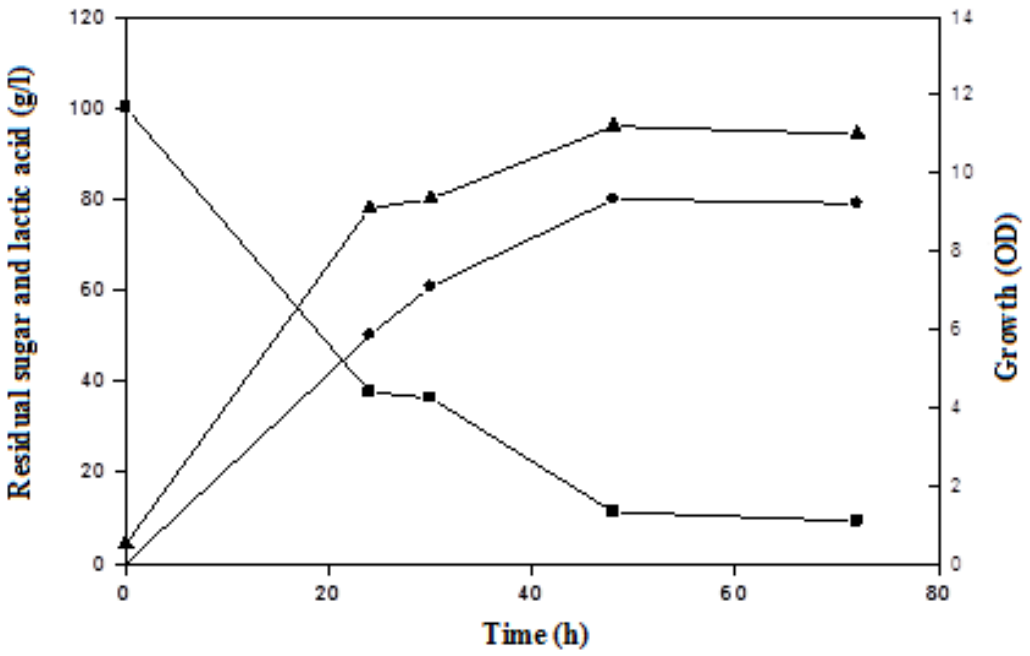


Figure 4.1: Profile of lactic acid production (●), residual sugar (■,) and growth (▲,) during fermentation by *Lactobacillus lactis* mutant RM2-24 using cellobiose (100 g/l).

Determination of enzyme activities

This mutant strain, RM2-24, was observed to utilize cellobiose, indicating the presence of cellobiose-degrading enzymes. Therefore, we attempted to detect the cellobiose degrading enzymes by using *p*-Nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-D-cellobioside (*p*NPC) and *p*-nitrophenyl-β-D-galactopyranoside (*p*NPgal), as substrates. We could not detect any enzyme activity in the supernatant.

Table 4.1 presents the data for all the enzyme activities in the mutant strain grown in liquid medium with different sugars. All activities were detected in lactose and cellobiose grown cells. However, a higher level of aryl-β-glucosidase was observed in cellobiose-grown cells. Other than cellobiose, lactose-grown cells also exhibited all activities. When cells were grown in glucose and a hydrolyzed sucrose-based medium, aryl-β-glucosidase and aryl-β-galactosidase activities were detected in small amounts. There was no activity detected using *p*NPC as substrate. These results show that the aryl-β-glucosidase or aryl-β–galactosidase gene could be constitutively expressed, and enhancement in activity was observed when the mutant was grown in either cellobiose or lactose, respectively. Activity with *p*NPC as substrate was detected only when the culture was grown in cellobiose or lactose. However, the induction in lactose-grown cells is less than that in cellobiose-grown cells.

None of these activities were detected in fermented broth suggesting the intracellular location of these enzymes. No activities were detected in the supernatant (cell extract) showing that the enzymes are not located in the cytoplasm. However, all activities (for lactose- and cellobiose grown cells) were detected in sonicated cells (Table 4.1), suggesting that the enzymes could be bound to the cell wall/membrane. The

activity levels analyzed in the sonicated cells were low compared to those of the intact cells.

Table 4.1: Detection of aryl-β-glucosidase activity from *Lactobacillus lactis* mutant RM2-24 grown in different sugar substrates

Enzyme source	Substrate	Enzyme activity (U/g of cells grown in)			
		Glucose	Lactose	Hydrolyzed sucrose	Cellobiose
Cells	*p*NPG	2.88	7.56	2.7	23.4
	*p*NPgal	5.18	6.6	5.1	4.41
	*p*NPC	ND	1.215	ND	6.075
Sonicated Cells	*p*NPG	ND	4.02	ND	10.5
	*p*NPgal	2.1	5.01	2	2.48
	*p*NPC	ND	0.81	ND	3.79

The cells were harvested at the onset of stationary phase for the determination of enzyme activity. Values are average of two independent experiments. ND, not detected.

Enzymatic hydrolysis of cellulosic substrates

Experiments were performed to evaluate the hydrolysis of various cellulose samples using cellulase enzyme preparations derived from *Penicillium janthinellum* EU2D-21. Hydrolysis of the most crystalline substrate, Avicel, was always lower than that of other cellulose samples due to its microcrystalline structure which prevents easy access to the enzyme. However, 71% hydrolysis was obtained in case of bagasse-derived cellulose, indicating its greater accessibility towards cellulase enzyme as shown in Figure 4.2. Solka-Floc and Sigma cellulose were hydrolyzed to approximately 50%, probably due to the more amorphous nature of these two substrates.

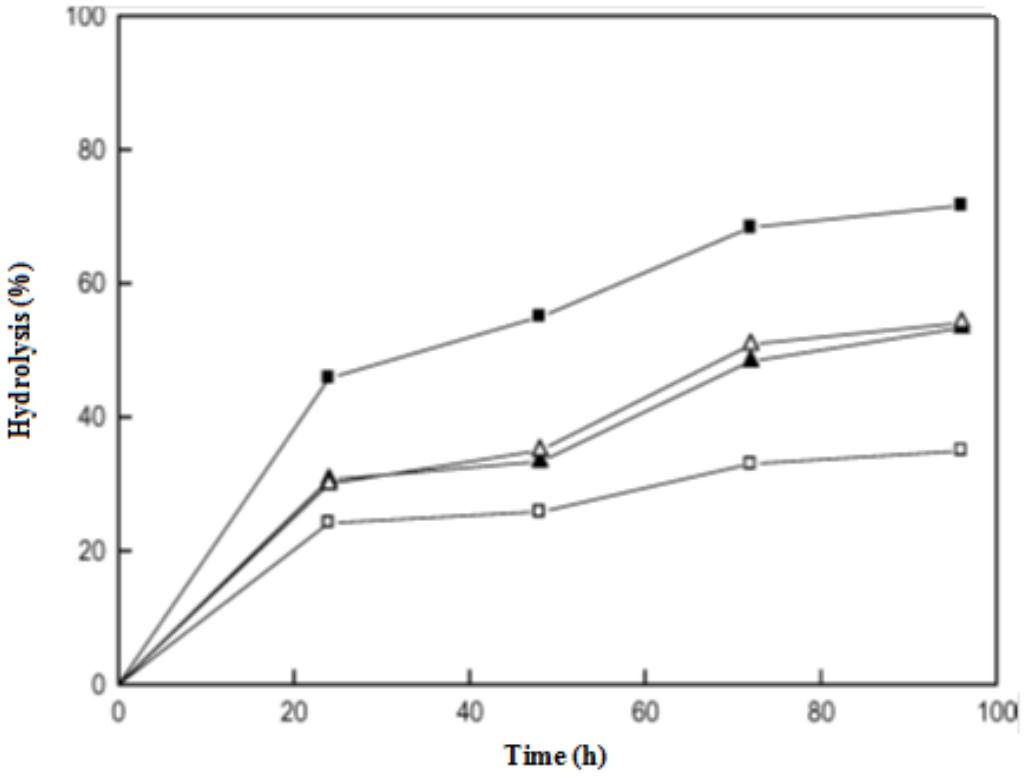


Figure 4.2: Profile of hydrolysis of Avicel (□), baggasse cellulose (■),Solka-Floc (Δ) and Sigma cellulose (▲) by mutant (EU2D-21) enzyme preparations. The hydrolysis was carried out using 10% substrate concentrations with mutant enzyme preparations (10 FPU/g).

Simultaneous Saccharification and Fermentation (SSAF)

SSAF experiments were performed under the selected conditions (42 ^{o}C, pH 6.5) because the organism used in this fermentation is a mutant of *Lactobacillus lactis* (RM2-24) and cannot grow at temperatures more than 42 ^{o}C. We carried out the SSAF at pH 6.5, at which the cellulase preparation used was active with retention of more than 50% activity. SSAF experiments were performed in production media containing cellulase (10 FPU/g of substrate). Different cellulosic substrates were evaluated for

lactic acid production using SSAF approach. The substrates used were Avicel, Sigma cellulose, Solka Flac and sugar cane bagasse derived cellulose (α-cellulose). Among all the substrates, α-cellulose yielded maximum lactic acid and the lowest yield was obtained when Avicel was used as substrate (Table 4.2).

Table 4.2: D-lactic acid production by *Lactobacillus lactis mutant* RM2-24 using different cellulosic substrates

Substrates	D-Lactic acid (g/l)			Productivity (g/l/h)		
	24h	48h	72h	24h	48h	72h
α-Cellulose	47.92	71.31	73.09	1.99	1.48	1.01
Sigma Cellulose	17.78	17.94	20.8	0.74	0.37	0.28
Solka Floc	30.96	31.13	32.62	1.29	0.64	0.45
Avicel PH-101	15.88	17.11	17.33	0.66	0.35	0.24

Figure 4.3 shows the lactic acid production and the pH profile of the fermentation broth. The pH of the fermentation broth dropped to 5.4 within 24 h, which is the pH at which enzymes are most active. No cellobiose accumulation was observed during the fermentation at any time. Cellobiose was either converted to glucose by β-glucosidase present in the cellulase preparations or utilized by the mutant strain to produce lactic acid. We obtained 73 g/l of lactic acid from 100 g/l of α-cellulose. The yield (g/g) and productivity (g/l/h) of lactic acid were 0.73 and 1.52, respectively.

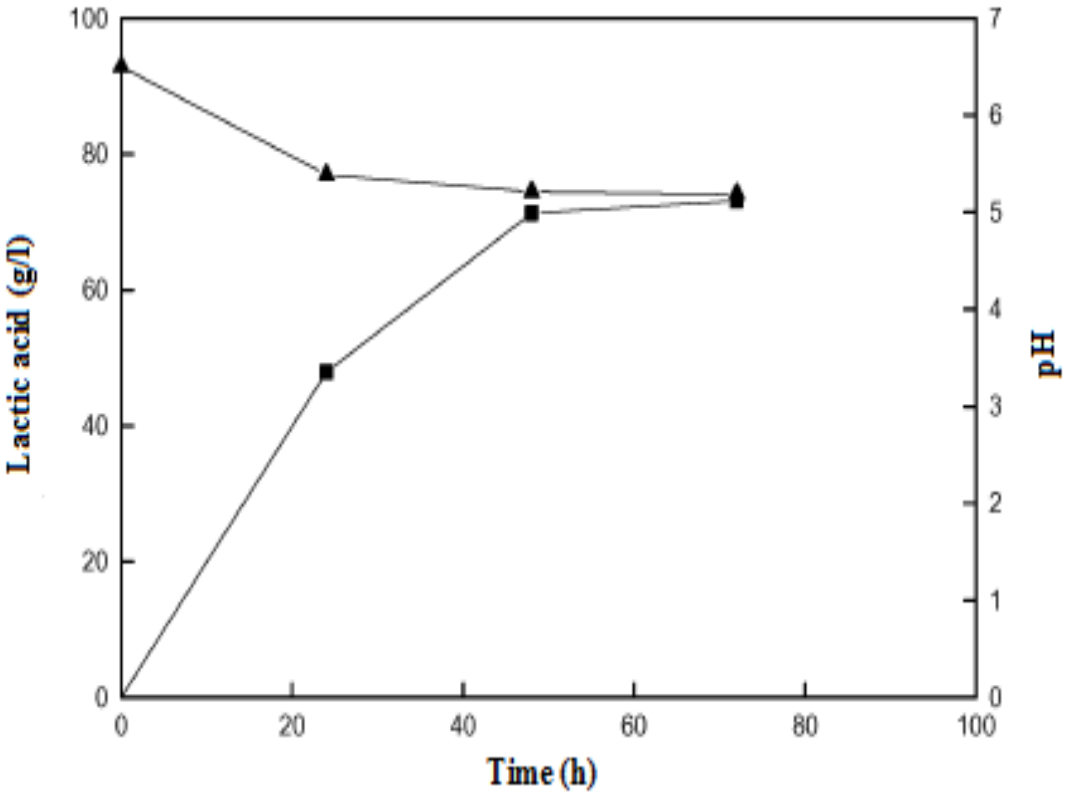


Figure 4.3: Course of lactic acid production (■) and pH (▲) during SSF of a-cellulose (100 g/ l) using mutant enzyme preparation (EU2D-21, 10 FPU/ g).

The mutant strain, *Lactobacillus lactis* RM2-24 produced maximum lactic acid concentration of 73 g/l was produced from a concentration of 100 g/l of bagasse-derived cellulose with the highest productivity 1.52 g/l/h. Table 4.3 shows the comparison of lactic acid production using different cellulosic substrates and also explains superiority of the *Lactobacillus lactis* RM2-24 as compared to other reports of D-lactic acid production.

Table 4.3: Comparison of recent data with the present work on SSAF production of D-(-)-lactic acid from cellulosic substrates

Substrate (g/l)	strain	Enzyme(FPU /g)	F.T.	C_{max}	$Y_{p/s}$	Q_p	Ref
Filter paper (33)	*L. coryniformis* ATCC 25600	Celluclast and Novozyme (28)	48	25	75	0.5	Yanez et al., (2003)
Pretreated cardboard (41)	*L. coryniformis* ATCC 25600	Celluclast and Novozyme (22.8)	48	23	56	0.49	Yanez et al., (2005)
Defatted rice bran (100)	*L. delbrueckii* IFO 3202	Cellulase-Y-NC	36	28	28	0.77	Tanaka et al., (2006)
Paper sludge (100)	*B. coagulans* strains 36D1	-	100	92	77	0.96	Budhavaram and Fan (2009)
Sugarcane bagasse	*Lactococcus lactis*	Celluclast	72	10.9	36	0.17	Laopaiboon et al., (2010)
Broken Rice (250)	*L. delbrueckii*	Glucoamylase	48	70	81	1.38	Nakano et al., (2012)
α-Cellulose (100)	*L. lactis RM2-24*	*P.janthinellum* EU2D-21 (10)	48	71	73	1.48	This work

F.T.-Fermentation time (h), C_{max} -Maximum lactic acid concentration (g/l), $Y_{p/s}$ -% product yield (g/g), Q_p -Lactic acid Productivity (g/l/h).

DISCUSSION

Lactobacillus lactis RM2-24, the mutant strain produced maximum amount of lactic acid by utilizing cellobiose within 48 h of fermentation. The maximum (80 g/l) amount of lactic acid was produced from 100 g/l of cellobiose with 1.66 g/l productivity and 0.8 g/g yield. This strain, therefore, is proved to be highly efficient for the conversion of cellobiose to lactic acid, and could be exploited at a commercial level. The efficient utilization of cellobiose by mutant RM2-24 indicated the presence of cellobiose-degrading enzymes in it. Therefore the fermented broth was analyzed for β-glucosidase activities. None of the activities were detected in the fermented broth suggesting the possibility of intracellular location of these enzymes. Hence cell extracts and sonicated cell debris were analyzed for these activities. No activities were detected in the supernatant (cell extract) showing that enzymes are not located in the cytoplasm. However, all activities (for lactose- and cellobiose grown cells) were detected in sonicated cells suggesting that the enzymes could be bound to the cell wall / membrane. It is noteworthy that aryl-β-glucosidase activities detected in *L. lactis* RM2-24 were much higher compared to those observed in *Lactobacillus delbrueckii* Uc-3 which produced L-lactic acid from cellobiose and cellotriose (Adsul et al. 2007c). There are reports on some recombinants and metabolically engineered strains which convert cellobiose to lactic acid. Tokuhiro et al. (2008) engineered a *Saccharomyces cerevisiae* strain that expresses the β-glucosidase gene derived from *Aspergillus aculeatus* in order to allow the yeast to utilize cellobiose as a substrate. The resulting engineered strain successfully produced approximately 80 g/l D-lactic acid from approximately 100 g/l cellobiose, with a yield of 0.70 g/g and a maximum production rate of 2.8 g/l/h.

Different cellulosic substrates have been evaluated for enzyme hydrolysis. Among these substrates, bagasse derived cellulose exhibited highest hydrolysis which may be due to its amorphous nature. The amorphous nature of substrate provides more accessibility towards enzyme attack hence the bagasse derived cellulose showed better hydrolysis than other substrates. We got approximately 71% hydrolysis at 10% bagasse cellulose concentration using EU2D-21 enzyme preparation (10 FPU/g of substrate) which showed the potentiality of cellulase produced by *P. janthinellum* EU2D-21.

SSAF experiments were carried out using different cellulosic substrates for lactic acid production. Among different substrates, the mutant strain, RM2-24 produced 73 g/l of lactic acid from 100 g/l of bagasse derived α-cellulose. This is, to our knowledge, the highest yield and productivity of D-lactic acid reported in the literature so far from cellulose. After fermentation there was no cellobiose accumulation at any time probably due to its conversion to glucose by β-glucosidase in the enzyme preparations or utilization by the mutant RM2-24 to produce lactic acid. The cellobiose accumulation could result in significant inhibition of cellulases, which could be removed by supplementation of media with additional cellobiase, leading to a remarkable improvement in lactic acid production in fed batch SSAF (Moldes et al., 2001). These results proved that the mutant strain RM2-24 has the ability to convert the lignocellulosic materials into D-lactic acid. The values of yield (g/g) and productivity (g/l/h) of D-lactic acid produced by RM2-24 are the highest values reported in the literature so far from cellulose. Table 3 reveals the reports on D-lactic acid production from different substrates by different *Lactobacillus* strains using SSAF approach. All these reports used commercial cellulase preparations with higher FPU activity/g of the

substrates. However, the yield and productivity of D-lactic acid are much lower than the values reported by us. Hence we feel that our mutant strains have great potential for conversion of cellulosic substrates to lactic acid which can be further used for PLA synthesis. Commercialization of the cellulose to chemicals such as biofuels and lactic acid has come a long way since the late 1990s. However, there is still scope for improvement as new and novel classes of lignocellulose degrading enzymes and robust microbes utilizing multiple substrates and producing value added chemicals are discovered. In addition, there is a need for improved biomass pretreatment processes which would solubilize the biomass polymers to monomer sugars to avoid the use costly pretreatments and costly enzymes.

CONCLUSION

Lactobacillus lactis mutant RM2-24 is a promising strain for the production of D-lactic acid from cellulosic materials in SSAF. Bottlenecks like feedback inhibition by glucose and cellobiose were removed by using this strain, leading to the complete conversion of cellulosic substrates to the value-added products like lactic acid. This strain utilized cellobiose effectively and produced lactic acid in a homo-fermentative manner. Such aryl β- glucosidase activities have not yet been reported for any of the D-lactic acid producing *Lactobacillus* strains so far. These studies show the potential of such a strain for producing value-added products from renewable biomass.

