Utilisation of agro residual biomass for L-Lysine production by *Corynebacterium glutamicum*

Thesis submitted under the Faculty of Science of the
Cochin University of Science and Technology

For the award of the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

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2016

To my family, friends and teachers

DECLARATION

I hereby declare that the the PhD thesis entitled **"Utilisation of agro residual biomass for L-Lysine production by *Corynebacterium glutamicum*"** is based on the original research work carried out by me under the supervision of Dr K Madhavan Nampoothiri in the Biotechnology Division of CSIR-NIIST, Thiruvananthapuram, India and that no part of this work has been submitted previously anywhere for the award of any degree.

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27th April 2016

CERTIFICATE

This is to certify that to the best of my knowledge and belief, the work embodied in the thesis entitled **"Utilisation of agro residual biomass for L-Lysine production by *Corynebacterium glutamicum*"** is a bonafide record of the original research work carried out by **Ms. ANUSREE M** under my supervision in the Biotechnology Division of CSIR-NIIST, Thiruvananthapuram and that no part of this work has been submitted previously anywhere for the award of any degree. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the doctoral committee of the candidate has been incorporated in the thesis.

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To whom it may concern

Bielefeld, 12. Apr. 2016

Letter of confirmation for Anusree Murali

Dear Madam or Sir,

this is to indicate that my lab has collaborated with Dr. K Madhavan Nampoothiri from the Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Industrial Estate P O, Pappanamcode, Thiruvananthapuram Kerala, India on a research programme titled as 'Construction of cellulose degrading *Corynebacterium glutamicum* strains for direct utilization of agro residual biomass for the production of amino acids and polyamines'. Under this programme, the PhD student Ms Anusree Murali from India stayed in my lab for research work and carried out a part of the recombinant strain constructions and trained in basic molecular biology techniques. Some of those results were included in her PhD work as well. Together, we published a manuscript in this joint project, which was funded by DST, New Delhi and DAAD, Germany.

If you have further questions, please feel free to contact me.

Sincerely,

Prof. Dr. Volker F. Wendisch

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Anusree M

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LIST OF ABBREVIATIONS

%	Percentage
&	And
˚C	Degree Celsius
<	Less than
≤	Less than or equal to
µF	Microfarad
µg	Microgram
µg/mL	Microgram per milliliter
µm	micrometer
BB	Box-Behnken
CB	Cassava bagasse
CCD	Central Composite Design
CGXII	*Corynebacterium glutamicum* twelve medium
COC	Coconut oil cake
DNS	3,5-dinitrosalicylic acid
DO	Dissolved oxygen
h	Hours
HPLC	High Pressure Liquid Chromatograpy
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JFS	Jackfruit seed powder
L	Liter
LB	Luria –Bertani medium
LBG	Luria –Bertani Glucose

M	Molar
min	Minutes
mL	Milliliter
mL/min	Milliliter/minute
mM	Millimolar
MOPS	3-(N-Morpholino)propanesulfonic acid
N	Normal
NADP	Nicotinamide adenine dinucleotide phosphate
nm	Nanometer
OD_{600}	Optical density at 600 nanometer wavelength
OPA	o-phthalaldehyde
PBD	Plackett–Burman Design
PDA	Photo Diode Array
pNPG	Para-nitrophenyl-beta-D-glucoside
PP	Potato peel
PUF	Poly urethane foam
RI	Refractive index
rpm	Revolutions per minute
RS	Rice straw
RSM	Response Surface Methodology
SEM	Scanning Electron Microscopy
SB	Sugarcane bagasse
SCT	Sugarcane tops
SmF	Submerged fermentation
SSF	Solid-state fermentation

U/gds	Units/gram dry substrate
UFLC	Ultra Fast Liquid Chromatograpy
V	Volt
v/v	Volume/Volume
w/v	Weight / Volume
WB	Wheat bran
Ω	Ohm

Chapter I

INTRODUCTION AND REVIEW OF LITERATURE

1. INTRODUCTION

Amino acids are the basic bio molecules of life and act as the protein building blocks of living beings and as intermediates of metabolism. More than three hundred amino acids are discovered in nature and twenty among them form major part of living systems. Amino acids, with its varied characteristics find applications in food and feed sector, pharmaceuticals, chemical intermediates and cosmetics and the increase in demand in each sector leads to mass production. The mass production is dominated by industrial amino acid production giants located in Japan, China, USA and Europe.

Amino acids production process mainly involves chemical synthesis, extraction from protein hydrolysates, enzymatic synthesis and microbial fermentation (Hermann, 2003). Chemical synthesis employs artificial synthesis of amino acids from chemical starting material. It usually produces an enantiomeric mixture of amino acids which will further require a step of optical resolution. The extraction method depends on the extraction of amino acids from the acid hyrolysates of proteins (Giacometti, 1979). Although the method was commercialized for amino acid production (proline, serine, tyrosine etc), the availability of raw materials like hair, feather, keratin, blood meal etc is usually a limiting factor. Moreover, the yield of amino acids depends on the amino acid composition of the raw material. Enzymatic synthesis involves the use of enzymes directly or in the form of cells to convert a precursor molecule to the amino acid of interest. Immobilized *E. coli* cells expressing aspartate or the immobilized enzyme have been used in the commercial production of aspartic acid from ammonia and fumaric acid. Chibata and co workers (Chibata et al., 1965) also produced alanine by microbial (*Pseudomonas dacunhae*) L-aspartate β-decarboxylase with aspartate as the starting material Phenylalanine was manufactured from trans-cinnamic acid and ammonia by enzymat

tic route by phenylalanine ammonia lyase as catalyst (Hamilton & Jackson, 1985) or from phenyl pyruvate and aspartic acid using transaminase.

The increasing demand and the want of stereo specificity of the amino acids led to microbial fermentation which is the most favoured means of amino acid production. The amino acid fermentation history has its roots in the discovery of monosodium glutamate (MSG) by Kikunae Ikeda in 1908. The flavor enhancer as found by Ikeda was extracted from sea weeds or kelps that flourished in Japanese cuisine. The production of MSG from soybean and wheat gluten by acid hydrolysis was soon patented (Giacometti, 1979) which was later used by Ajinomoto for the development of a seasoning. The increasing amino acid demand triggered the initiation of a large scale screening programme by the Kyowa Hakko Kogyo Co and led to the discovery of the glutamate overproducer *Corynebacterium glutamicum* (Kinoshita et al., 2004; Udaka, 1960). Further screening and random mutagenesis revealed the potential of *C. glutamicum* as an L-lysine overproducer (Kinoshita et al., 1958; Nakayama et al., 1961). The discovery by Kyowa Hakko Kogyo Co of a homoserine auxotroph of *C. glutamicum* overproducing L-lysine led to an important development in the amino acid fermentation industry which later led to its commercial industrial production.

Amino acid fermentation industry is mainly driven by modified strains of *C. glutamicum* and *Escherichia coli*. *C. glutamicum* is the major producer of glutamic acid and L-lysine, while *E. coli* is used production of threonine and branched chain amino acids (Ikeda & Takeno, 2013). Established amino acid producer strains were shaped by accumu lation of favorable genotypic and phenotypic characters over the decades by classical mutagenesis or recombinant DNA technology. Later developments in the strain improvement techniques, fermentation technology and deeper understanding of the microorganism led to the over production and further establishment of industrial scale production. In the last decade there has been tremendous improvement in characterizing *C. glutamicum* strains attributable to genomics, transcriptomics, metabolomics and proteomics. This has led to the deeper understanding of the underlying network of reactions that enable *C. glutamicum* for amino acid overproduction and profoundly affecting the strain improvement methods (Ikeda & Nakagawa, 2003; Kalinowski et al., 2003). Strain improvement methods are coupled with engineering expansion of substrate utilization has been a long leap in the amino acid overproduction in the last decade

(Anusree et al., 2016; Gopinath et al., 2011; Gopinath et al., 2012; Kawaguchi et al., 2008; Kawaguchi et al., 2006; Rittmann et al., 2008; Sasaki et al., 2008; Sasaki et al., 2009) . The thesis is in line with broadening the substrate utilization range of *C. glutamicum* and speaks about the use of agro residual substrates in different modes of fermentation for L-lysine production.

1.1. Major objectives of the present study

1. Screening of potent agro residual substrates and conversion to fermentable monomeric sugars.
2. Selection of the best agro residual hydrolysate by screening for growth and amino acid production by *Corynebacterium glutamicum.*
3. Optimization of process parameters for fermentative production of L-lysine.
4. Scale up studies in bioreactors.
5. Downstream processing involving purification and recovery.

The thesis is framed into 8 chapters. Chapter one deals with the introduction and summarizes the review of literature. Chapter two explains the general materials and methods. Selection of alternate carbon source and operational parameters for L-lysine fermentation using *C.glutamicum* DM 1729 is discussed in Chapter three. Chapter four describes the statistical optimization of process parameters for optimum L-lysine production. Chapter five depicts the application Solid-State Fermentation in L-lysine production. Downstream processing involving purification and recovery of L-lysine is presented in Chapter six. Chapter seven describes the co expression of endoglucanase and β-glucosidase genes in *C. glutamicum* DM 1729 towards the direct utilization of cellulose for L-lysine production. The summary and conclusions form the final chapter. Bibliography of the cited work is detailed in a separate section. The thesis also has supplementary files of list of tables, list of figures, major instruments used, media compositions and abbreviations.

2. REVIEW OF LITERATURE

2.1. Amino acids

The basic structure of all amino acids has a carboxyl group and an amino group and a side chain or R-group. Both L (levo) and D (dextro) forms of amino acids exist in nature among which the levo form is predominant. The carbon atoms in the amino acids are numbered according to the Greek alphabets and are termed as α-amino acid, β-amino acid depending on the carbon atom to which the amino group is attached. Amino acids are classified based on the essentiality in diet, the charge and polarity of the side chain, number of carboxyl groups and amino groups, glycogenic or ketogenic, proteogenic or non-proteogenic.

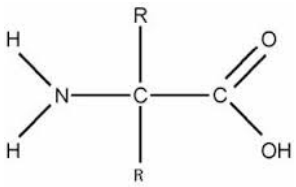
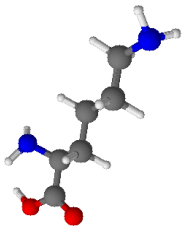


Fig 1.1. General amino acid structure

2.2. L-lysine

L-lysine is an essential, ketogenic, proteogenic, basic, aliphatic, α–amino acid.The basic facts about L-lysine are shown in **Table 1.1.** L-lysine was first discovered by the German dentist, E Drechsel from casein in 1889. Two years later in 1891, M Siegfried identified L-lysine from protein hydrolysates of conglutin, egg albumin, gluten etc. In the same year Drechsel identified the composition of the amino acid. The structure of L-lysine was predicted by E Ellinger in 1889 and the term L-lysine was coined by E Fischer to indicate the release of urea on alkaline hydrolysis. H B Vickery and C S Leavenworth were the first to prepare crystalline L-lysine.

Table 1.1. L-lysine-basic facts

Chemical name	α, ε-diamino caproic acid
Structural formula	
Molecular formula	$C_6H_{14}N_2O_2$
Three letter code	Lys
Abbreviation	K
R group	Lysyl group – $(CH_2)_4NH_2$
Molecular weight	146.19 g/mol
Major commercial form	L-lysine-HCl

2.3. L-lysine in industrial sector

L-lysine is an essential amino acid and has to be supplemented in human diet and animal feed. The major end use of L-lysine is in the animal feed sector and is the limiting amino acid while optimizing the growth of monogastric animals and poultry. L-lysine is also used in cosmetic industry (Barone et al., 1991; Song et al.,2001). L-lysine, in the form of lauryl lysine is used in the skin and hair conditioners, cosmetics or as viscosity controllers. In food and beverages, L-lysine is used either as flavor enhancers. It can also act as nutritional supplement (Attaelmannan & Reid, 1996; Flakoll et al., 2004) and as intermediates of chemicals and organic acids, in chemical industries. The varied applications of L-lysine are shown in **Fig 1.2.**

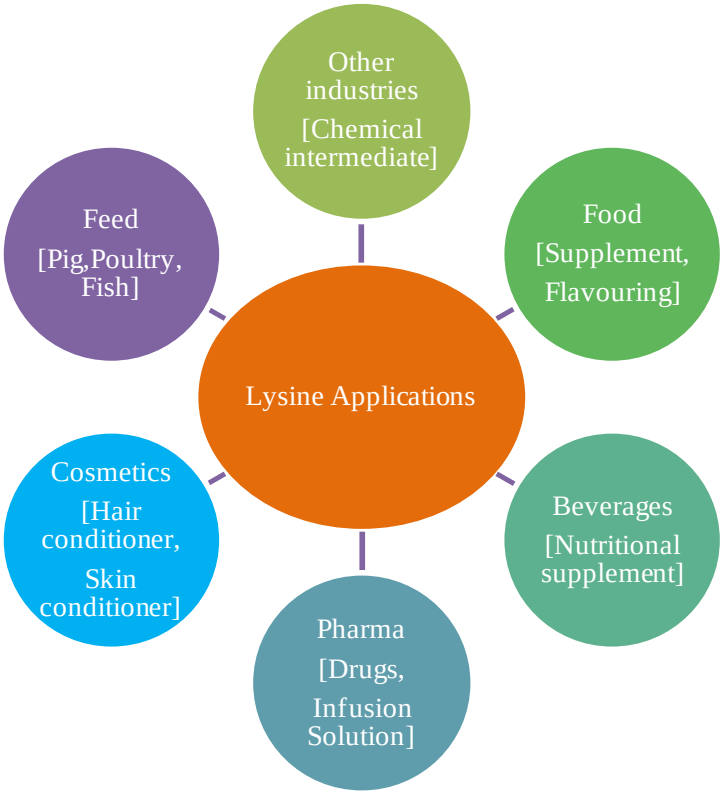


Fig 1.2. Wide spectrum industrial applications of L-lysine

2.4. L-lysine Market

According to TIFAC, India (Technology Information Forecast and Assessment Council), L-lysine requirements of India are completely met by imports. There are no production facilities in India as of now. However, Ajinomoto has plans of starting production plant in India soon. The main reason for not having production facilities in India are the lack of indigenous technology for production and huge financial burden for acquisition of foreign technology. Even the effort for development of indigenous technologies has been inadequate. The increase in L-lysine production over past 15 years and the fluctuations in the price are depicted in the **Fig 1.3**.

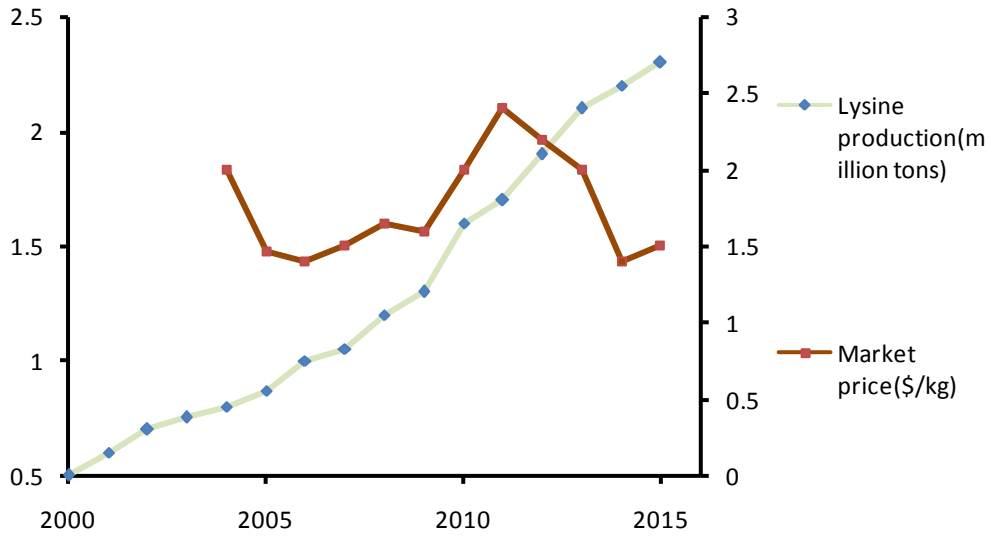


Fig 1.3. L-lysine market and price (2000 – 2015)(Eggeling & Bott, 2015)

The major players in the global amino acids market are Ajinomoto Co., Evonik Industries, Changchun Dacheng Group, Archer Daniel Midland (ADM), Global Bio-chem Technology Group Company Limited, Shandong Shaouguang Juneng Golden Corn Co. Ltd., Cheil Jedang Corporation, and COFCO Biochemical (Anhui) Co. Ltd. Among them four major giants manufacture L-lysine viz Ajinomoto Ltd. (Japan), Global Bio-Chem Tech (China), Cheil Jedang Corp (Korea) and Archer Daniel Midland (ADM, USA) and they capture over 60% of the market in terms of production capacity. These companies have firm competition with others and dominate the arena on the grounds of their technical expertise, strong capital investment, access to reliable raw material sources and supply, increased production capacities and brand names. The Asia Pacific region is the growth hub of the amino acid industry with the largest production plants and ever growing consumer markets of India and China. The extensive use of monosodium glutamate in food industry and L-lysine in the feed industry has contributed to the growth of amino acid market in these areas. The limitation in the market is the development of new protein rich feed additives. The amino acids prices were fluctuating in the last two decades with the market dynamics and tend to lower with increasing production capacities and easy availability of raw materials. Even though the amino acid production industry encountered a setback in late 2000s, it strongly regained the momentum soon .The industries have devised strategies to circumvent the supply and demand changes. The largest application of L-lysine is the animal feed sector and other application fields are food, pharma and

cosmetics. The major feed source of L-lysine is soya bean meal and with the supplementation of L-lysine the soya bean meal in the feed can be reduced or avoided. This checks the unnecessary amino acid uptake beyond the need and resultant ammonia emission in the manure. Thus L-lysine is advantageous over soya bean meal used in animal and rearing.

Table 1.2. Categories in global amino acid market

Global Amino acid market	Geography	China Europe North America Rest of Asia Pacific Rest of the world
	Product Type	Glutamic acid L-lysine Threonine, Methionine, Tryptophan
	Application	Food and dietary supplements Pharmaceuticals Feed
	Livestock	Poultry Swine/hog Others

The animal feed sector dominated the global amino acid market with a market share of 90% in the beginning of the decade. The categories of global amino acid market are shown in **Table 1.2.** In terms of revenue generated the swine feed sector scored the second largest position. In the same time, the poultry market dominated the global amino acids market with a 57% market share. Meanwhile, considering the amino acid demand China held 30.6% of the market share, followed by Europe with a 27% and North America with a share of 16.8% and is expected to have a rapid growth. China and Europe maintained their positions in the global amino acids market from 2011 to 2015 (Research, 2013).

L-lysine demand in the animal feed market is driven by the positive outlook on animal feed demand to accommodate the growing meat consumption, especially in rising markets of Latin America and Asia Pacific. Animal feed segment emerged as the single largest application segment for L-lysine in 2013. China is expected to be the one of the largest and fastest growing market for L-lysine. It occupied 92.4% of the total market share in 2013. Animal feed sector is also expected to be the fastest growing market and application segment for L-lysine at an estimated CAGR (Compound Annual Growth Rate) of 6.2% from 2014 to 2020. The global L-lysine was 1,902.3 kilo tons in 2013 and is expected to reach 2,854.9 kilo tons by 2020, growing at a CAGR of 6.0% from 2014 to 2020.Swine emerged as the leading livestock for L-lysine and accounted for about 58% of total market volume in 2013. Swine is also expected to be the fastest growing livestock for L-lysine, at an estimated CAGR of 6.5% from 2014 to 2020. China was the largest consumer for L-lysine and accounted for 31.2% total volume consumed in 2013. In terms of consumption, China was followed by Europe and North America. The global market for L-lysine is expected to reach USD 6.96 billion by 2020, according to a new study by Grand View Research, Inc (Grand view research, 2014).

2.5. Corynebacterium glutamicum

C. glutamicum is known as the work horse of amino acid production industry. The discovery of *C. glutamicum* led to the development of microbial amino acid fermentation industry. The microbe was isolated from avian faeces contaminated soil of the Ueno zoo in Japan. The isolation was a result of large scale screening program by the Kyowa Hakko Kogyo for a microorganism for microbial fermentation of amino acids. The isolated microbe was a natural producer of glutamic acid and was initially named as *Micrococcus glutamicus*. The amino acid production industry started its huge leap after the discovery of Micrococcus and the later developments are all connected with the strain improvements of this organism.

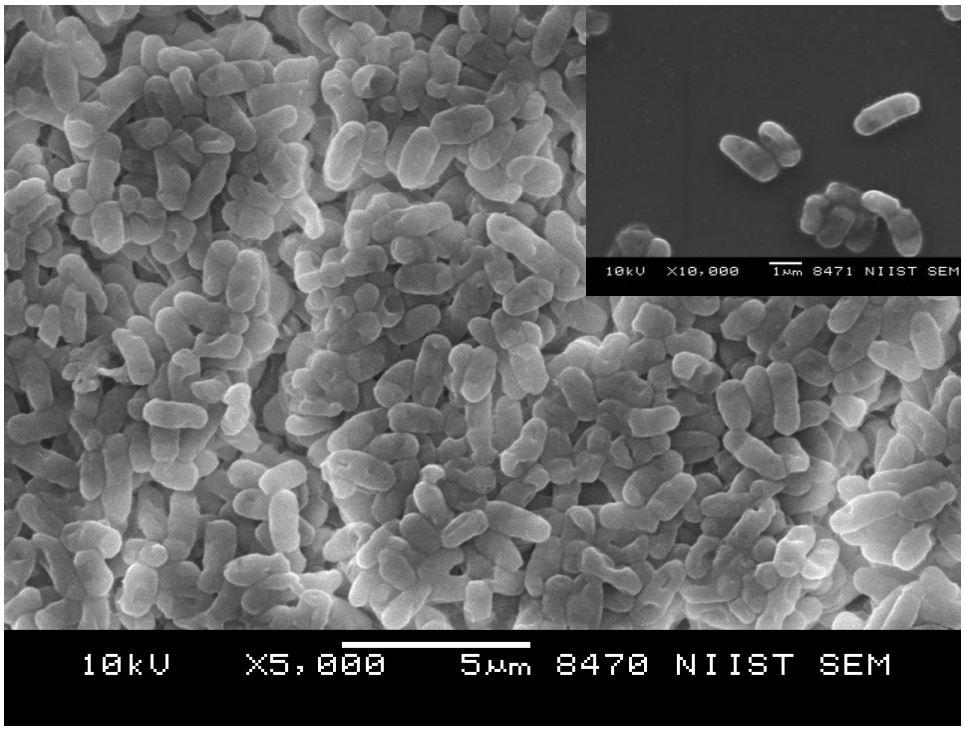


Fig 1.4. Scanning Electron Microscopic image of *C. glutamicum*. Dividing cells in the inset at a higher resolution

C. glutamicum is a soil dwelling, gram positive, rod shaped, non spore forming bacterium. It has high G+C content and belongs to Actinobacteria. On agar plates, the cells usually form small whitish transluscent colonies which increase in size and become yellowish on longer incubation. The bacterium has coryne –form shape or club shape as the name depicts. The shape of *C. glutamicum* cells are influenced by the culture conditions. The bacteria are characterized by a lipid-rich cell envelope surrounding the cell wall, which act as a permeability barrier and is described as an outer membrane of gram-positive bacteria (Minnikin, 1982; Zuber et al., 2008). The cells are then enveloped by the S-layer (Bayan et al.,2003; Chami et al., 1997; Peyret et al.,1993). In 2003, the mechanism of cell division of corynebacteria was reported (Daniel & Errington, 2003; Letek et al., 2008). During cell elongation, peptidoglycan synthesis occurs at the poles and then synthesized at the centre to produce daughter cells. After the bacteria undergo ‘‘snapping’’ cell division, the daughter cells remain joined together, forming a characteristic V-shape (Collins & Cummins, 1986) and often lie in clusters resembling Chinese letters or palisades. SEM image of *C. glutamicum* is shown in **Fig 1.4.**

So far, two wild type strains of *C. glutamicum* has been sequenced and annotated. The GRAS (Generally Recognized As Safe) status of *C. glutamicum* makes it the favorite of amino acid fermentation industry. The microbe is a natural overproducer of glutamic acid and has been engineered to overproduce amino acids like L-lysine, threonine, methionine, arginine etc. To meet the increasing demand of amino acids, three industrial giants BASF (Germany), Degussa (Germany) and Kyowa Hakko (Japan) sequenced the genome of *C. glutamicum* ATCC 13032 to necessitate easy strain improvement. Scientific research institutions also partnered up to sequence the genome. *C. glutamicum* ATCC 13032 has been sequenced independently by Nakagawa and Ikeda (2003) and Kalinowski (2003). According to Nakagawa, the genome sequence of *C. glutamicum* ATCC 13032 was 3309401 bp with a G+C content of 53.8% and there were 3099 genes on the genome. According to Kalinowski, the *C. glutamicum* genome consisted of a single circular chromosome comprising 3282708 bp. Several DNA regions of unusual composition were identified that were potentially acquired by horizontal gene transfer, e.g. a segment of DNA from *C. diphtheriae* and a prophage-containing region. About 3002 protein-coding genes were identified, and 2489 of these had functions similar to known proteins. *C. glutamicum* R strain (RITE strain of Japan) was sequenced independently of the others. Research Institute of Innovative Technology for the Earth (RITE) in Kyoto, Japan sequenced the genome of another wild-type strain of *C. glutamicum* and has reported that the genome comprises 3363299 bp and contains 3018 genes (Yukawa et al., 2007).

2.6. L-lysine biosynthesis

L-lysine belongs to the aspartate family of amino acids including homoserine, methionine, isoleucine and threonine. It is formed at the cost of 4 NADPH molecules from pyruvate, oxaloacetate and ammonia. There are 35 branch points in the L-lysine biosynthetic pathway. L-lysine biosynthesis occurs in organisms via two routes, alpha amino adipate route (fungi, archea etc) and the diaminopimelate route. The alpha aminoadipate route has two variants. The diaminopimelate route has four variants viz, Aminotransferase pathway, Succinylase pathway, Acetylase pathway and Dehydrogenase pathway. These four variants branch at tetrahydrodipicolinate. In *C. glutamicum*, the succinylase and dehydrogenase variants operate under different conditions. The dehydrogenase pathway is energetically cheaper than the succinylase variant and the operating pathway is selected based on the ammonium availability in the medium. Under

conditions of luxuriant free ammonium, the dehydrogenase variant is operated, while under high organic nitrogen concentrations, the succinylase variant operates. The choice of having two pathways with different energy requirements gives *C. glutamicum* flexibility under different environments. The sugar molecules assimilated by the microbe is transported through the PTS system and moves through the pyruvate node and oxaloacetic acid to aspartate.

The anaplerotic enzymes which replenish the NADPH supply to the TCA cycle are in the pyruvate node and are one of the important links between glycolysis and TCA cycle. This node act as a very important switch point for carbon flux distribution within the central metabolism as a set of reactions at this node direct the carbon flux into appropriate directions resulting in the production of product of interest (Sauer & Eikmanns, 2005). Phosphoenolpyruvate carboxykinase (PEPCK), phosphoenolpyruvate carboxylase (PEPC) and pyruvate carboxylase (PC) have been identified as anaplerotic enzymes, which catalyze the reaction of adding 1 mole of carbon dioxide to pyruvate and phosphoenol pyruvate, fulfilling the TCA with oxaloacetic acid (OAA) and playing an important role in supplying aspartic acid. The deamination of OAA results in aspartic acid production. All the enzymes in the pyruvate node are very important in L-lysine biosynthesis as is evident from the C-13 carbon flux analysis. Supply of OAA is a bottleneck in L-lysine overproduction and metabolic engineering at the pyruvate node has resulted in circumventing this. The strategies included either the overexpression or deregulation of feedback inhibition of PEPC (Chen et al.,2014; Cremer et al.,1991; O'Regan et al.,1989) or PC (Peters-Wendisch,V.F et al.,1998), deletion of pyruvate kinase (Becker et al.,2008), or PEPCK (Petersen et al., 2001).