Batch experiments were conducted for conversion of bagasse sample to lactic acid by simultaneous saccharification and fermentation using a cellulase preparation derived from a mutant of *P. janthinellum* (EU2D-21) and *L. lactis* RM2-24. Among the cellulosic substrates used for D-lactic acid production, α-cellulose yielded 73 g/l of

lactic acid from100 g/l of the substrate with a yield of 0.73 g/g of cellulose substrate and a productivity of 1.52 g/l/h. Applying SSAF to lactic acid production is advantageous since we would operate the SSAF at conditions suitable and optimum for both cellulose hydrolysis and growth of *L. lactis* mutant. The further improvements in batch SSAF to make it cost effective are necessary, as this work indicates great advantages from the industrial viewpoint. The work on utilization of other biomass materials with proper pretreatment and proper integrated saccharification and fermentation processes may lead to bio-recycling of biomass to produce value added chemicals. There is still need for improving the techno-economic efficiency of the sugarcane bagasse derived cellulose process, so as to obtain an optimum and inexpensive constitution (cellulose, xylan and lignin) that would be more amenable to enzyme attack. Thus these hydrolyzed materials can further be used for fermentation to lactic acid and other feedstock chemical. The next chapter describes the improvement of acid tolerance in *L. delbrueckii* mutant Uc-3. We used L-lactic acid producer since there is a major requirement of L-lactic acid compared to D-lactic acid for PLA synthesis which need to be produced at acidic pH. The production of L-lactic acid at acidic pH would reduce requirement of neutralizing agent thus reducing the cost of downstream processing to get pure and polymer grade L-lactic acid.

REFERENCES

Adsul M. G., Varma A. J., Gokhale D. V. (2007a) Lactic acid production from waste sugar cane bagasse derived cellulose. *Green Chemistry*, 9, 58–62.

Adsul M. G., Bastawade K. B., Varma A. J., Gokhale D. V. (2007b) Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. *Bioresource Technology*, 98, 1467-1473.

Adsul M. G., Khire J. M., Bastawade K. B., Gokhale D. V. (2007c) Lactic acid production from cellobiose and cellotriose by *Lactobacillus delbrueckii* mutant Uc-3. *Applied and Environmental Microbiology*, 73, 5055–5057.

Budhavaram N. K. and Fan Z. (2009) Production of lactic acid from paper sludge using acid-tolerant, thermophilic *Bacillus coagulans* strains. *Bioresource Technology*, 100, 5966–5972.

Carr F. J., Chill D., Maida N. (2002) The lactic acid bacteria: a literature survey. *Critical Review of Microbiology*, 8, 281-370.

Kadam S. R. Patil S. S., Bastawde K.B., Khire J. M., Gokhale D. V. (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. *Process Biochemistry*, 41, 120-126.

Laopaiboon P., Thani A., Leelavatcharamas V., Laopaiboon L.(2010) Acid hydrolysis of sugarcane bagasse for lactic acid production. *Bioresource Technology*, 101, 1036–1043.

Lu Z., Lu M., He F., Yu L. (2009) An economical approach for D-lactic acid production utilizing unpolished rice from paddy as major nutrient source. *Bioresource Technology*, 100, 2026-2031.

Moldes A. B., Alonso J. L., Parajo J. C. (2000) Multi-step feeding systems forlactic acid production by simultaneous saccharification and fermentation of processed wood. *Bioprocess Engineering*, 22, 175-180.

Moldes A. B., Alonso J. L., Parajo J. C. (2001) Strategies to improve the bioconversion of processed wood into lactic acid by simultaneous saccharification and fermentation. *Journal of Chemical Technology and Biotechnology*, 76, 279-284.

Nakano S., Ugwu C. U., Tokiwa Y. (2012) Efficient production of D-(–)-lactic acid from broken rice by *Lactobacillus delbrueckii* using $Ca(OH)_2$ as a neutralizing agent. *Bioresource Technology*, 104, 791–794.

Nakasaki K. and Adachi T. (2003) Effects of intermittent addition of cellulase for production of L-lactic acid from wastewater sludge by simultaneous saccharification and fermentation. *Biotechnology and Bioengineering*, 82, 263-217.

Patel M., Ou M., Harbrucker R., Aldrich H., Buszko M., Ingram L., Shanmugam K. (2006) Isolation and characterization of acid tolerant, thermophilic bacterial biocatalysts for effective fermentation of biomass-derived sugars to lactic acid. *Applied and Environmental Microbiology*, 72, 3228-3235.

Rivas B. A., Moldes, B., Dominguez J. M., Parajo J. C. (2004) Lactic acid production from corn cobs by simultaneous sacchrification and fermentation: A mathematical interpretation. *Enzyme Microbial Technology*, 34, 627-634.

Romani A. R., Yanez G., Garrote S., Alonso J.L. (2008) SSF production of lactic acid from cellulosic biosludges. *Bioresource Technology*, 99, 4247-4254.

Tanaka T. M., Hoshina S., Tanabe K., Sakai S., Ohtsubo S., Tainguchi M. (2006) Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation. *Bioresource Technology*, 97, 211-217.

Tsuji H. T. and Fukui I. (2003) Enhanced thermal stability of poly (lactide) in the melt by enantiomeric polymer blending. *Polymer*, 44, 2891-2896.

Vickroy T. B. (1985) *Comprehensive Biotechnology*, ed. M. Moo-Young,Pergamon Press, New York 3, 761–776.

Wyman C. E., Dale B. E., Elander R.T., Holtzapple M., Ladish M. R., Lee Y.Y. (2005) Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*, 96, 1959-1966.

Yanez R., Alonso J. L., Parajo J. C. (2005) D-lactic acid production from waste cardboard *Journal of Chemical Technology and Biotechnology*, 80, 76-84.

Yanez R., Moldes A. B., Alonso J. L., Parajo J. C. (2003) Production of D (-)-lactic acid from cellulose by simultaneous saccharification and fermentation using *Lactobacillus coryniformis subsp. torquens*. *Biotechnology Letters*, 25, 1161-1164.

Zhou S. D., Causey T. B, Hasona A., Shanmugam K. T., Ingram L. O. (2003a) Production of pure D-lactic acid in mineral salts medim by metabolically engineered *Escherichia coli* W3110. *Applied and Environmental Microbiology*, 69, 399-407.

Zhou S. D ., Shanmugam K. T., Ingram L. O. (2003b) Functional replacement of the *Escherichia coli* D-(-)-lactate dehydrogenase gene (ldhA) with the L-(+)-lactate dehydrogenase gene (ldhL) from *Pediococcus acidilactici Applied and Environmental Microbiology*, 69, 2237-2244.

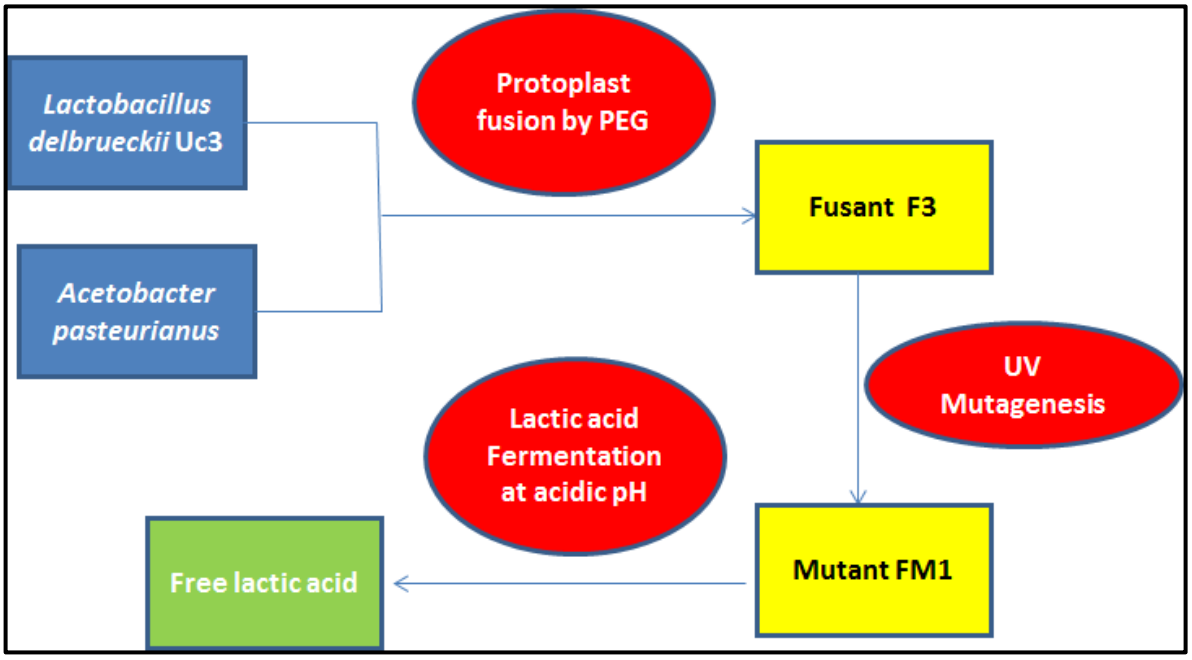
Zhu J. and Shimizhu K. (2004) The effect of pfl gene knockout on the metabolism for optically pure D-lactate production by *Escherichia coli. Applied Microbiology and Biotechnology*, 64(3), 367-375.

Tokuhiro K., Ishida N., Kondo A., Takahashi H. (2008) Lactic fermentation of cellobiose by a yeast strain displaying β-glucosidase on the cell surface. *Applied Microbiology and Biotechnology*, 79, 481–488.

CHAPTER 5

Improvement of acid tolerance in *Lactobacillus* through inter-generic protoplast fusion

Graphical Abstract



- Singhvi M., et al (2015) *RSC Advances* 5, 2024-2031.
- Singhvi M., et al (2013) *American J Bioeng. Biotech*. 1, 37-43.
- Singhvi M.S., et al (2010) *Ind J Microbiol* 50, 97-100.

SUMMARY

For the rapid development of industrially significant microbial strains, protoplast fusion technology is proficient approach. Fermentation based technologies depend comprehensively on biocatalyst development for commercialization. Here we describe the application of protoplast fusion technology followed by UV mutagenesis to improve the acid tolerance of ineffectively characterized *Lactobacillus delbrueckii* mut Uc3. We generated fusant F3 using protoplast fusion between *Lactobacillus delbrueckii* mut Uc3 and *Acetobacter pasteurianus* NCIM 2314 on solid media at pH4.0. The fusant, F3 was further treated by UV mutagenesis to generate the mutant, FM1 with elusive improvements in pH tolerance. We found that the mutant FM1 produced five-fold increase in lactic acid than the parent strain at acidic pH. The inheritance of *Lactobacillus delbrueckii* mut Uc3 and *Acetobacter pasteurianus* NCIM 2314 genomic chunks in fusant is validated using molecular techniques. We suggest that the modifications introduced by UV treatment in generic region or the regulatory regions of lactic acid production pathway control the high productivity of lactic acid in mutant FM1.

INTRODUCTION

Lactic acid is widely used in food, cosmetic, pharmaceutical, and chemical industries and has received increased attention for use as a monomer for production of biodegradable poly (lactic acid) (PLA) (Davison et al., 1995; Young et al., 2006). It is also an important platform chemical (Akerberg and Zacchi, 2002; Datta and Henry, 2006) as it can be converted into other chemicals such as acrylic acid, propylene glycol, acetaldehyde, and 2,3-pentanedione. In recent years, demands for lactic acid have

increased due to its application in biodegradable polymers and green solvents (Datta et al., 1995; Sodergard and stolt, 2002) and the greater demand is for the L isomer (Tsuji, 2002). The most important industrial microorganisms that produce lactic acid belong to the genus *Lactobacillus* (Benninga, 2002). Fermentative production of lactic acid offers a great advantage over the chemical route since pure L- or D-lactic acid can be produced by using selective strains of *Lactobacillus*. The present industrial production of lactic acid is based on microbial carbohydrate fermentation because it is economically more feasible compared with the chemical route (Benninga, 2002). The optical purity of the lactic acid is crucial during PLA production because small amounts of enantiomeric impurities drastically change the properties such as crystallinity or biodegradation rate of the polymer. Complexing poly L-lactic acid (PLLA) with poly D-lactic acid (PDLA), however, increases the melting point thus presenting an attractive solution to the heat sensitivity of PLA. Improved *Lactobacillus* strains have been developed which produce D- or L-lactic acid from hydrolyzed sucrose (Kadam et al., 2006), molasses (Dumbrepatil et al., 2008) and sugarcane bagasse derived cellulose (Adsul et al., 2007) in high amounts at neutral pH. Lactic acid fermentation at neutral pH produces calcium lactate, which need to be hydrolyzed by corrosive sulphuric acid to generate free lactic acid and gypsum. Hence it is essential to develop biocatalysts (*Lactobacillus* strains) which produce lactic acid at acidic pH thus making the whole down-stream process environment friendly. Considering this constraint, we concentrated on the development of improved *Lactobacillus* strains producing L-lactic acid in acidic environment which would make the process environmentally sound by avoiding the use of corrosive sulphuric acid. The downstream processing of lactic acid

plays a decisive role in the overall production route of PLA as the fermentation broth includes a complex mixture of impurities, nutrients and cell debris. The detection and removal of such impurities is essential since they can strongly deteriorate the properties of the produced polymer (Achmad et al., 2009).

Lactic acid is the fermented product of pyruvic acid, end-product of the glycolysis (Embden-Meyerhof) pathway, by which energy is supplied to most of the homo-fermentative lactic acid bacteria (Axelsson, 1993; Torriani et al., 1994). During typical lactic acid fermentation, low pH has an inhibitory effect on the metabolic activities of the producing organism (Buchata, 1983). Accumulation of lactic acid in an un-dissociated form inhibits both, cell growth and its production. Moreover, the lactic acid bacteria are not resistant to low pH and do not produce lactic acid at acidic pH (Hano et al., 1993). Therefore, during fermentation, a neutralizing agent such as NaOH, NH_4OH or $CaCO_3$ has to be added to minimize the inhibitory effects on cell growth and lactic acid production caused by free lactic acid. However, the neutralization of lactic acid during fermentation has disadvantages as the additional operations are required to regenerate un-dissociated lactic acid from its salt and to dispose of or recycle the neutralizing cation (Porro et al., 1999; Madzingaido et al., 2002). Furthermore, approximately one ton of crude gypsum, $CaSO_4$, is produced for every ton of lactic acid produced by the conventional fermentation (Prescot and Dunn, 1959). It is possible to reduce all the extra operations and expenses if the microorganism is able to grow and ferment the sugars at low pH levels (the pKa for lactic acid is 3.86). Such *Lactobacillus* strains capable of producing lactic acid at acidic conditions have not been yet developed. Use of acid tolerant *Lactobacillus* strain will change the entire scenario of

downstream processes for lactic acid purification. The effect of acid stress on bacterial physiology is not clearly known. Biochemical, proteomic and genetic analysis indicate that the responses of lactic acid bacteria to acid stress are intricate processes involving series of enzymes and genes (Arnold et al., 2001; Angelis and Gobbeti, 2004; Guchte et al., 2002; Paul and Hills, 2003).

Protoplasts are the cells without the cell wall and the cytoplasmic membrane is the outermost layer in such cells. Protoplast can be obtained by specific lytic enzymes to remove cell wall. Efficiency of fusion and appropriate phenotype transfer, therefore, is dependent on efficiency of protoplast formation and their regeneration. Specific conditions for protoplast isolation have been developed in plant cells (Vardi et al., 1989; Deng et al., 1992), fungi (Kim et al., 2000) and bacteria (Hopwood 1981). Protoplast fusion in *Lactobacilli* (Kanatani, 1989; Yeeh et al., 1996) has been reported. However, this technique has been applicable to certain strains of *Lactobacilli* mainly due to the difficulty in both protoplast isolation and regeneration. Though protoplast regeneration on agar media with 0.3 M raffinose has been successful, frequencies of regeneration often remained low (Connell et al., 1988). There are reports on regeneration of protoplasts of *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus rhamnosus* (Tanaka and Ohmomo, 2001) and *Lactobacillus casei* (Lee-Wickner and Chassy, 1984). There are no reports on systematic studies on protoplast isolation and regeneration in case of *L. delbrueckii and Acetobacter Pasteurianus.* Hence we have standardized the protocol for protoplast isolation and regeneration of both the strains.

Classical strain improvement methods have succeeded in obtaining many industrial strains. Recently, genome shuffling through protoplast fusion has made major

advances in the construction of strains with distinctly significant improved phenotypes. Protoplast fusion is the useful technique in the improvement of microorganisms. This technique allows exchange of entire genome between unrelated genera of microorganisms. It is considered as classical or fundamental method of gene transfer in which multiple genes can be simultaneously introduced in contrast to genetic engineering suitable for introducing only one or few genes. Successful transfer of hydrolase genes (multiple phenotype) using protoplast fusion approach led to changed properties of enzymes such as cellulase (Gokhale et al., 1984; Gokhale and Deobagkar, 1989) and amylase (Gokhale and Deobagkar, 1990). Genome shuffling through protoplast fusion has already been used to improve the acid tolerance in *Lactobacillus* (Patnaik et al., 2002; Wang et al., 2007) for L- lactic acid production. This approach was also used in *Streptomyces fradiae* to improve the production of the polyketide antibiotic, tylosin (Zhang et al., 2002) and in *Sphingobium* for the degradation of pentachlorophenol (Dai and Copley, 2004). We have isolated mutant strain, *Lactobacillus delbrueckii* Uc-3, producing L-lactic acid with high productivity (Kadam et al., 2006). We thought of using *L. delbrueckii* Uc-3 to improve its acid tolerance and ability to produce lactic acid at acidic pH since more than 90% of L-lactic acid is required for PLA synthesis. In this Chapter, we describe the application of protoplast fusion technique to generate acid tolerant strains of *Lactobacillus delbrueckii.* The protoplast fusion between *Lactobacillus delbrueckii* Uc-3 and acid tolerant strain of *Acetobacter pasteurianus* was performed to produce fusants with improved acid tolerance. One of the fusants was further subjected to UV mutagenesis to generate mutant producing enhanced levels of lactic acid at acidic pH. The fusant and mutant

strains were evaluated for lactic acid production at acidic pH and also validated using molecular techniques such as RAPD and RFLP.

MATERIALS AND METHODS

Chemicals

Yeast extract, sorbitol and MRS liquid medium were obtained from Hi-Media (India). Cane sugar was purchased from local market. Lysozyme and mutanolysin were purchased from Sigma (St. Louis, MO). Polyethylene glycol (PEG) 6000 was purchased from Fluka Chemicals. All the other chemicals were of analytical grade and obtained locally.

Microorganisms and growth media

Lactobacillus delbrueckii Uc-3 is a high lactic acid producing strain generated by UV mutagenesis (Kadam et al., 2006). *Acetobacter pasteurianus* NCIM 2314, *Acetobacter suboxydans* NCIM 2679 and all *Lactobacillus* strains were obtained from NCIM Resource Centre, National Chemical Laboratory, Pune, India. The *Lactobacillus* strains were maintained in liquid MRS medium supplemented with 0.1% $CaCO_3$. *Acetobacter* strains were maintained on Acetobacter medium. The cultures were transferred to respective fresh liquid medium every 4 weeks and were used as stock cultures for preparation of inoculum.

Acetobacter medium contained (g/l) sorbitol 20.0, yeast extract 5.0, with the pH adjusted at 6.2. *Acetobacter* Protoplast buffer (APB) with pH 8.0 consisted of Tris HCL (0.01M), EDTA (0.001M), NaCl (0.5M). *Lactobacillus* protoplast buffer (LPB) contained Tris-HCL (0.01M), $MgCl_2$ (0.02M) and sucrose (0.5M) with pH adjusted to

6.5. *Acetobacter* regeneration medium (ARM) consisted of *Acetobacter* agar medium with 0.5 M NaCl and *Lactobacillus* regeneration medium was MRS solid medium with 0.5 M sucrose. The HSYE medium containing (g/l) hydrolyzed sucrose 100, yeast extract 10 was used as fermentation medium. The selection of fusants was performed on HSYE solid medium at pH 4.0 with 0.5M sucrose.

Analytical methods

Reducing sugar concentration was determined by DNS (Dinitrosalicylic acid) method. L-lactate oxidase kit was used for the quantification of lactic acid as described in Chapter 2. The amount of glucose and lactic acid were determined using a high-performance liquid chromatography (HPLC) system (Dionex India Ltd.) equipped with UV- or RI-detectors. An ion exclusion column (Aminex, HPX-87H, Biorad, Hercules,CA) was used at a temperature of 30°C with 0.008N H_2SO_4 as a mobile phase at flow rate of 0.6 ml/min. An injection volume of the sample was 50µl. Optical density (growth) was determined spectrophotometrically at 660nm.

Protoplast formation and regeneration of *Acetobacter pasteurianus*

Cells grown in Acetobacter liquid medium were harvested by centrifugation at 5,000 rpm, washed twice with APB without osmotic stabilizer and suspended in APB to adjust the optical density at 660 to 0.6–0.8. A portion of cell suspension (5 ml) was treated with various concentrations of lysozyme and mutanolysin or combination of both and the mixture was incubated at 37 °C for 2 hr with shaking at 80 rpm. The formation of protoplasts was monitored by decrease in optical density at 660 nm. The protoplasts were also observed as spherical cells by microscopy or by enumeration of

osmotically resistant cells before and after protoplast formation. The same protocol was used for protoplast formation of other strain *Acetobacter suboxydans* NCIM 2679.

The cell pellet, after protoplast formation, was washed with APB to remove traces of lytic enzymes by centrifugation at 3,000 rpm for 10 min and the cell pellet was re-suspended in the same buffer. The cell suspension was suitably diluted in APB and 100 µl of each dilution was plated on ARM. The cell suspension was also plated on non-osmotically stabilized medium (ARM without osmotic stabilizers, NOS) to check the osmotically resistant cells. Plates were incubated aerobically at 37 °C and colonies were counted after 2 days of incubation. The frequency of protoplast regeneration was calculated from following equation:

$$\frac{[(\text{CFU/ml of protoplast on RM}) - (\text{CFU/ml of protoplast on NOS RM})]}{[(\text{CFU/ml of initial no. cells}) - (\text{CFU/ml of protoplast on NOS RM})]}$$

Protoplast formation and regeneration of *Lactobacillus delbrueckii*

Cells grown in MRS liquid medium were harvested by centrifugation at 5,000 rpm, washed twice with LPB without sucrose and suspended in LPB to adjust the optical density (OD_{660}) to 0.6–0.8. A portion of cell suspension (5 ml) was treated with various concentrations of lysozyme and mutanolysin or combination of both and the mixture was incubated at 37 °C for 2 h with shaking at 80 rpm. The formation of protoplasts was monitored by decrease in optical density at 660 nm. The protoplasts were also observed as spherical cells by microscopy or by enumeration of osmotically resistant cells before and after protoplast formation.

The cell pellet, after protoplast formation, was washed with LPB to remove traces of lytic enzymes by centrifugation at 3,000 rpm for 10 min and the cell pellet was

re-suspended in the same buffer. The cell suspension was suitably diluted in LPB and 100 µl of each dilution was plated on LRM. The cell suspension was also plated on non-osmotically stabilized medium (LRM without osmotic stabilizers, NOS) to check the osmotically resistant cells. Plates were incubated anaerobically at respective temperatures and colonies were counted after 2 days of incubation. The frequency of protoplast regeneration was calculated from following equation:

$$\frac{[(\text{CFU/ml of protoplast on RM}) - (\text{CFU/ml of protoplast on NOS RM})]}{[(\text{CFU/ml of initial no. cells}) - (\text{CFU/ml of protoplast on NOS RM})]}$$

Protoplast fusion followed by UV mutagenesis

Equal number of protoplasts of *L. delbrueckii* Mut Uc-3 and *A. pasteurianus* were mixed and treated with 30% Polyethylene glycol (PEG 6000) prepared in LPB for 10 min at room temperature. Then the fused protoplasts were centrifuged, washed with protoplast buffer (LPB), re-suspended in known volume of LPB. The fused protoplasts, after suitable dilution, were plated on HSYE regeneration medium at pH 4.0. The plates were incubated at 42 ^{o}C under anaerobic condition. Four fusants (F1, F2, F3, F4) were obtained which were evaluated for lactic acid production at pH 4.0. One of the fusants, F3, was further irradiated with UV at 254 nm at a distance 6 cm for 20 min. After UV-irradiation, cells were serially diluted in sterile saline and survivors determined by spreading 0.1 ml of diluted samples on a HSYE agar medium at pH 4.0. Only one mutant, FM1, was obtained after 72 h of incubation at 42 ^{o}C under anaerobic condition. The protocol for generation of fusant and mutant is shown in Figure 5.1.

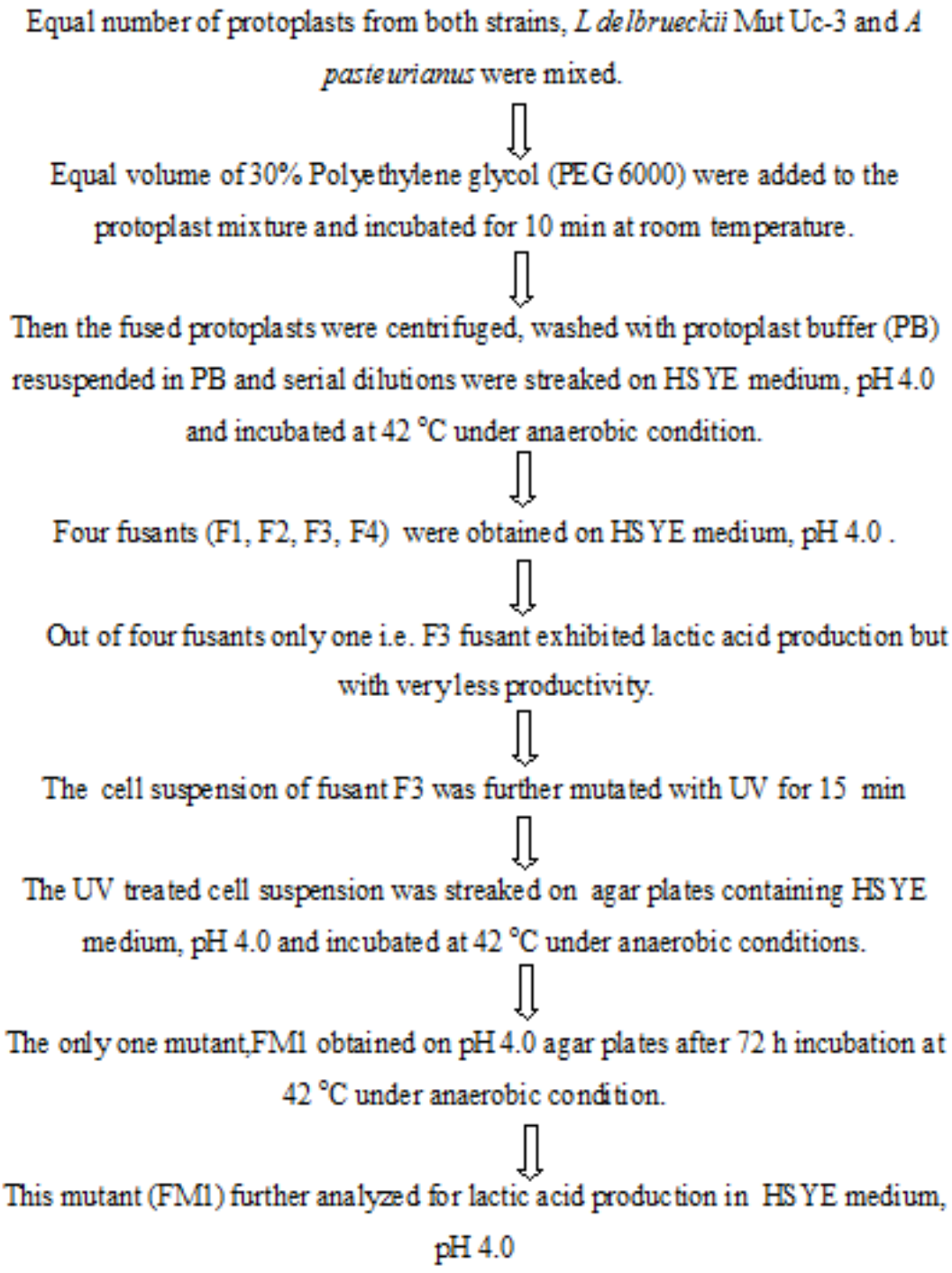


Figure 5.1: Schematic representation of generation of fusant and mutant strains

Shake flask analysis for lactic acid production

All the three strains include the parent strain, *L. delbrueckii Mut Uc-3*, **t**he fusant F3 and mutant FM1 were used to inoculate in 10 ml of MRS medium. Each strain was grown in HSYE medium with (50 g/l, 25 g/l $CaCO_3$) and without $CaCO_3$. An overnight culture (~ 5 ml) was transferred to 100 ml growth medium in 250 ml screw-cap conical flasks and then shaken at 42°C for 24 or 48 h. The 5 ml of culture was inoculated into fermentation media. Lactic acid and growth were periodically monitored up to 72 h. Each strain was grown in at least three separate shake flasks.

Analysis of genome fusion using molecular markers

DNA isolation

Various DNA extraction procedures were used for isolation of total genomic DNA of *Acetobacter, Lactobacillus,* F3 and FM1. CTAB DNA extraction method (Murray and Thompson, 1980) was used for *Acetobacter* and *Lactobacillus* while heat lysis of bacteria followed by DNA purification process was best suited for the F3 and FM1 DNA extraction. Two loopfull of cell pellet was dissolved in 1.0 ml of extraction buffer i.e. CTAB buffer (1 M Tris HCL,0.5M EDTA, 5.0M NaCl, 2.0 % [w/v] Cetyltrimethyl ammonium bromide (CTAB), 0.2 % (v/v) β-mercaptoethanol) and incubated at 60 °C in a water bath for 1hour. Equal volume of chloroform: Isoamyl alcohol (24:1) was added and mixed thoroughly and kept for at least 10 min at room temperature. It was centrifuged at 12,000 rpm for15 min at room temperature. The supernatant was collected and 0.6 volume of isopropanol was added and kept for incubation at 4 °C for 15 min. Then it was centrifuged at 12,000 rpm for 10 minutes at 4 °C. The pellet was washed with 70 % (v/v) ethanol and centrifuged at 10,000 rpm for

5 min at 4 °C. The pellet was air dried overnight and dissolved in sterile distilled water and RNase was added. The mixture was then incubated at 37 °C for 1 hr. The quality of DNA in the supernatant was determined by loading isolated DNA sample on agarose (0.8%) gel.

RAPD analysis

Genomic DNA of *Acetobacter, Lactobacillus, F3* and *FM1* was PCR amplified with RAPD primers (Operon technologies, USA) as detailed below. Each amplification reaction contained 20-30ng of genomic DNA, 200µM dNTPs, 1X PCR buffer, 1U Taq DNA Polymerase (Himedia) and 10µM RAPD primer in a 10µl reaction volume. The reactions were thermo-cycled using the MJ research machine PTC-200. PCR cycles were performed for 5 min at 94 ^{o}C followed by 40 cycles of denaturation at 94^{o}C, annealing at 50 ^{o}C to 54 ^{o}C (depending on the Tm of the primer) and extension at 72 ^{o}C for one min each, and final extension at 72 ^{o}C for 5 min. Amplification products were separated by electrophoresis in 2% agarose gels.

16S rDNA gene amplification

Genomic DNAs of *Acetobacter, Lactobacillus,* F3 and FM1 was PCR amplified with 16s rDNA primers designed to amplify 1521bp of *Lactobacillus delbrueckii* 16S rDNA gene. Each amplification reaction contained 40ng genomic DNA, 200 µM dNTPs, 1X PCR buffer, 1U Taq DNA Polymerase (Hi Media) and 10 µM 16s rDNA primers (both forward and reverse) in a 10µl reaction volume. The 16s universal primer sequence used was forward-5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse-5'-GGTTACCTTGTTACGACTT-3'. The reactions were thermo-cycled using the MJ

research machine. PCR cycles were performed for 5 min at 94 ^{o}C, followed by 35cycles of denaturation at 94 ^{o}C for 45 s, annealing at 52 ^{o}C for 45 s and extension at 72 ^{o}C for 1.5 min each, and final extension at 72 ^{o}C for 7 min. Amplification products were separated by electrophoresis in 2% agarose gels. Single band of amplification products thus confirmed were further used for restriction fragment length polymorphism (RFLP) using various restriction enzymes.

16S rDNA gene-RFLP

These amplification products were subjected to RFLP using various restriction enzymes chosen on the basis of virtual digestion. Accordingly the enzymes chosen for *in vitro* RFLP reaction were *Alu*I, *Fok*I, *Hha*I, *Mbo*I and *Taq*I. For RFLP reaction 1µg PCR product was digested with 1U of restriction enzyme (Promega, USA) in a cocktail containing 1X RE digestion buffer and deionized water. The reaction was incubated at 37 ^{o}C overnight for all the enzymes, except for *Taq*I which required incubation at 65 ^{o}C. The RFLP products were separated on 0.8% agarose gel run in TAE buffer system.

Analysis of RAPD and RFLP profiles

The RAPD and RFLP profiles of *A. pasteurianus*, *L. delbrueckii*, F3 and FM1 were compared and the presence or absence of band was scored as 1 or 0. The data thus generated was used to develop a dendrogram using online available software DendroUPGMA (http://genomes.urv.cat/UPGMA) (Garcia et al., 1999).

RESULTS

Protoplast formation and regeneration of *Acetobacter pasteurianus*:

Protoplasts of *Acetobacter pasteurianus* NCIM 2314 and *Acetobacter suboxydans* NCIM 2679 were obtained by adding freshly grown cells to protoplast buffer with osmotic stabilizer and lysozyme (0.5 mg/ml). Acetobacter medium with different osmotic stabilizers such as NaCl, KCl, Sucrose, and $MgCl_2$ of different pH and molarity were evaluated as potential osmotic stabilizers for protoplast formation. We found that the protoplast formation was favored at pH 8.0 (data not shown). Among all osmotic stabilizers tested, NaCl (0.5M) was found to be effective (Table 5.1). Fewer protoplasts were observed in MgCl2 at both 0.3 M and 0.5 M. The higher concentrations of lysozyme did not help in rapid protoplast formation. We also used the combination of lysozyme and mutanolysin for isolating protoplasts of *A. pasteurianus* which did not help in increased protoplast formation (Table 5.2). The profile of protoplast formation in protoplast buffer containing NaCl (0.5 M) and lysozyme (0.5 mg/ml) is given in Figure 5.2. The protoplast formation frequency for *Acetobacter pasteurianus* and *Acetobacter suboxydans* were around 70% and 65% respectively.

Table 5.1: Formation of *Acetobacter pasteurianus* NCIM 2314 protoplasts in different osmotic stabilizers

Osmotic stabilizers used	Protoplast formation frequency (%)
Sucrose (0.5 M)	29.72
NaCl (0.5 M)	70.66
KCl (0.5 M)	56.30
$MgCl_2$ (0.5 M)	13.75

The protoplasts were formed in PB using different osmotic stabilizers (0.5 M) containing lysozyme (0.5 mg/ml)) and the protoplast formation was checked after 2 h. The values are average of three independent experiments.

Table 5.2: Effect of mutanolysin and lysozyme concentration on the formation of protoplast of *Acetobacter pasteurianus* NCIM 2314 using 0.5 M NaCl buffer

Enzyme concentration	Protoplast formation frequency* (%)
Lysozyme (1 mg/ml)	61.11
Lysozyme (1 mg/ml)+ Mutanolysin (10 µg/ml)	60.55
Lysozyme (0.5 mg/ml)	62.19
Lysozyme (0.5 mg/ml)+ Mutanolysin (10 µg/ml)	60.51
Mutanolysin (10 µg/ml)	50.18

**The decrease in optical density (OD) at 660 nm or enumeration of the osmotically fragile cells (the difference between the number of osmotically resistant colonies before and after protoplast formation treatment) was used as indices of protoplast formation. The values are the average of three independent experiments.*

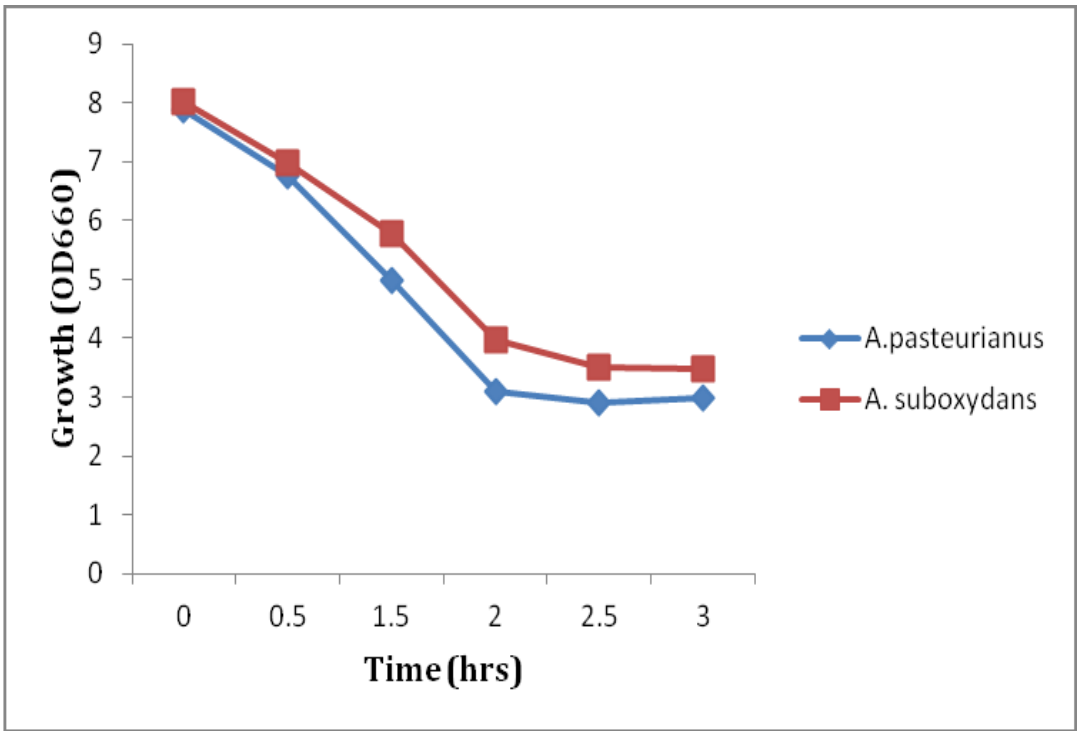


Figure 5.2: Profile of protoplast formation of *A. pasteurianus* and *A. suboxydans*

Acetobacter medium with different osmotic stabilizers such as NaCl, KCl, Sucrose and $MgCl_2$ at pH 4.5 were evaluated for protoplast regeneration. We found that NaCl at 0.5 M concentration was the best which gave maximum protoplast regeneration of *Acetobacter pasteurianus* NCIM 2314. It was also observed that NaCl derived protoplasts were regenerated better on NaCl containing ARM than the other osmotic stabilizers (Table 5.3). The regeneration frequency for *Acetobacter pasteurianus* was approximately 20%. We also got same results in case of *Acetobacter suboxydans* confirming the applicability of this protocol to other strains of *Acetobacter*

Table 5.3: Protoplast regeneration of *Acetobacter pasteurianus* NCIM 2314 on regeneration media containing different osmotic stabilizer

Protoplasts derived in	Protoplast regeneration (%) on Acetobacter regeneration medium with			
	NaCl (0.5M)	KCl (0.5M)	$MgCl_2$ (0.5M)	Sucrose (0.5M)
NaCl (0.5M)	20.01	14.33	8.91	10.21
KCl (0.5M)	12.31	13.21	9.88	9.00
$MgCl_2$ (0.5M)	8.90	8.33	9.21	7.01
Sucrose (0.5M)	7.60	7.33	6.00	8.10

The protoplasts were formed in PB using different stabilizers (0.5 M) and regenerated on ARM 0.5 M containing different osmotic stabilizers. The values are average of three independent experiments.