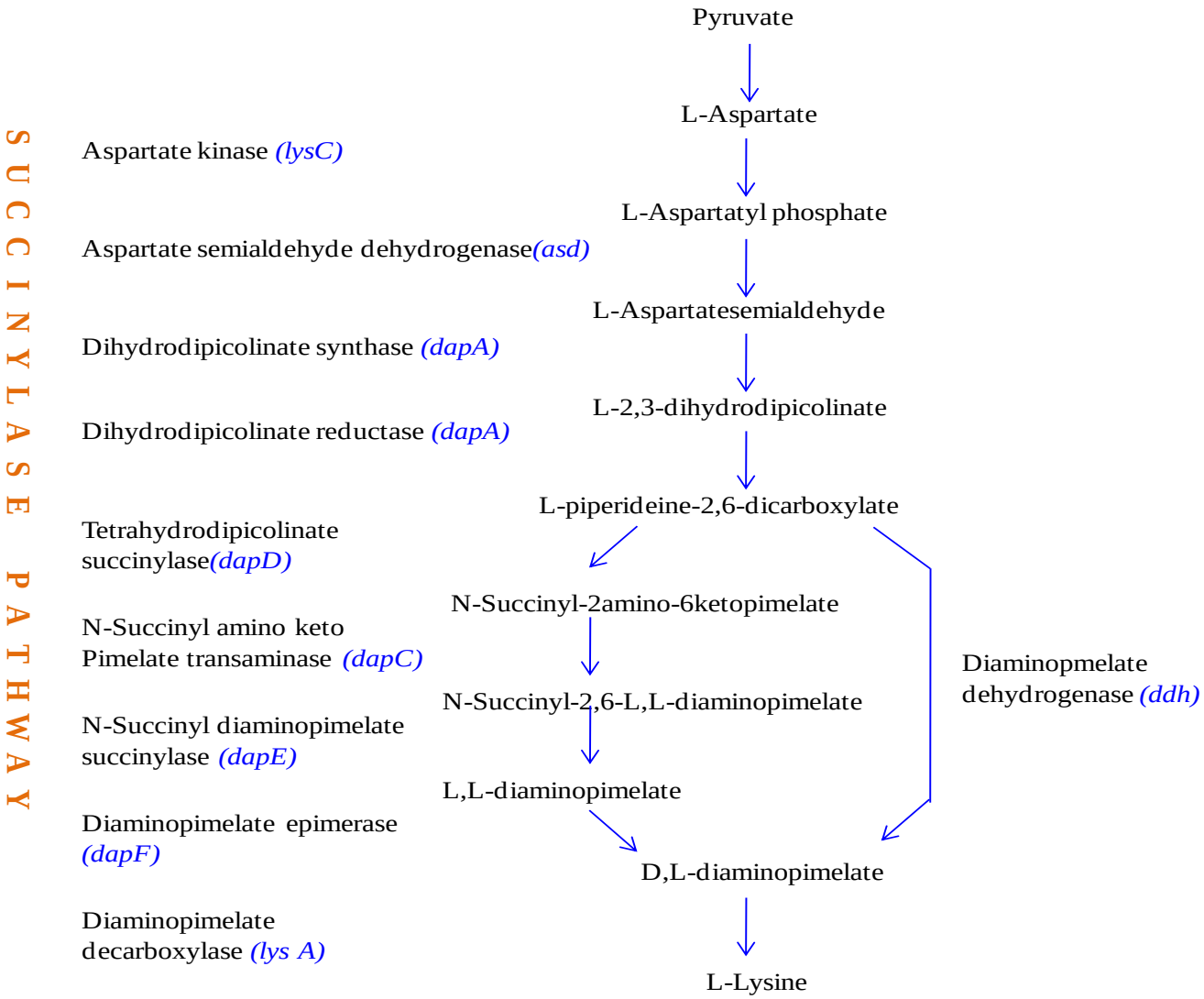


Fig1.5. L-lysine biosynthetic pathway in *C.glutamicum*

Aspartic acid forms the starting point of L-lysine biosynthesis (**Fig 1.5.**). From aspartic acid, the biosynthesis pathway proceeds through either succinylase or dehydrogenase pathways to produce L-lysine. L-lysine produced inside the cell is then excreted out through export carriers (**Fig 1.6**.) as explained by various authors (Bröer & Krämer, 1991; Erdmann et al., 1993; Vrljic et al., 1996). L-lysine export is crucial in production and is catalyzed by *LysE*. Deletion of *LysE* resulted in arrest of L-lysine excretion.

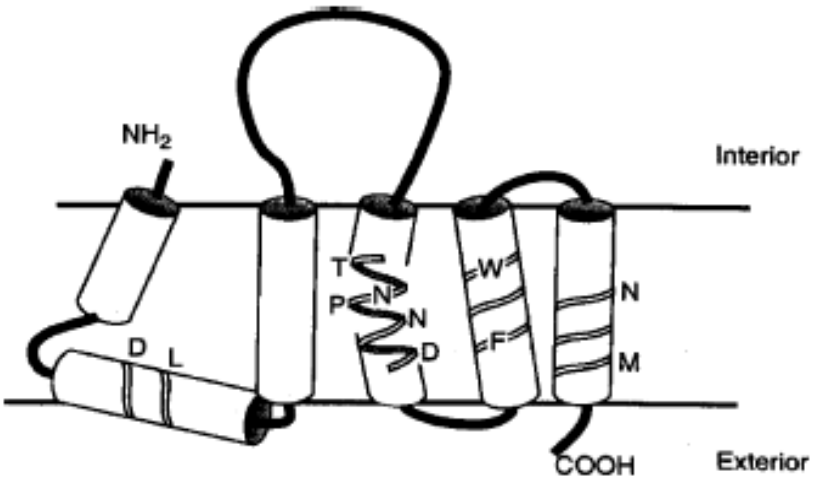


Fig 1.6. L-lysine export carrier (Eggeling & Sahm, 1999)

2.7. Microbial fermentation of L-lysine

Since microbial fermentation produces the optically active L-lysine, it is the most widely used method for amino acid production. The process economics includes raw material costs, location of the plant, fermentation yield, purification costs and overall productivity (Ikeda & Takeno, 2013). Even though majority of the amino acids in industries are produced by fermentation and follows a generalized pattern, the process flow in each industry are customized, trademarked and used exclusively by them. In amino acid production industries, the standard bioprocess involves upstream processing, fermentation downstream processing and treatment of waste water.

The industrial production starts with the procurement and testing of raw materials, and storage. The production strain maintained aseptically is tested for its purity, and its ability to produce the desired product in laboratory scale is validated at stipulated time intervals. Before initiating the seed train, the selected strain undergoes a number of vigorous purity, stability and product formation checks. The next step is enrichment in an optimal medium with the best nutrient components and physical conditions. Maintenance of sterility in enrichment process is of utmost importance as the chance of contaminants to flourish in the favorable environment is high. Once the enriched culture passes the purity and productivity test, it is transferred to fermentation vessels with subsequently increasing volumes called the seed tanks. The first seed tank usually has a volume of 1-2 m^3 (1 m^3 = 1000litres) (Hermann, 2003). The seed medium is formulated with extreme care so that it lessens the nutrient shift effects and sustains good vegetative growth of the cells. The inoculum age, size and incubation in each stage are determined so as to give optimum cell viability and density to the next seed step. The same step ensures that the cells don't have to pass through nutrient depletion and nutrient excess cycles that may hinder the optimum performance. After reaching sufficient growth a second seed tank with 10-20 m^3 capacity is seeded. The contents of the seed tank are harvested and used as inoculum for the production batch. In amino acid fermentations involving *Corynebacterium* strains, there are 1:10 steps between seed to seed (Hermann, 2003). The standard bioreactors in amino acid fermentation industries range from 50 m^3 to 500 m^3 (Eggeling & Bott, 2015; Ikeda & Takeno, 2013). The inoculum has to be monitored constantly for contamination risk or lowering of productivity which may otherwise lead to huge commercial loses. Fluctuating raw material composition from batch to batch and the change in the specifications of the production fermenter may lead to formation of unwanted or toxic products or accumulation of byproducts. Fermenters for amino acid production are usually equipped with rushton turbines alone or in combination with other mixing systems for agitation (Kelle et al., 2005). For process economics, the fermenters are scaled up to 500m^3 levels leading to high hydrostatic pressure and lower aeration rates or zones of insufficient mixing and oxygen depletion resulting in formation of unwanted products. Process modifications are done to circumvent these negative effects to some extent.

L-lysine fermentations are usually done in batch (Heng & Shiyi, 1996; Tam et al., 2015), fed batch (He et al., 2015; Modak & Lim, 1987; Schilling et al., 1999) or continuous (Hirao et al., 1989; Kiss & Stephanopoulos, 1992) modes of fermentation. But

industrial fermentations favor fed-batch or semi-continuous modes of fermentation. The production media employed are either defined media with refined pure chemicals (Kiefer et al., 2004) - inorganic salts, trace elements and biotin or alternatively complex media with natural substitutes for carbon (Xu et al., 2013) and nitrogen sources (Coello et al., 2000). The defined media for amino acid production include mineral media like CGXII (Eggeling & Bott, 2005). Some of the strains are genetically engineered to utilize mixed sugars (Cocaign et al., 1993), organic acids, alcohols, oils, glycerol etc (Meiswinkel et al., 2013; Rittmann et al., 2008). The complex carbon sources for amino acid fermentation include cane molasses, black strap molasses, starch hydrolysates or other biomass hydrolysates. Academic interest is now on utilizing waste stream from ligno cellulosic biomass pretreatments or direct utilization of the biomass. The nitrogen sources may be inorganic salts of ammonia like ammonium sulphate, ammonium nitrate , ammonium molybdate or organic sources like yeast extract, malt extract, beef extract, tryptone or corn steep liquor. Aeration, agitation and pH plays crucial role in large scale amino acid fermentations.

2.8. L-lysine overproduction

The initial L-lysine overproducers were the products of classical mutagenesis. Aspartate kinase (*lysC*) is an important enzyme catalyzing the formation of aspartyl phosphate from aspartate and is feedback inhibited by L-lysine and threonine (**Fig 1.7.**) .Deregulation of *lysC* from feedback control and overexpression of *dapA* encoding dihydropicolinate synthase resulted in L-lysine overproducers (Eikmanns et al., 1993; Sahm et al., 1995). Significant overproduction of L-lysine was first developed with a homoserine- (both threonine- and methionine-requiring) auxotrophs of *C. glutamicum* (Kinoshita et al., 1958). Since threonine and homoserine auxotrophs lack the ability to synthesize the particular amino acids, *lys C* is released from feedback inhibition by threonine resulting in L-lysine overproduction. Consequently, the aspartic semialdehyde synthesized proceeds through the L-lysine biosynthetic pathway as there are no branch points. Threonine and isoleucine auxotrophs also result in L-lysine production, but the production levels will be lower than homoserine auxotrophs (Kinoshita et al., 1958). Use of analogue resistant mutants also help in L-lysine overproduction. Mutants resistant to L-lysine analogues do not have feedback inhibition of *lysC* and hence aspartate semialdehyde is synthesized.

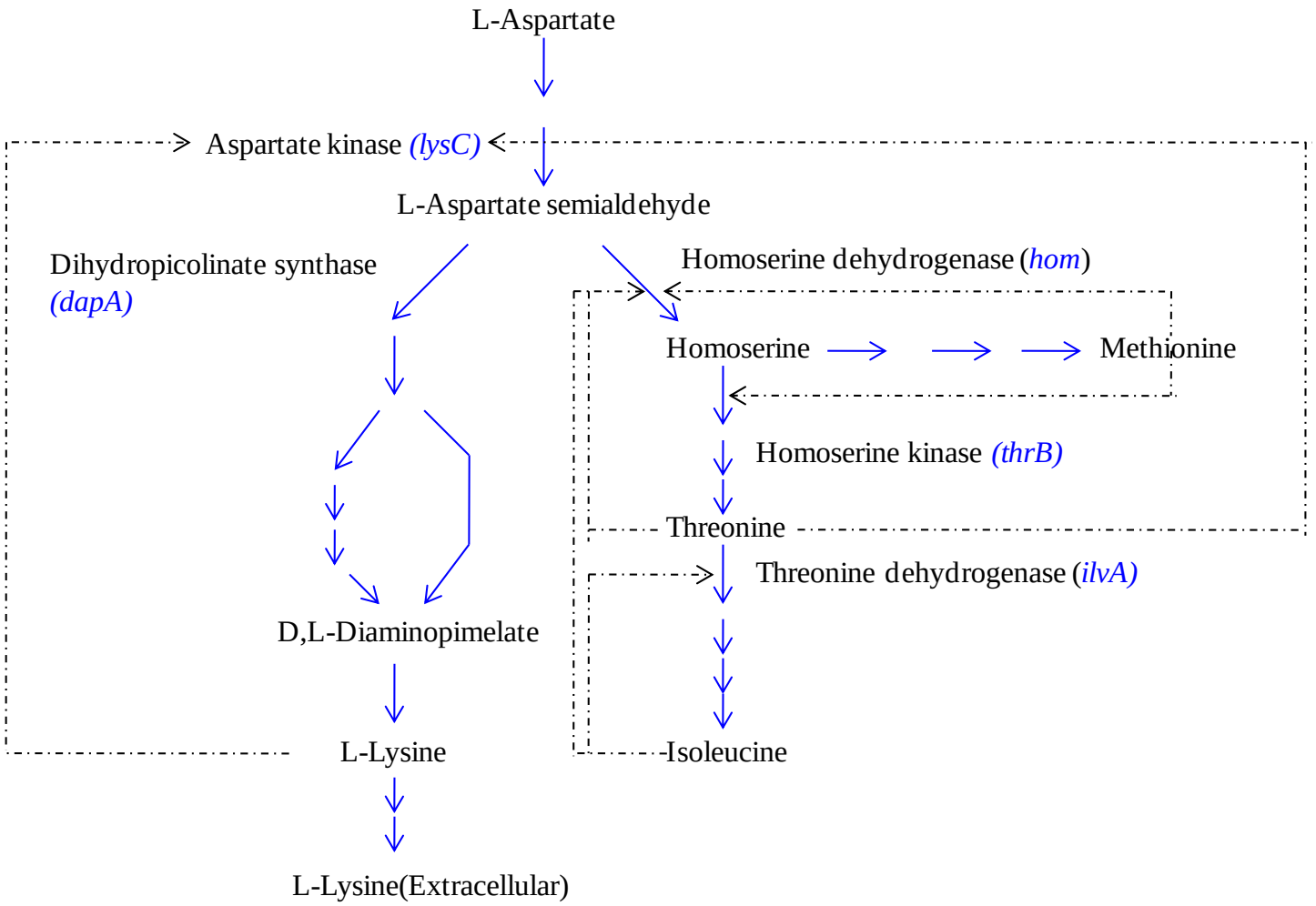


Fig1.7. Feedback regulation of aspartate family of amino acids in *C. glutamicum*. The solid arrows represent the biosynthetic pathways. Dotted arrows represent the feedback inhibition points.

Aspartate semialdehyde cannot be converted to threonine because of the feedback inhibition of threonine and thus the overproduced aspartate semialdehyde is channeled to L-lysine production (Nakayama & Araki, 1973). The production strains were further subjected to repeated mutations resulting in higher yields. But these led to general weakness of the production strain, unwanted nutritional requirements and lower stress tolerance. This led to the development of targeted mutations for product of interest. The release of aspartate kinase from feedback control is considered to be the most important feature of industrial L-lysine producers. The overexpression of enzymes in the biosynthetic pathway also led to improvements in production. Dihydropicolinate synthase (*dapA*) has been extensively studied for L-lysine overproduction (Bonnassie et al., 1990; Cremer et al., 1991). Other genes in the biosynthetic pathway *dapC*, *dapF* (Hartmann et al., 2003), *dapB* (Becker et al., 2011), *asd* (Cremer et al., 1991) were also studied. Another major breakthrough in L-lysine overproduction was the discovery and cloning of L-lysine exporter *lysE in C. glutamicum* (Vrljic et al., 1996). Metabolic engineering also aims at engineering the branch point enzymes, enzymes in anaplerotic pathways, engineering for increased precursor supply, increased NADPH or ATP supply and engineering for substrate broadening. The key driver for the developments in metabolic engineering is the availability of whole-genome analysis of the wild type and numerous conventional production strains in combination with post genomics technologies. The latter includes transcription profiling using DNA chip technology (Hayashi et al., 2002; Hayashi et al., 2006), proteome analysis using gel electrophoresis and mass spectrometry; C13 carbon flux analysis (Bartek et al., 2011; Wittmann et al., 2004; Yang et al., 2006) . The introduction of well defined gene manipulations in central carbon metabolism and L-lysine biosynthetic pathways has led to the production of L-lysine with a yield of 0.55 g/g glucose in a fed batch culture. This is the largest realized L-lysine yield from microbial fermentation (Becker et al., 2011).

2.9. Bioprocess improvement

Bioprocess improvement works by either strain improvement or process improvement. Strain improvement programmes have been instrumental even in the production of first L-lysine over producer. However, process modifications have also contributed to the overall increase L-lysine yields and technological developments. Fermentation parameters like media components; physical parameters like aeration or

oxygen concentration, pH, temperature, foaming etc were manipulated so as to realize improved L-lysine yields since the conception of the L-lysine overproducer till now (Nasri et al., 1989). Initial L-lysine fermentations used cane molasses or invert as carbon source (Nakayama & Araki, 1973). Scores of patents have been filed which claimed to use different sugar sources for L-lysine fermentation (Anastassiadis, 2007). Fermentation methods including batch (Heng & Shiyi, 1996), fed-batch (Weuster-Botz et al., 1997) and continuous cultures (Ensari & Lim, 2003; Nam-Soon & Manfred, 1993) were evaluated for L-lysine fermentation. Special reactor designs and mimicking industrial reactors for L-lysine production were developed for use in different fermentation modes (Neubauer, 2015; Schilling et al., 1999). Monitoring of physiological and metabolic states of the microorganisms led to better understanding of the fermentation process (Kiss & Stephanopoulos, 1991; Takiguchi et al., 1997) and hence improving production titres. Use of immobilized cells for L-lysine production also has been reported from the 1980s until recently (Nasri et al., 1989; Razak & Viswanath, 2015). L-lysine production has also been experimented with a number of carbon sources as described below.

2.9.1. L-lysine production from alternate carbon sources

Substrate range for L-lysine production is very vast including dextrose, sucrose, maltose and fructose. Organic acids such as acetic acid, propionic acid, benzoic acid, formic acid, malic acid, citric acid and fumaric acid; alcohols such as ethanol, propanol, inositol and glycerol and hydrocarbons, oils and fats such as soybean oil, sunflower oil, groundnut oil and coconut oil as well as fatty acids such as palmitic acid, stearic acid and linoleic acid were also used. These substances may be used individually or as mixtures (Anastassiadis, 2007). An array of non defined sugar substrates like cane and beet molasses, blackstrap molasses and starch hydrolysates were used for industrial fermentation (Ikeda, 2003). Use of cheap and easily available substrates which has low or zero competing value as food can provide an alternative carbon source. The experimental substrates used for L-lysine fermentation has evolved over the decades. The substrate utilization of *C. glutamicum* is depicted in **Fig 1.8.** The initial fermentation experiments used refined sugars. From glucose the reported yields of L-lysine are in the range of 50-55 %. Later on, hydrolysates of starchy biomass, molasses or sugar syrups were used. The starch hydrolysates were products of either enzymatic or acid hydrolysis. Molasses are

rich in glucose, sucrose and has 10-20% fructose and hence has more fermentable sugars resulting in a conversion of 30-40%.

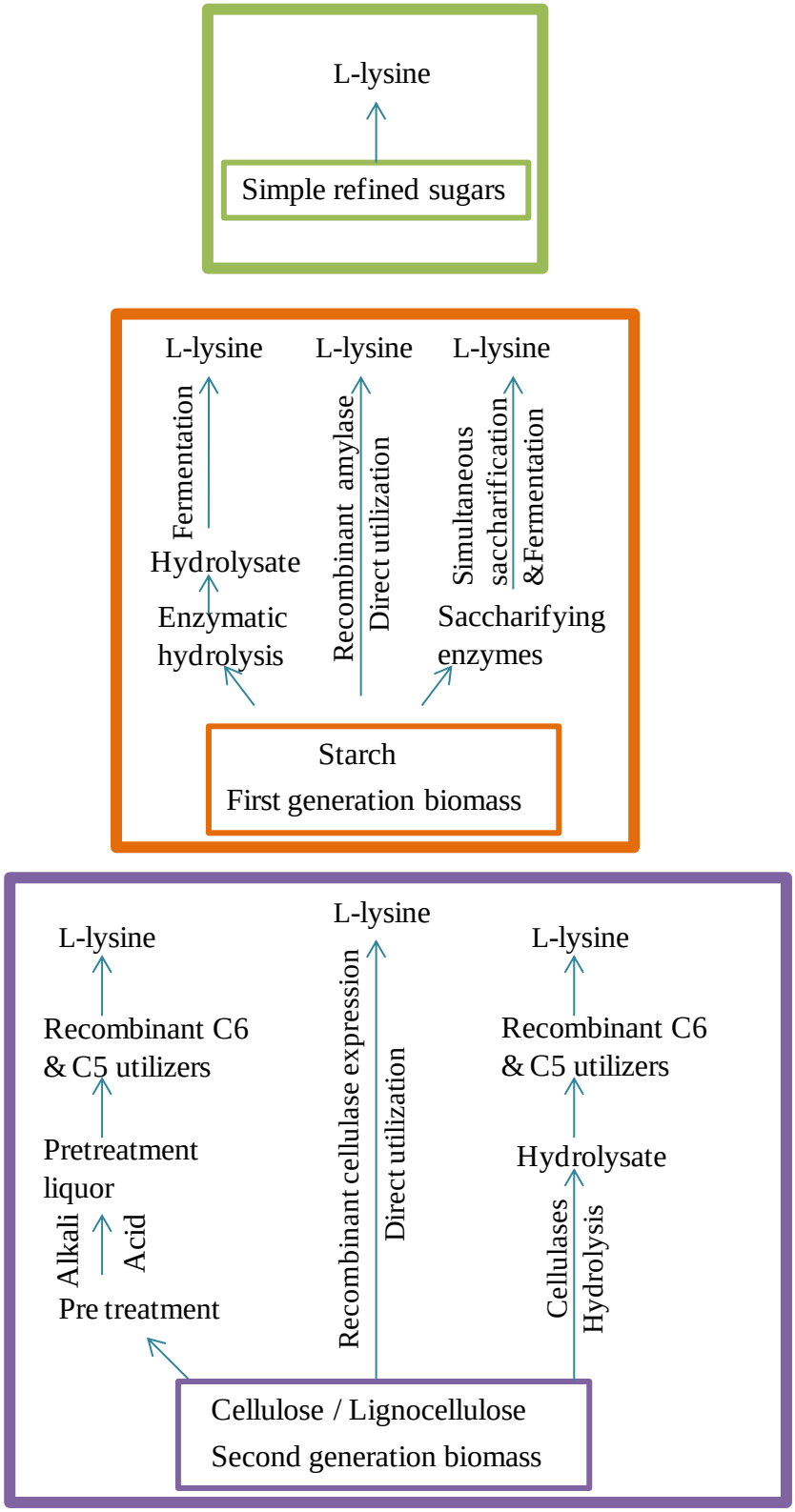


Fig1.8. Substrate utilization of *C.glutamicum* for L-lysine fermentation

Fermentation with starch hydrolysates of cereals, rap, flax and cotton accumulated 30-40 g/L of L-lysine in the medium. As next step, researchers cloned starch saccharifying enzymes in *C.glutamicum* which were expressed either extracellularly or on the cell surface (Seibold et al., 2006; Tateno et al., 2009). *C. glutamicum* engineered for starch utilization showed 19% conversion from soluble starch and 14% from raw corn starch (Tateno et al., 2007a; Tateno et al., 2007b). With the competition on food by the use of starchy biomass for L-lysine fermentation, the substrate range has been broadened to second generation biomass. Lignocellulosic biomass is considered as the cheap, sustainable and renewable feed stock of the decade as it generally avoids competition as food and fodder (Saxena et al., 2009). Concentrated efforts are not given for lignocellulosic biomass generation as it is usually agricultural, industrial or farmyard waste or forest forage. Due to the ease of access and low cost, there was always labored efforts towards utilization of lignocellulosic biomass for production of bio based fuels and chemicals. Lignocellulose derived sugars are a mixture of pentose and hexose sugars. Recently *C. glutamicum* has been engineered to co-utilize hexoses and pentoses. L-lysine yield by the pentose sugar utilizing constructs of *C. glutamicum* was 16% from wheat bran hydrolysate and 15% from rice straw hydrolysate (Gopinath et al., 2012). Most of the bacterial bioproduct producers lack the inherent ability to naturally degrade the cellulose chains to assimilable sugar monomers and hence lignocellulosic biomass is underutilized in this direction. On the other hand, the feed stock has to be extensively pretreated and hydrolysed so as to be accessible for the microbes. To fully capitalize on the available lignocellulosic feed stock for production of products of interest, the target microbes has to be engineered for heterologous expression of cellulose degrading enzymes or cellulases (Lynd et al., 2002). Expression of recombinant cellulases in *Corynebacterium glutamicum* for direct utilization of cellulose includes endoglucanase and betaglucosidase. Betaglucosidase displayed on cell surface showed 5% conversion from cellobiose (Adachi et al., 2013). Furthermore, endoglucanase and betaglucosidase has been co expressed in L-lysine producers (Kim et al., 2014). As cellulase is an enzyme complex , more component enzymes like cellobiohydrolases and xylanases has to cloned for complete conversion of cellulose to glucose and hence L-lysine fermentation.

For industrial fermentations, the substrates are selected based on the geographical location and availability of raw materials. Molasses are used in Europe, China and South

America whereas corn syrup is used in North America. However, other complex substrates like cane molasses, beet molasses, or starch hydrolysates of corn, wheat or cassava has been in use (Hermann, 2003; Ikeda, 2003; Kelle et al., 2005; Kimura, 2005). Since the budget head of the substrates and its logistics are the key variable costs in the industrial amino acid fermentations, there is always look out to use locally available, cheap and even complex raw materials more efficiently. The cost of carbon source is the most important variable in industrial amino acid fermentation. Even with the introduction of microbial biorefineries and organisms engineered to utilize broad spectrum of substrates, industrial L-lysine fermentation still use cereal starch hydrolysates or dextrose syrup. This indicates the efficacy, robustness and reproducibility of the production process. Apart from the strain improvement studies which has enabled substrate broadening (Gopinath et al., 2012) research interests are in lowering the production cost, increasing the productivity, decreasing co-product formation and better utilization of inexpensive carbon and nitrogen sources (Nielsen, 2001).

2.10. Downstream processing

Downstream processing of the amino acid from the fermentation broth is one of the important steps in the product development. The initial step is the removal of biomass by centrifugation, filtration or ultra filtration. Once the biomass-free liquor is obtained, it is purified by chromatographic techniques, crystallization methods, salting out, liquid phase pertraction (Boyadzhiev & Atanassova, 1992), reverse phase osmosis (Kaneko et al., 1986), concentration, and drying. Salting out crystallization is a process in which the product of interest dissolved in a liquid mixture is precipitated due to change in solubility by adding small amounts of salts (Sifniades & Tunick, 1975). The chromatographically purified amino acids have to be further recrystallized and decolorized to improve the quality and purity.

Industrial L-lysine purification uses ion exchange chromatography. There are several patents on L-lysine purification (Murata & Nagano, 2008; Norman, 1959; Walter, 1959). Ion exchange columns may be single or multiple connected in series, moving bed etc. Ion exchange chromatography works on the preferential adsorption of solute molecules on the ion exchange matrix and the selective elution based on change in ionic strength. Cation exchange resins have negative charges on the surface and have affinity to positively charged molecule. On the contrary, anion exchange resins bear positive charge.

The solute molecules bound to the ion exchange resins elute at a pH higher than the PI. L-lysine will have a net positive charge of 2 at a pH below 2 and can strongly bind to a cation exchange resin. As the pH increases, the hydrogen ions dissociate according to their pKa values and the net charge decreases. At a pH higher than the PI, L-lysine starts eluting. The dissociation constants of hydrogen ions in L-lysine are denoted in **Table 1.3.** Theoretically, L-lysine should adsorb on the resin at a pH below 9.7 (PI of L-lysine) and elute at any pH above it in cation exchange chromatography.

Table 1.3. Dissociation constants of L-lysine

Hydrogen ion	pKa value
αCOOH	2.18
αNH_3^+	8.95
εNH_3^+	10.53

Potassium, magnesium, calcium and sodium ions constitute a major share of ionic impurity in the L-lysine stream after primary ion exchange step. This mother liquor containing L-lysine is wasted after separating the L-lysine crystals. To avoid this, the eluate after the primary ion exchange step is passed through one or two ion exchange beds which may be stationary, simulated moving bed, countercurrent rotating fixed bed or counter current moving bed. This process reduces the loss of L-lysine in industries (Fechter et al., 1997; Itoh et al., 1994; Soper et al., 2002). Use of suitable primary and secondary ion exchange resins are used in the single column or multiple column L-lysine purifications wherein the solution is passed through ion exchange columns in series until the desired ratio of amino acid to impurities is reached. Strong acid cation exchange resins includes DOWEXXUS 43518, XUS 40406, DOWEX C500ES manufactured by The Dow Chemical Company; Amberlite IR 120, Amberlite IR 120 Plus, Amberlite 200, Amberlite IR 118H, Amberlite IR 122, Amberlite IR 130C by Rohm and Haas etc (Binder, 2008; Fechter et al., 1997; Itoh et al., 1994; Jaffari et al., 1989; Murata & Nagano, 2010; Murata & Nagano, 2008) .The strong acid cation resins are typically sulphonated copolymers of styrene and divinyl benzene. The weakly acidic cation exchange resins are usually acrylic acid-based or methacylic acid-based resins. Examples are Amberlite IRC50 and Amberlite IRC76 by Rohm and Haas, DIAION WK10, DIAION WK11, DIAION WK20 and

DIAION WK40 from Mitsubishi Chemical Corporation, MAC-3 from The Dow Chemical Company, CNP80, CNPLF and CNP105 from Bayer Corporation etc.

Following ion exchange purification, the purified fraction is crystallized using cooling crystallization, evaporation crystallization, vacuum crystallization etc. The purified solution is cooled at a rate of 3-6 ˚C per hour and ethanol is added to the stock solution to relax the environment and thus producing uniform high quality crystals (Wang Cheng, 2014). Alternatively, the ion-exchange purified liquid is subjected to concentration crystallization at 43-60 ˚C and the resultant product subjected to solid-liquid separation and then cooling crystallization in a tank (Luo Hu, 2014).The crystals thus obtained are of high purity and desired purity can be obtained by repeated crystallization and recrystallization.

Drying is another unit operation in amino acid downstream processing. Drying is accomplished by various drying equipments like the spray granulator, spray dryer, tray dryer, drum dryer, rotary dryer, and tunnel dryer etc. This results in the deactivation of microbes and a stable L-lysine preparation.

Different grades of amino acids, with varying purity are prepared by simplified downstream processing techniques. For example, an amino acid preparation called "Biolys" is made which is the evaporated and spray-dried fermentation broth including the biomass. It has application in the feed segment and has the advantage of increased feed value due to the other amino acids and minerals in the preparation. As the fermentation is carried out with *C. glutamicum*, with the GRAS status, the biomass in the feed does not pose any risk on animal health. Another L-lysine powder with 70 % L-lysine dry base powder is also commercialized (Choi et al., 2002). A liquid L-lysine-HCl preparation of Ajinomoto called LL-60 contains 60 % L-lysine. ADM sells feed grade L-lysine with a purity percentage of 98.5 % and also liquid L-lysine 50 with 50% L-lysine.

2.11. General methods for amino acid detection.

Amino acid analysis includes methods that use underivatized samples and derivatized samples. Analysis methods use Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Capillary electrophoresis, Electrochemical detection and amino acid detection kits.

Gas Chromatography (GC) method uses alkyl chloroformate (RCF) mediated derivatization of amino acids to GC amenable forms. Alkyl chloroformates in alcohol-pyridine based media is used for the dervatization process. The alkyl groups may be methanol, ethanol, butanol, isobutanol or propanol (Kim et al., 1997; Sobolevsky et al., 2003; Wang et al., 1994). The GLC (Gas-liquid chromatography) quantification of amino acids was established in the 1970s. In this method, liquid act as the stationary phase and gas is the mobile phase. The stationary phases used to be polar or non polar solvents adsorbed on matrices like Chromesorb W, Gaschrome Q etc. Amino acids were esterified to N-trifluoroacetyl *n*-butyl esters and quantified. A method was developed for the quantification of 20 protein amino acids in a single run (Gehrke et al., 1971). Protein hydrolysates are cleaned up using an ion exchange column, esterified and then quantified on a GLC column. GLC quantitation was used for amino acid profile of soil samples from the Apollo mission. GC for amino acids involves enantiomeric separation and quantification of the amino acids. Amino acids are first derivatized into GC resolvable compounds (Dołowy & Pyka, 2014). Either the –COOH or the -NH_2 group is derivatized.

HPLC is the most widely used and reliable method for amino acid quantification. Amino acids are non volatile compounds with little or no UV absorbance and are hence difficult to detect in their native form. So for HPLC the amino acids are derivatized into UV chromophores or fluorescent chromophores. However, detection of amino acids in the native form has been realized by ELSD (Evaporative Light Scattering Detector), CLN (Chemiluminescent Nitrogen), MS (Mass Spectrometry) and LC-MS (Liquid Chromatography-Mass Spectrometry), NMR (Nuclear Magnetic resonance), RID (Refractive Index Detector) etc (Petritis et al., 2002). Direct amino acid detection in its underivatized form has also been reported using C-8, C-18 or graphite column.

In HPLC detection, amino acids can be derivatized for improved detection. This can be done prior to elution called the ‘pre-column derivatization’ or after the elution step called ‘post-column derivatization’. Advantages and disadvantages of both methods are detailed in **Table 1.4.**

Table 1.4. Advantages and disadvantages of pre-column and post-column derivatization of amino acid.

Derivatization Type	Advantages	Disadvantages
Pre-column derivatization	Low reagent consumption Simpler instrument configuration High speed detection High sensitivity Allows selection of derivatizing agent Allows RP-HPLC	Unstable reaction products Sensitivity affected by sample matrix Suitable for known samples with limited components.
Post-column Derivatization	Automated reaction High reproducibility Good for quantitation Derivatization not affected by sample matrix	High reagent consumption Longer run time Low sensitivity Suitable derivatizing agents are limited Do not favor RP-HPLC

Derivatization with the chemical compounds offer advantages like improved detection, improved resolution, better selectivity and manipulation of the optical properties so as to use a preferred solvent which may result in lower retention times and better resolution.

PITC (Phenyl Isothiocyanate) is used as a derivatizing agent which converts amino acids into Phenylthiocarbamyl derivatives. Derivatization takes 5-10 minutes at room temperature. The phenylthiocarbamyl derivatives are dissolved in 0.05 M ammonium acetate, pH adjusted to 6.8 and injected onto the C8 or C18 column. Detection was at 254 nm. Elution was completed in 30 minutes with a gradient program of increasing concentrations of ammonium acetate and acetonitrile or methanol. This method is advantageous over the classical ion exchange separation and other methods which employ pre-column derivatization in precision and sensitivity. All amino acids, including proline,

are converted quantitatively to phenylthiocarbamyl compounds and these are stable enough to eliminate any need for in-line derivatization (Heinrikson & Meredith, 1984).

Dansyl chloride or 5-DimethylaminoNaphthalene-1-Sulfonyl chloride is a derivatizing agent that reacts with primary amino groups in both aliphatic and aromatic amines to produce stable blue- or blue-green–fluorescent chromophores. The method is called dansyl chloride derivatization or dansylation. Gradient elution of dansyl chloride derivatives on a C-18 column with acetonitrile/water resulted in the elution of amines in red wine. A Diode Array Detector is usually used for detection (Mo Dugo et al., 2006; Smith & Davies, 1985; Tapuhi et al., 1981). Dansyl amino acids absorb light in the UV region. Dansyl group is also fluorescent and dansyl amino acids can therefore be detected by a fluorimetric detector. The excitation and emission wavelengths for dansyl glycine are 324 and 559 nm, respectively.

Dabsyl chloride (4-N, N-Dimethylaminoazobenzene-4'-Sulfonyl chloride) that reacts with primary and secondary amines to form derivatives that is detectable in the visible range (420-460 nm). This detection range helps in eliminating other biological interferences. Dabsyl chloride derivatization is advantageous over other methods in simple derivatization procedure, stability, reproducibility, detection limit, separation of amino acids, and detection range. The dabsyl derivatives are separated on a C-18 column with a gradient of buffers like sodium phosphate or sodium acetate and acetonitrile or methanol (Krause et al., 1995; Lin & Lai, 1980; Lin & Wang, 1980; Vendrell & Avilés, 1986)

A carbamate derivatizing agent, 6-aminoquinolyl-*N*-hydroxy succinimidyl carbama te, is also used in amino acid derivatization for detection. The carbamate reacts with amino acids to form stable unsymmetric urea derivatives .These compounds can be eluted with reverse phase HPLC. The separation of the compound can be made on a C-18 column with a run time of 35 minutes and detection by a fluorescence detector with excitation at 250 nm and emission at 395 nm (Cohen & Michaud, 1993)

OPA (*O*-phthalaldehyde) is a widely used derivatization agent used in amino acid analysis (Dai et al., 2014). OPA reacts only with primary amines. It react with the amino group at a basic pH (9-11) resulting in an isoindole derivative. The reaction product is a fluorogen having an excitation wavelength of 340 nm and excitation wavelength of 455 nm. OPA derivatization can be employed in pre-column or post-column mode and the derivatized amino acids are detected using a fluorescence detector. The OPA derivatives

rapidly degrade to non-fluorescent degradation products and hence the derivatization reaction has to be done immediately before the detection. Using an automated amino acid pretreatment program and the use of stabilizers like 3-mercaptopropionic acid or N-acetyl L-cysteine provide stable fluorescent derivatives for analysis.

Ninhydrin is another oxidizing agent widely used in the detection of amino acids. Ninhydrin reacts with the free α-amino group of amino acids and produce a purple blue coloured compound. This dye is called Ruhemann's purple with maximum absorption at 570nm (Friedman, 2004). The major drawback is that all amino acids react with the reagent and individual quantitation is difficult. Moore and Stein developed the first amino acid analyzer employing post-column derivatization with ninhydrin (Moore & Stein, 1963). This method was later replaced by more sophisticated amino acid analytical instruments.

The amino acids can also be quantified by employing different colorimetric assays (eg.ninhydrin, 8-hydroxy quinoline), microbiological assays (John Boyd, 1948) or amino acid assay kits.

2.12. Future perspective

Researchers have devised various methods to understand the central carbon metabolism and L-lysine metabolic pathway and how to manipulate it for overproduction. The whole genome sequencing of *C. glutamicum* has been an important step in the last decade. But we have not yet understood the intricacies about why some specific genes over expressed in random mutagenesis outside the biosynthetic pathway, lead to amino acid overproduction. Also there is need to characterize the novel functions operating under amino acid overproduction. In addition, in the wake of awareness to tackle global warming, the amino acid industry is now moving towards sustainable and eco-friendly manufacturing processes. There is a strong positive drive towards developing strains that utilize agro residual biomass that does not compete with food and energy needs. Moreover, amino acid production strains with high production rates and process that can reduce effluents and wastes generated during fermentation have to be developed for environmental sustainability. These remain issues to be addressed in future.

2.13. Conclusion

The amino acid production industry will remain as a fast growing business enterprise until a cheaper and energy efficient process is commercialized. Microbial amino acid production will be exploited to the most possible extend of maximal production capacity and process economics by new innovations in strain improvement, metabolic engineering, and systems biology. Fermentative production of amino acids holds a promising position in the future of white biotechnology and studies on utilization of feedstock for production make it a part of the biomass biorefinery concept. The detailed understanding on the biosynthetic pathways, the enzymes and their mode of action, the sugar uptake systems, and the cell physiology of the amino acid producers has enabled to tailor the microorganisms for maximal production capacity.

Chapter 2
MATERIALS AND METHODS

The chapter describes the general materials and methods used throughout the study.

2.1. General Chemicals and Analytical Columns

All general chemicals were procured from Sigma (India), Merck (India), Himedia (India) and SRL (India) unless indicated otherwise. All dehydrated media and agar were procured from Himedia, India. Components of amino acid production medium, CGXII were procured from SRL, India.

The amino acid analysis column (Agilent Zorbax Eclipse AAA, 4.6 X 150 mm) was purchased from Agilent technologies, India. Amino acid derivatization agent ninhydrin was obtained from Sigma and OPA (o-phthalaldehyde) from Agilent Technologies. Amino acid standards, sodium phosphate monobasic and sodium hydroxide used in amino acid analysis were procured from Sigma and borate buffer from Agilent Technologies. HPLC solvents were from Merck.

The sugar analysis column (Rezex RPM Monosaccharide Pb^{+2} column 7.8 X 300mm) was purchased from Phenomenex, India. The sugar standards were purchased from Sigma. Molecular biology chemicals were procured from Thermo Fischer Scientific, Fermentas and New England Biolabs (NEB). Ion exchange resins were purchased from SRL, India.

2.2. Substrates and Processing

2.2.1. Substrates

Jackfruit seed powder (JFS), Cassva bagasse (CB), Coconut oil cake (COC), Potato peel (PP), Wheat bran (WB), Sugarcane bagasse (SB), Rice straw (RS), Sugarcane tops (SCT), Poly urethane foam (PUF) and Bamboo Ppowder (BP) were used as substrates for different studies conducted. All substrates except CB were procured from the local market. CB was obtained from Varalakshmi starch company, Tamil Nadu.

Among the substrates JFS, CB, COC, PP and WB were used for initial screening and selection of best substrate for L-lysine production. PUF, SCT, SB and bamboo powder were used as substrates in Solid-State Fermentation (SSF).SCT and RS were used as substrate for enzymatic hydrolysis by the recombinant *C. glutamicum* DM 1729 expressi ng cellulose degrading enzymes.

2.2.2. Substrate processing

2.2.2. i. Drying and milling

Jackfruit seeds were washed and dried at 70 °C for 24 hours in a drier (RRL-T NC drier, India). Seeds were powdered in a knife mill after removing the aril, and separated into desired size (≤ 0.425 mm) by particle size separating meshes. PP was peeled manually from potato and dried at 70 °C for 48 hours in a drier and powdered to particle size of ≤ 0.425 mm. COC and WB were powdered in a mill and separated into particles ≤ 0.425 mm by particle size separating mesh. Sugarcane bagasse (SB) was milled and washed with hot water to remove the adhering unwanted solid particles, soluble sugars and contaminants. It was then dried in hot air oven at 60 ˚C for 24 hours .The processed substrate was then separated into fine (< 0.5 mm) and coarse particles (0.5 mm -1 mm) by particle size separating meshes. This was used as the inert matrix for SSF. Rice straw, bamboo and sugarcane tops were dried and powdered in a knife mill to a particle size ≤ 1 mm. PUF was cut into cubes (1cm x 1 cm), washed thoroughly with distilled water and dried. All the substrates were sealed in polythene bags till further use.

2.2.2. ii. Hydrolysis of starchy agro residues

Commercial starch hydrolyzing enzymes, viz, alpha amylase (Termamyl 120L from Novozymes) and glucoamylase (Rashesh & Co. Mumbai, India) were used for starch hydrolysis. Starchy substrates (9 % w/v) JFS, CB, COC, PP and WB were gelatinized at 121 °C for15 minutes. Alpha amylase was used for liquefaction (90 °C for 40 minutes, pH 6.0) and the substrates were further saccharified with glucoamylase (60 °C for 60 minutes, pH 5.0).The hydrolysate so obtained was filtered through a muslin cloth. The filtrate was centrifuged at 8000 rpm for10 minutes to obtain the starch hydrolysate. The starch hydrolysates were used as sugar source for L-lysine fermentation as described in respective chapters of the thesis.

2.2.2. iii. Pretreatment of lignocellulosic biomass

Lignocellulosic biomass is hard to be broken down to monomeric sugars by direct enzymatic hydrolysis. Thus the biomass is pretreated by chemical or physical means so that the cellulose portion is accessible for enzymatic hydrolysis. Thus RS and SCT were pretreated, before subjecting to enzymatic hydrolysis by recombinant cellulase enzymes. The milled and processed biomass was subjected to dilute acid pretreatment with sulphuric acid (4 % w/w) at 121˚C for 60 minutes at a solid loading of 15 % w/w. The biomass so obtained was cooled, neutralized with 10 N sodium hydroxide, wet sieved and air dried. The pretreated biomass was used to test the hydrolytic efficiency of recombinant endoglucanase and betaglucosidase enzymes explained in Chapter 7.

2.3. Microbiological Methods

2.3.1. Bacterial strains-conservation and cultivation

Corynebacterium glutamicum DM1729 and derived strains were used throughout the study. It differs from the wild type strain, *C. glutamicum* ATCC13032 by point mutations in aspartokinase, pyruvate carboxylase and homoserine dehydrogenase genes (*lysC* ^{P458S}, *hom*^{V59A}, *pyc* ^{T311I}) (Georgi et al., 2005). The culture was procured as a gift in a collaborative project from Wendisch lab, University of Bielefeld, Germany.

For general propagation the strain was grown in LB with 0.5 % w/v glucose (LBG) for 18 hours at 30 ˚C. The derived strains were incubated with appropriate antibiotics and induction with IPTG concentrations up to 1 mM. The pre cultures grown in LBG was centrifuged at 6000 rpm and 4 ˚C for 10 minutes and transferred to CGXII minimal medium to an initial concentration of OD_{600} of 1.This was used as the inoculum in all the studies described throughout the thesis unless otherwise mentioned.

Bacterial strains were preserved in 40 % glycerol at - 80 ˚C for long term storage and maintained on LB agar plates supplemented with 0.5 % w/v glucose for short term preservation at 4˚C.

All the bacterial strains used in the study were grown in LB medium supplemented with 0.5 % w/v glucose and incubated at either 37 ˚C or 30 ˚C and 200 rpm or on agar plates. Strains harboring plasmids were incubated with appropriate antibiotics like ampicilin (100 µg/mL), spectinomycin (100 µg/mL) or kanamycin (25 – 50 µg/mL).

2.3.1. i. Bacterial growth measurement

Growth of the bacterial strains was followed by reading absorbance of suitably diluted samples at 600 nm in a spectrophotometer (Shimadzu 160 A) and represented as OD_{600}. For *C. glutamicum*, the weight of biomass was calculated by an experimentally determined correlation factor of 0.25 g dry cell weight for OD_{600} of 1 (Wendisch et al., 2000).

2.3.2. Gram staining

Grams' staining was used to visualize the bacterial strains under microscope and check for possible contamination before each set of experiments (Gram, 1884). A thin film of the culture was smeared on a glass slide and heat fixed. The slide was flooded with crystal violet and incubated for 60 seconds. This was washed off in indirect stream of running water and fixed in iodine for 60 seconds. The excess stain was decolorized in absolute ethanol and counter stained with safranin for 30 seconds. After clearing the slide in indirect stream of running water, it is air dried and visualized under microscope (Leica DM 2000). The gram positive bacteria appeared purple.

The difference in the cell wall composition of gram positive and negative bacteria distinguishes between the two in this staining procedure. Gram positive bacterial cell wall has thick peptidoglycan layer which retains the purple color of crystal violet. Crystal violet in aqueous solutions dissociates into ions and the positively charged ions interact with the cell membrane and cell wall of bacteria and stain them purple. The mordant, iodine reacts with crystal violet to form crystal violet-iodine complex which are leached out of gram negative cells on decolorization with ethanol/acetone. While in gram negative bacteria, the peptidoglycan layer is dehydrated on acetone/ethanol treatment and the crystal violet – iodine complex gets blocked and visualized as purple cells.

2.3.3. Scanning Electron Microscopy

The growth of *C. glutamicum DM1729 in* submerged condition and growth on sugarcane bagasse in solid state fermentation was examined using a scanning electron microscope (JEOL JSM 5600LV, 115 Japan). The respective culture samples were diluted and smeared on a glass cover slip. The smear was fixed using gluteraldehyde in phosphate buffer and subsequently dried in increasing concentrations of ethanol from 10 % v/v to 100 %. The preparation was mounted on a brass stud with the bacterial cell preparation

facing outward. This was subjected to a mild gold coating (0.01 μM) and was subjected to electron microscopy at an accelerating voltage of 10 kV. The scanning electron microscopic photograph of *C. glutamicum* are shown in chapter 1 (**Fig 1.4.)** & (**Fig.5.3.)**

2.3.4. L-lysine production medium and fermentation

CGXII medium (Eggeling & Bott, 2005) supplemented with glucose or different carbon sources or starch hydrolysates of agro residues was used as the fermentation or production medium which will be discussed in the respective chapters. A complex amino acid production medium, M2 was used in the initial screening for L-lysine production. M2 medium was used in the native form with 4 %w/v glucose or starch hydrolysates of different agro residual substrates for fermentation. All the media compositions are outlined in Annexure 1.

Unless otherwise mentioned fermentation was carried out in CGXII medium supplemented with different carbon sources at 200 rpm and 30 ˚C with an initial OD_{600} of 1. The flask level experiments were carried out in 250 mL Erlenmeyer flasks with a working volume of 25 mL. The process conditions when the fermentations were scaled up are described in the respective chapters.

2.4. Analytical and Biochemical Methods

2.4.1. Moisture analysis

Moisture measurements of the substrates were done on a moisture analyzer which worked on the change in weight of the substrate on heating (AND MX-50, Japan)

2.4.2. Sugar estimation

2.4.2. i. Total Reducing Sugar estimation by DNS assay

Total reducing sugar was analyzed by DNS assay according to (Miller, 1959). DNS assay works on the principle of reduction of 3,5-dinitrosalicylic acid by the aldehyde group of the sugar molecule to 3-amino,5-nitrosalicylic acid under alkaline conditions. Appropriately diluted samples are treated with DNS reagent and boiled in a water bath for 15 minutes. The chemical reaction result in the formation on an orange-red complex whose absorbance is read at 540 nm. Glucose was used as the standard for the assay (**Fig 2.1.**).

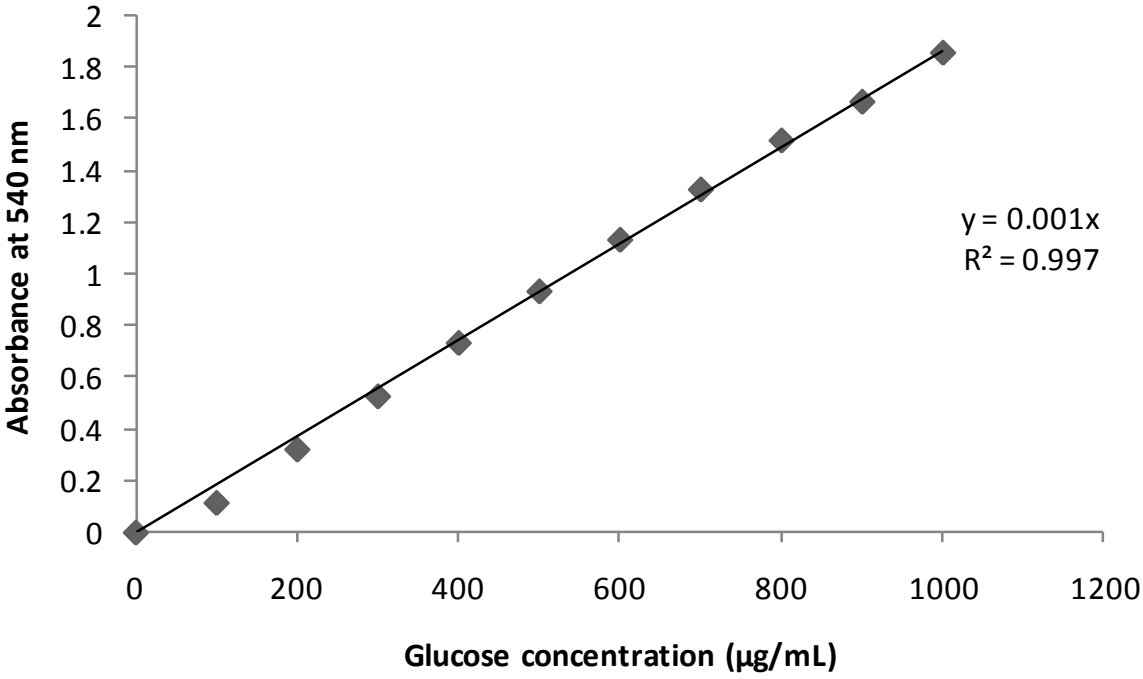


Fig.2.1. Standard graph of DNS assay for total reducing sugar

2.4.2. ii. Sugar estimation by HPLC

The monomeric sugars were also analyzed by Shimadzu Prominence UFLC using Rezex® RPM Monosaccharide Pb^{+2} column (300 x 7.8mm, Phenomenex, India). Appropriately diluted and filtered samples were injected using an auto sampler program and eluted with deionized water as the mobile phase. The column temperature was maintained at 80 $^{\circ}$C and the flow rate was 0.6 mL/min. The sugars were analyzed by RI detector connected to the HPLC. The monosaccharides and disaccharides released during starch hydrolysis and the sugars released during cellulose hydrolysis by recombinant cellulases were quantified using the above method.

2.4.3. Protein estimation

Soluble proteins were analyzed using the Bradford assay (Bradford, 1976). The assay depends on the amino acid composition of the measured protein and is quantified on the development of blue color shift developed by Coomasie Brilliant blue G-250 on binding with the amino acids. The absorbance was read at 595 nm (**Fig 2.2.**). Bradford assay was used to determine the soluble protein concentration of jackfruit seed powder.

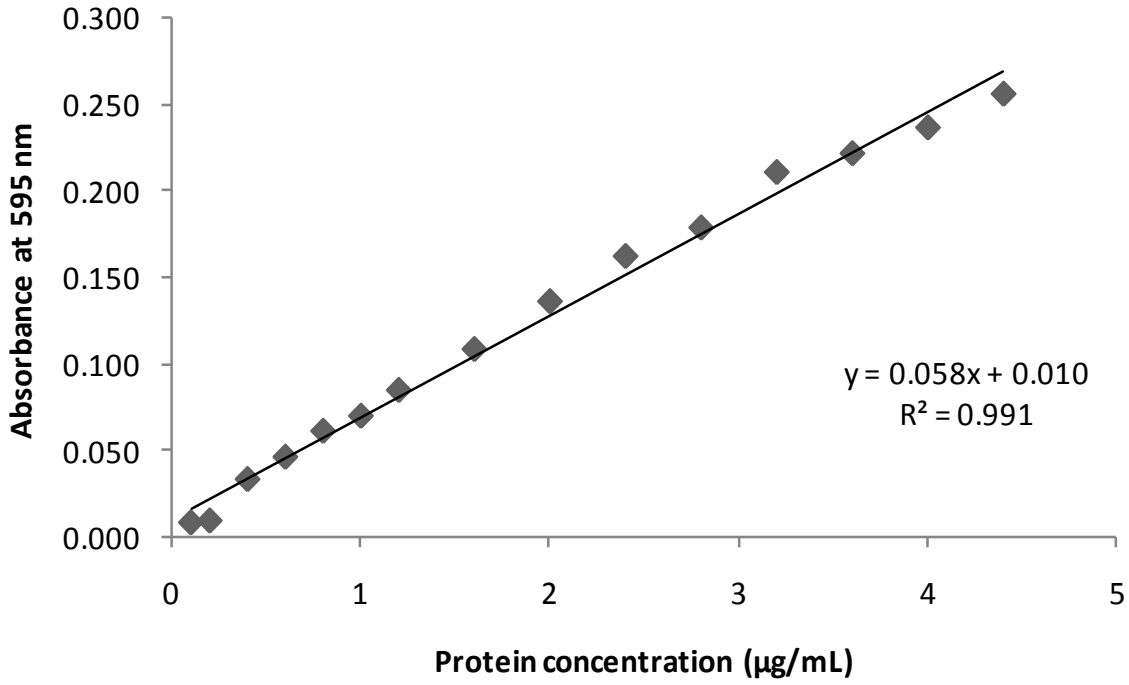


Fig.2.2. Standard graph of Bradford assay for soluble protein

2.4.4. Enzyme assays

2.4.4. i. Amylases

Alpha amylase and gluco amylase activity was determined according to Okolo et al. (1995). The reaction mixture had a total volume of 2 mL and consisted of 1.25 mL of 1 %w/v soluble starch, 0.5 mL of 0.1 M sodium acetate buffer (pH 5.0), and 0.25 mL of enzyme. The mixture was incubated at 60 °C for 10 minutes and the resultant reducing sugars were estimated by DNS assay. Glucose was used as standard. The blank contained 0.75 mL of 0.1 M acetate buffer (pH 5.0) and 1.25 mL of 1 %w/v starch solution. One unit of alpha amylase activity was defined as the amount of enzyme releasing one µmol of glucose equivalent per minute under the assay conditions. The assay was used to calculate enzyme activity of amylases used for starch hydrolysis of agro residual substrates.

2.4.4. ii. β - glucosidase assay

β-glucosidase assay was performed using p-nitrophenyl β-D glucopyranoside (pNPG) (Sigma-Aldrich, India) as substrate with modifications from the standard protocol

(Ghose & Bisaria, 1987). The assay was carried out in a total volume of 250 µL in a microtitre plate. Appropriately diluted enzyme sample was incubated with 10 mM pNPG in citrate buffer (0.05 M, pH 4.8) at 50 °C for 10 minutes. The reaction was terminated by adding 1M Na_2CO_3 solution. Appropriate blanks devoid of enzyme or substrate were also run in parallel to the enzyme assay. The color developed due to liberation of p-Nitrophenol (pNP) was read at 400 nm and the amount of pNP liberated was calculated by comparing to a standard curve generated using varying concentrations of pNP. One unit of β-glucosidase activity was defined as the amount of enzyme needed to liberate 1µmol of pnitrophenol pNP) per minute under the assay conditions (**Fig 2.3.**). The assay was used for the β-glucosidase activity assay of recombinant *C. glutamicum* DM 1729 expressing β-glucosidase.

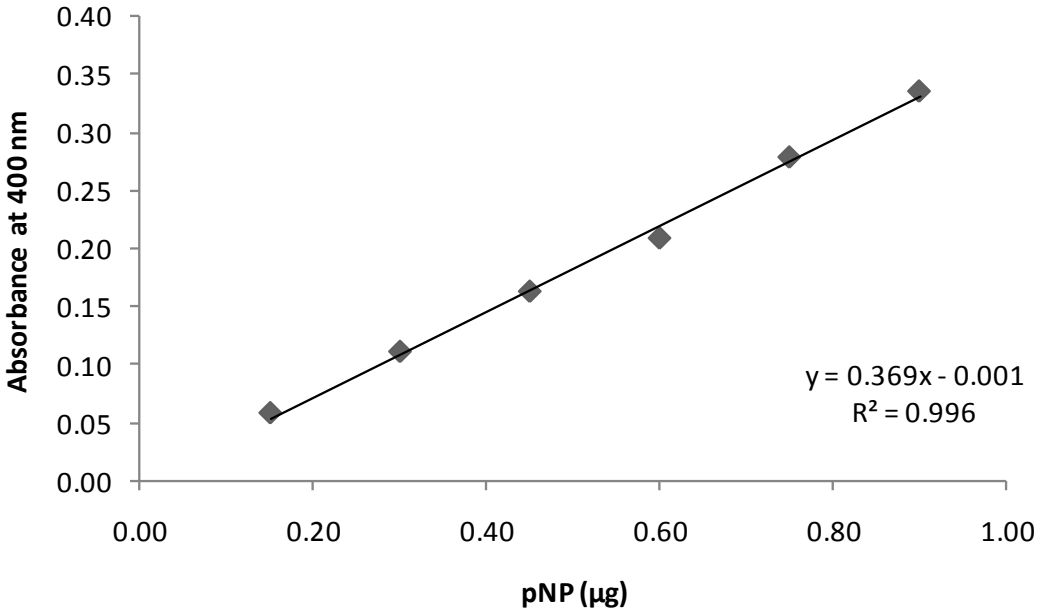


Fig.2.3. Standard graph of β-glucosidase assay

2.4.4. iii. Endoglucanase assay

Endoglucanase activity was determined by measuring the glucose yield from 2 % w/v carboxymethylcellulose (CMC) using a modified protocol (Xiao et al., 2005). CMC was dissolved in citrate buffer (0.05 M, pH 4.8) and reacted with an equal volume of

enzyme solution at 50 ˚C for 30 minutes. The reaction was stopped by the addition of DNS reagent. Enzyme blank and substrate blank were also run to blank the individual effect of enzyme or substrate on the assay. The amount of sugars released from CMC was calculated based on color development which is read at 540 nm and compared to a standard graph of glucose. One unit of endoglucanase activity was defined as the amount of enzyme needed to liberate 1 µmol of glucose per minute under the assay conditions. The assay was used for the endoglucanase activity assay of recombinant *C. glutamicum* DM 1729 expressing endoglucanase.

2.4.5. L-lysine detection

2.4.5. i. Thin Layer Chromatography

TLC was used for qualitative analysis of amino acids. Samples were loaded along with the corresponding standards on a TLC silica plate (TLC silica gel 60G F_{254}) and air dried. The samples were run in a solvent system of butanol /acetic acid /water in the ratio 4:1:1 and developed using ninhydrin spray reagent consisting of 300 mg ninhydrin dissolved in 3 mL acetic acid and 100 mL n-butanol. Ninhydrin reacted with the primary amines and gave a purple complex indicative of presence of amino acids and the reaction is shown in **Fig 2.4.** The plates were visualized on CAMAG TLC scanner.