Protoplast formation and regeneration of *Lactobacillus* strains

We attempted to standardize the protocol for protoplast formation for *L. delbrueckii* mutant Uc-3 and the same procedure was adapted to different species of *Lactobacillus* genus. Lysozyme or mutanolysin individually was not suitable for protoplast formation but mixture of lysozyme (1 mg/ml) and mutanolysin (10 μg/ml) produced maximum protoplasts (Table 5.4). Higher concentrations of lysozyme and mutanolysin did not help in increasing the protoplast formation efficiency. The growth of *L. delbrueckii* Uc-3 in presence of glycine did not enhance the protoplast isolation significantly (data not shown). Table 5.5 showed the effect of different osmotic stabilizers on isolation of protoplasts from *L. delbrueckii* Uc-3 cells and it was found that sucrose was almost as effective as mannitol at 0.5 M concentration

Table 5.4. Effect of lysozyme and mutanolysin concentration and time of incubation on production of protoplasts of *Lactobacillus delbrueckii* Uc-3

Enzyme concentrations	Protoplast formation* (%)			
	0 h	1 h	2 h	3 h
Control	0	0	0	0
Lysozyme (10mg/ml)	0	48 ± 3	63 ± 3.5	62 ± 3.0
Lysozyme (10mg/ml) + Mutanolysin (10 ug/ml)	0	70 ± 4.0	81 ± 5.0	82 ± 4.5
Lysozyme (5mg/ml) + Mutanolysin (10 ug/ml)	0	74 ± 3.5	82 ± 4.0	83 ± 4.0
Lysozyme (1mg/ml) + Mutanolysin (10 ug/ml)	0	73 ± 3.0	82 ± 4.5	82 ± 5.0
Mutanolysin (10 ug/ml)	0	42 ± 2.5	63 ± 4.5	65 ± 4.0

**The decrease in Optical density (OD) at 660 nm or enumeration of the osmotically fragile cells (the difference between the number of osmotically resistant colonies before and after protoplast formation treatment) was used as indices of protoplast formation. The values are the average of three independent experiments.*

Table 5.5: Formation of protoplasts in different osmotic stabilizers

Osmotic stabilizers used	Protoplast formation (%)
Sucrose (0.5M)	83 ± 5.0
Sorbitol (0.5M)	78 ± 4.5
Mannitol (0.5M)	83 ± 3.0
Raffinose (0.3M)	33 ± 3.5
Raffinose (0.5M)	35 ± 4.0

The protoplasts were formed in PB with different stabilizers containing lysozyme (1mg/ml)) and mutanolysin (10 ug/ml) and the protoplast formation was checked after 2 h. The values are average of three independent experiments.

Table 5.6 summarizes the effect of MRS with different osmotic stabilizers such as sucrose, sorbitol and mannitol on regeneration of *L. delbrueckii* Uc-3 protoplasts. We found that sucrose at 0.5 M concentration was the best which gave maximum protoplast regeneration. It was also observed that sucrose derived protoplasts regenerated well on sucrose based LRM. We also used hydrolyzed sucrose based medium which was as effective as MRS-based LRM (data not shown). MRS-based LRM with sucrose as an osmotic stabilizer was found to be effective for all strains of *Lactobacillus* used for protoplast regeneration studies (Table 5.7).

Table 5.6: Protoplast regeneration of *Lactobacillus delbrueckii* mutant Uc-3 on regeneration media containing different osmotic stabilizers

Protoplasts derived in	Protoplast regeneration (%) on MRS regeneration medium with		
	Sucrose (0.5M)	Sorbitol (0.5M)	Mannitol (0.5M)
Sucrose (0.5M)	25.5 ± 2.5	25.0 ± 3.0	21.0 ± 3.5
Sorbitol (0.5M)	23.5 ± 3.0	11.0 ± 3.0	19.0 ± 4.0
Mannitol (0.5M)	20.0 ± 3.0	25.0 ± 4.0	6.0 ± 2.5

Table 5.7: Protoplast formation and regeneration of different *Lactobacillus* strains

Strains used	Protoplast regeneration (%) on MRS with 0.5M sucrose
L. delbrueckii mutant Uc-3	25.5 ± 3.5
L. lactis NCIM 2368	30.6 ± 3.0
L. brevis NCIM 2090	38.0 ± 4.5
L. casei NCIM 2364	36.5 ± 4.5
L. plantarum NCIM 2083	30.5 ± 3.0
L. fermentum NCIM 2165	33.0 ± 4.0
L. delbrueckii NCIM 2025	25.0 ± 2.5

Protoplast fusion followed by UV-mutagenesis

Protoplast fusion was carried out in presence of PEG 6000 which resulted in isolation of four fusants (F1, F2, F3 and F4). These fusants grew well initially on HSYE agar medium at pH 4.0 and 42 ^{o}C under anaerobic condition. The stability of these fusants for their growth at pH 4.0 was tested and it was found that only F3 continued to grow at pH 4.0 after several transfers indicating that it was a stable fusant. Hence the fusant F3 was evaluated further for the lactic acid production at acidic pH (4.0) at 42 ^{o}C. It produced 5.5 g/l of lactic acid within 24 h with trace amount of acetic acid. Fusant F3 was further mutated by exposing it to UV irradiation for 20 min which resulted in selection of mutant FM1 showing enhanced lactic acid production (11.0 g/l) with trace amounts of acetic acid. Hence the mutant FM1 was selected for further studies and compared with the parent and the fusant strains.

Constant monitoring of fermentation pH

The profile of pH and lactic acid production by parent, fusant F3 and mutant strain FM1 in fermentation medium at pH 4.0 is shown in Figure 5.3. The improved acid tolerant strain, FM1, was compared with the parent strain and the fusant F3 in a100 ml fermentation medium without $CaCO_3$. Initial pH of the fermentation medium was kept at 4.0. The pH of the medium and the lactic acid production were monitored at various time intervals. The mutant strain grew faster, produced higher amount of lactic acid, and brought the pH of the medium to 3.55. Within 36 h of fermentation, the mutant FM1 produced more lactic acid (10.99g/l) and brought the pH of the medium lower than the parent and the fusant strain (3.90 and 3.75, respectively).

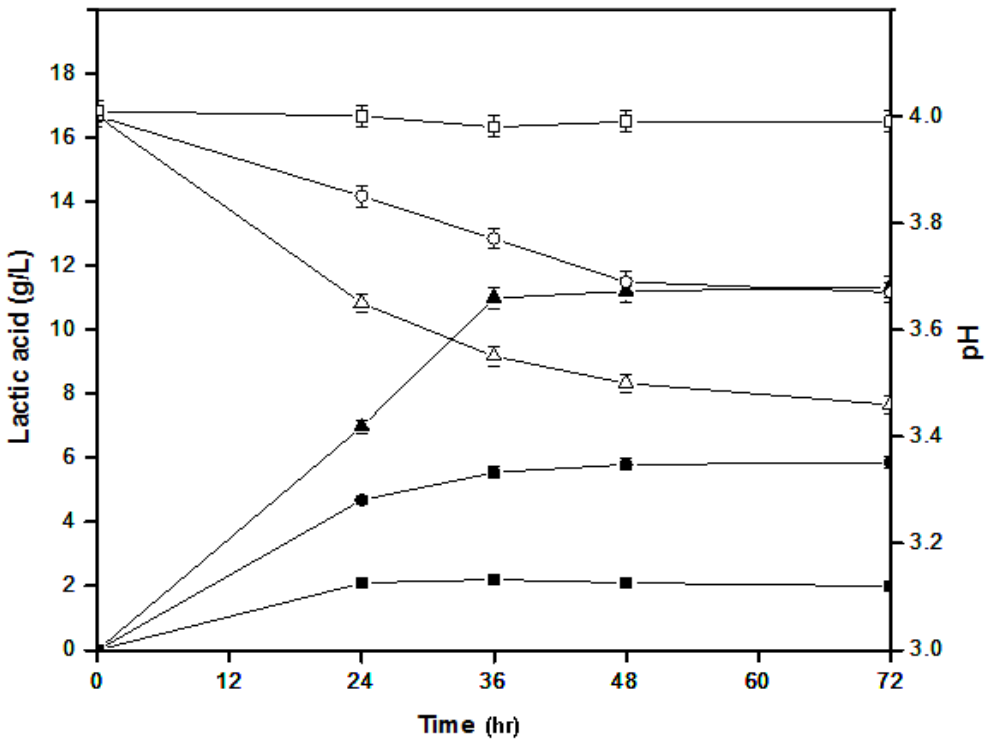


Figure 5.3: Characterization of the parent strain, *L. delbrueckii* Uc3, fusant F3 and mutant FM1 strains in 100 ml fermentation medium of pH 4.0.All the above mentioned strains were compared in HSYE medium at pH 4.0.Profile of continuously monitored pH and lactic acid were plotted.

—■— LA by Parent, —●— LA by fusant (F3), —▲— LA by Mutant (FM1), —□— pH of Parent , —○— pH of fusant (F3), —△— pH of mutant FM1.

Characterization of growth and lactic acid production using various concentrations of neutralizing agent ($CaCO_3$)

To illustrate performance of all the strains in liquid culture, small scale fermentations were carried out in HSYE medium using various concentrations of $CaCO_3$. The growth and lactic acid production studies of all the strains in HSYE medium using various concentrations (0.0%, 2.5%, 5.0%) of $CaCO_3$ has been carried out and the results are given in Table 5.8.

Table 5.8: Comparison of lactic acid and growth between parent *L. delbrueckii* Uc-3, fusant F3 and mutant FM1 using different concentrations of $CaCO_3$ in production medium containing hydrolyzed cane sugar (100g/l) and yeast extract (10g/l) after 48h of fermentation

$CaCO_3$ (g/l)	Lactic acid (g/l)			Growth (OD_{660})		
	Parent	Fusant (F3)	Mutant (FM1)	Parent	Fusant (F3)	Mutant (FM1)
50	81.90	78.99	83.11	9.80	9.88	11.00
25	38.11	56.11	72.21	4.00	6.90	7.90
0	1.02	5.55	11.20	0.90	2.77	5.52

All the values are the average of three independent experiments.

The Figures 5.4 and 5.5 showed the profiles of lactic acid production and growth of parent and mutant FM1 using different $CaCO_3$ concentrations. Growth and lactic acid were monitored throughout the fermentation. Although growth and lactic acid production by the parent, the fusant and the mutant strains were almost similar in the presence of 5% $CaCO_3$, these parameters were more diverse in the presence of half the amount of $CaCO_3$ (2.5%) and in the absence of $CaCO_3$. The Fusant F3 and the mutant FM1 grew to higher cell densities and produced more lactic acid than the parent strain, *L.delbrueckii* Mut Uc-3, in media containing less (2.5%) or no $CaCO_3$. The well performing fusant and the mutant strains produced 5.20 g/l and 11.20 g/l of lactic acid respectively compared to the parent which produced only 2.5 g/l of lactic acid in a medium without $CaCO_3$. The mutant FM1 produced more lactic acid (70 g/l) in comparison to the parent strain which produced only 38 g/l in medium with lesser amount (2.5%) of $CaCO_3$. Thus, the requirement of $CaCO_3$ is reduced to almost half using the acid tolerant FM1 strain.

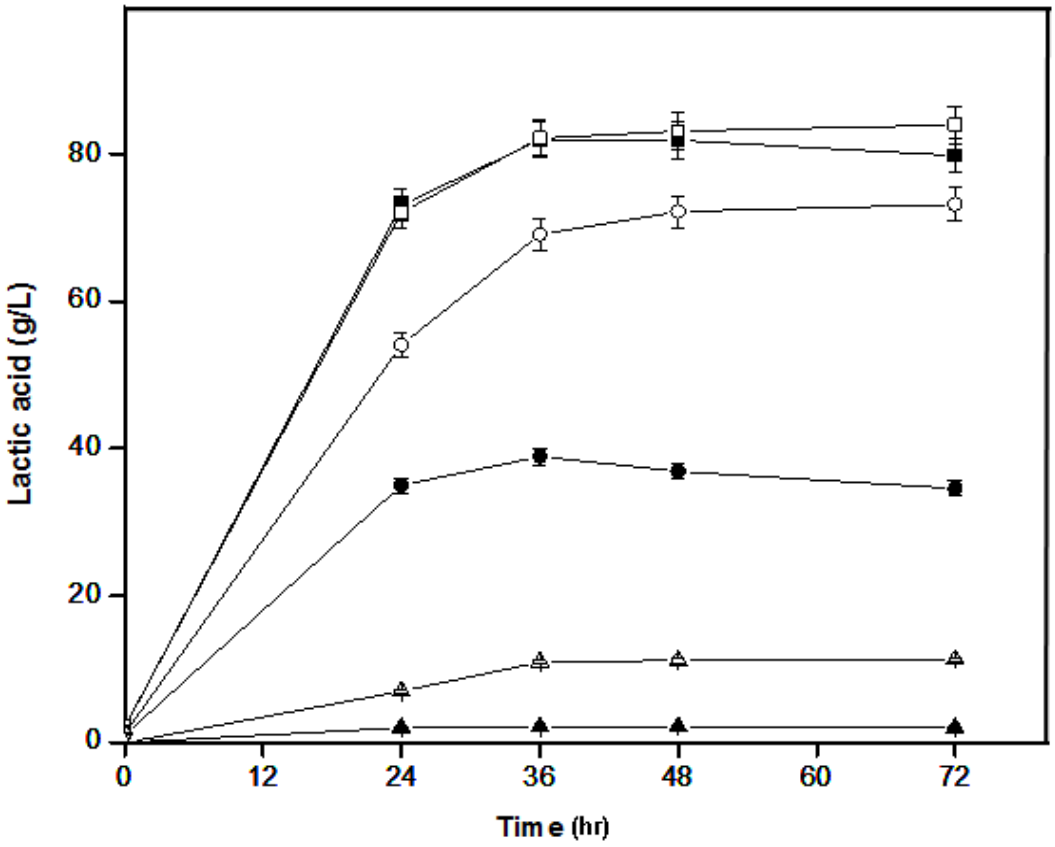


Figure 5.4: Fermentations in shake flasks at pH 4.0 using different concentrations of $CaCO_3$. The parent strain *L. delbrueckii* Uc-3 (closed symbols) and mutant FM1 (open symbols) were evaluated for lactic acid production 250 ml shake flask containing 100ml of fermentation medium (100g/l) in the presence of different concentrations of $CaCO_3$.Lactic acid production data were plotted as averages from three independent experiments.

—■— 5.0g $CaCO_3$, —●— 2.5g $CaCO_3$, —▲— 0.0g $CaCO_3$, —□— 5.0g $CaCO_3$, —○— 2.5g $CaCO_3$, —△— 0.0g $CaCO_3$.

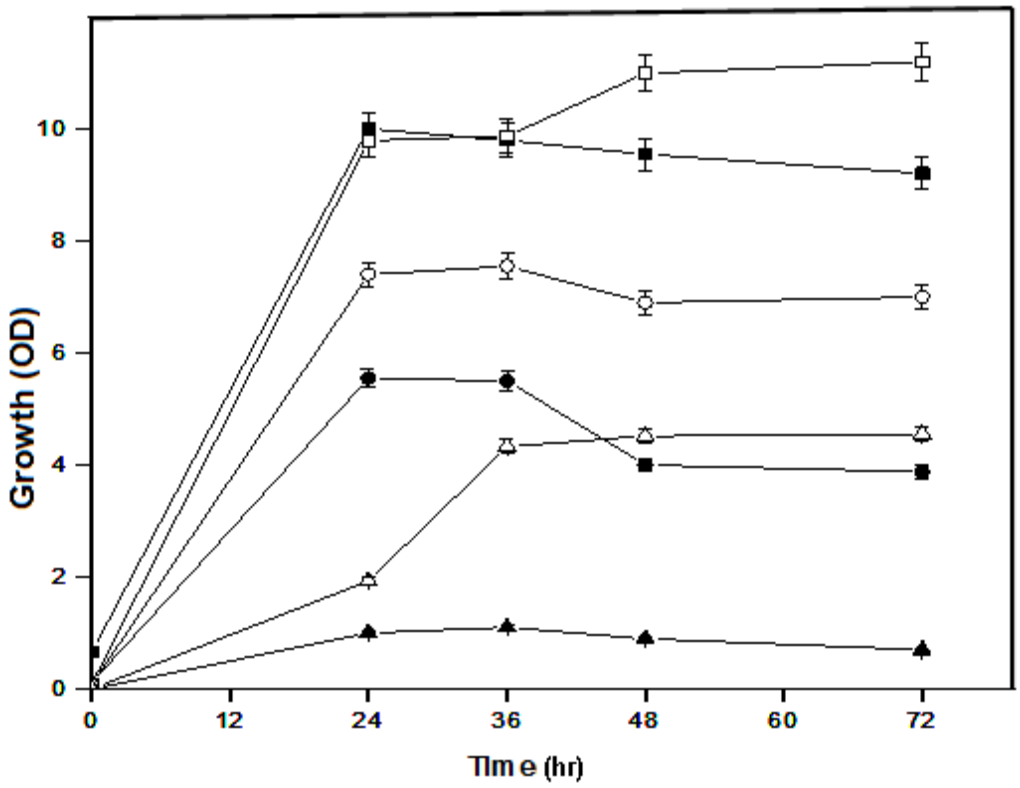


Figure 5.5: Fermentations in shake flasks at pH 4.0 using different concentrations of $CaCO_3$. The parent strain *L. delbrueckii* Uc-3 (closed symbols) and mutant FM1 (open symbols) were evaluated for growth in 250 ml shake flask containing 100ml of fermentation medium (100 g/l) in the presence of different concentrations of $CaCO_3$. OD660 data were plotted as averages from three independent experiments.

—■—5.0g $CaCO_3$, —●— 2.5g $CaCO_3$, —▲— 0.0g $CaCO_3$, —□— 5.0g $CaCO_3$, —○— 2.5g $CaCO_3$, —△— 0.0g $CaCO_3$.

Molecular characterization of individual strains

A total of 80 RAPD primers were screened for amplification in all the four strains namely, the parental strains *Acetobacter* and *Lactobacillus,* the fusantF3 and the mutant FM1. Most of the RAPD primers did not amplify reproducibly with all the four bacterial samples. Only 6 primers which showed reproducible amplification were further used for generating RAPD profiles and data scoring. Out of these six primers, OPAK4, OPAK9 and OPAK17 showed no amplification in either one of the four bacteria (Figure 5.6). Three primers, namely, OPAA2, OPAA3 and OPAA4 depicted amplicons in all the four samples with size polymorphism. It is interesting to note that in case of OPAA4, the band of size in the fusant F3 is identical to the *Lactobacillus* while the second band of size is identical to *Acetobacter* while the third band of size is novel whereas the mutant FM1 shows one band of size similar to the band of size of the fusant F3. In case of all the other primers at least one band of the fusant is similar in size to that of *Acetobacter* with one primer while another band of the fusant is similar in size to that of *Lactobacillus* with another primer. Moreover, some of the bands seen in the lane of mutant FM1 are similar in size to the either of the strains with the respective primers.

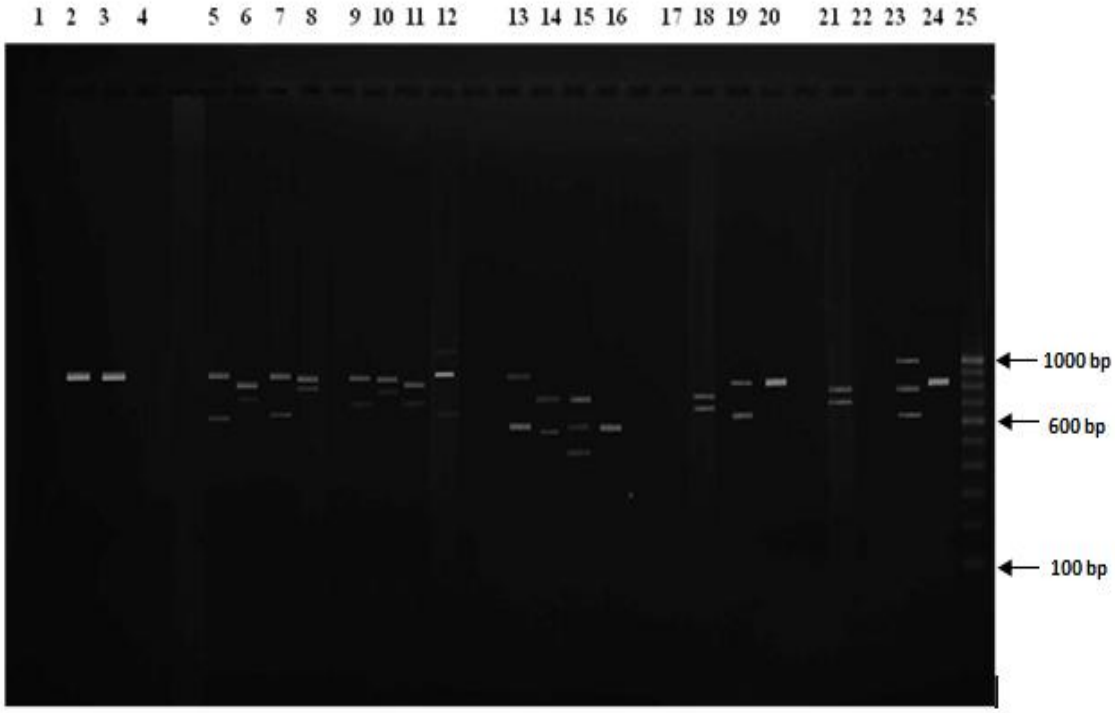


Figure 5.6: RAPD profiles of *Acetobacter, Lactobacillus,* F3 and FM1 generated using primers OPAK17(lane no.1-4),OPAA2 (lane no.5-8), OPAA3 (lane no.9-12), OPAA4(lane no.13-16), OPAK4 (lane no.17-20) , OPAK9 (lane no. 21-24) in the same order and lane no.25 represents 100bp marker.

Primers designed to amplify 1521bp of *Lactobacillus delbrueckii* 16S rDNA gene were used for amplification of all the four bacterial DNA samples which yielded a product of about 1.2 kb. These amplification products were subjected to RFLP using various restriction enzymes chosen on the basis of virtual digestion profiles generated for 1521 bp fragment of *Lactobacillus delbrueckii* 16S rDNA gene. Five enzymes namely, *Alu*I, *Fok*I, *Hha*I, *Mbo*I and *Taq*I yielded, reproducible and scorable but polymorphic profiles (Figure 5.7). In case of *AluI* digestion, FM1 showed bands of size similar to those of the *Acetobacter* and the *Lactobacillus* while in case of *Hha*I

digestion, the fusant F3 showed only two bands (700 & 800bp) similar to *Acetobacter* while the third band (300bp) was missing. In this case, the FM1showed pattern similar to that in the *Lactobacillus*. In case of *FokI*, the fusant F3 and the mutant FM1 showed similar profile except one band (450bp). Interestingly, in case of *MboI* digestion, the *Acetobacter* and the *Lactobacillus* showed variation in their banding pattern, the fusant F3 showed bands similar to *Acetobacter* with one novel band (300 bp) while the mutant FM1 showed one band (600bp) same as that of *Acetobacter* and one as that of *Lactobacillus*. *TaqI* digestion also showed similar pattern in case of the four strains.

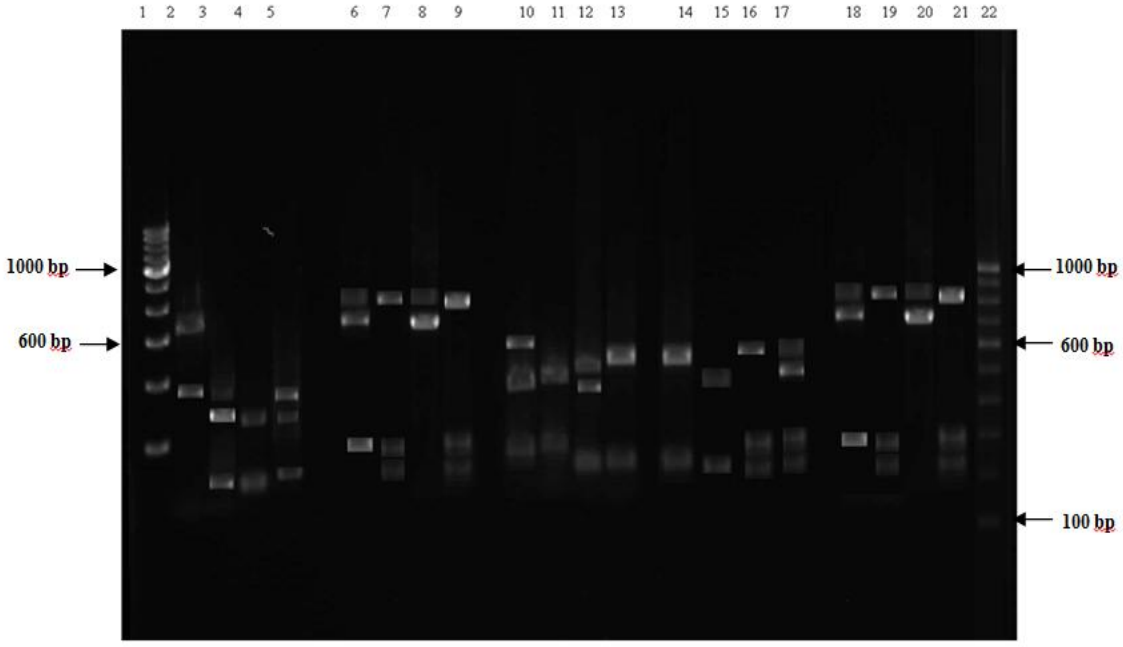


Figure 5.7: 16S rDNA gene-RFLP profiles of *Acetobacter*, *Lactobacillus*, *F3* and *FM1* generated using restriction enzymes AluI (lane no.2-5) , HhaI (lane no.6-9), FokI (lane no.10-13) , MboI (lane no.14-17) , TaqI (lane no.18-21) and line no.1 and 22 represents 200 and 100 bp marker.

The presence or absence of the DNA fragments in RAPD or RFLP profiles were scored as 1 or 0 in all the four strains. Dendrogram was generated using DendroUPGMA software which indicated that the *Acetobacter* and the *Lactobacillus* were closer to each other as compared to the F3 followed by the FM1 which was the most diverse (Figure 5.8).

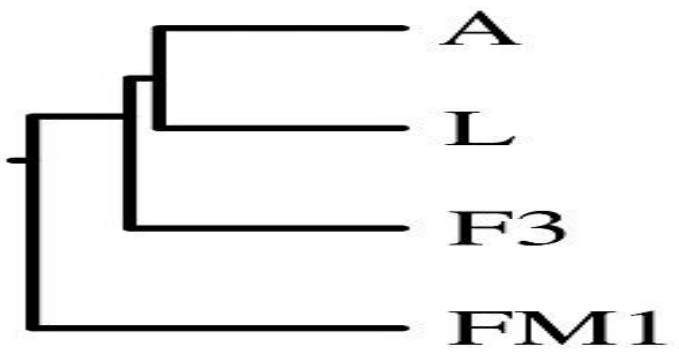


Figure 5.8: Dendrogram generated using RAPD and RFLP profiles of *Acetobacter*, *Lactobacillus*, F3 and FM1 .

DISCUSSION

The tolerance towards extreme process conditions such as pH, temperature during fermentation is the usual constraint for many industrially important microbial strains. The improvement in tolerance towards acidic pH in lactic acid producing strains could change the entire scenario of economics of lactic acid production. However genetic manipulation of strains involving the expression of large number of genes responsible for such complex traits is difficult. On the contrary, the protoplast fusion technique allows the exchange of entire genome between unrelated genera of microorganisms leading to transfer and expression of multiple phenotypes. Genome shuffling approach through protoplast fusion has been used for the isolation of recombinant strains of *Lactobacillus* (Patnaik et al., 2002) and *Rhizopous* (Miura et al., 2004) with acid tolerance and ammonia tolerance, respectively. In the present study, we employed inter-generic protoplast fusion approach followed by UV mutagenesis to address this common challenge and improved the acid tolerance in *Lactobacillus delbrueckii*. This approach provides an innovative methodology to alter the acid tolerance of *Lactobacillus* strain with commercial importance. The pH profile during fermentation clearly illustrates the improved acid tolerance of the fusant and the mutant strains over the parent (Figure 5.3). The mutant strain grows faster and produces more lactic acid at acidic pH (4.0) and brings down the pH more quickly to 3.5. However, the pH of the fermentation medium almost remained same in case of the parent strain.

For improvement in acid tolerance of *Lactobacillus delbruekii* we have used inter-generic protoplast fusion approach. For inter-generic protoplast fusion, we have selected *Acetobacter Pasteurianus* (growing profusely at acidic conditions and does not

produce lactic acid) and *Lactobacillus delbrueckii* Mut Uc3 (producing L-lactic acid and does not grow at acidic conditions). Protoplast isolation and regeneration studies in case of these two bacteria are very limited and hence we optimized conditions for protoplast isolation and regeneration for *A. pateurianus* and *L. delbrueckii*. Among all the osmotic stabilizers (NaCl, KCl, Sucrose, Mannitol, Sorbitol) tested, NaCl at 0.5M was found to be effective for protoplast isolation from *A. pasteurianus* cells. It was also found that maximum regeneration of *A. pasteurianus* was obtained on ARM containing 0.5M NaCl. Usually, sugars and sugar alcohols proved to be the best osmotic stabilizers for isolation and regeneration of bacterial protoplasts. We found that the inorganic osmotic stabilizers such as NaCl and KCl are suitable for isolation and regeneration of *Acetobacter pasteurianus.* This is, to our knowledge, first report to show that the inorganic compounds support both isolation and regeneration of bacterial protoplasts. In case of *Lactobacillus*, mixture of lysozyme (1mg/ml) and mutanolysin (10µg/ml) produced maximum number of protoplasts in buffer containing 0.5M sucrose as a stabilizer. Such requirement of mixture of lysozyme and mutanolysin was found to be necessary for formation of protoplasts from various *Lactobacillus* strains (Tanaka and Ohmomo, 2001; Lee-Wickner and Chassy, 1984). Sucrose as an osmotic stabilizer was observed to be effective for both *Lactobacillus* protoplast isolation and regeneration. Fewer protoplasts were observed in raffinose at both 0.3 M and 0.5 M. It is reported that raffinose at 0.3 M concentration favors the protoplast formation in *Lactobacillus* strains (Tanaka and Ohmomo, 2001). Complex media supplemented with bovine serum albumin, gelatin, polyvinylpyrrolidone and raffinose were used for regeneration of *Lactobacillus* protoplasts which led to more than 10–99% regeneration of protoplasts

(Tanaka and Ohmomo, 2001). We performed protoplast fusion and the fused protoplasts were streaked on the selection medium which allowed only the fusants to grow and still produce lactic acid albeit with low productivity at pH 4.0. The selected fusant (F3) was further subjected to UV mutagenesis which resulted in selection of FM1 mutant which grew well at pH 4.0 and produce improved levels of lactic acid compared to both parent and fusant.

To exemplify the parent, the fusant and the mutant strains, small scale fermentations were carried out in HSYE medium using various concentrations (5%,2.5% and 0.0%) of $CaCO_3$. The mutant FM1, grew very well and produced more lactic acid than the parent strain, *L. delbrueckii* Uc-3. This difference is very significant when less amount of $CaCO_3$ was used in the fermentation medium. Especially, the mutant FM1 grew faster than the parent in a medium without $CaCO_3$ suggesting its capability of growing and fermenting even at acidic pH. The growth profiles also demonstrated that the growth of the mutant FM1 did not decline even after 72 h of fermentation indicating its improved acid tolerance compared to the growth of the parental strain which declines after 24 h of fermentation. Similar results were obtained using 2.5% $CaCO_3$ where the FM1 produced higher amount of lactic acid (72 g/l) than the parent (38 g/l). Shake flask experiments further highlighted the improvements in the mutant strain as compared to the parent one.

To elucidate these results, it must be considered that when the medium is acidified below the pKa value of lactic acid (3.86), the lactic acid in the culture supernatant mainly remains un-dissociated and can diffuse back through the plasma membrane. Once inside the cells, the acid dissociates due to the presence of a more

neutral environment, causing acidification of the cytosol (Holyoak et al., 1996; Brul and Coote, 1999). The cells counteract this intracellular acidification by proton extrusion through the increased H^+-ATPase activity which resulted in a significant loss of available energy for growth and other essential metabolic functions. However, with time it will not be possible for the cell to maintain its pH within a physiological acceptable range leading to growth inhibition and finally cell death. On the basis of a pKa of 3.8 for lactic acid, the mutant strain appears to be more tolerant compared to the parent strain. The comparison of H^+-ATPase activity in these strains is necessary to show the role of enzyme in acid tolerance of the FM1 as well as the fusant F3.

We have further demonstrated the genetic changes that have taken place in the fusant F3 and the mutant FM1 using three molecular approaches, random marker profiling, 16S rDNA-RFLP. The resistance towards CTAB extraction procedure demonstrated by both the F3 and the FM1 as against the parental strains, *Acetobacter* and *Lactobacillus,* showed some significant variation/mutation caused in them because of genome fusion or UV treatment or both, which may have resulted in a change in their cell / cell wall structure or cellular contents that inhibited DNA extraction by CTAB method. The procedure was otherwise successful in the *Acetobacter* and *Lactobacillus* strains. This led us to use heat treatment for cell lysis and DNA isolation in the fusant F3 and the mutant FM1. Further studies using RAPD markers also indicated that at various random genetic loci, the fusant F3 and the mutant FM1 were different from their parent strains as well as from each other. Use of more random markers may further identify diverse loci in the fusant and the mutant as compared to the parental strains. However, considering all the primers together which showed amplification in all the

four or some of the four samples used for present analysis, it is clear that the fusant F3 shows a few bands with same size as those of the *Acetobacter* while some bands with same size as those of the *Lactobacillus* and the mutant FM1 shows few bands with same size as those of the fusant. Thus, it is clear that the fusant is derived from both the parental strains, *Acetobacter* and *Lactobacillus* and the mutant is derived from the fusant. The tolerance of low pH as well as lactic acid production, both these characteristics have been acquired by the fusant F3 due to PEG mediated fusion of *Acetobacter* and *Lactobacillus*. Further, UV treatment of the fusant F3 led to the making of the FM1 which had the quality of producing lactic acid at a five-fold higher rate than the parent strain. This modification can be attributed to DNA modifications which may have occurred in the genes of lactic acid production pathway or regulatory genes which govern this pathway due to UV treatment. Identification and sequence characterization of genes involved in the pathways might throw more light on this observation and will open further avenues of the characterization of the fusant and the mutant strains. However, the 16S rDNA-RFLP has clearly brought out the genetic variation created in the fusant and the mutant strains. The dendrogram generated from RAPD and RFLP analysis further indicates that the F3 is closer to the parental strains, *Acetobacter* and *Lactobacillus* than the FM1 which stands farther away from the parent strains, since it has undergone two DNA modification events (PEG treatment and UV treatment) unlike F3 which had undergone only PEG treatment. UV treatment has been proposed to induce photolysis of pyrimidines to form dimers. UV treatment can also cause error in DNA replication leading to accumulation of mutation in further generations. It is also known to cause an increase in gene copy number causing high

enzyme production and activity (Shafique and Bajwa, 2009). All these molecular approaches used in the present studies clearly indicate genomic variation in the fusant and the mutant compared to their parental strains and with each other. Further, there have been genetic variations created due to DNA modification event leading to acid tolerance. Earlier Yu et al. (2008) have reported the glucose tolerance in *Lactobacillus* strain using intraspecific genome shuffling approach. We used non-homologous recombination through inter-generic protoplast fusion to address the challenge of acid tolerance in *Lactobacillus* which may prove to be the appropriate approach to develop biocatalysts of industrial importance.

CONCLUSIONS

The genome shuffling approach using protoplast fusion has been employed in case of transfer of multiple genotypes through protoplast fusion since usual genetic engineering techniques fail to transfer such multiple genotypes. Genome shuffling approach has been used for generating acid tolerant and glucose tolerant *Lactobacillus* strains using intra-specific protoplast fusion. We employed inter-generic protoplast fusion to transfer acid tolerant phenotype from *Acetobacter* in *Lactobacillus* strain. This work provides a basis for the genetic improvements using protoplast fusion. The fusant F3 and mutant FM1, thus obtained, produced more lactic acid at acidic pH (without $CaCO_3$) than the parent strain. The mutant FM1 produced more lactic acid (72.21 g/l) in comparison to the parent strain which produced only 38.11 g/l in medium with lesser amount (25 g/l) of $CaCO_3$. Thus, the requirement of $CaCO_3$ is reduced to almost 60% using the acid tolerant FM1 strain. In conclusion, the mutant FM1 has a potential for lactic acid production in acidic environment which would reduce the requirement of

neutralizing agent and thus will ease the down-streaming process. As there will be no use of corrosive sulphuric acid and no generation of gypsum, the lactic acid fermentation process will be much greener if such acid tolerant strains are employed. The downstream processing of lactic acid plays key role in PLA production route. There is no information available on PLA production using natural strain. However, engineered strains have been used extensively for producing LA based polyesters. Overall, configuration of enantiomers of the LA unit in the LA-based polyester is mainly determined by the enantio-selectivity of LDH and LPE. In that case, *Lactobacillus* strain could be useful in establishing the one step process for synthesis of LA-based polyesters. The utilization of such acid tolerant strains could be able to produce free lactic acid without using any kind of neutralizing agents at acidic pH which can directly diverted to the synthesis of PLA thereby reducing the requirement of neutralizing agent to maintain the pH during fermentation.

REFERENCES

Achmad, F. K., Yamanishi Z., Liu S., Kokugan T. (2009) The effect of the impurities in refinery process from fermentation broth on lactic acid polymerization. *Journal of Chemical Engineering of Japan*, 42, 632–635.

Adsul M. G., Varma A. J., Gokhale D. V. (2007) Lactic acid production from waste sugar cane bagasse derived cellulose. *Green Chemistry*, 9, 58–62.

Akerberg C. and Zacchi. G. (2000) An economic evaluation of the fermentative production of lactic acid from wheat floor. *Bioresource Technology*, 75, 119-126.

Angelis M. and Gobbetti M. (2004) Environmental stress responses in *Lactobacillus*: a review. *Proteomics*, 4, 106–122.

Arnold C.N., McElhanon J., Lee A, Leonhart R., Siegele D. (2001) Global analysis of *Escherichia coli* gene expression during the acetate-induced acid tolerance response. *Journal of Bacteriology*, 183, 2178–2186.