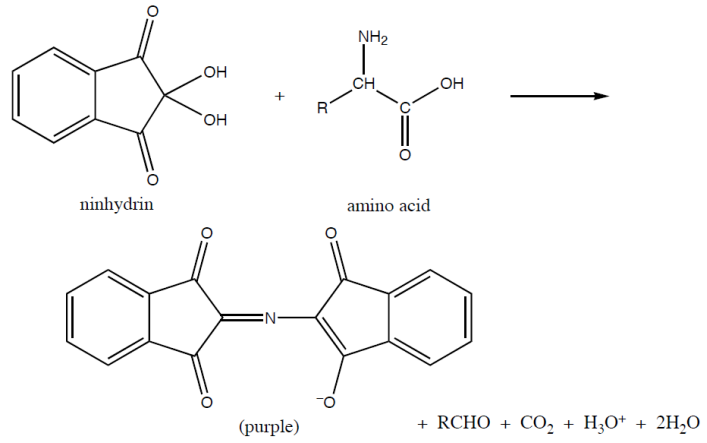


Fig2.4. Reaction of ninhydrin with amino acids

2.4.5. ii. HPLC analysis

HPLC analysis is based on the pre-column derivatization with OPA using a Shimadzu UFLC system. The protocol followed was from Agilent Technical Note (Henderson et al., 1999).

a. Sample preparation

Samples were centrifuged at 12000 rpm for 5 minutes and the supernatant is diluted to desired concentrations and filtered through a 0.22 µm syringe driven filters. This preparation was used in the reaction mixture (**Table 2.1.**)

b. Analysis

Amino acids are less sensitive chromophores whose proper quantification and visualization need complexing with other chemicals which form easily detectable compounds by a process called derivatization. OPA reacts with primary amino acids at a buffering pH of 10.2 to produce a fluorescent derivative exciting at 338 nm and emitting fluorescence at 450 nm which is recorded by a fluorescence detector. The protocol used automated derivatization of the sample solution with OPA and subsequent detection, the buffering pH being provided by borate buffer. The sample is separated and detected using a binary gradient elution program employing 40 mM sodium phosphate buffer (pH 7.8) and Acetonitrile/ methanol/ Water in a ratio of 45:45:10 as mobile phase and Agilent Zobax Eclipse AAA column (4.6 x 150 mm ,5 µm) operating at a temperature of 40 ˚C (**Table 2.2.**). The chromatograms were obtained and parameters analyzed using LC solution software.

Table2.1. Reaction mixture for L-lysine analysis

Components	Volume (µL)
Amino acid sample	5
Borate buffer (pH 10.2)	25
Deionized water	320
Total reaction volume	350

Table 2.2. Gradient elution program for L-lysine analysis

Time(min)	%B
0	0
1.9	0
18.1	57
18.6	100
22.3	100
23.2	0
32.0	0

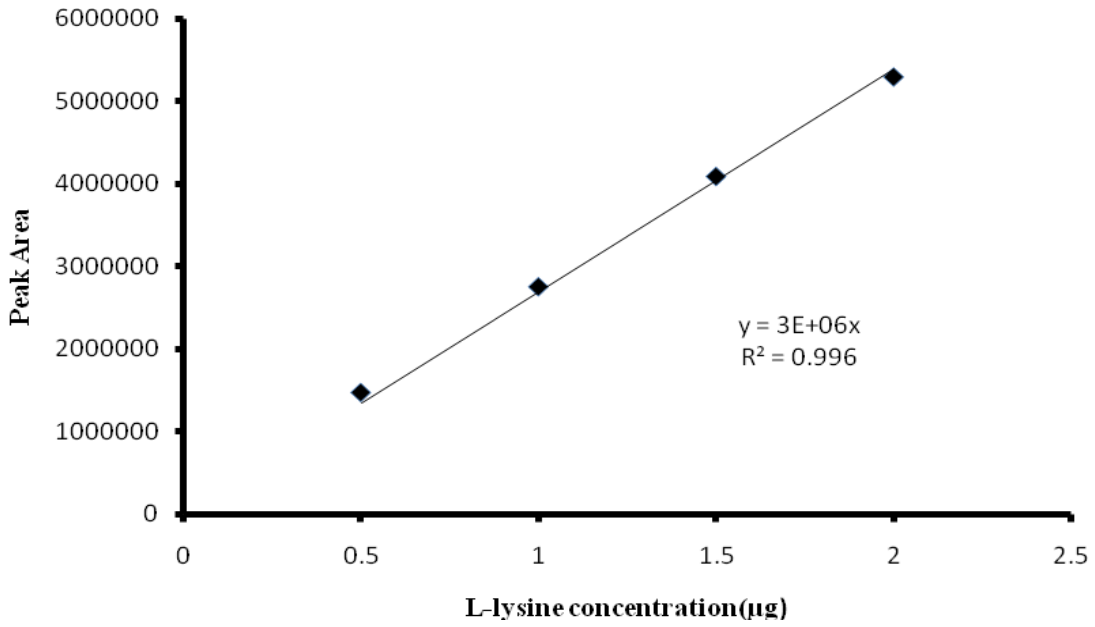


Fig 2.5. Standard graph for L-lysine quantification

2.5. Molecular biology Methods

Standard molecular biology reactions including PCR, restriction digestion and ligation, were executed according to standard protocols (Sambrook & Russell David, 1989). All other molecular biology methods are described in detail in Chapter 7.

2.6. Statistical optimizations

All the statistical data analysis was done using Minitab statistical software. Where ever applicable, the factors affecting fermentation were screened by Plackett-Burman design. The combinatorial effect of the factors significantly affecting the response was analyzed by Response Surface Methodology. The methods are explained in the respective chapters.

2.7. Conclusion

The general methods and methodologies employed in the study were explained in the chapter. More elaborate version of the methodologies employed is provided in the respective chapters. Standard protocols were employed for microbiological, analytical and molecular biology methods and appropriate references were cited. Major instruments used in the study are listed in Annexure II.

Chapter 3

SELECTION OF CARBON SOURCE AND OPERATIONAL PARAMETERS FOR L-LYSINE FERMENTATION USING *CORYNEBACTERIUM GLUTAMICUM* DM1729

3.1. Introduction

India is an agricultural economy with agriculture contributing a major share to the GDP (Gross Domestic Product) of the country. Over 50 % of the country's land is arable. Thus, India has large amounts of agricultural residues produced every year. But, the available statistics are on the agricultural production and forest coverage and not on the agro residues or biomass produced .The biomass generated are either used as fodder or burned off in the fields and are usually unavailable for value addition. Moreover, the figures available on annual biomass generation are usually hazy. The agro residual biomass generation is usually calculated in proportion to the respective crop production. Agro residual biomass can be defined as all natural and organic materials which are either wasted on surplus or during harvesting and processing of crops in natural or managed ecosystems. Food processing industries also generates considerable amounts of byproducts which can be effectively used for value addition.

Utilization of agro residual biomass may in turn help to reduce the cost of an industrial production process which otherwise uses more refined raw materials. This in turn helps to add value to the agro residues and reduce environmental burden. In this study cassava bagasse, wheat bran, potato peel, coconut oil cake and jackfruit seed powder has been tested as substrates for microbial amino acid fermentation. Cassava bagasse is the starchy fibrous residue after starch extraction from cassava tubers. It does not have cyanide content and has low ash content which favors microbial growth. But the low protein content makes it undesirable as animal feed (John, 2009).Wheat bran is the byproduct of dry milling of wheat and consists of outer layers of wheat together with small amount of the endosperm. There are no universally accepted definitions for the wheat bran composition and it varies from place to place. The mixture of fine and coarse biomass is used as livestock feed (Heuze, 2015). Potato peel is mainly generated from the food

processing industries for fast food and convenience food. The wet peels forms a major issue in the processing industry as it is susceptible to rapid microbial spoilage. Coconut oil cake is the residue left after extraction of oil from the dry coconut. The cake after de oiling is usually used as cattle feed. Jackfruit seeds vary in size from 2-4 centimeters and make up around 10 to 15% of the total fruit weight and are rich in carbohydrates and proteins (Madruga et al., 2014). Except for some local culinary preparations, most of the seeds go wasted. Frying industry also generates jackfruit seeds as the major waste. Jackfruit seed and powder are shown in **Fig 3.1.**

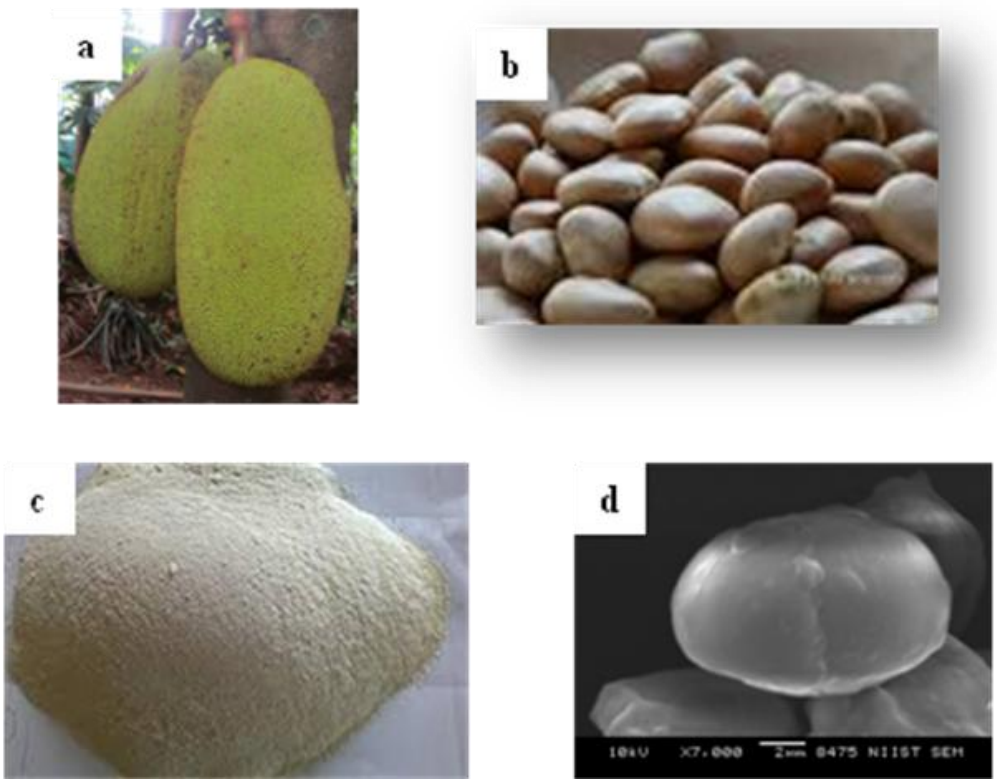


Fig 3.1. Jackfruit, jackfruit seed and powder.(a) Jackfruit (b) jackfruit seed (c) jackfruit seed powder (d) Scanning Electron Micrograph of jackfruit seed powder

In Kerala (India) alone 350 million jackfruits are wasted annually (Civil Society, 2009). Fresh jackfruit seeds are reported to be rich in starch. The starch granules are round and bell shaped similar to cereal starches with an amylose content of 28.1%. The starch isolated from the seeds had a D-glucose composition of more than 99 % (Bobbio et al.,

1978). The starch granular structure similar to cereals and the rich starch content makes the seeds desirable substrate for amino acid fermentation.

Starch hydrolysis of the agro residual biomass for generation of fermentable sugars is an important step while using starchy biomass. Starch is a homopolymer of glucose and consists of two types of molecules called amylose and amylopectin (**Fig 3.2a. & 3.2b**.)

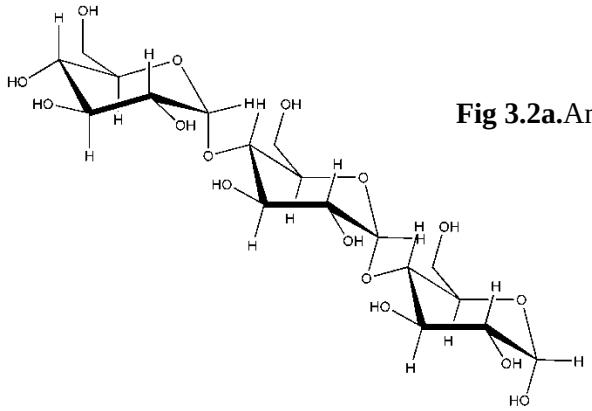


Fig 3.2a. Amylose unit of starch

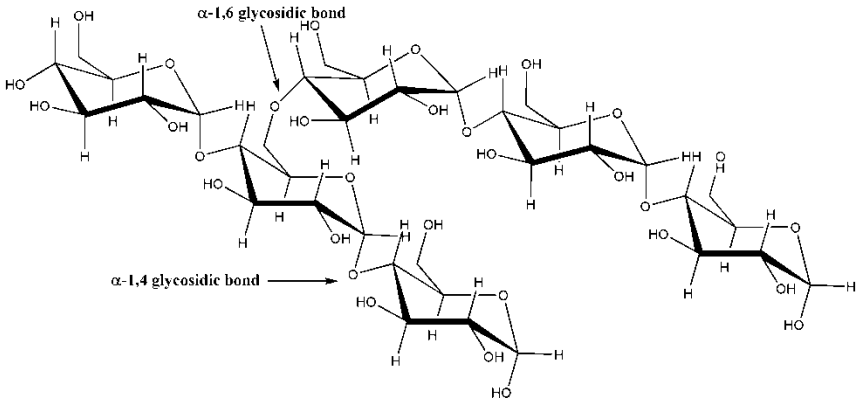


Fig 3.2b. Amylopectin unit of starch

Amylose is formed of α-1,4 linked glucose residues and in amylopectin one in every 20 residues has a α-1,6 linked branch points. Amylose has 500-20000 glucose units depending on the source and amylopectin has more than a million residues. The starch is gelatinized before enzymatic hydrolysis and amylose reduce the crystallinity of amylopectin and allow penetration of water. The gelatinized substrate is treated with starch saccharifying enzymes like alpha amylase and glucoamylase. Alpha amylases are endoamylases that cleaves internal α-1, 4 glycosidic bonds and releases fragments of

polysaccharides. Glucoamylases are exoamylases that cleave the external α-1, 4 and α-1, 6 glycosidic bonds and release disaccharides or glucose monomers (**Fig 3.3.**).

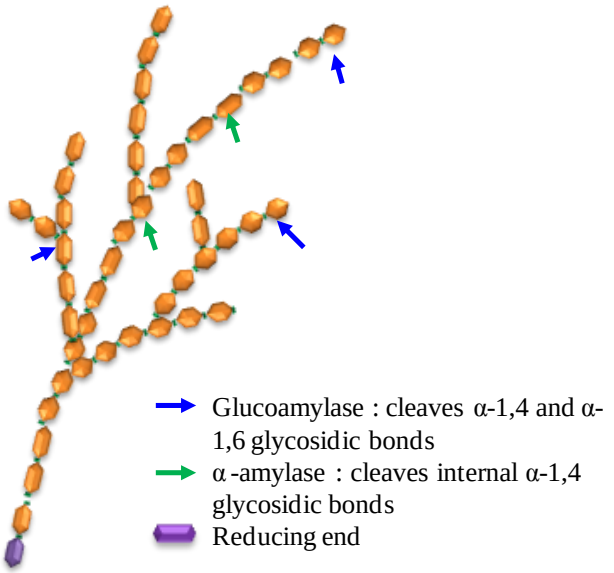


Fig 3.3. Representative structure of amylopectin with the cleavage site for starch hydrolyzing enzymes

Commercial amino acid fermentation use either refined sugars or hydrolysates of starchy biomass. Starch hydrolysates are made either by enzymatic hydrolysis or acid hydrolysis. The main carbon sources of L-lysine fermentation are starch hydrolysates or sugar syrups of corn or tapioca. Even though the bioprocess is commercially established, this may pose competition on food and fodder. Thus search for an alternate carbon source is of essence.

The effect of using agro residual biomass as sugar source has to be analyzed for efficient fermentation, as selection of the best conditions for microbial growth and metabolite secretion is the key in the success of any fermentation process. The selection of best conditions ensures the optimal growth of the microbe, good metabolite excretion and low byproduct formation. Thus, a suitable agro residual biomass was selected as raw material for L-lysine fermentation after screening. The process parameters were further optimized using the selected hydrolysate based production medium and subsequently scaled up in bioreactor and evaluated the L-lysine yield

3.2. Materials and Methods

3.2.1. Microorganism, media and culture conditions

C. glutamicum DM 1729 was used for L-lysine fermentation. The culture maintenance and inoculum preparation was carried out as mentioned in the general materials and methods (section 2.3.1.). The culture was visualized using Gram staining and phase contrast microscopy using Leica DM2000 microscope. The result is depicted in **Fig 3.4a. and 3.4b**. The fermentation studies in the chapter used CGXII and M2 medium (Annexure 1) supplemented with starch hydrolysates of different agro residues.

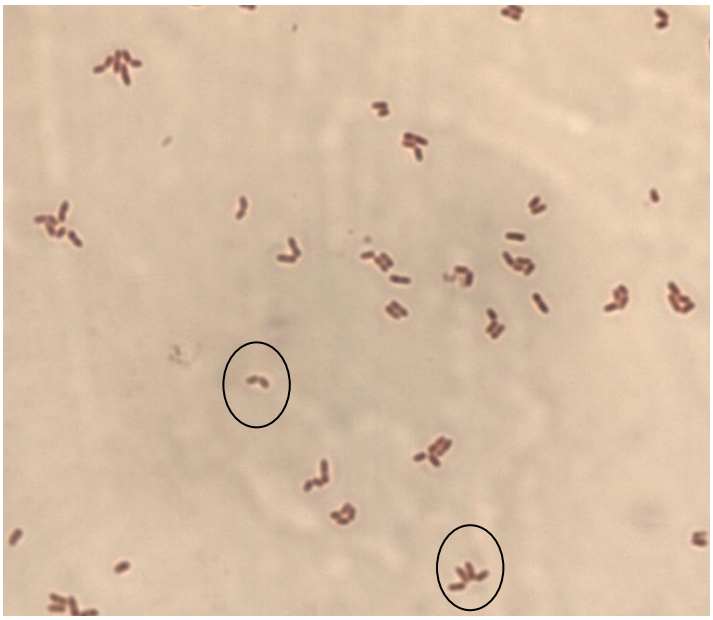


Fig 3.4a.Gram stained image of *C. glutamicum* DM1729 cells showing the characteristic V-shape

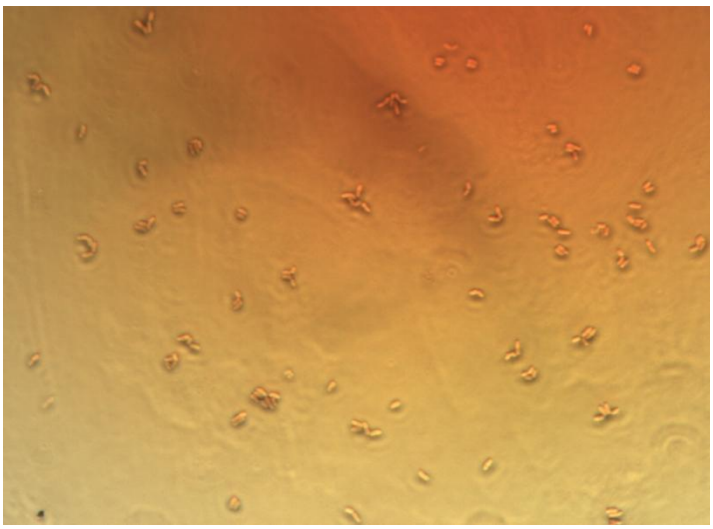


Fig 3.4b.Phase contrast image of *C. glutamicum* DM1729 cells

3.2.2. Substrates

Jackfruit seed powder, Cassava bagasse, Potato peel, Wheat bran and Coconut oil cake were the substrates used in the study.

3.2.2.1. Substrate-starch hydrolysis

All substrates were hydrolysed with commercial starch hydrolyzing enzymes. The enzyme activity was quantified as described in section 2.4.4.i. Both alpha amylase and glucoamylase had an enzyme activity of 2000 IU/ mL. The procedure for starch hydrolysis was conducted as in section 2.2.2.ii.

3.2.2.2. Screening of substrate and media

The starch hydrolysates of the substrates were supplemented as carbon sources in either M2 medium or CGXII medium. The media composition is given in Annexure 1.The initial pH was adjusted to 7.0. The fermentation was run in a volume of 25 mL in 250 mL flask and continued for 120 hours with intermittent sampling. The temperature for incubation was set at 30 ˚C and agitation was 200 rpm. The best hydrolysate was selected as the sole carbon source to formulate the production medium based on the L-lysine yield from the respective fermentations.

3.2.2.3. Proximate composition of jackfruit seed powder

The proximate composition of jackfruit seed was carried out by analysis of moisture, ash, fat, starch and protein. Moisture measurements were done by moisture analyzer which determines the moisture percentage based on the difference in weight. Ash and fat were quantified according to AOAC protocol (AOAC, 2000). Protein was extracted according to Sirisha et al. (Sirisha et al., 2014) and quantified by Bradford method (Bradford, 1976). Starch was isolated according to the modified protocol of Bobbio et al (Bobbio et al., 1978) and quantified on the basis of the difference in weight.

3.2.3. Submerged fermentation

Nutrient and process parameter optimization was done by screening one factor at a time in 250 mL Erlenmeyer flasks with 25 mL working volume. The individual effects of different inorganic nitrogen sources, viz, ammonium nitrate, ammonium chloride, ammonium carbonate, sodium nitrate and ammonium sulphate on L-lysine production were screened. The contribution of initial pH (6.0, 6.5, 7.0 and 8.0) towards L-lysine production was checked by adjusting the pH with 1N NaOH and 1N HCl before autoclaving. Different temperatures (30, 32 and 37 ˚C) and agitation (200,220 and 250 rpm) were set in an orbital shaker. The fermentation was carried out, with varying one

factor at a time and started with varying inoculum sizes (initial OD_{600} of 0.5, 0.75, 1, and 1.25). Fermentations were continued up to 120 hours and samples were withdrawn at 24 hour intervals and analyzed for L-lysine level. The varying parameters attempted and the ranges were mentioned in Table 3.1

To check whether the amino acid production titres in shake flasks could be sustained in a fermenter, the best of each factor was combined and tested in a parallel fermenter (Infors HT, Switzerland, **Fig 3.5.**).The experimental set up had six reaction vessels with jackfruit seed hydrolysate based CGXII medium devoid of MOPS buffer. The reactor vessels consisted of three way inlets, inoculation port, exhaust gas cooler and sampling device. The bioreactors were equipped with rushton type impellers; aeration was maintained by an air sparger and an air flow meter. Agitation was maintained by a stirrer controlled with a magnetic base. The working volume was kept at 300 mL; agitation set to cascade between 200-500 rpm, gas flow from 0.2-1 vvm such that the pO_2 varied between 60, 70, 80 and 90 %. The DO probe was calibrated at 100 % with atmospheric air sparged through the medium. Foaming was controlled online by antifoam O-30 (Sigma) and water suspension. pH was measured continuously using standard pH electrode (Mettler Toledo) and maintained at 7.0 with 1N HCl and 1N NaOH .

Fig 3.5. Experimental set up of the parallel fermenter for L-lysine production.

3.2.4. Analytical methods

Growth was monitored by measuring absorbance at 600 nm by spectrophotometer (Shimadzu 160A, Japan). Sugar concentrations were determined by DNS assay (Miller, 1959). Amino acids were quantified using Shimadzu UFLC system employing fluorescence detector as described in general materials and methods (Section 2.4.5.ii.).

3.3. Results and Discussion

3.3.1. Substrate – starch hydrolysis

The stages of hydrolysis and resultant reducing sugar concentrations of different substrates are depicted in **Fig 3.6., Fig 3.7.** and **Fig 3.8**. Hydrolysis of wheat bran and coconut oil cake released lower amounts of sugars compared to the other substrates. The reducing sugars released after jackfruit seed hydrolysis was in the range of 55 to 60 mg/mL from 9 %w/v substrate with a conversion efficiency of 60-67%. During the stages of hydrolysis, there was an increase disaccharide concentration after liquefaction with alphaamylase. During saccharification the disaccharides are broken down into glucose resulting in decrease in disaccharide concentration and increase in glucose concentration during saccharification (**Fig3.9.**)

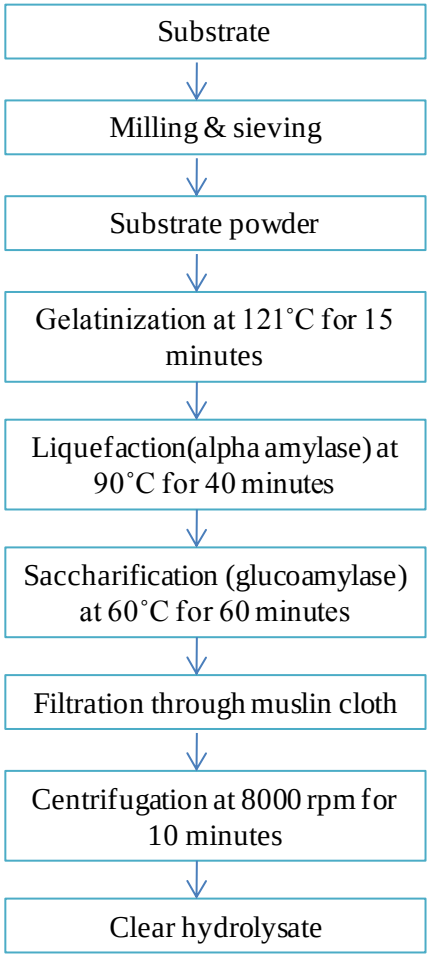


Fig 3.6. Flow chart of starch hydrolysis

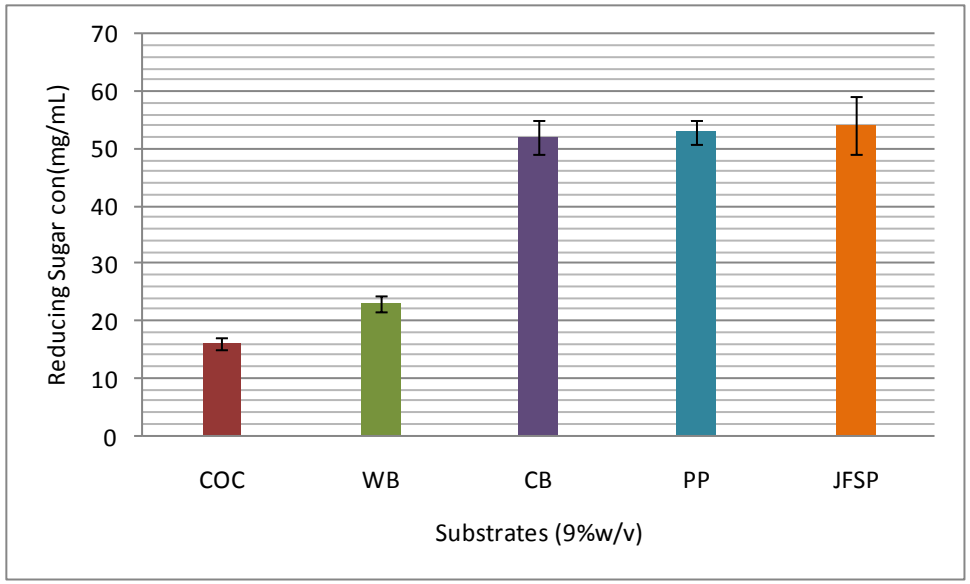


Fig 3.7. Reducing sugar concentrations of starch hydrolysate of different substrates

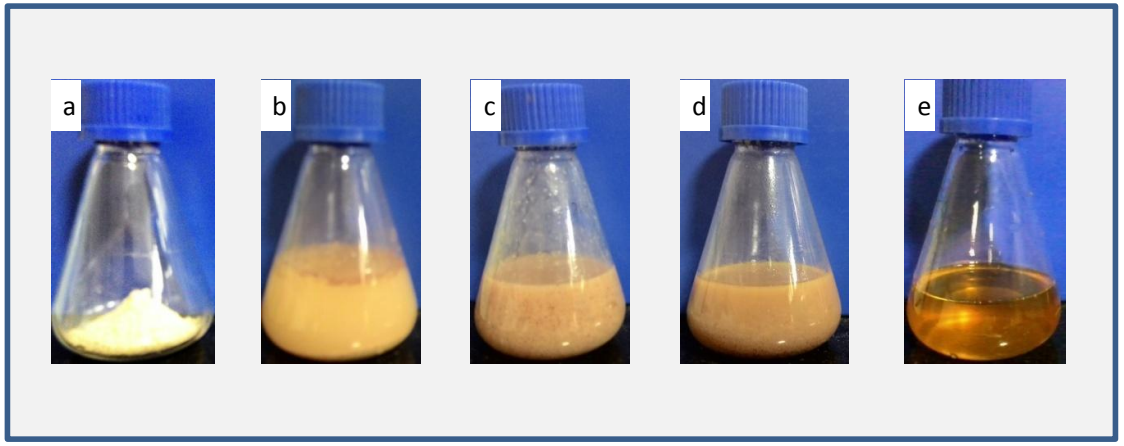


Fig 3.8. Stages of hydrolysis of jackfruit seed powder. (a) Jackfruit seed powder (b) jackfruit seed powder suspended in water (c) Gelatinized jackfruit seed powder (d) After α-amylase treatment and (e) After glucoamylase treatment and centrifugation

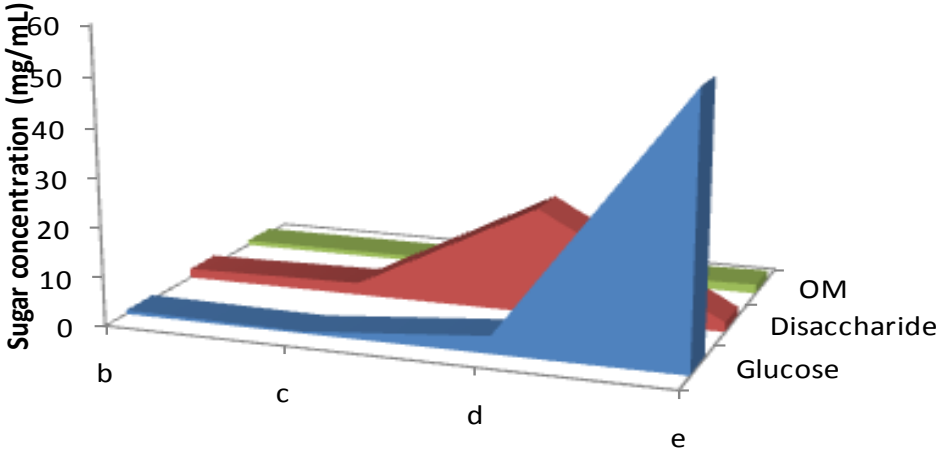


Fig 3.9. Sugar composition of jackfruit seed powder hydrolysate at various stages of hydrolysis. (b) Jackfruit seed powder suspended in water (c) Gelatinized jackfruit seed powder (d) After α-amylase treatment and (e) After glucoamylase treatment and centrifugation, OM represents other sugar monomers in hydrolysate

The hydrolysate so obtained had a golden brown color and pH near 5. The hydrolysate was either diluted or concentrated under pressure on a rotary evaporator to obtain desired reducing sugar concentrations.

3.3.2. Selection of fermentation medium and starch hydrolysate.

Based on the L-lysine yields in two different media and five different starch hydrolysates, the best medium and substrate was selected. The resultant L-lysine yields are depicted in Fig 3.10. Jackfruit seed powder hydrolysate gave the best L-lysine yield of 0.12 g $_{lysine}$/g $_{reducing\ sugar}$. The best medium was CGXII medium and gave better yields with all other hydrolysates than the M2 medium. Hence CGXII medium supplemented with jackfruit seed hydrolysate was used as the production medium for L-lysine fermentation.

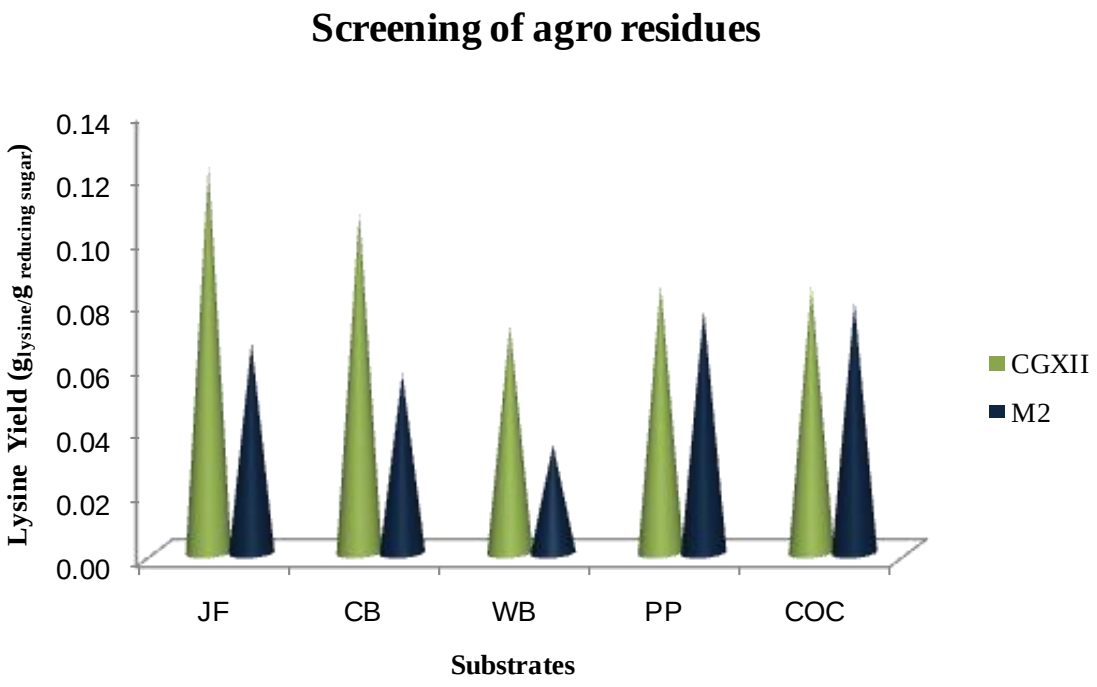


Fig 3.10. Screening of agro residual biomass hydrolysate and media for L-lysine fermentation. JFS –jackfruit seed powder, CB-Cassava Bagasse, WB - Wheat Bran, PP- Potato Peel, COC – Coconut Oil Cake, CGXII-L-lysine production medium,M2- L-lysine production medium

3.3.3. Proximate composition of jackfruit seed powder

Jackfruit seed powder contained 9.2% moisture, 2.5% ash, 0. 23% fat, 42 % starch and protein11mg /g substrate. The proximate composition of jackfruit seeds changes from variety to variety. It is also influenced by the geographical location and climatic conditions. Understanding the composition of jackfruit seed played an important role as it is used as the substrate for fermentation. Bobbio et al (1978) reported starch content of 25-40 % of the total solids of jackfruit seeds. The yield of starch extracted from fresh seeds varied from 12.7-15.4 % (Oates & Powell, 1996) to 16.9 % (Kittipongpatana & Kittipongpatana, 2011) and 18.2 % (Tongdang, 2008) on dry weight basis. On the contrary Tulyatan et al (2002) reported 77 % of starch extracted from jackfruit seeds which was higher than maize (70 %). Even though there was a difference in starch yield, the carbohydrate composition of the extracted starch was more than 99 % in all reports. These reports emphasize the possible utilization of starch hydrolysate of jackfruit seeds for amino acid fermentation. The soluble sugars released by hydrolysis depended on the hydrolysis conditions and composition of the seeds which varied with variety.

3.3.4. Submerged fermentation

Varying factors affecting L-lysine fermentation was checked (Table 3.1) for its contributory effects on L-lysine production. Among the different nitrogen sources, 2 % of ammonium sulphate gave the maximum L-lysine titre. Table 3.1 indicated that inoculum at OD_{600} of 1(0.25 g/L) was most desirable for fermentation. Other optimized parameters are, pH of 7.0, temperature of 30 ˚C and agitation of 200 rpm. The best conditions gave maximum L-lysine titre of 8.8 ± 0.6 mg/mL with a conversion rate of 0.14 $g_{lysine}/g_{reducing\ sugar}$. Submerged fermentation is the most favored means of amino acid fermentation since its discovery in the 1950s. The fermentation efficiency and L-lysine titres depend greatly on the fermentation conditions. During fermentation temperature change leads to overall change in cell metabolism. It will also alter the substrate utilization pattern leading to unbalanced nutrients in the medium with respect to

Table 3.1: One factor at a time optimization of L- lysine fermentation

Parameter	**Screening range and L-lysine produced (mg/mL)**								**Optimum**
Initial sugar concentration(%w/v)	2	4	**6**	8	10				6
L-lysine	0.04±0	2.6±0.2	8.2±0.2	8.2±0.3	8.6±0.1				
Incubation period (hours)	12	24	36	48	60	**72**	84	96	72
L-lysine	2.6±0.1	3.4±0.1	5.0±0.2	7.7±0.3	8.1±0.1	8.3±0.1	8.0±0.2	8.0±0.2	
pH	6	6.5	**7**	8					7
L-lysine	8.2±0.1	7.2±0.3	8.8±0.6	7.98±0.1					
Temperature (˚C)	**30**	32	37						30 ˚C
L-lysine	8.8±0.5	7.2±0.2	2.4±0.1						
Agitation (rpm)	**200**	220	250						200
L-lysine	8.3±0.1	7.4±0.08	7.3±0.1						
Nitrogen source(% w/v)	A	B	C	D	**E**				E
L-lysine	1.3±0.6	6.2±0.2	6.5±0.1	4.9±0.1	8.3±0.1				
Inoculum size (OD_{600})	OD 0.5_{600}	OD 0.75_{600}	**$OD1_{600}$**	OD 1.25_{600}					$OD1_{600}$
L-lysine	7.0±0.2	7.6±0.1	8.3±0.02	6.6±0.2					

A-Ammonium nitrate, B- ammonium chloride, C-Ammonium bicarbonate, D- Sodium nitrate, E- Ammonium sulphate,

the growth rate of *C. glutamicum* cells. The present results corroborate previous reports (Hilliger et al., 1984) stating that 30 ˚C was the most favoured temperature for L-lysine fermentation by *C. glutamicum* and beyond 32 ˚C there was a decrease in production. Agitation maintains aeration and homogenous distribution of nutrients during fermentation. Increased agitation causes shear stress and decreased agitation causes insufficient aeration and nutrient distribution. Hence, establishment of optimum agitation (200 rpm) aided in better product yield. Among the nitrogen sources tested for submerged fermentation, ammonium sulphate was selected for further experiments. Earlier reports said that *C. glutamicum*, there are two pathways for L-lysine biosynthesis viz the succinylase and dehydrogenase pathways and are governed by ammonium availability (Wehrmann et al., 1998). Dehydrogenase pathway is energetically cheaper than the succinylase variant and contributes to 72% of the flux and operated during the initial stages of fermentation. Availability of ammonium ions at the beginning of the fermentation shifts the flux towards the dehydrogenase pathway and hence L-lysine accumulation (Tryfona & Bustard, 2005). The inoculum concentration and its robustness affect the fermentation pattern and end product formation. *C. glutamicum* have prolonged lag phases if inoculated below an inoculum size of 0.1 g/L (Kelle et al., 2005) and hence low production rates. The shake flask experiments when scaled up in a parallel fermenter gave comparable L-lysine titres and conversion.

The best conditions in the shake flask were adopted and ran in a parallel fermenter in batch to examine the sustainability of L-lysine titres. L-lysine concentration increased from 0.5 to 8 mg/mL during 12- 60 hours of fermentation when the aeration was maintained 90 % pO_2. The aeration and agitation were adjusted to give dissolved gas concentrations of 60, 70, 80 and 90%. Fermentations at 60 and 70% pO_2 gave similar L-lysine titres with the maximum of 5 mg/mL.The bioreactor with 90 % dissolved air gave the maximum L-lysine production (8 mg/mL) with a conversion of 0.13 $g_{lysine}/g_{reducing\ sugar}$ after 60 hours of fermentation (Fig 3.11). The biomass (OD_{600} of 24) concentration was also at the peak at this time period (Fig 3.12.).

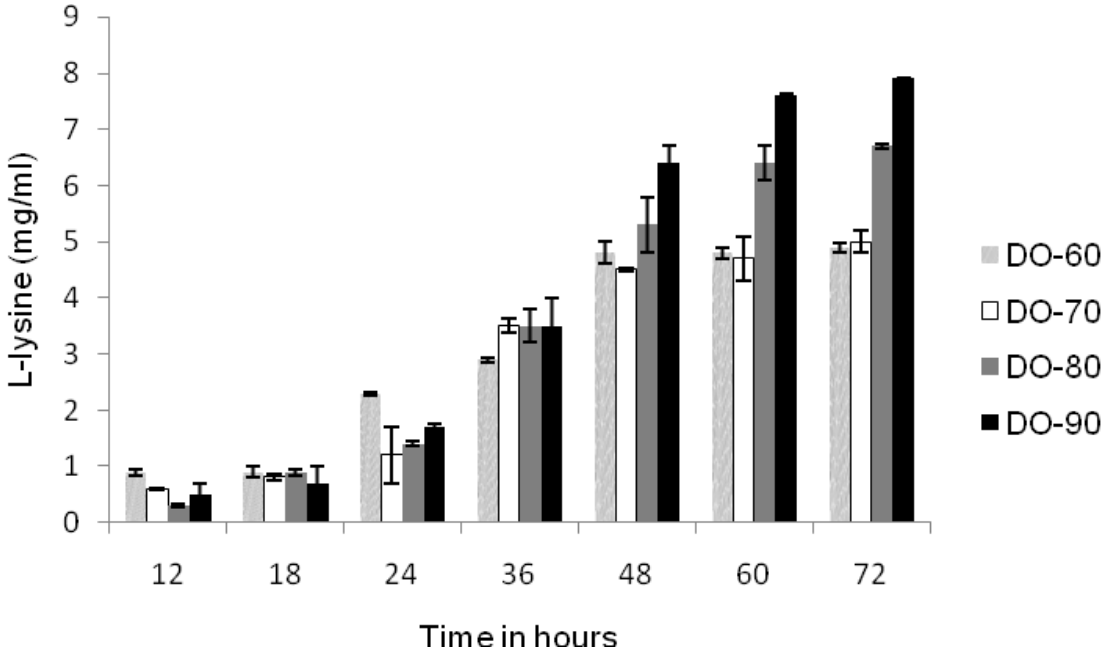


Fig 3.11. L-lysine production under different aeration conditions in a parallel fermenter

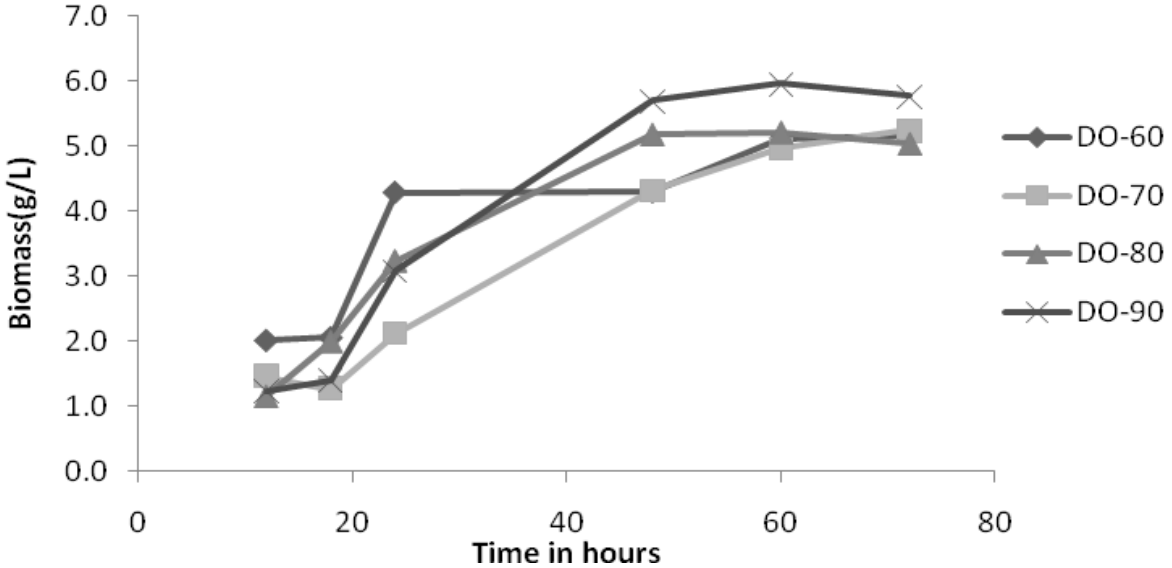


Fig 3.12. Growth of *C. glutamicum* DM 1729 in parallel fermenter with different air saturations

Under the experimental conditions, the L-lysine yields were comparable in shake flask and in fermenter except the fact that the incubation time to get the maximum production reduced to 60 hours in fermenter from 72 hours. L-lysine fermentations were favoured during high aeration rates (Ahmed et al., 2013; Sassi et al., 1996; Yao et al., 2001). *C. glutamicum* can also produce L-lysine at low aeration, but there will be decreased conversion rates, purity and increase in byproduct formation.

3.4. Conclusion

C. glutamicum DM 1729 was able to grow and utilize all the starchy substrates tested with a preference to the hydrolysate of jackfruit seed for L-lysine production. The best conditions gave maximum L-lysine titre of 8.8 ±0.6 mg/mL with a conversion rate of 0.14 g/g reducing sugar. The fermentation process was scaled up in a parallel fermenter with a conversion rate of 0.13 g/g reducing sugar. The starch and amylose content of jackfruit seeds made it an exceptional raw material for amino acid fermentation. Certain varieties of jackfruit seeds were reported to have starch content higher than maize and the carbohydrate content of isolated starch higher than potato starch and corn starch which are used in commercial amino acid fermentation. Jackfruit seeds are usually wasted during fruit processing and consumption which makes it a low cost raw material and adds to the cost effectiveness of the overall production process. With L-lysine dominating the animal feed market and the industrial manufacturing giants were on the quest for renewable sources as fermentation starting material, there will always be academic interest on L-lysine fermentation process improvements.

Chapter 4

STATISTICAL OPTIMIZATION OF L-LYSINE FERMENTATION USING JACKFRUIT SEED POWDER AS CARBON SOURCE

4.1. Introduction

Biological systems are dynamic and complex and so does the bioprocess. Understanding the biological system and bioprocess in a quantifiable way is quite intricate. Either of them cannot be understood completely in a unit operation as they interact constantly with the physical, biochemical and physiological parameters and hence affect operation under study. However, it is essential to quantify the biological systems and bioprocess in physical terms, so as to have a better understanding of the process and hence translating it into a successful bioprocess (Panda et al., 2015). Unlike a physical process, it is very difficult to evaluate and predict a bioprocess with 100% confidence as there is a score of factors affecting it. A complete understanding of the biological system requires knowledge in mathematics, chemistry, biochemistry etc. Thus, statistical tools employing mathematical models will be helpful to some extent in this understanding.

Design of Experiments (DoE) is a general approach involving collection of data, its analysis and generating information (Lee & Gilmore, 2006). This system allows generation of mathematical relationships between the input variable and the response. DoE is carried out when there is constraints on time and resources. The experiments are carried out such that it is completed in less number of experimental runs saving cost and time. While optimizing a bioprocess, the suspected factors affecting the process is screened and significant factors are selected. Further the levels of significant factors are optimized (Arutchelvi et al., 2011; Reddy et al., 2008). Screening design allows finding the main effects of the factors on the response and selects the significant ones. Thus, the screening design shows relative significance of the main effects. Screening designs are of resolution III to V. Plackett- Burman design is one of the most commonly used resolution III designs. Resolution III designs allow users to understand the significant factors with relatively low number of factors compared resolution IV and V designs.

Response surface designs (RSM) are used when the significant factors are determined using the screening designs and there is chance of curvature in the response. There are two main types of response surface designs: Central Composite designs (CCD) are used to fit a full quadratic model. CCD can also accommodate information from a factorial experiment. CCD is often used when the experimental design demands sequential experimentation.CCD has been widely used for process parameter optimizations (John et al., 2006; Lee et al., 2011; Rahulan et al., 2009). Box-Behnken designs usually have fewer design points than central composite designs, thus, they are less expensive to run with the same number of factors. They can efficiently estimate the first- and second-order coefficients; however, they can't include runs from a factorial experiment. Box-Behnken designs always have 3 levels per factor, unlike central composite designs which can have up to 5. Bioprocess optimizations have been extensively done employing Box-Behnken designs (Abdel-Fattah, 2002; Ismail & Nampoothiri, 2010; John et al., 2007; Sharma et al., 2009).

The chapter summarizes statistical optimization of both 'submerged fermentation' and 'simultaneous saccharification and fermentation' processes for L-lysine production from jackfruit seed powder by *C. glutamicum* DM1729. In submerged fermentation, the starch hydrolysate of jackfruit seed powder was supplemented to the fermentation medium as the sole carbon source and the sugars were assimilated by *C. glutamicum* for L-lysine fermentation. In simultaneous saccharification and fermentation, jackfruit seed powder and starch saccharifying enzymes were added to the medium and inoculated with *C. glutamicum* DM1729. The starch saccharifying enzymes released the sugars by hydrolysis and was subsequently used by *C. glutamicum* for growth and amino acid production. By avoiding the hydrolysate preparation step of jackfruit seed powder, simultaneous saccharification and fermentation reduced the unit operations in fermentation.

4.2. Materials and Methods

4.2.1. Statistical Optimization

Statistical optimization of both fermentation processes involved Plackett Burman Design and Box Behnken design.

4.2.2.1. Plackett –Burman Design

Fermentable sugar concentration, incubation period and media components were screened for L-lysine production by PBD (Plackett- Burman Design)(Plackett & Burman, 1946) .With the conservation of the factors optimized with one factor at a time approach the variables in the study were screened in experimental runs as per the design. The PBD works on a first order polynomial model

$$Y = \beta_o + \sum \beta_i X_i \qquad (1)$$

The individual effect of variables was determined using the equation 2

$$E(X_i) = \frac{\sum M_{i+} - \sum M_{i-}}{N} \qquad (2)$$

Where, E(Xi) is the calculated effect, ΣM i+ is the sum of the high level response values and ΣMi- is the sum of low level response values and N is the number of experimental runs.

4.2.2.2. Response Surface Design

Box-Behnken experimental design was used to understand the interaction effects contributing to the production of L-lysine. In submerged fermentation, three significant factors viz sugar concentration, ammonium sulphate concentration and incubation period were studied in 15 experimental runs. In simultaneous saccharification and fermentation, the factors- jackfruit seed powder concentration, enzyme concentration and incubation period were studied in 15 experimental runs. To correlate the response variables and independent variables a second order quadratic equation was used. It is expressed as in equation 3.

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_{i^2} + \sum \beta_{ij} X_i X_j \qquad (3)$$

where Xi and Xj are the independent variables influencing the response variable Y, βo is the interception coefficient, βi is the coefficient of linear effect, βii is the coefficient of quadratic effect and βij is the ijth interaction coefficient.

The regression analysis and statistical significance of the models were tested with ANOVA using the Minitab statistical software. The R^2 value predicted the wellness of the fit of the sample data to the regression equation; the adjusted R^2 indicated the unnecessary predictor variables that do not improve the model. Predicted R^2 was used to measure the amount of variation in the experimental data to the predicted response and showed the wellness of prediction. Fischer F-test was used in testing the statistical significance and t-test for testing regression coefficient.

4.2.3. Microorganism, media and culture conditions

C. glutamicum DM1729 was used throughout the study. The general culture conditions were maintained as described in general materials and methods (section 2.3.1.).

4.2.4. Fermentation

4.2.4. i. Submerged fermentation

In submerged fermentation, CGXII mineral medium supplemented with jackfruit seed powder hydrolysate was used as the sole carbon source. Nutrient and process parameter optimization was done by statistical method as per experimental design. Fermentable sugar concentration, according to the design was adjusted by varying concentration of the starch hydrolysate. Each run was performed in 250 mL Erlenmeyer flasks with 25 mL working volume. All runs were performed at an initial inoculum concentration of OD_{600} of 1, initial pH of 7 and agitation speed of 200 rpm and incubated at 30 ˚C for stipulated time periods. In the screening design using PBD, eleven experimental factors were screened, viz, initial sugar concentration, ammonium sulphate concentration, concentrations of urea, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, magnesium chloride, calcium chloride, trace elements, biotin and protocatechuate. The incubation period were varied from 24 hours to 72 hours. The interaction between initial sugar concentration, ammonium sulphate concentration and incubation period were studied using Box- Behnken experimental design. The optimum conditions were scaled up in a bioreactor at 1 L level. The experiment set up is detailed in section 4.2.5.Results of all the experiments are shown as average values of three independent experiments, unless otherwise stated.

4.2.4. ii. Simultaneous saccharification and fermentation

In simultaneous saccharification and fermentation, instead of jackfruit seed powder hydrolysate, jackfruit seed powder was used as the sole carbon source. Starch saccharifying enzymes α amylase and glucoamylase were sterilized separately using 0.22 µm filter membranes and were added to the medium as per the experimental runs. Each run was performed in 250 mL Erlenmeyer flasks with 25 mL working volume. All runs were performed at an initial inoculum concentration of OD_{600} of 1, initial pH of 7, agitation speed of 200 rpm and incubated at 30 ˚C for stipulated time periods. Fourteen factors were screened to determine the factors affecting simultaneous saccharification and fermentation. The factors were jackfruit seed powder concentration, the concentrations of ammonium sulphate concentration, concentrations of urea, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, magnesium chloride, calcium chloride, trace elements, biotin, protocatechuate, α amylase and glucoamylase. The incubation periods were varied from 24 hours to 120 hours. Box-Behnken experimental design was to study the interaction between factors, viz, jackfruit seed powder concentration, enzyme concentration and incubation period. Results of all the experiments are shown as average values of three independent experiments, unless otherwise stated.

4.2.5. Bioreactor Experiments

The selected conditions in the submerged fermentation were scaled up in a bioreactor (Infors Minifors) of 2.5 L capacity. The reactor vessels consisted of three way inlets, inoculation port, exhaust gas cooler and sampling device. The bioreactors were equipped with rushton type impellers; aeration was maintained by an air sparger and an air flow meter. Agitation was maintained by a stirrer controlled with an external motor. The working volume was kept at one litre; agitation set to 200 rpm, pO_2 of 90%. The DO probe was calibrated at 100% with atmospheric air sparged through the medium for 2 hours and calibrated as DO of 100%. Foaming was controlled online by antifoam O-30 (Sigma) and water suspension. pH was measured continuously using standard pH electrode (Mettler Toledo) and maintained at 7.0 with 1N HCl and 1N NaOH .

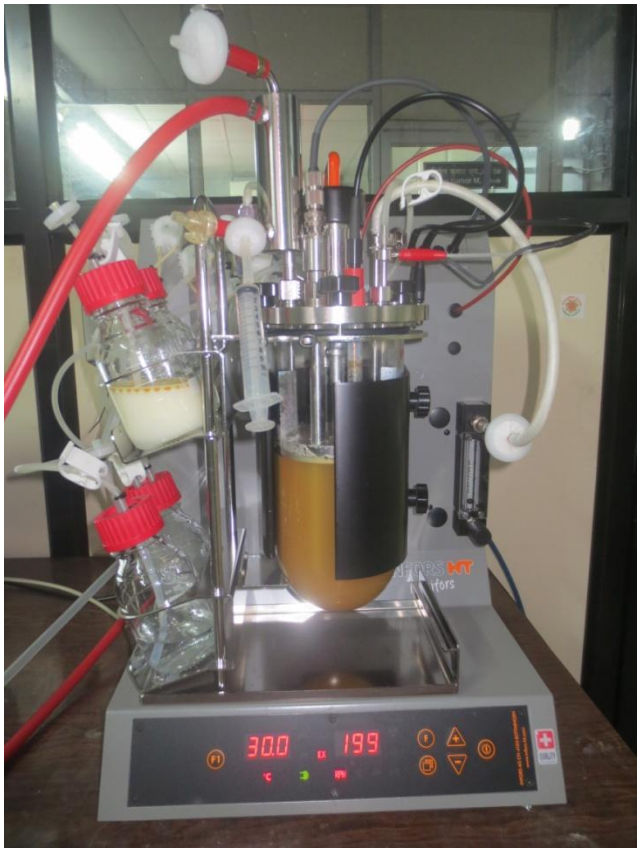


Fig 4.1.Experimental set up of 3L bioreactor for L-lysine production

4.2.6. Analytical methods

Growth was monitored by measuring absorbance at 600 nm by spectrophotometer (Shimadzu 160A, Japan). Sugar concentrations were determined by DNS assay (Miller, 1959). Amino acids were quantified using Shimadzu UFLC system employing fluorescence detector. The details were described in section 2.4.5.

4.3. Results and Discussion

The Results and Discussion part of this chapter is divided into two sections A and B. Section A discusses the results of submerged fermentation and Section B discusses the results of simultaneous saccharification and fermentation.