Axelsson L. (1993) Lactic acid bacteria: classification and physiology,in *Lactic Acid Bacteria*, Ed by Salminen S and von Wright A, Marcel Dekker, New York. pp 1-61

Benninga H. A. (1990) A History of Lactic Acid Making (Kluyver Academic Publishers,Dordrecht, The Netherlands).

Brul S. and Coote P. (1999) Preservative agents in food: mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, 50,1 –17 .

Buchata K. (1983) Lactic acid. In H. Dellweg (ed.), Biotechnol. Verlag Chemie, Weinheim, Federal Republic of Germany.3, 409-417.

Connell H., Lemmon J., Tannock G. W. (1988) Formation and regeneration of protoplasts and sphaeroplasts of gastrointestinal strains of lactobacilli. *Applied and Environmental Microbiology*, 54, 1615–1618.

Dai M. H. and Copley S.D. (2004) Genome Shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. *Applied and Environmental Microbiology*, 70, 2391–2397.

Datta R. and Henry M. (2006) Lactic acid: recent advances in products, processes and technologies – a review. *Journal of Chemical Technology and Biotechnology*, 81, 1119–1129.

Datta R., Tsai S. P., Bonsignor P., Moon S., Frank J. (1995) Technological and economical potential of polylactic acid and lactic acid derivatives. *FEMS Microbiology Review*, 16, 221–231.

Davison B. E., Llanos R. L., Cancilla M. R., Redman N. C., Hillier A.J. (1995) Current research on the genetics of lactic acid production in lactic acid bacteria. *International Dairy Journal*, 5, 763–784.

Deng X. W., Matsui M., Wei N., Wagner D., Chu A. M., Feldmann, K. A., Quail, P. H. (1992) An arabidopsis regulatory gene encodes a protein with both a zinc-binding motif and a homologous domain. *Cell*, 71, 791-801.

Dumbrepatil A., Adsul M., Chaudhari S., Khire J., Gokhale D. (2008) Utilization of molasses sugar for lactic acid production by Lactobacillus delbrueckii mutant Uc-3 in batch fermentation. Applied and Environmental Microbiology, 74, 333-335.

Garcia-Vallve S., Palau J., Romeu A. (1999) Horizontal gene transfer in glycosyl hydrolases inferred from codon usage in *Escherichia coli* and *Bacillus subtilis. Molecular biology and Evolution,* 9, 1125-1134.

Gokhale D. V. and Deobagkar D. N. (1989) Different expression of xylanases and endoglucanases in the hybrid derived from intergeneric protoplast fusion between *Cellulomonas sp.*and *Bacillus subtilis. Applied Environmental Microbiology*, 55, 2675-2680 .

Gokhale D. V. and Deobagkar D. N. (1990) Secretion of thermostable β-glucosidase by an intergeneric bacterial hybrid between *Cellulomonas sp*. and *Bacillus subtilis. Applied Biochemistry and Biotechnology*, 26, 207-216 .

Gokhale D. V., H. Eun Soo-Han Srinivasan S. R., Deobagkar D. N. (1984) Transfer of DNA coding for cellulase from *Cellulomonas species* to *Bacillus subtilis* by protoplast fusion. *Biotechnology Letters*, 6, 627-632.

Guchte M., Serror P., Chervaux C., Smokvina T., Stanislav D. (2002) Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek*, 82, 187–216.

Hano T., Matsumoto M., Uenoyama S., Ohta T., Kawano Y., Miura S. (1993) Separation of lactic acid from fermented broth by solvent extraction. *Bioseparation*,3, 321-326.

Holyoak C.D., Stratford M., McMullin Z., Cole M., Crimmins K., Broen A., Coote P. (1996) Activity of the plasma membrane H+-ATPase and optimal glycolytic flux are required for rapid adaptation and growth in the presence of the weak acid preservative sorbic acid. *Applied and Environmental Microbiology*, 62, 3158–3164.

Hopwood A. (1981). Uses of protoplasts in the genetic manipulation of *streptomycetes. Zentrulblatt fur Bakteriologie* , 11, 523-531.

Kadam S. R. Patil S. S., Bastawde K.B., Khire J. M., Gokhale D. V. (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. *Process Biochemistry*, 41, 120-126.

Kanatani K., Miura H., Sakamoto M., Oshimura M. (1989) Protoplast formation and regeneration of *Lactobacillus plantarum*. *Agricultural Biology and Chemistry*, 53, 1185–1187.

Kim C. Y., Lee S. H., Park H. C., Bae, C. G., Cheong Y. H., Choi Y. J., Cho, M. J. (2000) Identification of rice blast fungal elicitor-responsive genes by differential display analysis. *Molecular plant-microbe interactions*, 13, 470-474.

Lee-Wickner L. J. and Chassy B. M. (1984) Production and regeneration of *Lactobacillus casei* protoplasts. *Applied and Environmental Microbiology* ,48, 994–1000 .

Madzingaido L., Danner H., Braum R. (2002) Process development and optimization of lactic acid purification using electrodialysis. *Journal of Biotechnology*, 96, 223–239.

Miura S., Dwiarti L., Arimura T., Hoshino M., Tiejun L., Okabe M (2004) Enhanced production of L-lactic acid by ammonia-tolerant mutant strain *Rhizopus sp. Journal of Bioscience and Bioengineering*, 97, 19–23.

Murray M. G. and Thompson W. F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, 8, 4321-4325.

Patnaik R., Louie S., Gavrilovic V., Perry K., Willem P. C., Stemmer M., del Cardayré S. (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nature Biotechnology*, 20, 707–712 .

Paul D. and Hill C. (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Review*, 67, 429–453.

Porro D. M., Bianchi L., Brambilla R., Menghini D., Bolzani V., Carrera J., Lievense C. L., Liu B. M., Ranzi L., Frontali L., Alberghina L. (1999) Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. *Applied and Environmental Microbiology*, 65, 4211–4215.

Prescott S. C. and Dunn C. G. (1959) Lactic acid and other organic acids. *Indian Microbiology 3rd edn*. Mc-Graw-Hill,New York.271-292.

Shafique S., and Bajwa R. (2009) Mutation of *Alternaria tenuissima* FCBP-252 of hyper-active α amylase. *Indian Journal of experimental botany*, 47, 591-596.

Sodergard A. and Stolt M. (2002) Properties of lactic acid based polymers and their correlation with composition. *Progress of Polymer Science*, 27, 1123–1163 .

Tanaka O. and Ohmomo S. (2001) Efficient protoplast regeneration for some homofermentative *Lactobacilli* and P*ediococci. Archives Microbiology*, 177, 36–40.

Torriani S., Vescovo M., Scolari G. (1994) An overview on *Lactobacillus helveticus*. *Annuals of Microbiology and Enzymology*, 44, 163-191.

Tsuji F. (2002) Autocatalytic hydrolysis of amorphous-made polylactides:effects of l-lactide content, tacticity, and enantiomeric polymer blending. *Polymer*, 43,1789–1796.

Vardi A., Arzee-Gonen P., Frydman-Shani A., Bleichman S., Galun, E. (1989) Protoplast-fusion-mediated transfer of organelles from Microcitrus into Citrus and regeneration of novel alloplasmic trees. *Theoretical and applied genetics*, 78, 741-747.

Wang Y. H., Li Y., Pei X. L., Yu L., Feng Y. (2007) Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity in *Lactobacillus rhamnosus. Journal of Biotechnology*, 129, 510–515.

Yeeh Y., Jo Y. B., Kwon O. C. (1996) Protoplast fusion between *Lactobacillus casei* and *Lactobacillus acidophilus. Biotechnology Letters*, 18, 805–808.

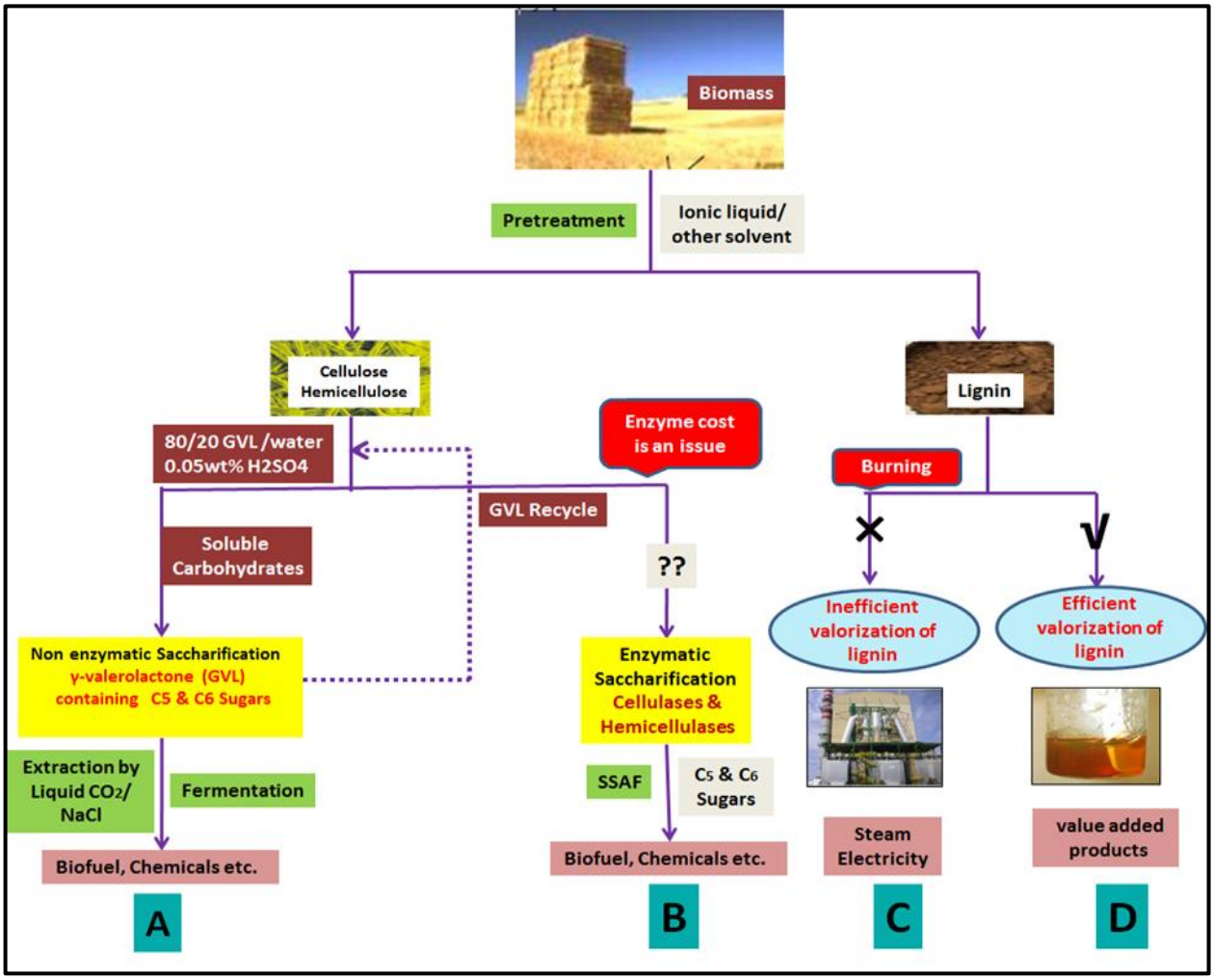
Young-Jung W., Jin-Nam K., Hwa-Won R. (2006) Biotechnological Production of Lactic Acid and Its Recent Applications. *Food Technology and Biotechnology*, 44 (2), 163–172.

Yu L., Pei X., Lei T., Wang Y., Feng Y. (2008) Genome shuffling enhanced L-lactic acid production by improving glucose tolerance of *Lactobacillus rhamnosus*. *Journal of Biotechnology*, 134, 154-159.

Zhang Y., Perry K., Powell K., Stemmer P. C., del Cardayré S. (2002) Evolution of *Streptomyces fradiae* by whole genome shuffling. *Nature*, 415, 644–646.

CHAPTER 6

Conclusions and Future perspectives



Singhvi M. and Gokhale D.V. (2015). ***Current Science*** **Communicated**

CONCLUSIONS

1. The improved strain of *Lactobacillus lactis* has been isolated which produced pure D-lactic acid with high productivity using hydrolyzed sucrose, molasses.
2. This strain also utilized cellobiose very efficiently which prompted us to exploit it for the production of lactic acid from cellulosic substrates. The strain, *L. lactis* RM2-24, produced D-lactic acid very efficiently using cellobiose and sugarcane bagasse derived cellulose using *P. janthinellum*, EU2D-21 cellulases. Hence the strain proved to be a promising strain for the production of D-lactic acid from cellulosic substrates.
3. The optimization studies on cellulase production by *P. janthinellum* mutant, EU2D-21 revealed that it produced two types of β-glucosidases. The SmF derived enzyme contained both thermo-stable and thermo-labile β-glucosidases whereas SSF derived enzyme contained predominantly thermo-labile species.
4. We employed inter-generic protoplast fusion approach to improve the acid tolerance of *L. delbrueckii* Uc-3 mutant. The improved acid tolerant strain produced L-lactic acid at acidic environment reducing the requirement of neutralizing agent which may ease the down-streaming process of lactic acid.
5. These studies show the potential of the strains developed in our laboratory that are capable of converting biomass to lactic acid which can be polymerized to biodegradable and biocompatible PLA polymers. PLA could be the better substitute for many petroleum based polymers. Owing to the environmental concerns and the limited availability of petroleum feedstock, a completely green process would be preferred method for the production of lactic acid and their derivatives. At present, the biomass is burnt as an inefficient energy source leading to the release of poisonous gases causing environmental pollution. This study reveals the possibility of converting biomass to value products via greener technologies which would certainly reduce the environmental pollution problems.

FUTURE PERSPECTIVE

The abundantly available lignocellulosic biomass consists of almost 70% carbohydrates made up of fermentable sugars which can be converted to second generation biofuels and chemicals. The complete utilization of carbohydrates derived from inexpensive biomass is essential for sustainably producing liquid biofuels and chemicals. These carbohydrates can be de-polymerized and converted to their respective monomers, glucose, xylose and arabinose. Isolation of individual components of biomass in pure form is rather tricky requiring appropriate pretreatments using solvents such as ionic liquids (IL) which disrupt the lignin and hemicellulose network. These individual components will be sustainably available in abundance as renewable feedstock biopolymers and bio-composites with properties matching to petroleum-based compounds. In an oil refinery, a crude product is converted into individual compounds of high purity. Similarly, in the bio-refinery concept, the biomass is converted to a variety of products such as fuels and chemicals including high cost specialty chemicals. The lignocellulosic biomass needs to be subjected to different pretreatments to separate individual components of the biomass. Pretreatment processes are supposed to be the entry point for any bio-refinery scheme.

All biomass pretreatments reported till today, produce insoluble solids that need to be hydrolyzed using costly enzyme cocktails which is another threat to the economics of biomass refinery. Hydrolysis of treated biomass results in less concentrated sugar syrups along with the glucose and xylose degradation products causing problems in sugar fermentation. In recently published article in Science, Luterbatcher et al. (2014) reported laboratory scale production of soluble carbohydrates from biomass such as

corn stover, hardwood and softwood using a solvent mixture of biomass derived γ-valerolactone (GVL), water, and dilute H_2SO_4. GVL is known to solubilize biomass components including lignin. The soluble carbohydrates in the solvent mixture can be recovered as a concentrated solution (127 g/l) by addition of NaCl or liquid CO_2. The liquid CO_2 recovered monomer fraction was converted to ethanol by a robust *Saccharomyces cerevisiae* strain with 87% yield. Considering the preliminary economics based on various assumptions, the proposed approach appears to be economically competitive to present biomass derived processes involving pretreatment followed by simultaneous saccharification and fermentation (SSAF). One of the major advantages in this approach is the synthesis of GVL from levulinic acid and recycling of this solvent back to the system. These results could be a turning point in the biomass conversion technologies which do not require costly enzymes to hydrolyze the biopolymers in the pretreated/untreated biomass to their respective monomers. In addition, this approach can be used for any type of biomass including hardwood and softwood unlike other pretreatments in which the type of biomass decides the suitability of the pretreatment. The only disadvantage is that the major solid obtained in all streams in the process is lignin which is utilized for burning. Hence we feel that the fate of very important biomass component, lignin, still remains unclear.

Lignin conversion to value added products is still in its infancy due to its complex and irregular structure and even now lignin is incinerated as an inefficient energy source. It is also the most abundant carbon source available in nature and hence its valorization is necessary to improve the economics of biomass conversion. Many of those working on bio-refinery pay more attention to carbohydrate conversion. However,

lignin is also a major fraction potentially valuable if recovered in useable form. Commercial success of bio-refinery depends on the conversion of all lignocellulosic components into high value products. Lignin can serve as a potential source of aromatic platform chemicals although such conversion is still challenging. There are many opportunities for companies and research institutions to develop processes that convert lignin into higher-value chemicals. These chemicals would hit markets by 2021 (http://biomassmagazine.com/articles/10662/lux-research-lignin-derived-chemicals-to-hit-market-in-2021). Deconstruction of biomass with specific ionic liquids (IL) is promising because lignin extracted in IL can be precipitated and recovered by the addition of anti-solvent, usually water. This recovered lignin can be used in the manufacture of wood adhesives, cement additives or lignin derived products such as vanillin and cinnamic acid that are commercially used as sweeteners and precursors of pharmaceuticals. In addition, lignin can be de-polymerized and the released substituted monomers can act as precursors of wide range of products including fuels such as cyclohexane. Very recently, Sathitsuksanoh et al (2014) used non-toxic and recyclable ionic liquid, [C2mim][OAc], for pretreatment of wheat straw, Miscanthus, and Loblolly pine followed by enzymatic hydrolysis of the treated material. They found that ionic liquid pretreatment process conditions play a major role in obtaining lignin structures suitable for its valorization thereby improving the economics of bio-refinery. Though the ionic liquids are costly, they can be recycled to make the IL-pretreatment processes economically feasible. We suggest that the IL-pretreated biomass (rich in cellulose with low crystallinity and hemicellulose) can be treated with the mixture of GVL and water to depolymerize to their respective sugars without the use of costly enzymes. This may

help in utilization of all the three biomass components for production of biofuels and value added chemicals. The developments in the use of solid acids in biomass hydrolysis may also establish the efficient and economically viable process for biomass hydrolysis in near future.

Brunecky et al. (2013) also reported an unusually large multidomain cellulase that may give a complete solution to biomass conversion technology. I think the CelA described by Brunecky et al. could be the ideal biocatalyst since it acts preferably on raw/native biomass releasing cellobiose and glucose. In addition, it is active at even higher temperatures (80 ^{o}C) which will avoid the bacterial contamination problems during hydrolysis of raw biomass. This enzyme has great potential in biomass to fuel technologies compared to most commercial biomass degrading enzymes that are enzyme cocktails with extremely less specific activity on raw biomass. The biomass consists of cellulose, hemicellulose and lignin bound together in a complex structure. The utilization of all biomass components is essential to develop biomass conversion technologies with economics. Hence there is a scope for development of new classes of enzymes such as hemicellulases preferentially degrading hemicellulose in raw or celA treated biomass. Specific xylanases capable of producing xylo-oligosaccharides (XOS) from xylan in raw biomass could add new dimensions to the economics of hemicellulose (biomass) conversion to value added products. Thus the search for the existence of such unknown enzymes may provide new avenues for efficient utilization of lignocellulosic biomass.