Section A- Submerged Fermentation

4.3.1. Optimization of submerged fermentation conditions using Plackett-Burman and Box-Behnken Design

Eleven variables that may influence the submerged fermentation were screened in twenty experimental runs designed by the Minitab software and L-lysine (mg/mL) production was recorded as the response (**Table 4.1.**). Plackett-Burman design was used as the screening design here. The range of variation of L-lysine produced was from 1 to 9 mg/mL suggesting the feasibility of the model. The p-value lower than 0.05 helped to understand the independent variables having significant linear main effect on determining the response (**Table 4.2.**). The significant factors affecting fermentation were sugar concentration, ammonium sulphate concentration and incubation period (**Fig 4.2.**). The significance was identified from the pareto chart as bars extending beyond the critical t-value, the red line on the chart**.**

Table 4.1. Plackett-Burman experimental design matrix with L-lysine (mg/mL) produced as response

Run	A	B	C	D	E	F	G	H	I	J	K	R
1	8	0.5	0.1	200	200	5	200	200	25	1.25	24	3.3
2	8	4	1	200	25	5	200	200	25	10	72	9.2
3	8	4	0.1	25	200	40	25	200	200	1.25	24	7.0
4	8	0.5	1	200	200	40	25	25	200	10	24	3.3
5	2	0.5	0.1	200	25	40	25	200	200	10	72	1.2
6	8	4	0.1	200	200	5	25	25	25	10	24	3.3
7	8	0.5	1	200	25	5	25	25	200	1.25	72	5.2
8	2	0.5	0.1	25	25	5	25	25	25	1.25	24	0.5
9	2	4	1	200	200	5	25	200	200	1.25	72	2.4
10	2	4	0.1	200	200	40	200	25	25	10	72	2.4
11	2	0.5	1	25	200	5	200	200	200	10	24	0.5
12	2	4	0.1	200	25	40	200	200	200	1.25	24	1.1
13	2	0.5	0.1	25	200	5	200	25	200	10	72	1.2
14	8	0.5	0.1	25	25	40	25	200	25	10	72	5.0
15	8	0.5	1	25	200	40	200	200	25	1.25	72	7.8
16	2	4	1	25	25	5	25	200	25	10	24	1.1
17	2	0.5	1	200	25	40	200	25	25	1.25	24	0.5
18	8	4	1	25	25	40	200	25	200	10	24	6.5
19	8	4	0.1	25	25	5	200	25	200	1.25	72	8.5
20	2	4	1	25	200	40	25	25	25	1.25	72	2.4

A-Sugar (%w/v), B-$(NH_4)_2SO_4$ (%w/v), C- Urea (%w/v), D-KH_2PO_4 (mg/dL), E-K_2HPO_4 (mg/dL), F-$MgSO_4$ (mg/dL) G-$CaCl_2$ (mL), H-Trace elements (mL), I-protocatechuate (mL),J-Biotin (mL),K-Incubation (hours), R-L-lysine (mg/mL)

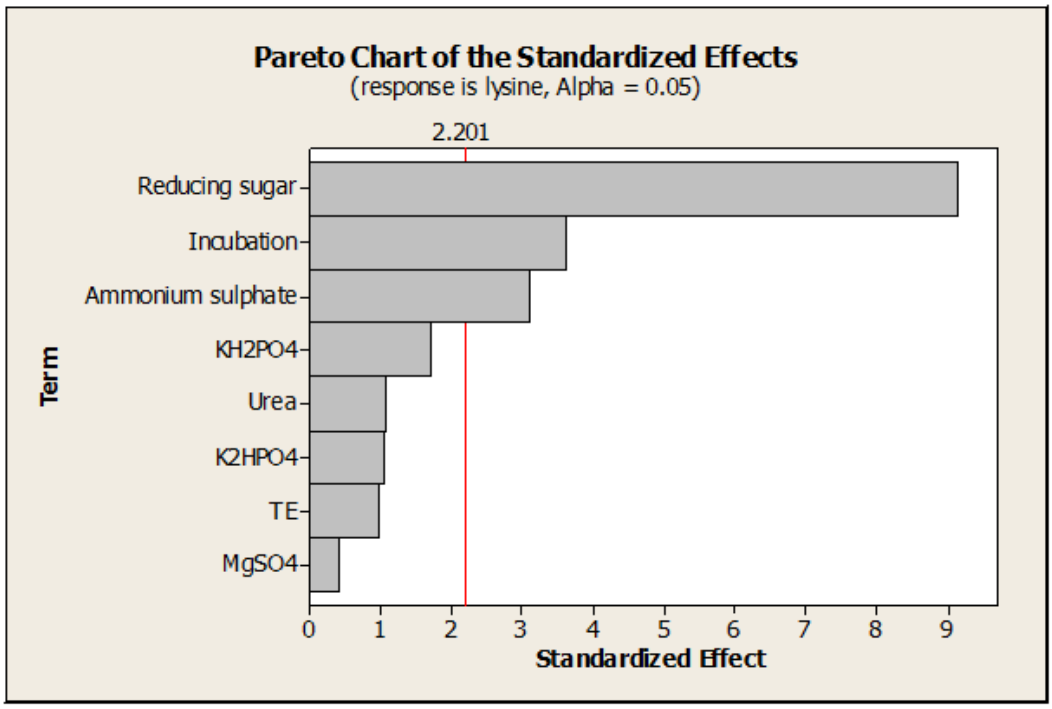


Fig 4.2. Pareto chart showing significant factors affecting L-lysine production

Table 4.2. PBdesign: Estimated Effects and Coefficients for L-lysine (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		3.6200	0.2502	14.47	0.000
Sugar	4.5800	2.2900	0.2502	9.15	0.000
$(NH4)_2SO_4$	1.5400	0.7700	0.2502	3.08	0.011
Urea	0.5400	0.2700	0.2502	1.08	0.304
KH_2PO_4	-0.8600	-0.4300	0.2502	-1.72	0.114
K_2HPO_4	-0.5200	-0.2600	0.2502	-1.04	0.321
$MgSO_4$	0.2000	0.1000	0.2502	0.40	0.697
TE	0.4800	0.2400	0.2502	0.96	0.358
Incubation	1.8200	0.9100	0.2502	3.64	0.004

Effect: The estimated effects of variables, Coef: The coefficients of interception and estimated slopes, SE Coef: The standard error coefficient, T: The t-test statistic, P: The calculated probability

A Box-Behnken experimental design (Box & Behnken, 1960) was conducted to evaluate the contributory and interaction effects of different variables on L-lysine fermentation by *Corynebacterium glutamicum* DM1729. The design matrix was generated using Minitab software after inputting the variables and the levels (**Table 4.3.**). The design analyzed the effect of sugar concentration, ammonium sulphate concentration and incubation period on the response. The experiments were conducted, and the response values were analyzed to generate a polynomial equation which could be used to predict the L-lysine titres.

$$Y = 21.6250 + 2.18750A + 8.62500B - 1.16667C - 0.343750A^2 - 0.875000B^2 + 0.00954861C^2 - 0.125000AB + 0.0416667AC - 0.0416667BC \quad (4)$$

Table 4.3. Box-Behnken experimental design and the response

Run	Sugar (%)	$(NH_4)_2SO_4$ (%)	Incubation (Hours)	L-lysine (mg/mL)
1	6	2	48	9
2	4	2	60	7
3	6	3	60	10
4	6	4	72	11
5	4	3	72	10
6	8	3	48	8
7	6	2	72	12
8	8	4	60	8
9	8	2	60	8
10	4	3	48	9
11	8	3	72	13
12	6	4	48	10
13	4	4	60	8
14	6	3	60	10
15	6	3	60	10

The constants in the equation provide the estimated regression coefficients for L-lysine using data in uncoded units. Y was the response, ie, L-lysine produced per experimental run. The regression analysis showed that the model terms- initial sugar concentration and incubation period were significant (**Table 4.4.**).

The ANOVA was carried out by default by the software as depicted in **Table 4.5.** Three dimensional surface graphs were plotted as the graphical representation of the polynomial equation. The interactions between sugar concentration and incubation period were plotted in **Fig 4.3.(a)** and **Fig 4.3.(b)** represents the interactions between ammonium sulphate and incubation period with L-lysine production as the response. The interaction between ammonium sulphate and sugar concentration was depicted in **Fig 4.3.(c).** The optimization steps led to the sugar to L-lysine yield of 0.3 $g_{\text{lysine}}/g_{\text{reducing sugar}}$ with 4% w/v initial reducing sugar concentration and incubation period of 72 hours.

Table 4.4. Box –Behnken Design- Estimated Regression Coefficients for L-lysine

Term	**Coef**	**SE Coef**	**T**	**P**
Constant	10.0000	0.2236	44.721	0.000
Sugar	0.3750	0.1369	2.739	0.041
Ammonium sulphate	0.1250	0.1369	0.913	0.403
Incubation	1.2500	0.1369	9.129	0.000
Sugar x Sugar	-1.3750	0.2016	-6.822	0.001
$(NH_4)_2SO_4$x $(NH_4)_2SO_4$	-0.8750	0.2016	-4.341	0.007
Incubation x Incubation	1.3750	0.2016	6.822	0.001
Sugar x $(NH_4)_2SO_4$	-0.2500	0.1936	-1.291	0.253
Sugar x Incubation	1.0000	0.1936	5.164	0.004
$(NH_4)_2SO_4$ x Incubation	-0.5000	0.1936	-2.582	0.049

Effect: The estimated effects of variables, Coef: The coefficients of interception and estimated slopes, SE Coef: The standard error coefficient, T: The t-test statistic, P: The calculated probability

Statistical tools have been used in the optimizations and media engineering for a plethora of products and micro organisms (Garyali et al., 2014; Gupta et al., 2014; Sathiyanarayanan et al., 2013). PBD (Plackett & Burman, 1946) was used to screen the factors affecting fermentation and studying their main effects. The significant factors were identified from the pareto chart as bars extending beyond the critical t- value, the red line on the chart. These factors also had positive regression coefficients suggesting an increase in the factors leading to increased response values. The main effects could be identified from the main effects plot (not shown). The farther the line is from the centre, the more pronounced was the effect. The most significant main effect was of reducing sugar followed by the incubation period and ammonium sulphate concentration. The explanatory power of the model was assessed by the R^2 values. The R^2 was 91%, and adjusted R^2 was 86%. There was an increase in the predictability of the model when the model terms were reduced to reducing sugar, ammonium sulphate, urea, potassium dihydrogen orthophosphate, dipotassium hydrogen phosphate, magnesium sulphate, trace elements and incubation.

The interaction effects among the variables were studied using the Box-Behnken experimental design. The variables A, B and C represented the initial sugar concentration, ammonium sulphate concentration and incubation period. The second order variables (A^2, B^2 and $C^{2)}$ depicted the squared interactions and the two way interactions between the variables were represented as AB, AC and BC.

Table 4.5. Box-Behnken Design –ANOVA

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Regression	9	36.9833	36.9833	4.1093	27.40	0.001
Linear	3	13.7500	13.7500	4.5833	30.56	0.001
Square	3	17.9833	17.9833	5.9944	39.96	0.001
Interaction	3	5.2500	5.2500	1.7500	11.67	0.011
	R^2 98.01				Adj R^2 94.43	

DF: Degrees of freedom, Seq SS: Sequential sum of squares, Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean square, F; F static,P: The calculated probability

ANOVA is used to test the variability in a given data set .It divides the data set into two groups- random factors and statistically significant systematic factors and calculates the effect of independent variables on the response. The ANOVA showed that the overall regression, the linear, squared and two way interactions were significant with p value less than 0.05. The regression analysis showed that the most effective factor influencing L-lysine production was the incubation period with its lowest p value.

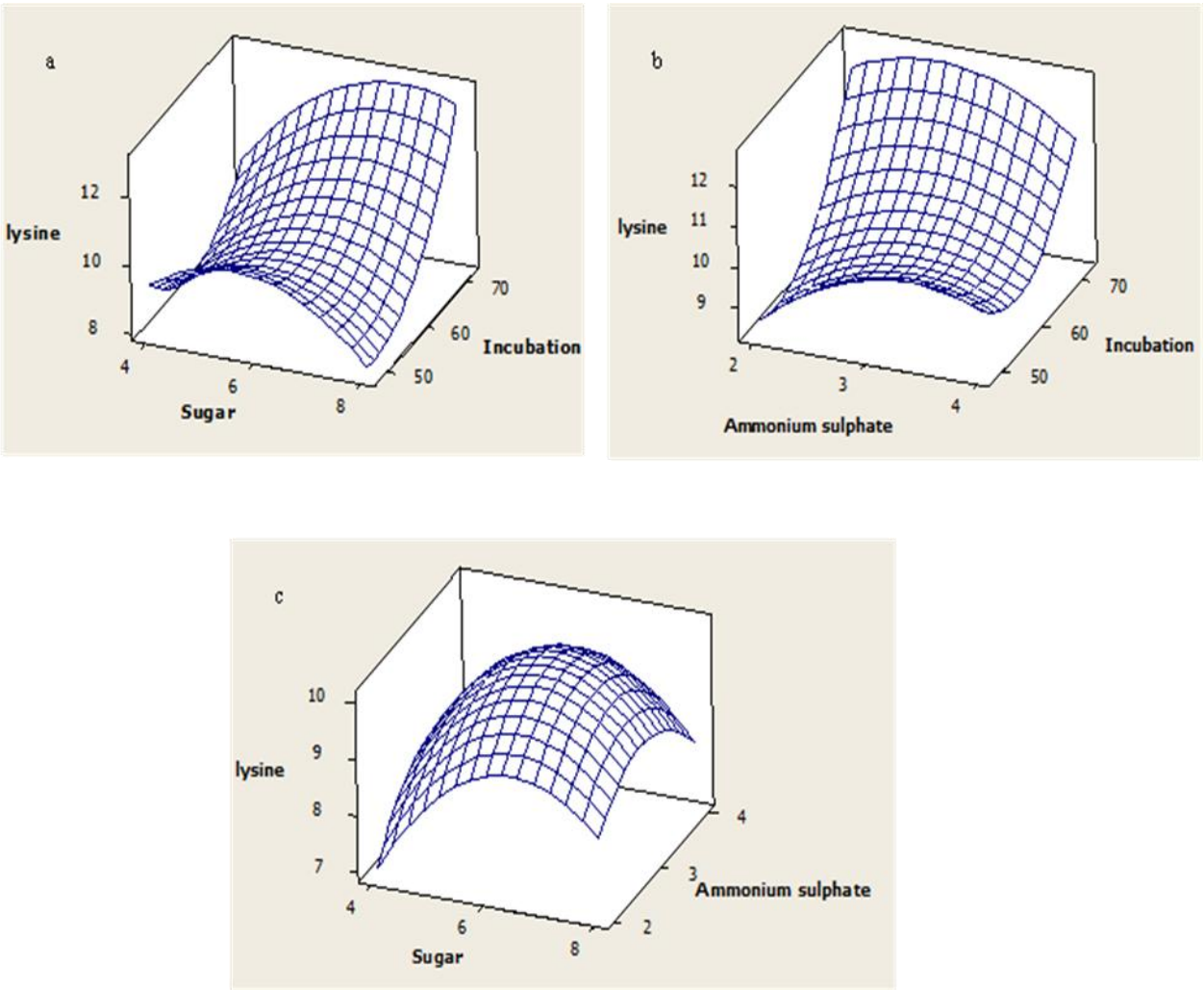


Fig 4.3. Response surface graphs of interactions of (a) sugar concentration and incubation period (b) ammonium sulphate and incubation (c) sugar concentration and ammonium sulphate concentration towards L-lysine production

The squared interactions A^2, B^2, C^2 and the two way interactions AC and BC had a significant effect on the response. The regression coefficient predicts the goodness of fit of the model to the equation. The R^2 value of 98.01% (0.98) showed that the model could explain 98 % of the variability around its mean. Adjusted R^2 value of 94.43% was in good relation with the R^2 showing that the increase in R^2 is not due to increasing number of predictors.

4.3.2. Submerged fermentation in a bioreactor

The optimized bioprocess conditions in the shake flask were adopted and ran in 2.5L fermenter in batch to examine the sustainability of L-lysine titres. L-lysine concentration increased from 0 to a maximum of 10 mg/mL during 0-72 hours of fermentation when the aeration was maintained 90% as shown in **Fig 4.4.**

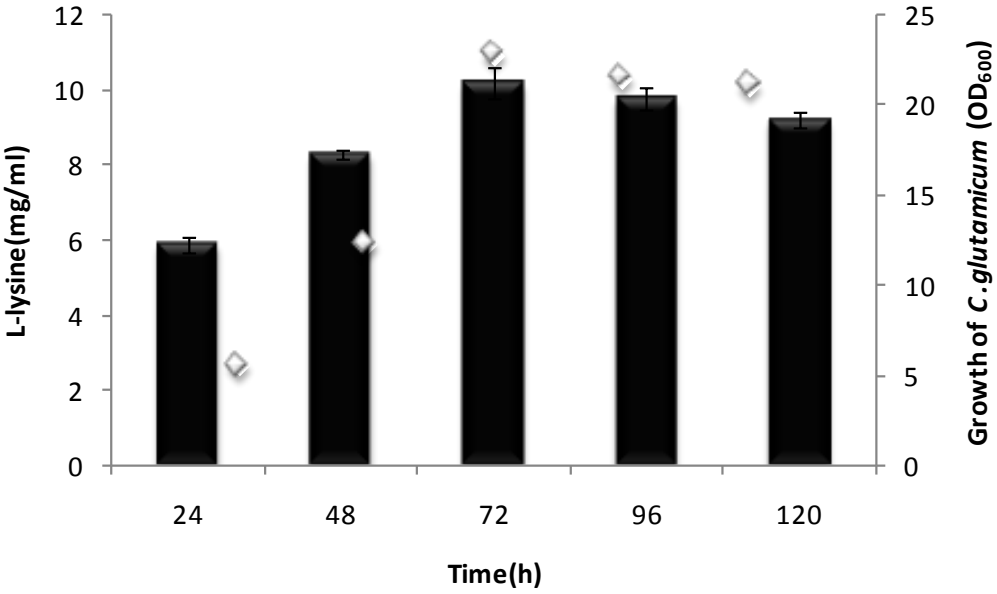


Fig 4.4.L-lysine fermentation in the bioreactor. The solid bars represent the L-lysine titre. The diamonds represents growth of *C. glutamicum* DM1729 (OD_{600})

Section B- Simultaneous Saccharification and Fermentation

Simultaneous Saccharification and Fermentation studies used jackfruit seed powder (expressed as %w/v in DoE) as the carbon source for L-lysine production. The starch saccharifying enzymes were added in respective concentrations to the media immediately after sterilization to avoid hardening.

4.3.3. Optimization of simultaneous saccharification and fermentation conditions using Plackett-Burman and Box-Behnken Design

Fourteen experimental factors were screened in twenty eight runs using PB experimental design.The experimental design and the response are shown in **Table 4.6** .The results showed that jackfruit seed powder concentration, glucoamylase concentration and incubation period in the order of decreasing intensity, affected the response L-lysine titre. This is evident from the pareto chart with the bars extending beyond the critical t-value (**Fig4.5.**).The main effects plot showed that increase in the factors increased the L-lysine titre (**Fig4.6.**). Main effect plot examines the difference in the response between a single factor at different levels. A line is drawn connecting the response means of the factor levels. If the resultant line is horizontal to the X axis, it indicates that the factor does not affect the response. There is considerable main effect of the factor when the line moves farther away from the X axis and has significance as it gets steeper.

Table 4.6. Plackett-burman design matrix with L-lysine produces as the response (Simultaneous saccharification and fermentation)

Run	A	B	C	D	E	F	G	H	I	J	K	L	M	N	R
1	4	1	25	200	40	200	25	25	1.25	5	0.1	1.5	0.5	48	3.8
2	0.5	0.1	25	200	5	200	200	200	10	5	0.1	1.5	0	120	5.2
3	0.5	1	200	200	40	25	200	200	1.25	10	1.5	0.1	0.5	120	4.6
4	4	0.1	25	200	5	25	25	25	10	10	1.5	0.1	0.5	120	7.2
5	0.5	1	200	25	40	200	200	25	10	10	1.5	1.5	0	48	5.4
6	0.5	0.1	200	200	5	25	200	25	1.25	5	1.5	1.5	0.5	120	4.2
7	4	0.1	200	200	40	200	25	25	1.25	5	1.5	0.1	0	48	4.4
8	4	1	25	25	40	200	200	200	1.25	5	0.1	0.1	0.5	120	3.6
9	4	1	200	25	5	25	200	200	1.25	10	0.1	0.1	0.5	48	5.1
10	0.5	1	200	200	40	200	25	25	1.25	10	0.1	0.1	0	120	9.1
11	4	0.1	200	200	5	200	200	200	1.25	10	1.5	1.5	0	48	5.1
12	0.5	1	25	25	5	200	25	25	10	10	1.5	0.1	0.5	48	4.7
13	4	0.1	200	200	40	25	200	25	10	5	0.1	0.1	0.5	48	3.2
14	0.5	0.1	25	200	40	25	200	200	10	10	0.1	0.1	0	48	2.9
15	0.5	0.1	200	25	5	200	25	200	1.25	10	0.1	1.5	0.5	48	8.9
16	4	1	200	25	5	25	25	200	10	5	1.5	0.1	0	120	4.8

17	4	0.1	25	25	40	25	25	200	1.25	10	0.1	1.5	0	120	9.8
18	4	1	25	200	40	25	25	200	10	10	1.5	1.5	0	48	3.2
19	4	0.1	200	25	40	200	25	200	10	5	1.5	1.5	0.5	120	4.8
20	4	1	200	25	5	25	200	25	10	5	0.1	1.5	0	48	4.2
21	0.5	1	25	200	5	25	25	200	1.25	5	1.5	1.5	0.5	48	4.4
22	0.5	0.1	25	25	5	25	25	25	1.25	5	0.1	0.1	0	48	1.2
23	0.5	1	25	25	40	25	200	25	1.25	5	1.5	1.5	0	120	4.6
24	4	1	25	200	5	200	200	25	10	10	0.1	1.5	0.5	120	9.4
25	4	0.1	25	25	5	200	200	25	1.25	10	1.5	0.1	0	120	1.1
26	0.5	0.1	25	25	40	200	200	200	10	5	1.5	0.1	0.5	48	2.1
27	0.5	0.1	200	25	40	25	25	25	10	10	0.1	1.5	0.5	120	9.6
28	0.5	1	200	200	5	200	25	200	10	5	0.1	0.1	0	120	4.4

A--$(NH_4)_2SO_4$ (%w/v), B- Urea (%w/v), C-KH_2PO_4 (mg/dL), D-K_2HPO_4 (mg/dL), E-$MgSO_4$ (mg/dL) F-$CaCl_2$ (mL), G-Trace elements (mL), H-protocatechuate (mL),I-Biotin (mL),J- JFS concentration(%w/v),K-α-amylase(mL), L-Glucoamylase (mL),M- Surfactant (mL), N-Incubation (hours), R-L-lysine (mg/mL)

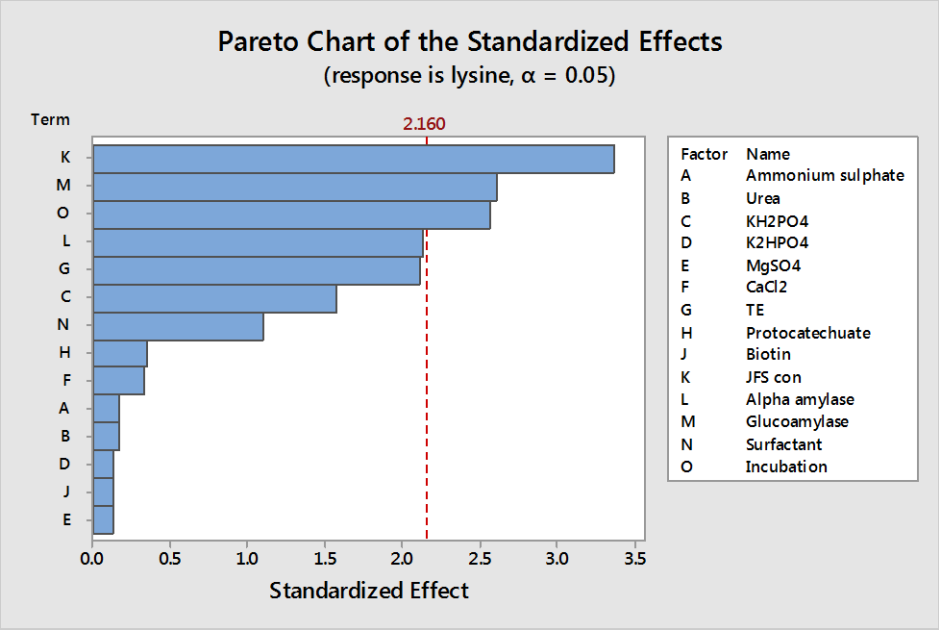


Fig 4.5. Pareto chart showing the significant variables affecting simultaneous saccharification and fermentation of L-lysine.

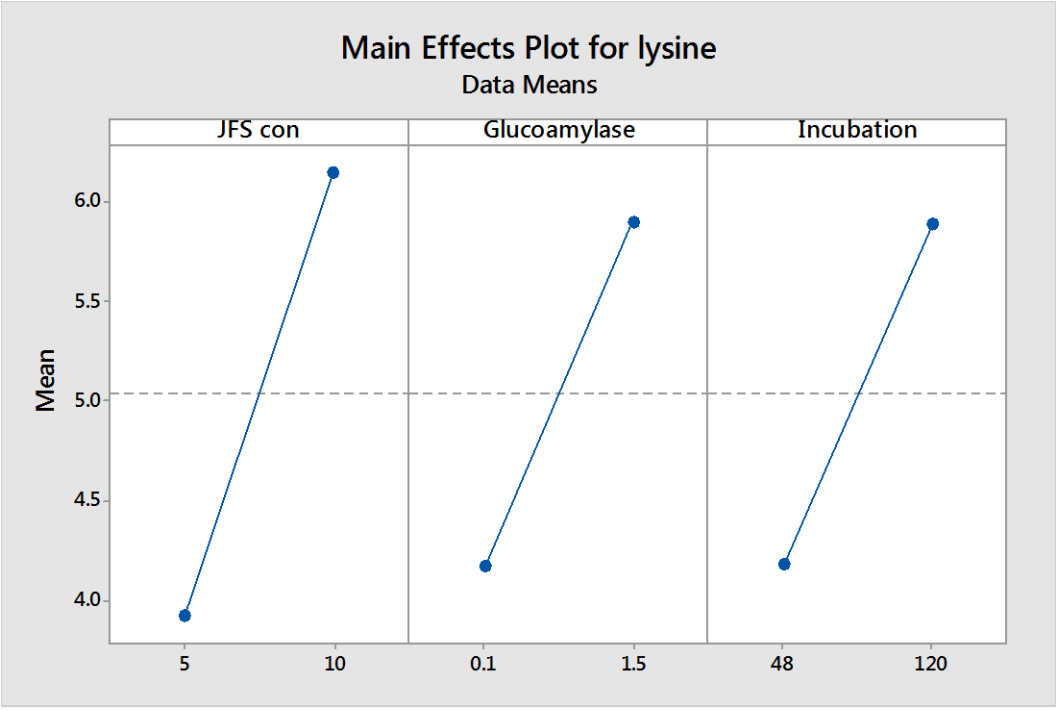


Fig 4.6. Main effect plot of factors significantly affecting L-lysine production in simultaneous saccharification and fermentation of L-lysine

The interaction effects among the significant variables were studied using Box-Behnken Design. The design and the response is shown in **Table 4.7.**The method studied the linear, squared and two-way interactions between the factors jackfruit seed powder concentration, glucoamylase concentration and incubation period. ANOVA (**Table 4.8.**) showed that the there was significant linear, squared and two- way interactions among the variables. This was concluded by p value less than 0.05. But, the only significant two –way interaction was between jackfruit seed powder concentration and incubation period. The corresponding response surface graph is shown in **Fig 4.7.** The model showed an R^2 value of 96.5 % and an adjusted R^2 value of 90.3%. The model was not very accurate in predictions, but was effective in studying the interactions. The maximum L-lysine titre obtained was 10 mg/mL (0.1g $_{lysine}$ /g $_{substrate}$) at an incubation period of 96 hours and initial substrate concentration of 8% w/v.

Table 4.7. Box-Behnken experimental design for simultaneous saccharification and fermentation of L-lysine

Run	JFS (%w/v)	Enzyme (mL)	Incubation (h)	L-lysine (mg/mL)
1	2	1	96	1.0
2	5	0.5	96	3.0
3	5	1	72	3.0
4	8	0.5	72	4.0
5	8	1.5	72	6.0
6	8	1	96	9.5
7	5	1	72	3.0
8	2	1	48	3.0
9	5	1.5	48	3.0
10	5	0.5	48	2.0
11	2	0.5	72	1.0
12	5	1.5	96	2.0
13	5	1	72	3.0
14	8	1	48	4.0
15	2	1.5	72	1.0

Table 4.8. Box-Behnken Design - ANOVA (Simultaneous saccharification and fermentation)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9.0000	64.1208	7.1245	15.40	0.004
Linear	3.0000	40.3125	13.4375	29.00	0.001
JFS	1.0000	38.2812	38.2812	82.77	0.000
Enzyme	1.0000	0.5000	0.5000	1.08	0.346
Incubation	1.0000	1.5313	1.5313	3.31	0.128
Square	3.0000	7.7458	2.5819	5.58	0.047
JFS*JFS	1.0000	3.2452	3.2452	7.02	0.045
Enzyme*Enzyme	1.0000	3.2452	3.2452	7.02	0.045
Incubation*Incubation	1.0000	0.7067	0.7067	1.53	0.271
2-Way Interaction	3.0000	16.0625	5.3542	11.58	0.011
JFS*Enzyme	1.0000	1.0000	1.0000	2.16	0.201
JFS*Incubation	1.0000	14.0625	14.0625	30.41	0.003
Enzyme*Incubation	1.0000	1.0000	1.0000	2.1600	0.2010

DF : Degrees of freedom, Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean square, F; F static, P: The calculated probability

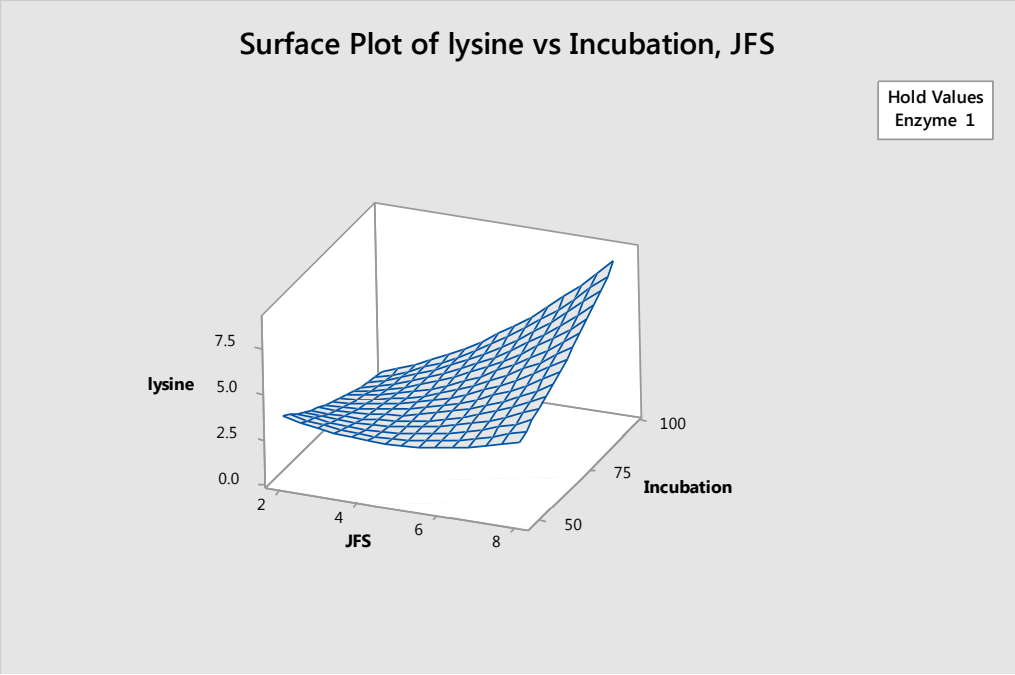


Fig 4.7. Response surface graph of interaction between jackfruit seed powder concentration and incubation period in simultaneous saccharification and fermentation for L-lysine production

Use of statistical tools has been advantageous in the present study, which helped to compare and analyze the factors affecting two different modes of fermentation. L-lysine production usually used molasses like cane molasses, black strap molasses and various starchy substrates. From glucose the reported yields of L-lysine are in the range of 50-55%. Molasses are rich in glucose, sucrose and has 10–20% fructose and hence has more fermentable sugars resulting in a conversion of 30-40%. Fermentation with starch hydrolysates of cereals, rap, flax and cotton accumulated 30-40 g/L of L-lysine. A homoserine auxotrophic mutant of *C. glutamicum* produced 25 g/L L-lysine from cane molasses based medium at 60 g/L total sugar concentration. All the other sugar concentration above 60 g/L gave lower L-lysine concentrations (Nelofer et al., 2007). L-lysine production from pulpy date waste, molasses and glucose at the same experimental conditions yielded different concentrations of L-lysine (Moosavi-Nasab et al., 2008). These indicate that the L-lysine fermentation pattern and production changes with change in substrates and thus there is a necessity to optimize the experimental conditions for different substrates used as sugar sources.

Here, the maximum L-lysine yield under submerged fermentation was 0.3 $g_{lysine}/g_{reducing\ sugar.}$ In simultaneous saccharification and fermentation mode, the maximum L-lysine yield was $0.1g_{lysine}/g_{substrate.}$ Hence considering L-lysine yield, submerged fermentation was better than simultaneous saccharification and fermentation. However, simultaneous saccharification and fermentation was advantageous in reducing steps in the fermentation process flow and may limit the inhibitory effects of high sugar concentration by slow release of reducing sugars. As *C. glutamicum* prefers temperature around 30 ˚C, the optimum temperature for enzyme activity cannot be applied for the process. Construction of recombinant strains with temperature tolerance or introduction of recombinant thermostable enzymes may solve the problem (Kim et al., 2014; Ohnishi et al., 2003).

While considering the downstream processing steps, the fermentation broth from simultaneous saccharification and fermentation may pose problems during stages of cell separation. A lot of comparative studies, techno economic analysis and in depth analysis of the process flow will only decide the better process. However, considering merits and the demerits and the yield, submerged fermentation was considered advantageous over simultaneous sachharification and fermentation in the present context.

4.4. Conclusion

Corynebacterium glutamicum DM1729 was able to grow and utilize the hydrolysate of JFS containing reducing sugars for L-lysine production under submerged fermentation. Besides, the culture was also able to utilize the sugars released during simultaneous saccharification for L-lysine production. Statistical tools like PBD and BB were successfully used used to study the effect of each parameter on L-lysine production in both modes of fermentation. The statistical tools enabled screening of numerous parameters in limited experimental runs to study the impact on L-lysine fermentation.The efficacy of jackfruit seed powder as a nutrient source for L-lysine production in the two modes of fermentation is established with the study. Plackett –Burman and Box-Behnken experimental designs were successfully employed in the parametric evaluation of L-lysine production resulting in an increase in yield from 0.14 $g_{lysine}/g_{reducing\ sugar.}$ to 0.3 $g_{lysine}/g_{reducing\ sugar.}$ in submerged fermentation. The optimum conditions when scaled up in a 2.5 L bioreactor gave a L-lysine titre of 10 mg/mL. Simultaneous saccharification and fermentation also resulted in L-lysine titres of up to 10 mg/mL with yield of 0.1 $g_{lysine}/g_{substrate.}$

Chapter 5

SOLID-STATE FERMENTATION FOR L -LYSINE PRODUCTION

5.1. Introduction

Microbial fermentation has become one of the major industrial production processes for amino acids since its discovery in the 1950s (Kinoshita et al., 2004). The increasing demand in the end use market has been fuelling the rapid growth of the industry. To counterbalance the demand and supply, the major focus has always been directed towards either the improvement of the production strain or technical advancement in the production process. Strain improvement targets classical mutagenesis or engineering the biosynthetic pathways for amino acid production or even broadening the substrate utilization range (Wittmann & Becker, 2007). On the other hand, process improvement aims at the improvement of existing production process or trying the alternate processes (Anastassiadis, 2007).

Solid -State Fermentation (SSF) is the growth of microorganisms on solid material in the absence or near absence of free water (Pandey et al., 2008). SSF system is employed in the production of a plethora of compounds with high production rates. It is considered economical than submerged fermentation as the overall production technology is simple and require lower investment, low energy consumption and waste water output (Pandey et al., 2008; Pandey et al., 2000). India, with almost 60% agricultural land (Theworldbank, 2016) has copious amount of plant biomass and agro residues which could be used as substrates for solid-state fermentation. SSF processes are reported to upgrade the quality of the agro residual biomass to be used as animal feed (Tripathi & Yadav, 1992; Villas-Bôas et al., 2002). In SSF, the solid support can be an inert solid substrate (solid-state fermentation) moistened with the production medium or use substrates which act as both nutrient and support (solid-substrate fermentation). SSF process for the production of amino acids (Nampoothiri & Pandey, 1996) and other bioproducts such as enzymes, pigments etc are reported (Pandey et al., 2000; Soccol et al., 1994). Even though SSF process is usually used for fungal fermentation, bacterial fermentations have also been

successful (Baysal et al., 2003; John et al., 2007; John et al., 2006; Nampoothiri & Pandey, 1996; Prakasham et al., 2006; Rojan et al., 2005) for the production of organic acids, amino acids and enzymes.

The present study attempts L-lysine production under solid-state fermentation by *C. glutamicum* DM 1729 using sugarcane bagasse as an inert substrate moistened with starch hydrolysate of jack fruit seed.

5.2. Materials and Methods

5.2.1. Microorganism and culture conditions

The L-lysine producer strain *C. glutamicum* DM1729 was used throughout the study. The pre-inoculum and inoculum were prepared as in Section 2.3.1.The bacterial biomass was collected and used as the inoculum in desired concentrations as described in the experimental designs for the solid-state fermentation.

5.2.2. Substrate screening and processing

Sugarcane bagasse, Polyurethane foam (PUF), Sugarcane tops, Bamboo powder were procured locally. All substrates were washed, dried and processed to suitable particle sizes before fermentation. Sugarcane bagasse, which was selected as the inert support for SSF was procured locally, milled and processed as described in section 2.2.2.i. This was used as the inert matrix for fermentation. The surface morphology of sugarcane bagasse was shown in **Fig 5.1a.** and **5.1b.** Starch hydrolysate of jackfruit seed powder (JFS) was used as the moistening agent. Jack fruit seeds were procured locally, dried and the outer white aril was removed. The cotyledons with the brown spermoderm were powdered in a knife mill and screened to obtain particles of size < 0.425 mm and were used for hydrolysate preparation. The substrates were screened for their capacity to act as inert substrate in solid state fermentation. Fermentation was carried out with an initial moisture content of 90% for 96 hours. CGXII mineral medium supplemented with 4% w/v glucose was used as the moistening agent. The best substrate was selected based on the L-lysine titre.

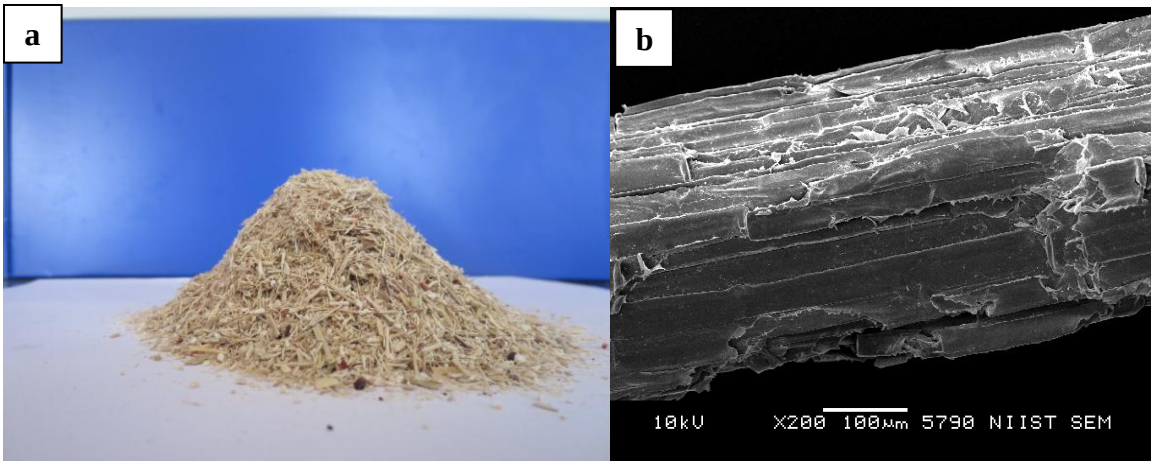


Fig 5.1 (a) Sugarcane bagasse (b) SEM image of sugarcane bagasse showing the surface morphology.

5.2.3. Preparation of starch hydrolysate of JFS

Jack fruit seed powder (9% w/v) was gelatinized at 121 ˚C for 15 minutes, liquefied with a commercially available alpha amylase and further saccharified with glucoamylase. The hydrolysate thus prepared was filtered through muslin cloth and centrifuged at 8000 rpm for 10 minutes to obtain a clear liquid. Methodology is detailed in Section 2.2.2.ii.

5.2.4. Solid -State Fermentation

All the experiments were carried out in 250 mL Erlenmeyer flasks. In each experimental run, 3 grams of the sterilized sugarcane bagasse was moistened with CGXII medium where the pure sugar was replaced with starch hydrolysate of jack fruit seed powder. The CGXII salts composition was kept constant and only the variables like sugar concentration or initial moisture percentage was varied as per the experimental design. Unless otherwise specified, the flasks were inoculated with cells pellet from 25 mL of seed medium at OD_{600} of 1, mixed thoroughly and incubated at 30 ˚C in static condition till the stipulated period of time in the experimental design and harvested as a whole.

5.2.5. Extraction

After the desired period of incubation, extraction of the fermented matter was done by adding 10% w/v deionized water to each flask and agitating at 200 rpm for three hours. The extract was centrifuged at 6000 rpm for 10 minutes and the supernatant was then used for various analyses.

5.2.6. Analytical procedures

Moisture measurements were made using moisture analyzer (AND MX-50, Japan) and the reducing sugar concentrations were measured by DNS (3, 5-Dinitro salicylic acid) assay (Miller, 1959). L-lysine was quantified according to Agilent protocol employing pre column derivatization with OPA (Henderson et al., 1999)**.** Protocols were discussed in section 2.4.5.ii.

5.2.7. Experimental designs

All the experiments were designed and analyzed using Minitab statistical software. Plackett-Burman Design (PBD) was used as the screening design. Using a limited number of experiments, the PB design determined the significance of each input variable or factor and subsequently the selected factors or variables were studied in combinations by Response Surface Methodology

5.2.7.1. Plackett- Burman experimental design (PBD)

PBD (Plackett & Burman, 1946) served as an effective tool for understanding the main effects of the variable or factor on the design, assuming that the interactions were negligible. The variables under consideration were screened in a total of twelve experimental runs with each variable tested at a high level coded as +1 and low level coded as -1.The factors or variables screened were initial sugar concentration, particle size of the solid support matrix, initial moisture, inoculum size, incubation period and ammonium sulphate concentration. The PBD works on a first order polynomial model

$$Y=\beta o+\Sigma\beta iXi. \qquad (1)$$

The effect of each variable is determined by the equation

$$E(Xi) = \frac{\Sigma M_{i+} - \Sigma M_{i-}}{N} \qquad (2)$$

where $E(Xi)$ is the calculated effect, ΣM_{i+} is the sum of the high level response values *and* ΣM_{i-} is the sum of low level response values and N is the number of experimental runs. The experimental design and the responses are summarized in **Table 5.1.**

5.2.7.2. Response Surface Design

To understand the interaction effects contributing to the production of L-lysine, Central Composite Design experiments were conducted (Box & Wilson, 1951). Three significant factors viz sugar concentration, moisture and incubation period were studied in 20 experimental runs. To correlate the response variables and independent variables a second order quadratic equation was used which is generally expressed as in equation 3.

$$Y = \beta o + \Sigma \beta i Xi + \Sigma \beta ii Xi^2 + \Sigma \beta ij Xi Xj \qquad (3)$$

where *Xi and Xj* are the independent variables influencing the response variable *Y*, *βo* is the interception coefficient, *βi* is the coefficient of linear effect, *βii* is the coefficient of quadratic effect and *βij* is the ijth interaction coefficient. The experimental design and the response are summarized in **Table 5.3.**

The regression analysis and statistical significance of the models were tested with ANOVA using the Minitab statistical software. The R^2 value predicted the wellness of the fit of the sample data to the regression equation; the adjusted R^2 indicated the unnecessary predictor variables that do not actually improve the model. Predicted R^2 was used to measure the amount of variation in the experimental data to the predicted response and showed the wellness of prediction. Fischer F test was used in testing the statistical significance and t test for testing regression coefficient.

5.3 Results and Discussion

5.3.1. Substrate screening

Sugarcane bagasse, Polyurethane foam (PUF), Sugarcane tops, Bamboo powder were randomly screened to be used as inert carrier in L-lysine production by SSF. The substrates used for screening are depicted in **Fig 5.2**. Under the experimental conditions, the L-lysine production was maximum (2 mg/gds) when sugar cane bagasse was used as the support substrate and thus it was selected for further experiments. Bacterial growth on sugarcane bagasse was shown in **Fig 5.3.**

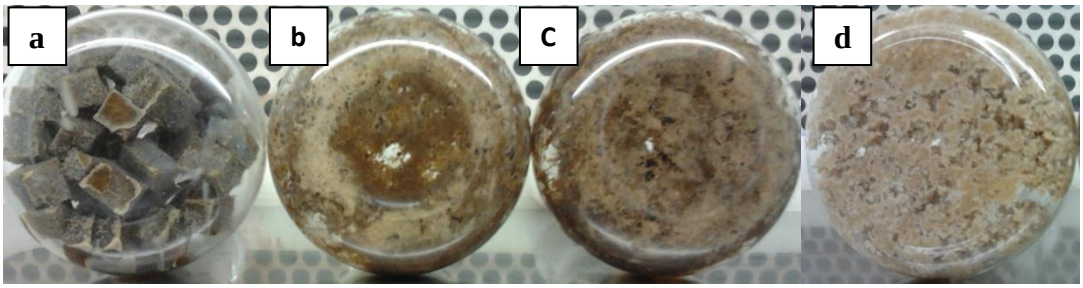


Fig5.2. Solid-state fermentation using inert supports for L-lysine production with (a) PUF (b) Sugarcane tops (c) bamboo powder (d) Sugarcane bagasse

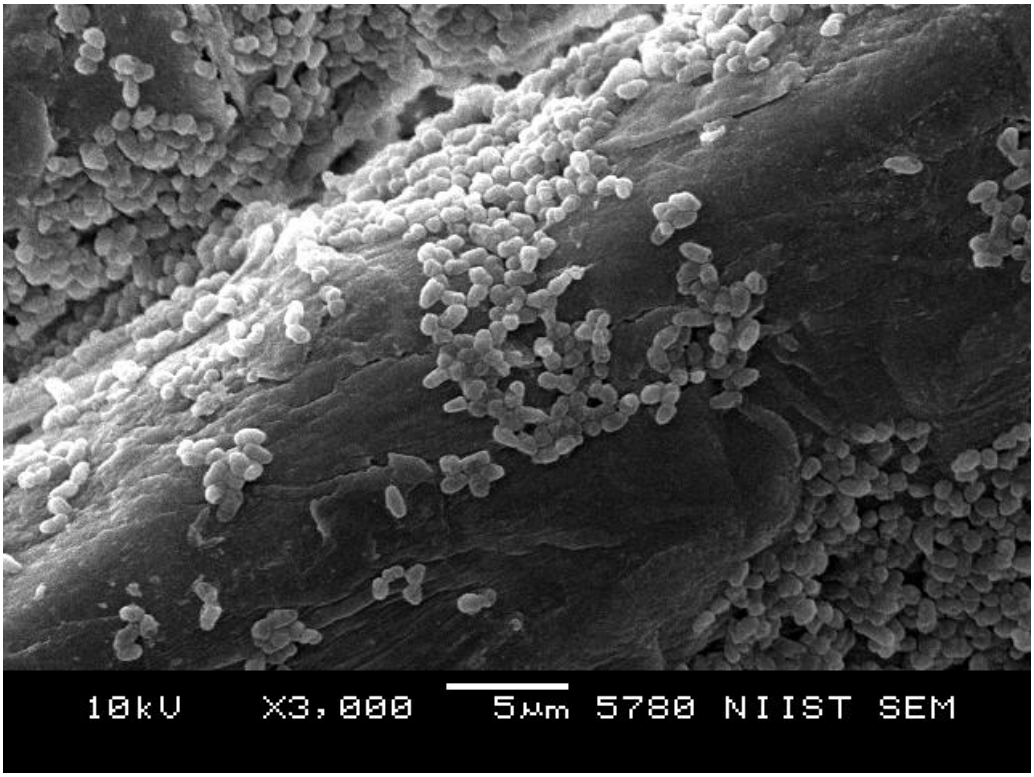


Fig5.3. Scanning Electron Micrograph of *C. glutamicum* adhered on the surface of sugarcane bagasse in SSF.

5.3.2. Screening for significant factors in L-lysine fermentation using Plackett-Burman experimental design

In different experimental runs of the PB design, the scale of variation in L-lysine production was from 2.3 mg/gds to 13.9 mg/gds. This suggested the significance of the factors

Table5.1. Plackett- Burman experimental design of variables (uncoded levels) with L- lysine produced as response values in SSF

Run Order	**PtType**	**Blocks**	**Moisture (%)**	**Particle size (mm)**	**Incubation (hours)**	**Inoculum (OD_{600})**	**Sugar concentration (%w/v)**	**$(NH_4)_2SO_4$ (%w/v)**	**L-Lysine (mg/gds)**
1	1	1	90	≤ 0.5 mm	96	2	2	4	3.9
2	1	1	90	0.5-1 mm	36	2	2	0.5	2.3
3	1	1	70	0.5-1 mm	36	1	2	4	8.3
4	1	1	70	≤ 0.5 mm	36	1	2	0.5	2.9
5	1	1	70	0.5-1 mm	96	1	8	0.5	**13.9**
6	1	1	70	≤ 0.5 mm	36	2	8	4	4.9
7	1	1	90	0.5-1 mm	96	1	8	4	11.7
8	1	1	90	≤ 0.5 mm	36	1	8	4	4.6
9	1	1	90	≤ 0.5 mm	96	1	2	0.5	3.9
10	1	1	70	0.5-1 mm	96	2	2	4	8.2
11	1	1	90	0.5-1 mm	36	2	8	0.5	4.6
12	1	1	70	≤ 0.5 mm	96	2	8	0.5	13.5

involved and the variations caused in the response at the higher and lower levels. The PBD design and responses are described in **Table 5.1.** The regression analysis of the model is as shown in **Table 5.2.** and the significant factors affecting the response were identified by the corresponding p value. A p value lower than the selected significance (<0.05), showed that the factor and the corresponding response has a statistically significant relationship. It had lower chances of accidently rejecting the null hypothesis that was true. On this basis, the factors viz, moisture, initial sugar concentration and incubation period were identified as the significant factors having a linear effect on L-lysine fermentation. The significant factors were also identified from a pareto chart. The bars that extend beyond the critical t value described the significant factors on the pareto chart (**Fig 5.4.**). The positive and negative values of the coefficients were indicative of their influence on the overall

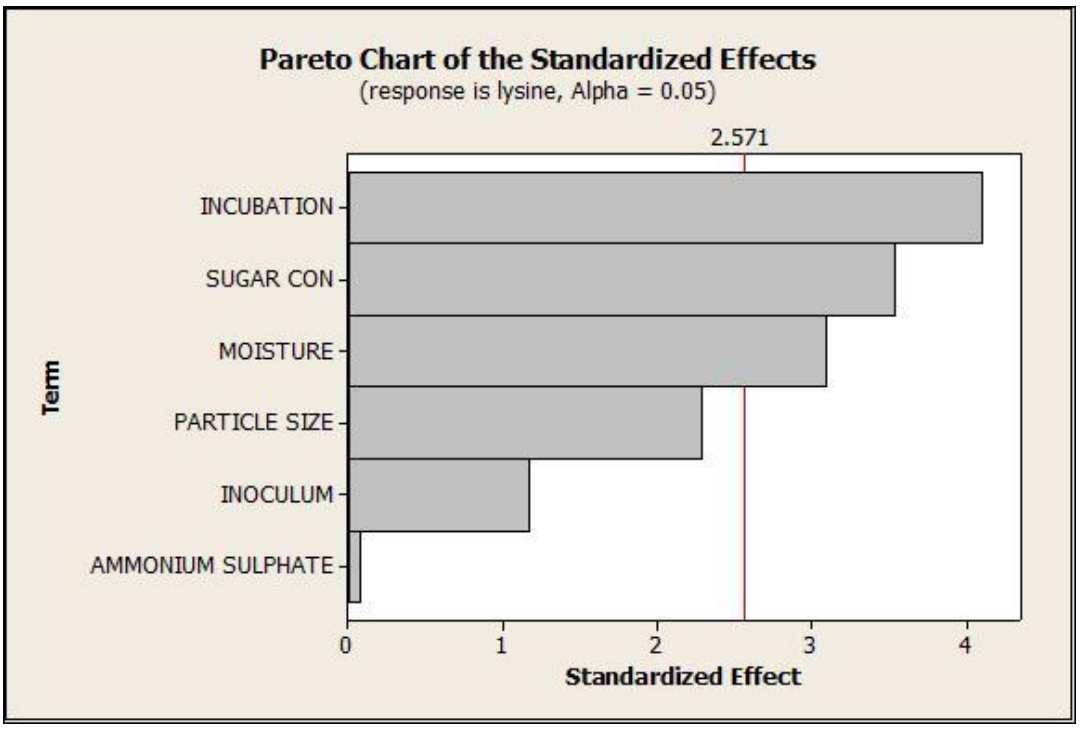


Fig5.4. Pareto chart of standardized effects showing significant variables
(PBD) for L-lysine production in SSF

L-lysine production process, the positive factor on increase in the concentration and negative factors on decrease in concentrations, increased the production. The main effect of each factor on the response was also depicted by the main effects plot (**Fig 5.5.**). The farther the plotted lines were from the X axis, more pronounced was the effect of the

factor and vice versa. Evidently decrease in moisture; higher incubation periods and higher sugar concentrations helped higher L-lysine production, when considered independently.

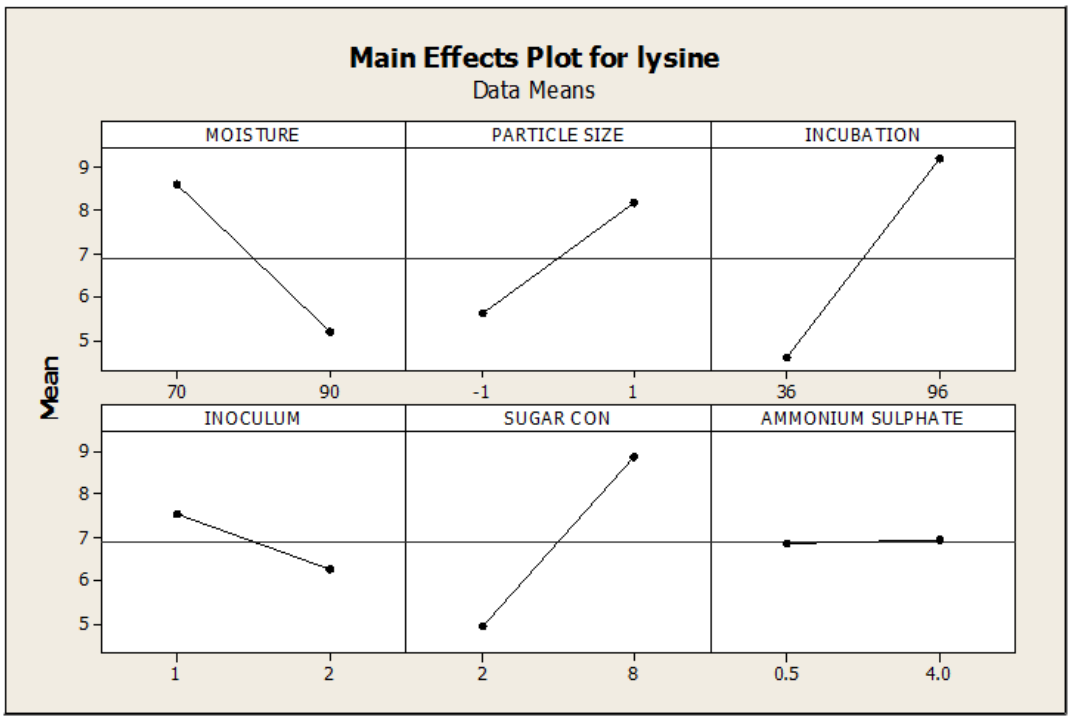


Fig5.5. Main effects plot for L-lysine production

Table5.2. PBD-estimated effects and coefficients for L-lysine (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		6.892	0.5586	12.34	0.000
Moisture	-3.450	-1.725	0.5586	-3.09	0.027
Particle size	2.550	1.275	0.5586	2.28	0.071
Incubation	4.583	2.292	0.5586	4.10	0.009
Inoculum	-1.317	-0.658	0.5586	-1.18	0.292
Sugar conc.	3.950	1.975	0.5586	3.54	0.017
$(NH4)_2SO_4$	0.083	0.042	0.5586	0.07	0.943

Effect: The estimated effects of variables, Coef: The coefficients of interception and estimated slopes, SE Coef: The standard error coefficient, T: The t-test statistic, P: The calculated probability

5.3.3. Optimization of the process variables by Response Surface Methodology

The factor levels and the effect of their interaction on L-lysine production were studied by Central Composite Design (CCD). Twenty experimental runs were conducted with varying levels and combinations of the factors under consideration. The response values ranging from 2.3mg to 16 mg/gds accounted for the feasibility of the selected factors and levels. The experimental design is shown in **Table 5.3.** The regression analysis depicted the fit of the model, the significance of the factors and their linear, squared and interaction effects (**Table 5.4.**).

Table5.3.CCD experiment with three variables and the responses in uncoded levels

Std. order	Run order	Blocks	Sugar conc. (%w/v)	Incubation (hours)	Moisture (%)	L-lysine (mg/gds)
16	1	1	7.5	96	70	16.0
8	2	1	9	120	75	8.3
20	3	1	7.5	96	70	**16**
7	4	1	6	120	75	2.1
13	5	1	7.5	96	61	8.0
10	6	1	10	96	70	14.4
1	7	1	6	72	65	3.4
15	8	1	7.5	96	70	16.0
4	9	1	9	120	65	6.9
9	10	1	5	96	70	2.3
14	11	1	7.5	96	78	13.6
17	12	1	7.5	96	70	16.0
18	13	1	7.5	96	70	16.0
5	14	1	6	72	75	3.1
12	15	1	7.5	136	70	12.3
6	16	1	9	72	75	12.0
11	17	1	7.5	55	70	3.2
3	18	1	6	120	65	12.4
19	19	1	7.5	96	70	16.0
2	20	1	9	72	65	5.0

.

The analysis showed the positive linear effect of the factors and their impact on the response. The coefficient values depicted the magnitude of impact of each factor on response. The p values were used to test the statistical significance of the model and the factors influencing the response. Sugar concentration and incubation period had significant

effect on L-lysine production. The squared interactions of sugar concentration, incubation period and moisture were significant with low p values and had negative quadratic effects. Some terms also had significant interactions effects. Percentage of moisture had significant interaction with both sugar concentration and incubation period, but the interactions between sugar concentration and incubation period was not significant as shown by a higher p value. The significance of the model was explained using ANOVA (**Table 5.5**). The calculated p value of the model 0.001 indicates the good fit of the model. The coefficient R^2 was calculated as 90% which is in reasonable agreement with the adjusted R^2 value of 81.6%. The model could explain 90% of the variability in the experiments with an adjustment in statistical shrinkage of 81.6%. The ANOVA also showed significant linear, squared and interaction effects of the variables on L-lysine production indicated by the p values less than α (0.05).