In our previous article (Singhvi et al., 2014), we stated that suitable biomass pretreatment and low cost production of biomass degrading enzymes still remain as

challenges in biomass processing. However, recent publications on GVL-pretreatment and CelA enzyme acting on raw biomass would probably meet these challenges to make the biomass conversion technologies ecofriendly as well as cost effective. The work performed in this thesis may pave the way to emergence of possible technology for producing D-lactic acid from α-cellulose derived from sugar cane bagasse. We are pursuing the conversion of C5 sugars derived from hemicellulose component of the biomass to lactic acid. Intact lignin has been recovered during the patented process of α-cellulose production which can be further processed for value added products. Thus all the components of sugar cane bagasse can be fully converted to value added products which may lead to development of technology for L- and D-lactic acid from sugar cane bagasse. Currently the carbon from CO_2 feedstock is fixed and converted in to simple plant sugars, known as "First generation materials". These plant sugars are starch and cane sugar which are being used for producing commodity chemicals. The second generation materials are cellulosic sources such as sugarcane bagasse and nonfood plant materials which are being assessed for technology development to produce bulk chemicals such as biofuels and lactic acid. Recent news report that Calysta Energy and Nature-Works have entered in to collaboration to develop a practical, world-scale process for fermenting methane, a potent green-house gas (Biogas) in to lactic acid. Methane is generated through natural decomposition of plant materials and also from society's organic wastes through anaerobic digestion. The feasibility of the process for producing lactic acid from methane will take approximately five years (http://biomassmagazine.com/articles/9113/partnership-to-investigate-biogas-to-lactic-acid-conversion).

Brunecky R., et al., (2013). *Science* **342,** 1513-1516.

Luterbacher, J. S. et al., (2014). *Science*, **343**, 277 – 280.

Sathitsuksanoh, N., et al., (2014). *Green Chem*. DOI: 10.1039/c3gc42295j.

Singhvi et al., (2014). *RSC Advances,* **4,** 8271-8277.

Biotechnol Lett (2010) 32:517–520
DOI 10.1007/s10529-009-0187-y

ORIGINAL RESEARCH PAPER

Strain improvement of *Lactobacillus lactis* for D-lactic acid production

D. S. Joshi · M. S. Singhvi · J. M. Khire · D. V. Gokhale

Received: 1 October 2009 / Revised: 26 November 2009 / Accepted: 30 November 2009 / Published online: 24 December 2009

Abstract Three mutants, isolated by repeated UV mutagenesis of *Lactobacillus lactis* NCIM 2368, produced increased D-lactic acid concentrations. These mutants were compared with the wild type using 100 g hydrolyzed cane sugar/l in the fermentation medium. One mutant, RM2-24, produced 81 g lactic acid/l which was over three times that of the wild type. The highest D-lactic acid (110 g/l) in batch fermentation was obtained with 150 g cane sugar/l with a 73% lactic acid yield. The mutant utilizes cellobiose efficiently, converting it into D-lactic acid suggesting the presence of cellobiase. Thus, this strain could be used to obtain D-lactic acid from cellulosic materials that are pre-hydrolyzed with cellulase.

Keywords Cellobiose utilization · *Lactobacillus lactis* · D-Lactic acid production · Mutant · Sucrose (cane sugar)

Introduction

Lactic acid and its derivatives are widely used in the food, pharmaceutical and cosmetic industries (VickRoy 1985). It is also a major raw material for the production of polylactic acid (PLA) that is a biodegradable, environmentfriendly polymer which could be a substitute for synthetic plastics derived from petroleum feedstocks. Fermentative production of lactic acid offers the great advantage of producing optically pure L-or D-lactic acid depending upon the strains selected for fermentation. The optical purity of lactic acid is crucial for the physical properties of PLA. Though L-lactic acid can be polymerized to give crystalline (PLLA) suited to commercial uses (Sodegard and Stolt 2002), its application is limited by its low melting point. Complexing PLLA with poly D-lactic acid (PDLA), however, increases the melting point thus presenting an attractive solution to the heat sensitivity of PLA (Tsuji and Fukui 2003). This finding has increased the importance of the synthesis of D-lactic acid. Presently, L-lactic acid is widely used in the food and pharmaceutical industries and hence its production by fermentation is well established (Yu and Hang 1989). However, fermentation of sugars to D-lactic acid is little studied and its microbial productivity is not well known (Zhou et al. 2003). Therefore, we decided to investigate D-lactic acid fermentation with a view to obtaining improved strains capable of producing D-lactic acid with enhanced productivities.

India is one of the largest producers of cane sugar (more than 20 million tonnes). During this process, large amounts of molasses are generated as a byproduct which contains 40–60% (w/v) sucrose.

D. S. Joshi · M. S. Singhvi · J. M. Khire · D. V. Gokhale (✉)
NCIM Resource Center, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411 008, Maharashtra, India
e-mail: dv.gokhale@ncl.res.in

D-(−)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-2†4

Mamta Singhvi,[a] Dipti Joshi,[a] Mukund Adsul,[a] Anjani Varma[b] and Digambar Gokhale*[a]

Received 9th December 2009, Accepted 1st April 2010
First published as an Advance Article on the web 14th May 2010
DOI: 10.1039/b925975a

Lactobacillus lactis mutant RM2-24 utilizes cellobiose efficiently, converting it into D-(−)-lactic acid. Cellobiose-degrading enzyme activities were determined for whole cells, cell extracts and disrupted cells. Aryl-β-glucosidase activity was detected in whole cells and disrupted cells, suggesting that these activities are confined to the cells. The mutant produced 80 g l^{-1} of lactic acid from 100 g l^{-1} of cellobiose with 1.66 g l^{-1} h^{-1} productivity. Production of D-lactic acid from different cellulose samples was also studied. The cellulose samples at high concentration (10%) were hydrolyzed by cellulase enzyme preparation (10 FPU g^{-1} cellulose) derived from *Penicillium janthinellum* mutant EU1 generated in our own laboratory. We obtained a maximum 72% hydrolysis, yielding glucose and cellobiose as the main end products. Lactic acid was produced from these cellulose samples by simultaneous saccharification and fermentation (SSF) in a media containing a cellulase enzyme preparation derived from *Penicillium janthinellum* mutant EU1 and cellobiose utilizing *Lactobacillus lactis* mutant RM2-24. A maximum lactic acid concentration of 73 g l^{-1} was produced from a concentration of 100 g l^{-1} of bagasse-derived cellulose, the highest productivity and yield being 1.52 g l^{-1} h^{-1} and 0.73 g g^{-1}, respectively. Considering that bagasse is a waste material available in abundance, we propose to use this biomass to produce cellulose and then sugars, which can be fermented to valuable products such as ethanol and lactic acid.

Introduction

Lactic acid and its derivatives have been widely used in food, pharmaceutical, cosmetic and industrial applications.[1] It has a potential to become a commodity chemical for production of biodegradable polymers, oxygenated chemicals, plant growth regulators and special chemical intermediates. It has been receiving great attention as a feedstock for manufacture of polylactic acid (PLA), a biodegradable polymer used as environmentally friendly biodegradable plastic. Lactic acid is manufactured either chemically or by microbial fermentation. Chemical synthesis always results in racemic mixture of lactic acid, which is a major disadvantage. Microbial fermentation offers the advantage in both utilization of renewable carbohydrates and production of pure L- or D-lactic acid depending on the strain selected. The physical properties of PLA depend on the isomeric composition of lactic acid. Poly(L-lactide) (PLLA) obtained by polymerization of L-lactic acid or L-lactide has a melting temperature of 175 °C. The melting point of this polymer can be increased by blending with poly (D-lactide) (PDLA) in a solvent. Recently, it was found that the polymer blend of PLLA and PDLA produces a stereo-complex with melting temperature around 230 °C.[2] This finding has attracted more attention to the production of D-lactic acid.

Cellulosic substances are abundantly available resources of renewable biopolymer which can be utilized as a feedstock for producing a number of bulk chemicals like ethanol or lactic acid through fermentation processes. The production of such value-added products from renewable feedstocks is a present need, and there is a demand to make it an economically and environmentally feasible process. Such bioprocesses involving cellulosic residues not only provide alternative substrates but also help solve their disposal problems. With the advent of biotechnological innovations, mainly in the area of enzyme technology and fermentation technology, many new avenues have opened up for their exploitation as value-added products. Currently, optically pure lactic acid is produced mainly from corn starch. However, the use of agro-waste materials for lactic acid production appears to be more attractive because they do not then have any impact on the human food chain.

Unfortunately, the process for converting cellulosic material into lactic acid is not yet feasible due to the high cost of cellulase enzymes involved in cellulose hydrolysis[3,4] and also to the use of fastidious organisms.[5] The utilization of cellulosic wastes such as cardboard and corn cobs as substrates for lactic acid fermentation, simultaneous saccharification and fermentation (SSF) has been considered to be a promising approach.[6] However, there have been many technical problems – for instance, the enzymes of cellulose hydrolysis are inhibited by the intermediate products such as cellobiose, and lactic acid biosynthesis is inhibited by lactic acid.[7] Many investigations have been carried out to relieve such inhibitions, for example, *in situ* product removal technology

[a]*NCIM Resource Center, National Chemical laboratory, Dr Homi Bhabha Road, Pune, 411008, India. E-mail: dv.gokhale@ncl.res.in; Fax: 91-20-25902671; Tel: 91-20-25902670*
[b]*Polymer Science and Engineering, National Chemical laboratory, Pune, 411008, India*

† Electronic supplementary information (ESI) available: Additional experimental information. See DOI: 10.1039/b925975a

Indian J Microbiol (March 2010) 50(1):97–100
DOI: 10.1007/s12088-010-0008-y

ORIGINAL ARTICLE

Protoplast formation and regeneration in *Lactobacillus delbrueckii*

Mamta Singhvi · Dipti Joshi · Shalaka Gaikaiwari · Digambar V. Gokhale

Received: 8 December 2008 / Accepted: 31 December 2008

Abstract Method for production and regeneration of *Lactobacillus delbrueckii* protoplasts are described. The protoplasts were obtained by treatment with a mixture of lysozyme and mutanolysin in protoplast buffer at pH 6.5 with different osmotic stabilizers. The protoplasts were regenerated on deMan, Rogosa and Sharpe (MRS) with various osmotic stabilizers. Maximum protoplast formation was obtained in protoplast buffer with sucrose as an osmotic stabilizer using a combination of lysozyme (1 mg/ml) and mutanolysin (10 µg/ml). Maximum protoplast regeneration was obtained on MRS medium with sucrose (0.5 M) as an osmotic stabilizer. The regeneration medium was also applicable to other species of lactobacilli as well. This is, to our knowledge, the first report on protoplast formation and efficient regeneration in case of *L. delbrueckii.*

Keywords *Lactobacillus delbrueckii* · Protoplast formation · Protoplast regeneration

M. Singhvi · D. Joshi · S. Gaikaiwari · D. V. Gokhale (✉)
NCIM Resource Center,
National Chemical Laboratory,
Pune – 411 008, Maharashtra, India

E-mail: dv.gokhale@ncl.res.in

Introduction

Protoplast fusion is a useful technique in the improvement of microorganisms. This technique allows the exchange of entire genomes between unrelated genera of microorganisms. It is considered as classical or funda mental method of gene transfer in which multiple genes can be introduced in contrast to the advanced methods in molecular biology which are suitable for introducing only one or few genes. We have been successful in transferring cellulase genes (multiple phenotype) using protoplast fusion approach leading to changed properties of enzymes such as cellulases [1, 2] and amylases [3]. Recent reports on genome shuffling in *Lactobacillus* proved the success of protoplast fusion approach which generated new population of strains with additional improvements in acid [4, 5] and glucose tolerance [6]. This genome shuffling approach was also used to obtain *Lactobacillus delbrueckii* strain capable of converting starch to lactic acid [7].

Protoplast fusion in lactobacilli [8, 9] has been reported. However, this technique has been applicable to certain strains of lactobacilli mainly due to the difficulty in both protoplast isolation and regeneration. Though protoplast regeneration on agar media with 0.3 M raffinose has been successful, frequencies of regeneration often remained low [10]. There are reports on regeneration of protoplasts of *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus rhamnosus* [11] and Lactobacillus casei [12]. There are no reports on systematic studies on protoplast isolation and regeneration in case of *L. delbrueckii.*

We have isolated several mutants of *L. delbrueckii* using acclimatization and ultraviolet irradiation that produced increased lactic acid concentrations [13]. One of these mutants, Uc-3 was used to produce lactic acid from

Bioresource Technology 102 (2011) 6569–6572

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Comparative production of cellulases by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose

M.S. Singhvi, M.G. Adsul, D.V. Gokhale *

NCIM Resource Center, National Chemical Laboratory (CSIR), Pune 411008, Maharashtra, India

ARTICLE INFO

Article history:
Received 20 September 2010
Received in revised form 24 December 2010
Accepted 7 January 2011
Available online 14 January 2011

Keywords:
Mutant EU2D-21
Submerged fermentation
Solid state fermentation
β-Glucosidase thermostability

ABSTRACT

Mutants of *Penicillium janthinellum* NCIM 1171 were evaluated for cellulase production using both submerged fermentation (SmF) and solid state fermentation (SSF). Mutant EU2D-21 gave highest yields of cellulases in both SmF and SSF. Hydrolysis of Avicel and cellulose were compared using SmF and SSF derived enzyme preparations obtained from EU2D-21. Surprisingly, the use of SSF derived preparation gave less hydrolysis compared to SmF derived enzymes. This may be due to inactivation of β-glucosidase at 50 °C in SSF derived enzyme preparations. SmF derived enzyme preparations contained both thermostable and thermosensitive β-glucosidases where as SSF derived enzyme preparations contained predominantly thermosensitive β-glucosidase. This is the first report on less thermostability of SSF derived β-glucosidase which is the main reason for getting less hydrolysis.

1. Introduction

Lignocellulosic substances are abundantly available sources of renewable biopolymer for the production of biofuels or other bio-based products. The bioconversion of cellulosic materials has been receiving a great attention in recent years. Hydrolysis of lignocellulosic materials by cellulases and hemicellulases could be the most efficient method for the release of fermentable sugars (Lynd et al., 2002). Cellulases are the key enzymes required for the degradation of lignocellulosic polysaccharides into the simple monomeric sugars that are converted through microbial fermentation processes to biofuels or other value added products. Development of large scale bioconversion process would alleviate shortages of food and animal feeds, solve modern waste disposal problems and also diminish the dependence on fossil fuels by providing an energy source in the form of glucose. Much work has been done on the production of cellulases from different microorganisms (Depaula et al., 1999; Solomon et al., 2000). The application of these enzymes for biofuel production is hindered by the high cost of enzyme production and the suitability of pretreatment of biomass which makes it amenable to cellulase attack.

Cellulose is degraded by synergistic action of three types of enzymes in the cellulase complex: exo-1,4-β-d-glucanase (EC 3.2.1.91), endo-1,4-β-d-glucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). Among fungi, *Trichoderma* and *Aspergillus* have been extensively studied particularly due to their ability to secrete cellulose degrading enzymes. We have reported the hyper-production of β-glucosidase (Gokhale et al., 1984) and β-xylosidase (Gokhale et al., 1986) by *Aspergillus niger* NCIM 1207 isolated in our laboratory. However the search for an efficient and possibly better source of cellulase continues due to the low activity of β-glucosidase in *Trichoderma* which limits the rate and extent of hydrolysis. Thus the conversion of waste cellulose to glucose is not yet commercially feasible. Hence, efforts are needed to produce cellulases at affordable cost which can be used for hydrolyzing biomass to monomers with high economical potential. *Penicillium janthinellum* NCIM 1171 was identified as cellulase producer which produces cellulases using baggase as carbon source (Adsul et al., 2004) and for its application in baggase hydrolysis (Adsul et al., 2005). Mutants of *P. janthinellum* NCIM 1171 were isolated that are capable of producing enhanced level of cellulases (Adsul et al., 2007). The present communication describes the comparative enzyme production by the wild type *P. janthinellum* NCIM 1171 and its mutant, EMS-UV-8 and EU2D-21 using different substrates like Avicel, cellulose powder, solka floc and tissue paper under submerged and solid state fermentation conditions. The hydrolysis of Avicel and cellulose powder using mutant EU2D-21 enzyme preparations produced by solid state and submerged fermentation conditions is also described.

2. Methods

2.1. Chemicals

Avicel PH 101 was obtained from Fluka AG, Switzerland. Solka Floc SW44 was purchased from Brown Co., Berlin, NH. Tissue paper

* Corresponding author. Tel.: +91 20 25902670; fax: +91 20 25902671.
E-mail address: dv.gokhale@ncl.res.in (D.V. Gokhale).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.01.014

Bioresource Technology 102 (2011) 4304–4312

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Review

Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass

M.G. Adsul, M.S. Singhvi, S.A. Gaikaiwari, D.V. Gokhale *

NCIM Resource Center, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India

ARTICLE INFO

Article history:
Received 25 October 2010
Received in revised form 30 December 2010
Accepted 2 January 2011
Available online 7 January 2011

Keywords:
Biomass utilization
Commodity chemicals
Ethanol
Lactic acid
Microbial cellulases

ABSTRACT

Lignocellulosic biomass is recognized as potential sustainable source for production of power, biofuels and variety of commodity chemicals which would potentially add economic value to biomass. Recalcitrance nature of biomass is largely responsible for the high cost of its conversion. Therefore, it is necessary to introduce some cost effective pretreatment processes to make the biomass polysaccharides easily amenable to enzymatic attack to release mixed fermentable sugars. Advancement in systemic biology can provide new tools for the development of such biocatalysts for sustainable production of commodity chemicals from biomass. Integration of functional genomics and system biology approaches may generate efficient microbial systems with new metabolic routes for production of commodity chemicals. This paper provides an overview of the challenges that are faced by the processes converting lignocellulosic biomass to commodity chemicals. The critical factors involved in engineering new microbial biocatalysts are also discussed with more emphasis on commodity chemicals.

1. Introduction

Presently, petroleum represents a basic feedstock for production of commodity chemicals and fuels. Rapid depletion of this finite resource and increase in emission of CO_2 levels encouraged a replacement of petroleum with renewable resources such as lignocellulosic biomass as feedstock (Dukes, 2003). With the advent of conversion technologies, biomass resources have regained the potential as feedstock for fuels and chemicals. Many countries are engaged in research and development programs that can provide biofuels from lignocellulosic biomass. US Department of Energy Office developed a program which may provide 60 billion gallons per year of biofuels by 2030. Corn starch and sugars from sugarcane and beets are currently being used directly for biofuels such as ethanol. Brazil has been using sugarcane as raw material for large scale bio-ethanol production for more than 30 years (Goldemberg, 2007). Chemicals such as 5-hydroxymethylfurfural (HMF) obtained by dehydration of glucose is a platform chemical for synthesis of variety of useful acids, aldehydes, alcohols and amines, as well as 2,5-dimethylfuran which is similar to gasoline. In fact, all biofuels and commodity chemicals are based on such food resources and oils because it is easy to convert them into valuable products. Utilization of structural sugars in biomass is comparatively difficult due to its recalcitrance nature. Biomass processing can commence only when we improve the slow kinetics of breaking down biomass to release sugars with high yields. Recent advances in synthetic biology (Pleiss, 2006), metabolic engineering (Lee et al., 2006; Keasling and Chou, 2008) and system biology approach (Mukhopadhyay et al., 2008) have generated microbial cell factories for synthesis of commodity chemicals. These microbial cell factories are constructed by modulating gene expression to fine tune microbial metabolism and also by engineering the proteins to impart new catalytic activities or to improve native properties. The concept of conversion of biomass into biofuels or commodity chemicals is very well discussed in many reviews and conference meetings. Bio-ethanol from lignocellulosic materials such as sugarcane bagasse has been studied for more than two decades but its production is not economically feasible at industrial scale (Clomburg and Gonzalez, 2010; Rodriguez-Moya and Gonzalez, 2010).

Sugarcane bagasse is the major lignocellulosic feedstock available in great amounts in tropical countries. India is one of the largest sugar cane growing countries, producing approximately 300 million tons per year, which generate about 75 million tons of sugarcane bagasse on dry weight basis. It is one of the main byproducts of the sugar industry which is already available at plant site. This could be the sustainable raw material in India that can be considered for the production of commodity chemicals. In addition, the production of commodity chemicals from sugarcane bagasse can share part of its infrastructure where bio-ethanol is produced, such as fermentation and distillation units. Methods of pretreatments of sugarcane bagasse followed by detoxication are well

* Corresponding author. Tel.: +91 20 25902670; fax: +91 20 25902671.
E-mail address: dv.gokhale@ncl.res.in (D.V. Gokhale).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.01.002

Columbia International Publishing
American Journal of Bioengineering and Biotechnology
(2013) Vol. 1 No. 2 pp. 37-43
doi:10.7726/ajbebt.2013.1004
Research Article

Protoplast Formation and Regeneration in *Acetobacter Pasteurianus*

Mamata Singhvi, Shalaka Gaikaiwari and Digambar Gokhale*

Received 7 May 2013; Published online 3 August 2013

Abstract

Method for production and regeneration of *Acetobacter pasterianus* protoplasts is described. The protoplasts were obtained by treatment of cells with lysozyme in protoplast buffer at pH 8.0 with different osmotic stabilizers. The protoplasts were regenerated on Acetobacter medium with various osmotic stabilizers. Maximum protoplast formation was obtained in protoplast buffer with NaCl (0.5M) as an osmotic stabilizer and lysozyme (0.5mg/ml). Addition of mutanolysin did not show enhancement in protoplast formation. Maximum protoplast regeneration was obtained on Acetobacter medium with NaCl (0.5M) as an osmotic stabilizer. This is, to our knowledge, the first report on protoplast formation and efficient regeneration in case of *Acetobacter pasteurianus.*

Keywords: Acetobacter pasteurianus; Protoplast formation; Protoplast regeneration

1. Introduction

Protoplast fusion is a useful tool in the improvement of microorganisms. This technique allows the exchange of entire genomes between unrelated genera of microorganisms. It is considered as classical or fundamental method of gene transfer in which multiple genes can be introduced in contrast to the advanced methods in molecular biology which are suitable for introducing only one or few genes. We have been successful in transferring cellulase genes (multiple phenotype) from *Cellulomonas* to *Bacillus subtilis* using protoplast fusion approach which generated intergeneric fusion products. These fusants produced cellulases with changed properties such as extracellular secretion and thermostability (Gokhale et al., 1984, Gokhale et al., 1989). Recent reports on genome shuffling in *Lactobacillus* proved to be the successful approach which generated new population of strains with additional improvements in acid (Patnaik et al., 2002, Wang et al., 2007) and glucose tolerance (Yu et al., 2008). This genome shuffling approach was also used to obtain *Lactobacillus*

*Corresponding e-mail: dv.gokhale@ncl.res.in
NCIM Resource Center, CSIR-National Chemical Laboratory Pune 411 008, Maharashtra, India

Bioresource Technology 146 (2013) 736–739

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Supplementation of medium with diammonium hydrogen phosphate enhanced the D-lactate dehydrogenase levels leading to increased D-lactic acid productivity

Mamata Singhvi [1], Akanksha Jadhav [1], Digambar Gokhale *

NCIM Resource Center, CSIR-National Chemical Laboratory, Pune 411008, India

HIGHLIGHTS

- Addition of $(NH_4)_2HPO_4$ and peptone in media enhanced D-lactic acid production.
- $(NH_4)_2HPO_4$ induces L-lactate dehydrogenase in D-lactic acid producing strain.
- This is the novel finding and new microbiological information in D-lactic acid fermentation.
- These findings are very useful to industries engaged in lactic acid production.

ARTICLE INFO

Article history:
Received 17 March 2013
Received in revised form 9 July 2013
Accepted 12 July 2013
Available online 20 July 2013

Keywords:
D(-) lactic acid production
Lactate dehydrogenase
Lactobacillus delbrueckii Uc-3
Lactobacillus lactis RM2-24

ABSTRACT

The production of D-lactic acid by *Lactobacillus lactis* RM2-24 was investigated using modified media to increase the efficiency of the fermentation process. The results indicated that the addition of 5 g/l peptone and 1 g/l $(NH_4)_2HPO_4$ enhanced D-lactic acid production by 32%, as compared to that obtained from non supplemented media, with a productivity of 3.0 g/l/h. Lactate dehydrogenase (LDH) expression profile in these different media was studied which resulted in appearance of additional LDH isoform produced by cells when they were grown in HSYE supplemented with $(NH_4)_2HPO_4$. The additional LDH appears to be L-LDH contributing to production of L-lactic acid in the fermented broth. This is totally new information in the lactic acid fermentation and could be very useful to industries engaged in D-lactic acid production.

1. Introduction

In recent years, demand for lactic acid in production of biodegradable plastics and coating for application in biocompatible materials used in medical implants, has increased (Cheng et al., 1991; Goncalves et al., 1992). It is a raw material for the production of polylactic acid (PLA) that is biodegradable and eco-friendly polymer which could be the substitute for synthetic plastics derived from petroleum feedstock. It is also a product that has found immense applications in diverse fields as an acidulant in the food and pharmaceutical industries (Schepers et al., 2002). The chemical synthesis of lactic acid leads to racemic mixture, which is a major disadvantage. Fermentative production of lactic acid offers a great advantage since D- or L-lactic acid is produced depending upon the strains selected for fermentation. The optical purity of the lactic acid is crucial factor for the physical properties of PLA. Though L-lactic acid can be polymerized to get crystalline poly L-lactic acid (PLLA), its application is limited due to its low melting point. The attractive solution to increase the heat stability of PLLA is the complexing of PLLA with poly D-lactic acid (PDLA) (Tsuji et al., 1991). This finding has generated a great interest in the production of D-lactic acid. L-Lactic acid production by fermentation is well established (Yu and Hang, 1989). However, fermentation of sugars to D-lactic acid is very little studied and its microbial productivity is not well known (Zhou et al., 2003). Therefore, we decided to work on D-lactic acid fermentation using indigenously improved strains capable of producing D-lactic acid with enhanced productivities. We have isolated a mutant, *Lactobacillus lactis* RM2-24, which produced D-lactic acid (110 g/l) with high productivity (Joshi et al., 2010). The mutant also found to be utilizing cellobiose efficiently proving its potential to produce D-lactic acid from bagasse derived cellulose (Singhvi et al., 2010).

By manipulating the medium composition (media engineering), one can achieve a reduction in fermentation time and thus increasing the lactic acid productivity. The present study describes the effect of addition of different inorganic and organic nitrogen sources into fermentation medium on lactic acid production. It was found that the addition of peptone and $(NH_4)_2HPO_4$ increased the growth

* Corresponding author. Tel.: +91 20 25902670; fax: +91 20 25902671.
E-mail address: dv.gokhale@ncl.res.in (D. Gokhale).
[1] These authors contributed equally to this work.

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.07.057

RSC Advances

RSC Publishing

REVIEW

Biomass to biodegradable polymer (PLA)

Mamata Singhvi and Digambar Gokhale*

Cite this: *RSC Advances*, 2013, **3**, 13558

Received 3rd April 2013,
Accepted 30th April 2013

DOI: 10.1039/c3ra41592a

www.rsc.org/advances

Lignocellulosic biomass is renewable and cheap, and it has the potential to displace fossil fuels for the production of fuels and chemicals. Biomass derived lactic acid is an important compound that can be used as a chemical platform for the production of a variety of important chemicals on a large scale. The quality of the monomers of lactic acid and lactide, as well as the chemical changes induced during polymerization and processing, are crucial parameters for controlling the properties of the resulting polylactic acid (PLA) products. In this review, we outline the process of exploiting biomass for the production of polylactic acid, a biodegradable polymer which is well-known as a sustainable bioplastic material.

Introduction

Biomass, being renewable, is the only sustainable source of energy and organic carbon for our industrial society.[1] Nature produces a vast amount of biomass by photosynthesis, 75% of which is assigned to the class of carbohydrates. Surprisingly, only 3–4% of these compounds are used by humans for food and non-food purposes.[2] Biomass carbohydrates are the most abundant renewable resources available, and they are currently viewed as feedstock for the green chemistry of the future.[3] The production of fuels and chemicals from biomass is beneficial concerning an environment which is associated with the reduction of the net emissions of CO_2 (a greenhouse gas) into the atmosphere. In contrast to fossil fuels, biofuels are considered to be carbon neutral because any CO_2 produced during fuel combustion is consumed by a subsequent biomass re-growth.[4] A biomass based energy system would improve the economy of those countries determined to accept the challenges. In addition, the use of the lignocellulosic biomass does not affect the food supplies, thereby permitting a sustainable production of fuels (so-called second-generation fuels) and chemicals. Additionally, the lower cost and faster growth of the lignocellulosic biomass compared with food crops[5] and its ample availability[6] make this resource an attractive raw material suitable for the substitution of fossil fuels.

Lactic acid (2-hydroxypropionic acid), CH_3–CHOHCOOH, is the most widely occurring hydroxyl-carboxylic acid in nature. Lactic acid is frequently used in the food industry, especially for beverage production and in the pharmaceutical and chemical industry, or in medicine.[7] Because lactic acid has both carboxylic and hydroxyl groups, it can also be converted into different and potentially useful chemicals such as pyruvic acid, acrylic acid, 1,2-propanediol and lactate esters.[8] The recent growing interest for the manufacture of biodegradable plastic necessitates a high demand for lactic acid as the raw material for PLA production.[9] Another very promising lactic acid application is the production of environmentally friendly ''green'' solvents (lactate esters) which can replace traditional solvents made from petrochemical feedstock.[10]

Poly(lactic acid) is one of the most promising biodegradable plastics.[11] Much research effort is currently focused on the modifications of polylactide to make it suitable for a wider range of applications. Optically pure lactic acid is necessary to obtain high crystalline poly(lactic acid) which leads to the high strength, chemical and heat resistances properties of the polymer.[12] In this review, we focus on the utilization of the lignocellulosic biomass for the production of PLA, a biodegradable polymer. Environmental, economic and safety challenges have pushed towards the partial replacement of petrochemical-based polymers with bio-based ones. The general purpose of this review is to introduce PLA, a compostable and biodegradable thermoplastic made from renewable sources (Fig. 1).

Biomass structure

Lignocellulose, a carbohydrate source, is an interesting raw material for biotechnological processes, owing to its renewable character, widespread distribution, abundance and low price. Lignocellulosic biomass (plant biomass) is a prodigious potential resource for the production of fuels and chemicals because it is abundant, inexpensive and the production of such resources is environmentally sound. Agricultural residues are a great source of lignocellulosic biomass which is renewable, mainly unexploited and inexpensive. Such resources include: leaves, stems and stalks from corn fibre, corn stover, sugarcane bagasse, rice hulls, woody crops and forest residues. Also, there are multiple sources of lignocellulosic waste from industrial and agricultural processes, *e.g.* citrus peel waste,

NCIM Resource Centre CSIR-National Chemical Laboratory, Pune-411008. E-mail: dv.gokhale@ncl.res.in; Fax: 91-20-25902671; Tel: 91-20-25902452

RSC Advances

REVIEW

Lignocellulose processing: a current challenge

Cite this: *RSC Adv.*, 2014, **4**, 8271

Mamata S. Singhvi, Shivani Chaudhari and Digambar V. Gokhale*

Lignocellulosic biomass, of which inedible crops are a renewable source, is expected to become one of the key renewable energy resources in the near future, to deal with global warming and the depletion of conventional fossil fuel resources. It also holds the key to supplying society's basic needs for the sustainable production of chemicals and fuels without impacting the human food supply. Despite this, the production of 2nd generation biofuels and chemicals has not yet been commercialized. Therefore, the challenges involved in the production of lignocellulosic biomass derived fuels and chemicals must be addressed. The search for economic pretreatment methods has been recognized as one of the main hurdles for the processing of biomass to biofuels and chemicals. The conversion of all biomass components, lignin in particular, would greatly contribute to the economic viability of biomass based processes for 2nd generation biofuels and chemicals. The highly organized crystalline structure of cellulose presents an obstacle to its hydrolysis. Hydrolysis of lignocellulose carbohydrates into fermentable sugars requires a number of different biomass degrading enzymes such as cellulases and hemicellulases. Still, a number of technical and scientific issues within pretreatment and hydrolysis remain to be solved. Depending on the raw material and pretreatment technology, the enzyme mixtures must be designed to degrade biomass carbohydrates. Rapid advances in enzyme, microbial and plant engineering would provide the necessary breakthroughs for the successful commercialization of biomass derived fuels and chemicals.

Received 25th October 2013
Accepted 2nd December 2013

DOI: 10.1039/c3ra46112b

www.rsc.org/advances

Introduction

At present, society faces the difficulty of searching for alternative and renewable energy sources to the widely used conventional sources (fossil fuels). The current energy crisis necessitates the development of processes that are based on renewable substrates. Conversion of lignocellulosic biomass to fermentable sugars may serve as a viable approach for the production of renewable fuels. Fuels and chemicals derived from biomass are regarded as an environmentally friendly alternative to petroleum based products. The exploitation of lignocellulosic biomass as a source of commodity chemicals, especially fuels, has been encouraged by governments to alleviate dependence on the volatile depleting petroleum reserves. Global warming due to emission of CO_2 emerging from the combustion of fossil fuels, depletion of finite carbon fossil fuel resources and unreliability of petroleum supplies has prompted us to think about biofuels and biomaterials.[1] First generation biofuels, consisting of ethanol and biodiesel, are derived from food stocks such as sugars, starches and oils and crops that are used for human consumption. The use of these food stocks for biofuel production was linked to an increase in food prices. In response to the "food for fuel" debate in 2008, the World Bank and other international agencies advised governments to cease biofuel subsidies in the USA in 2011. The "food for fuel" debate also compelled governments to encourage second generation biofuel production that focuses on the extraction of energy from lignocellulosic biomass, which consists of inedible parts of plants, grasses and forest waste. A major advantage of using these materials is that they are not grown on agricultural land which is being used for food production. In many biorefineries it has been proposed that the non-food portions of crops are processed for biofuels and chemicals, therefore making the agricultural land useful for the production of biofuel.[2] Despite the large amount of R & D input for almost five decades, there has not been much successful economic conversion of lignocellulosic biomass to fuels and chemicals. Beyond second generation biofuels, scientists are also engaged in so-called third generation biofuels which are derived from microalgae and cyanobacteria, but this field has not been explored as extensively as lignocellulosic feed stock. Microalgae convert sunlight, CO_2 and water into biomass-containing oil which can then be converted into biodiesel using existing refining processes. However, the actual amount of oil produced in the biomass depends on many critical factors, such as CO_2 levels and sunlight, that require further research relating to land use options for ponds and farms and the development of costly and as-yet imperfect photobioreactors. In addition, genetic engineering of microalgae with increased biomass yields and oil content is still a challenge.[3] Similarly, microalgae and

NCIM Resource Center, CSIR-National Chemical Laboratory, Dr Homi Bhabha Road, Pune 411008, India. E-mail: dv.gokhale@ncl.res.in; Fax: +91-20-25902671; Tel: +91-20-25902670

RSC Advances

PAPER

Cite this: *RSC Adv.*, 2015, **5**, 2024

Biocatalyst development for lactic acid production at acidic pH using inter-generic protoplast fusion

Mamata Singhvi,[a] Gayatri Gurjar,[b] Vidya Gupta[b] and Digambar Gokhale*[a]

Received 25th September 2014
Accepted 27th November 2014

DOI: 10.1039/c4ra11104d

www.rsc.org/advances

Global warming and environmental problems force us to develop sustainable processes based on the use of biocatalysts that are eco-friendly with the least potential toxicity. Lactic acid fermentation at neutral pH generates a large amount of gypsum during down-stream processing. Hence it is essential to develop *Lactobacillus* strains which produce lactic acid at acidic pH thus making the whole downstream process environmentally friendly. Fusant F3 was generated using protoplast fusion between *Lactobacillus delbrueckii* Mut Uc-3 and *Acetobacter pasteurianus* NCIM 2314 on solid media at pH 4.0. Fusant F3 was further treated by UV irradiation to generate a mutant, FM1, with improvements in acid tolerance which produced five-fold more lactic acid than the parent strain at acidic pH. The molecular studies using RAPD markers demonstrated that the fusant is derived from both the parental strains, *Acetobacter* and *Lactobacillus* and the mutant is derived from the fusant. The utilization of such acid tolerant strains could be able to produce free lactic acid at acidic pH without using neutralizing agents and will offer an effective means for designing environmentally benign processes for lactic acid production.

Introduction

Lactic acid is widely used in food, cosmetic, pharmaceutical, and chemical industries and has received increasing attention for use as a monomer for production of biodegradable poly-(lactic acid) (PLA).[1,2] It is also an important platform chemical[3,4] as it can be converted into other chemicals such as acrylic acid, propylene glycol, acetaldehyde, and 2,3-pentanedione. In recent years, demand for lactic acid has increased due to its application in biodegradable polymers and green solvents[5,6] and the greater demand is for the L isomer.[7] Approximately 90% of the total lactic acid produced worldwide is by bacterial fermentation which offers a great advantage over the chemical route since pure L- or D-lactic acid can be produced by using selective strains of lactic acid bacteria (LAB). In addition, chemical synthesis of lactic acid always leads to racemic mixture which is a major disadvantage. The present industrial production of lactic acid is based on microbial carbohydrate fermentation because it is economically more feasible compared with the chemical route.[8] The optical purity of the lactic acid is crucial during PLA production because small amounts of enantiomeric impurities drastically change the properties such as crystallinity or biodegradation rate of the polymer. Complexing poly L-lactic acid (PLLA) with poly D-lactic acid (PDLA), however, increases the melting point thus presenting an attractive solution to the heat sensitivity of PLA. Improved strains of LAB have been developed which produce D- or L-lactic acid from hydrolyzed sucrose,[9,10] molasses[11] and sugarcane baggase derived cellulose[12,13] in high amounts at neutral pH. Lactic acid fermentation at neutral pH produces calcium lactate, which need to be hydrolyzed by corrosive sulphuric acid to generate free lactic acid and gypsum. Hence it is essential to develop biocatalysts (LAB) which produce free lactic acid at acidic pH without generation of gypsum thus making the whole down-stream process environment friendly. Considering this constraint, we concentrated on the development of improved LAB producing L-lactic acid in acidic environment which would make the process environmentally sound by avoiding the use of corrosive sulphuric acid.

Lactic acid is the fermented product of pyruvic acid, end-product of the glycolysis (Embden–Meyerhoff) pathway, by which energy is supplied to most of the homo-fermentative LAB.[14,15] During a typical lactic acid fermentation, low pH has an inhibitory effect on the metabolic activities of the producing organism.[16] Accumulation of lactic acid in an un-dissociated form inhibits both, cell growth and its production. Moreover, the lactic acid bacteria are not resistant to low pH and do not produce lactic acid at acidic pH.[17] Therefore, during fermentation, a neutralizing agent such as NaOH, NH_4OH or $CaCO_3$ has to be added to minimize the inhibitory effects on cell growth and lactic acid production caused by free lactic acid. However, the neutralization of lactic acid during fermentation has disadvantages as the additional operations are required to generate free lactic acid and to dispose off or recycle the neutralizing cation.[18,19] It is possible to reduce all the extra operations and expenses if the microorganism is able to grow

[a]*NCIM Resource Centre, Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune-411008, Maharashtra, India. E-mail: dv.gokhale@ncl.res.in; Fax: +91 (020)25902671; Tel: +91 (020)25902670*

[b]*Plant Molecular Biology, Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune-411008, Maharashtra, India*