Table5.4. Estimated regression coefficients for L-lysine (CCD)

Term	**Coef**	**SE Coef**	**T**	**P**
Constant	16.061	0.9534	16.846	0.000
sugar	2.3102	0.6326	3.652	0.004
incubation	1.5746	0.6326	2.489	0.032
moisture	0.5285	0.6326	0.836	0.423
Sugar × sugar	-3.1035	0.6158	-5.040	0.001
incubation × incubation	-3.3157	0.6158	-5.384	0.000
moisture × moisture	-2.2373	0.6158	-3.633	0.005
Sugar × incubation	-1.2250	0.8265	-1.482	0.169
Sugar × moisture	2.3750	0.8265	2.874	0.017

Coef: The coefficients of interception and effects, SE Coef: The standard error coefficient, T: The t-test statistic,P: The calculated probability

Table5.5. Analysis of variance for L-lysine (CCD)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	508.883	508.883	56.543	10.35	0.001
Linear	3	110.561	110.561	36.854	6.74	0.009
Square	3	310.771	310.771	103.590	18.96	0.000
Interaction	3	87.550	87.550	29.183	5.34	0.019

R^2 = 90.30% R^2 (adj) = 81.6%

DF : Degrees of freedom, Seq SS: Sequential sum of squares, Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean square, F; F static,
P: The calculated probability

5.3.4. Response surface plots

Three dimensional graphs were generated for studying the significant interaction effect of the variables or factors on L-lysine production. Three diamensional plots were created with the predictor variables on the X and Y axes and response on the Z axis. **Fig 5.6(a)** showed the interaction effect of incubation period and moisture on L-lysine production when the sugar concentration kept as constant. L-lysine production increased with the increase in moisture up to 70% and further increase in moisture showed a decrease in production. The maximum production was at 70% moisture and 96 hours of fermentation. Moisture percentage affects the physical properties of the substrate. At very low moisture levels, there is improper distribution of the nutrients and

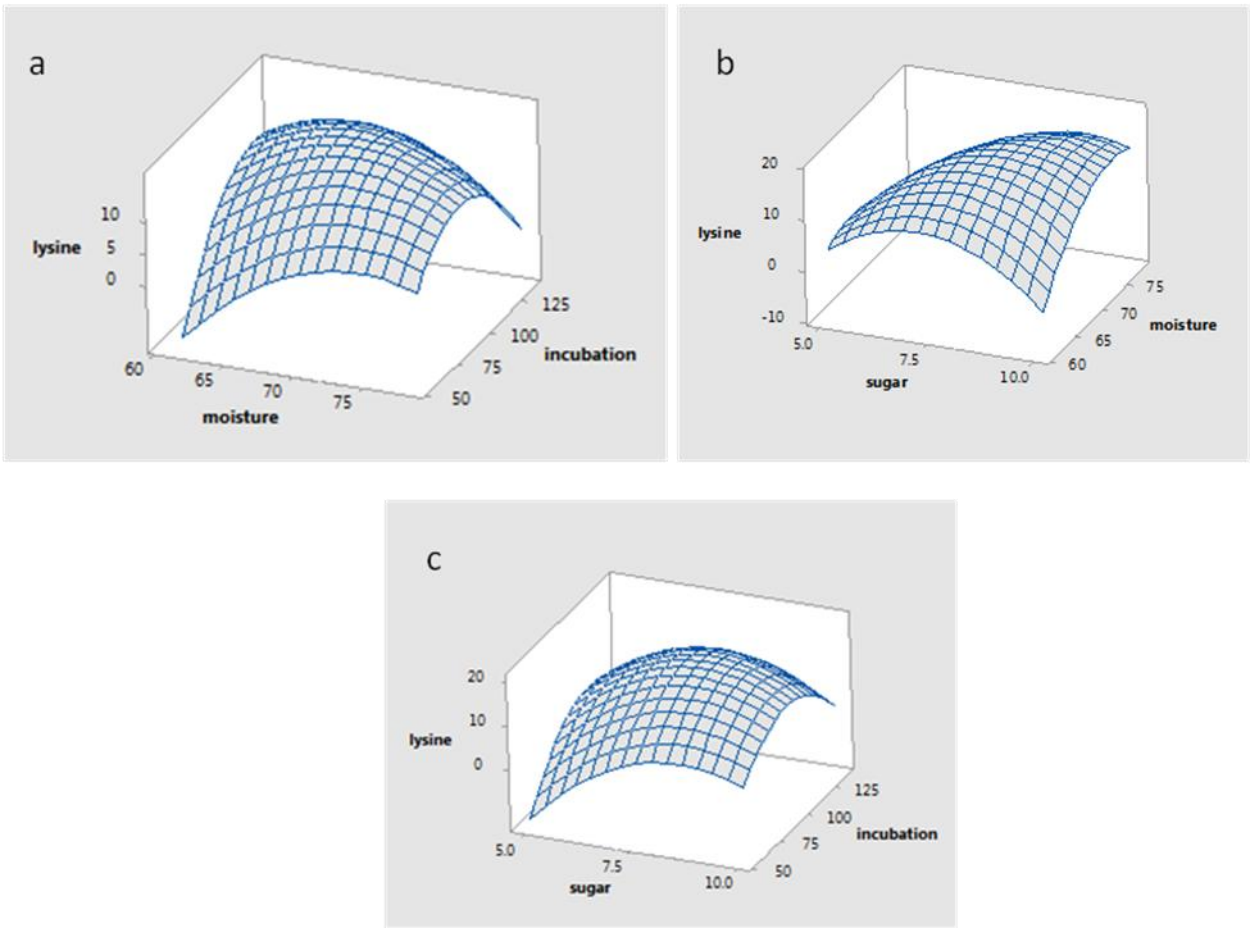


Figure 5.6. Surface plot of L-lysine produced (mg/gds) against (a) the incubation period and moisture (b) sugar and moisture (c) sugar and incubation

decreased availability to the organism and hence decrease in production. On the other hand, at high moisture levels, there is easy availability of nutrients, but agglomeration of the particles reduce the overall porosity and hinder proper heat transfer, mass transfer and product formation (Divakar et al., 2006; Kumar et al., 2003).This explains the decreased amino acid production at high moisture percentage. **Fig 5.6(b).** showed the interaction effect of sugar concentration and moisture on L-lysine production with incubation period kept as constant. L-lysine produced increased up to a sugar concentration of 7.5% with the maximum production at 70% moisture. Further increase in sugar concentration led to decreased production .This can be explained by the osmotic stress factor. In SSF, the water activity is low resulting in osmotic stress (Ruijter et al., 2004). *C. glutamicum* is prone to changes in energy requirements under osmotic stress (Guillouet & Engasser, 1995). Metabolic flux analysis showed that increased osmolalities increase specific fluxes via

PTS and TCA cycle (Varela et al., 2003). This in turn trigger increased ATP production rate which requires elevated maintenance energy for the organism resulting in decreased L-lysine production. **Figure 5.6(c)** showed the response surface graph of L-lysine production when there is no significant interaction between the sugar concentration and incubation period.

Present study exemplifies the possible blend of starchy agro residual hydrolysate and inert substrate for the successful production of L-lysine by SSF. Sugarcane bagasse has been successfully used as substrate for SSF for production of organic acids and enzymes (da Penha et al., 2012; dos Santos et al., 2003; Kumar et al., 2003). At the perfect moisture percentage and sugar concentration, sugarcane bagasse acted as an excellent inert substrate. The suitability is evident from the growth of bacterial cells on the surface of sugarcane bagasse in the SEM image (**Fig5.3.**). Even though amino acid production is reported using SSF (Ahmad et al., 2011; Nampoothiri & Pandey, 1996), submerged fermentation is usually preferred in large scale production processes. But different grades of L-lysine are produced and marketed targeting various end use markets. Since L-lysine has the largest end use application in animal feed market which does not demand high purity grades, the SSF product can find direct application. L-lysine produced by SSF may be directly used as feed for cattle, swine, poultry or fish depending on the compatibility of substrates. L-lysine produced by SSF may help to cut down or eliminate extraction and downstream processing steps imparting consequential economic advantages.

5.4. Conclusion

Corynebacterium glutamicum DM 1729 was able to grow and produce L-lysine on inert sugarcane bagasse supplemented with jack fruit seed hydrolysate as the moistening and nutrient agent. Plackett-Burman experimental design and Central Composite Design were applied effectively for the improvement of the production process and identified sugar concentration, moisture and incubation period as the significant factors affecting fermentation. Central Composite Design was used to study the contributory and interaction effects of these factors on L-lysine fermentation. Since the amino acid production by *C. glutamicum* in solid-state fermentation is convincingly proved, the future prospects to exploit more of such agro industrial residues may lead to better production rates and feed formulations. Capitalizing on the GRAS (Generally Recognized as Safe) status of this well studied industrial microbe, the fermented matter can be further dried and

directly used as animal feed. Furthermore, a clear understanding of the intricacies underlying the SSF process will lead to technological advancement and commercialization in diverse fields of application.

Chapter 6

ION EXCHANGE PURIFICATION OF L-LYSINE

6.1. Introduction

Product separation and purification is the culminating point of any industrial bioproduct manufacturing process. Fermentation products can be recovered from the fermentation broth with varying degrees of purity depending on the end use. L-lysine is an essential amino acid produced by fermentation and having an increasing share in the feed market. Industrially, many methods are applied for the purification of L-lysine. There has been extensive research on L-lysine recovery processes as it is one of the steps that reflect on the overall production cost of the product.

The steps in industrial L-lysine recovery are cell separation followed by ion exchange chromatography or crystallization. Cell separation is usually accomplished by centrifugation or filtration. Crystallization yields highly pure form of L-lysine whereas products of lower purity for use in animal feed have been developed by direct spray drying, granulation or pelleting of the fermentation broth. However, ion exchange chromatography has been the industry favorite owing to the ease of operation, technological knowhow, purity of the product, recovery rate etc. The advantages of ion exchange chromatography include selectivity, speed and ease of detection of eluted compounds (Khopkar, 1998). Ion exchange chromatography coupled with other methods like reverse osmosis (Kaneko et al., 1986) or modifications of the native method were also developed (Mortaza et al., 2004). Griessbach (1939) is one of the pioneers in separation of amino acids using ion exchange chromatography.

Ion exchange chromatography depends on the selective adsorption of charged solute molecules on the oppositely charged ligands on the ion exchanger and subsequent elution with a strong eluents (Williams & Frasca, 2001). In cation exchange chromatography, the support matrix will be negative and positively charged molecules are attracted to it (**Fig 6.1.**). In anion exchange chromatography, negatively charged molecules are attracted to positively charged support matrix. In both cases, the support matrix will

have charged ligands of opposite charges which are replaced with solute molecules during chromatographic separation (Helfferich, 1962). The support matrix is usually styrene divinylbenzene with the sulphonic acid functional group (SO_3H). The sulphonic acid functional group hold the ion exchangeable group (H^+, NH_4^+ etc).

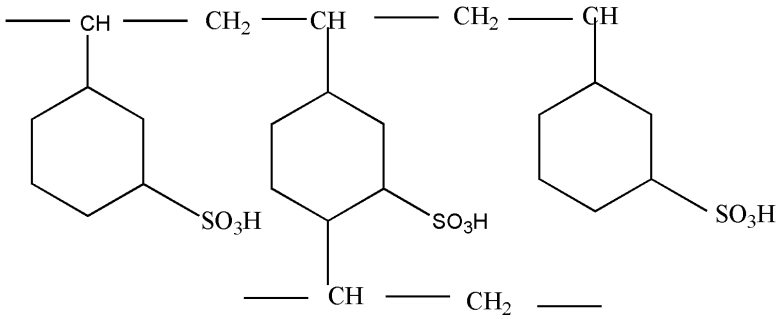


Fig 6.1. Cation exchange resin structure

In the present study, strongly acidic cation exchange resin seralite SRC 120 and weakly acidic cation exchange resin Seralite WRC50 were investigated for their capacity to purify L-lysine from fermented broth. The adsorption and elution conditions were optimized using analytical grade L-lysine and were applied in purification and recovery.

6.2. Materials and Methods

6.2.1. Microorganisms and culture conditions

C. glutamicum DM1729 was used in the study. The microorganism was cultured as described in section 2.3.1. L-lysine fermentation was done in CGXII medium supplemented with jackfruit seed powder hydrolysate. Bacterial cells from the inoculum are added to the production medium so as to get an initial OD_{600} of 1.The fermentation was continued for 96 hours and the broth was used for purification studies.

6.2.2. Ion exchange resins

In the present study, two cation exchange resins were used. One is strongly acidic and the other is weakly acidic cation exchange resin. Both were procured from SRL, India. Seralite SRC120 is the strongly acidic cation exchange resin and Seralite WRC 50 is the weakly acidic cation exchange resin. The resins had a styrene divinyl benzene backbone. The properties of the resin are described in **Table 6.1.**The moisture content of the resin was determined by moisture analyzer (AND MX50, Japan).

Table 6.1. Properties of cation exchange resins

Type	**Seralite SRC 120**	**Seralite WRC 50**
Ion exchange type	Strongly acidic cation exchanger	Weakly acidic cation exchanger
Standard mesh size	20-50	20-50
Ionic form	H+	H+
Moisture	45-50%	45-50%
pH range	0-14	5-14
Ion exchange capacity	4.5meq/g dry resin	10 meq/g dry resin
Color	Yellow brown	White

6.2.3. Adsorption capacity of resins

For adsorption experiments, Seralite SRC 120 and Seralite WRC 50 were used. L-lysine stock solutions (Sigma ≥98% pure) were prepared .One gram of moisture corrected resin samples were taken in stoppered glass tubes and shaken in aqueous solution of standard L-lysine for desired time intervals and concentrations. The L-lysine concentrations were 200mg, 100mg, 75mg, 50mg and 25 mg, per gram of resin. The glass tubes were shaken such that the resin was always in a suspension. The amount adsorbed onto the resin was calculated using the equation

$$q = \frac{l_0 - l_f}{w}$$

where l_f is the final concentration of L-lysine in aqueous solution, l_0 is the initial concentration of L-lysine and w is the weight of the resin. The equation gives the amount

of L-lysine adsorbed per gram of resin (John et al., 2008). The adsorption capacity is then expressed in percentage adsorption for comparison between the resins.

6.2.4. Effect of pH on adsorption of L-lysine by ion exchange resins.

The experiment was conducted in stoppered tubes at a controlled temperature of 30˚C and an initial L-lysine load of 5mg. The pH under study for Seralite WRC 50 was from 5- 9 and for Seralite SRC 120 was from 2-9. The pH for the standard L-lysine solution was adjusted with either 10N NaOH or 10N HCl.

6.2.5. Effect of contact time on adsorption

The effect of contact time of the weakly acidic cation exchange resin and strongly acidic cation exchange resin on the adsorption of L-lysine was studied. For the experiment one gram of the dry weight of the resin was shaken in a stoppered glass tube for varying intervals of time at pH 5 and 30 ˚C in a temperature controlled environment with a total L-lysine load of 5mg per gram of resin.

6.2.6. Selection of elution conditions

The conditions of maximum recovery of the adsorbed L-lysine were studied by elution with 1M sodium phosphate buffers with pH 10, 11, 12 and 2M ammonia solution. The recovery was also plotted under varying time intervals. Swelling of the resins on ion exchange is calculated on the basis of difference in the initial and final bed volume of the resins after elution.

6.2.7. Ion exchange chromatography for purification of L-lysine from fermented broth

The conditions optimized for the recovery of standard L-lysine were applied in the purification of L-lysine from the fermentation broth employing Seralite SRC 120 and Seralite WRC 50. Fermentation broth was centrifuged to obtain clear filtrate. The pH of the cell free fermentation broth was 6 ± 0.5 and was subsequently adjusted to 5.0 with 1M sulphuric acid. The cell free culture supernatant is now called the feed solution. The feed solution was charged on to a bed of cation exchange resin. The experiment was carried out at ambient temperature. The resin was packed in a stoppered glass column (27 cm X 1.85 cm) with the ends secured by glass wool. L-lysine from fermented broth was adsorbed on 20 g of the resin and packed in a glass column with stoppered end. The column was

washed with deionized water till the pH of the eluate reached 7.0. The L-lysine concentrations in the effluent were determined by amino acid analysis with pre column derivatization with OPA. L-lysine was thus adsorbed on the cation exchange column. The adsorbed L-lysine was eluted with 2M ammonia water, and the purity of the eluate was checked by TLC and HPLC as per the details given in section 2.4.5.i.and 2.4.5.ii. respectively.

6.3. Results and Discussion

6.3.1. Adsorption capacity of resins

The adsorption capacity of Seralite WRC 50, the weakly acidic cation exchange resin was better than Seralite SRC 120. Seralite WRC 50 bound 97% of total L-lysine in the solution whereas SRC120 bound 87% of the total L-lysine. It was noted that irrespective of initial L-lysine load, Seralite WRC 50 was better compared to SRC 120. The results are shown in **Fig 6.2.**

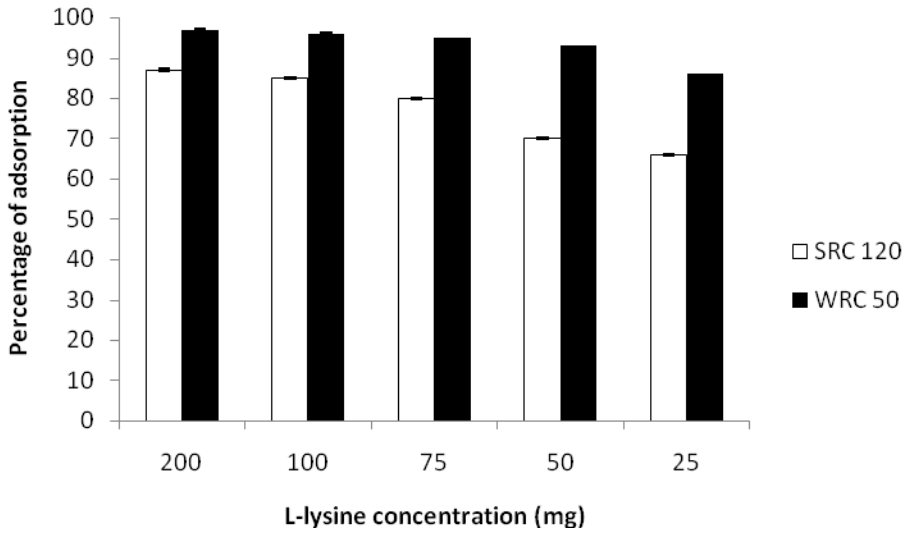


Fig 6.2. L-lysine adsorption capacity of Seralite SRC 120 and Seralite WRC 50

6.3.2. Effect of pH on adsorption of L-lysine by ion exchange resins.

pH played an important role in ion exchange chromatography, and the optimum was determined. Seralite SRC 120 showed L-lysine adsorption over a wide pH range of 2-9 and Seralite WRC 50 around pH 5 and 6 which is near the pH of fermentation broth.The results are shown in **Fig 6.3.**and **Fig 6.4.** L-lysine molecule possesses two amino groups

one at α position, one at ε position, and one α-carboxyl group. The degree of ionization is characterized by the dissociation equilibrium constant of each of these groups and the equilibrium constant is dependent on the pH value of the solution. Since the pKa values for the dissociations of the α-carboxyl group and the α-amino group are 2.18 and 8.95 respectively, the dominant ionic forms of L-lysine are divalent (+2) and monovalent (+1) ions in solutions with an acidic pH below 6. The ionic composition of L-lysine under other pH conditions can be calculated utilizing the definitions and equilibrium constant values given in **Table 6.2.** The calculated ionic compositions of L-lysine are given in **Table 6.3.** Since the ion exchange resin used has monovalent hydrogen ion (H^+) as the exchangeable group, the pH at which the solution has more monovalent ions is desirable for maximum adsorption (**Table 6.3.)**. The final pH of the fermentation broth is also near 6 ± 0.3. Hence, the pH 5.0 is chosen for further studies based on the observations mentioned above. L-lysine has a charge of +2 at pH below 2 and has more lys^{2+} ions at pH 2. Theoretically, divalent L-lysine ions occupy two positions at the exchangeable group in the ion exchanger whereas monovalent ions occupy only one position and hence there is an increase in adsorption capacity with the increase in pH of the solution. To maintain the monovalent L-lysine ion concentration in the feed solution, a neutral pH is preferred. But on the basis of loss of anti contamination property, pH above 5 is not preferred for L-lysine adsorption.

Table 6.2. Equillibrium constant and dissociation constants of L-lysine

Dissociation reaction	**Equilibrium constant**	**pKa**
$lys^{2+} = lys^{+}+H^{+}$	$Ka1=[lys^{+}][H^{+}]/[lys^{2+}]$	2.18
$lys^{+} = lys + H^{+}$	$Ka2=[lys][H^{+}]/[lys^{+}]$	8.95
$lys = lys^{+}+H^{+}$	$Ka3=[lys^{+}][H^{+}]/[lys]$	10.53

Table 6.3. Calculated percentage of lys^{2+} to lys^{+} at different pH

pH	**percentage of lys^{2+} to lys^{+}**
1	94,6
2	60,40
3	13,87
4	1.5,98.5
5	0,100

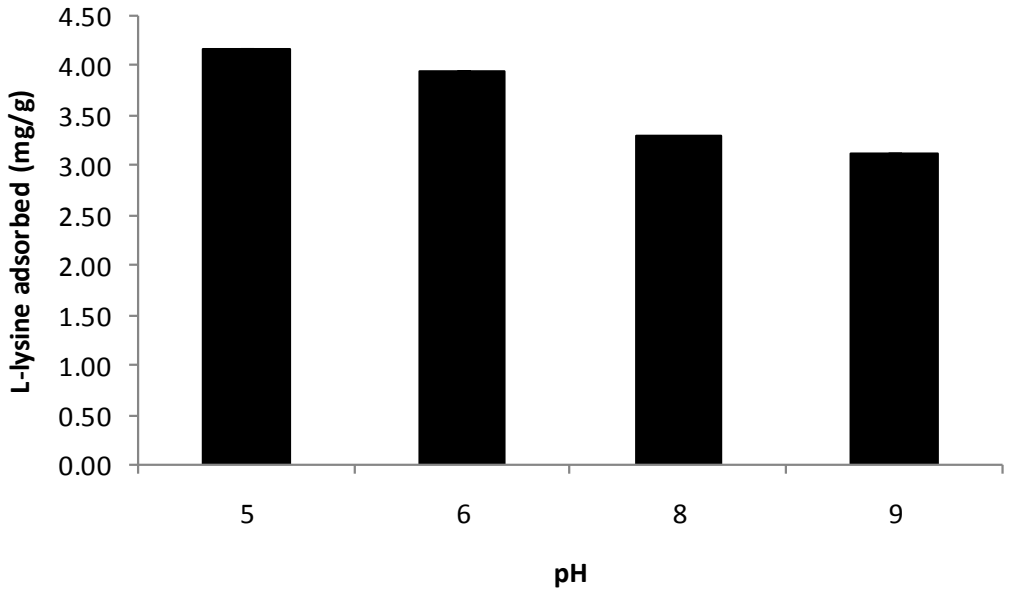


Fig 6.3. L-lysine adsorbed at different pH to Seralite WRC50

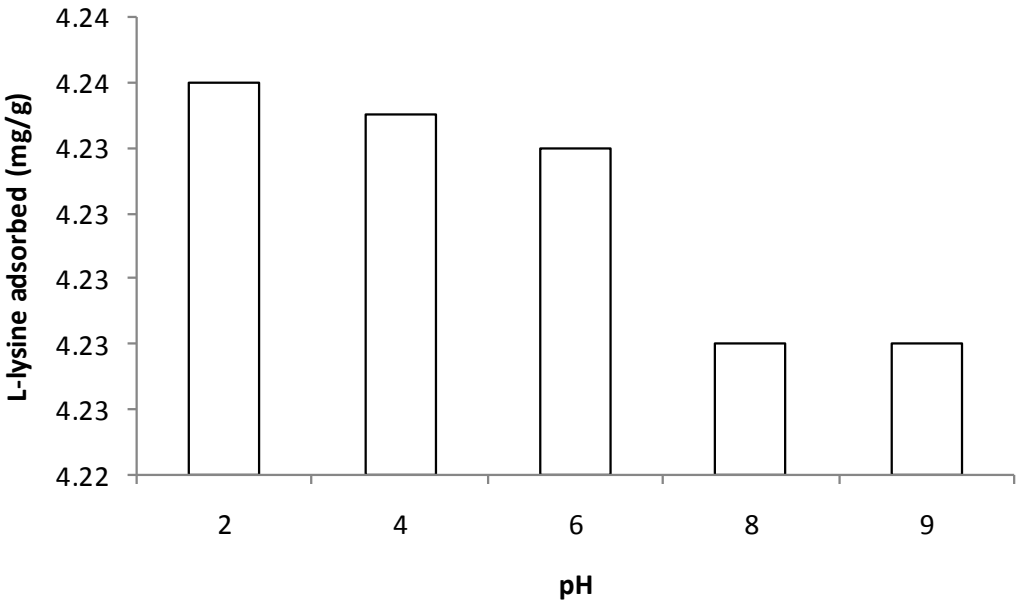


Fig 6.4. L-lysine adsorbed at different pH to Seralite SRC 120

6.3.3. Effect of contact time on adsorption

The contact time required for adsorption of standard L-lysine on the resin surface was determined by mixing and sampling at intervals. Seralite SRC120 adsorbed 86% of L-lysine present in the solution of which 82% of the adsorption took place in the first 20 minutes. On the other hand with Seralite WRC 50, the maximum adsorption was 82%, and there was no significant adsorption after 50 minutes of incubation. The results are shown in **Fig 6.5.** and **Fig 6.6.**

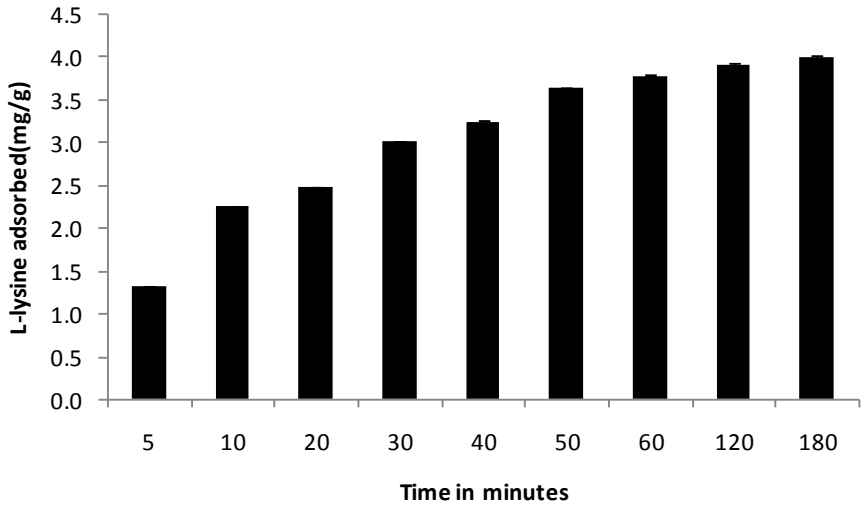


Fig 6.5. Adsorption of L-lysine to Seralite WRC 50 at different time intervals

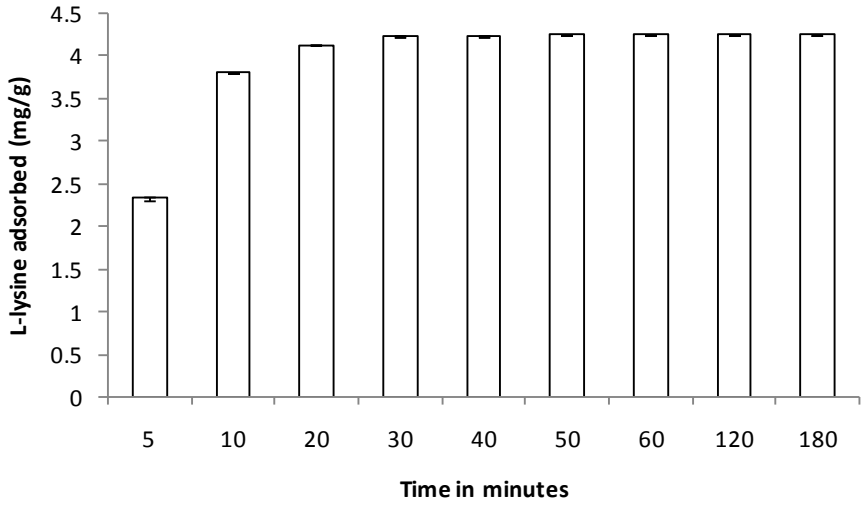


Fig 6.6. Adsorption of L-lysine to Seralite IR 120 at different time intervals

6.3.4. Selection of elution conditions

For the elution of adsorbed L-lysine, 1M phosphate buffers of pH 11, 12 and 13 and 2 M ammonia water was used. Among the eluents used ammonia gave the best result with 92 ± 2% recovery from Seralite SRC120 and 86 ± 2% from Seralite WRC 50.The results are shown in **Fig 6.7** and **Fig 6.8**. Elution or desorption of L-lysine from the cation exchanger can be affected by majorly two factors in the ion exchange mechanism called the ion exchange factor and the pH factor. Elution effected by the displacement of adsorbed L-lysine with ammonium ions is due to the ion-exchange factor and by the loss of positive charge on the adsorbed L-lysine owing its association with hydroxide ions is the pH factor. Both of these factors may contribute jointly to L-lysine desorption. If the ion-exchange factor is more influential, the L-lysine desorption will be more sensitive to changes in the ammonium ion concentration than to pH changes. If the pH factor is more important, L-lysine desorption will be more dependent on changes in eluant pH than on changes eluant concentration. The conversion of adsorbed L-lysine (either Lys+ orLys2+) to its negatively charged form (Lys-) is influenced by the pKa values for the L-lysine molecule and by the pH of the surroundings. If the eluant pH values are high enough to guarantee the dominance of Lys- (such as above pH 11), it will result in a smooth elution.

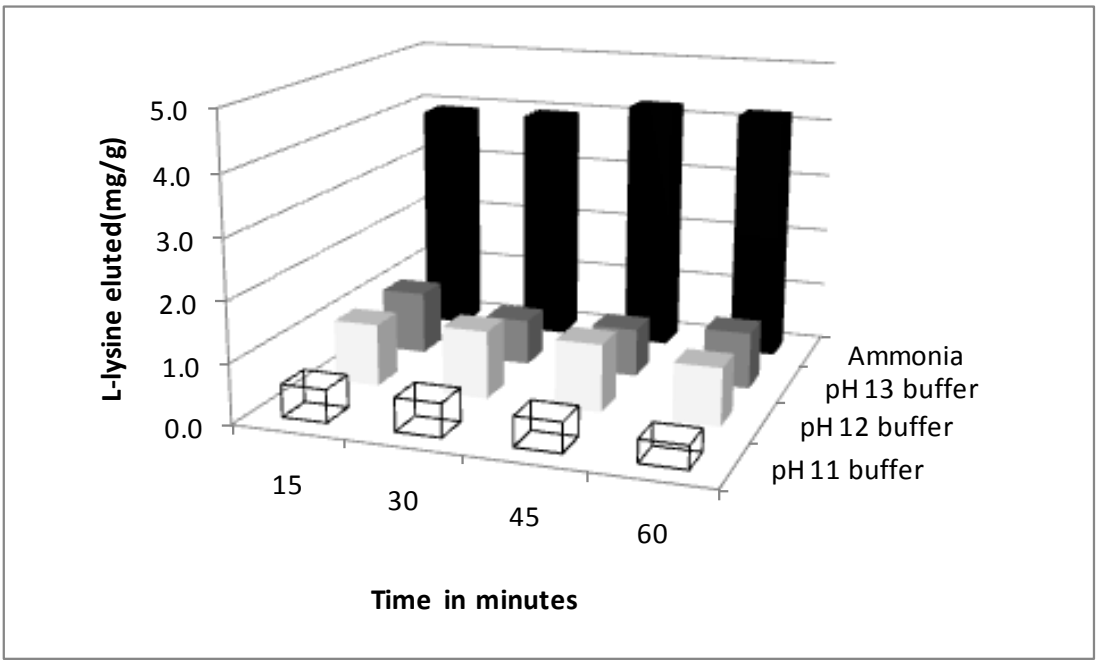


Fig 6.7.Elution of L-lysine at different pH and time intervals by Seralite WRC50

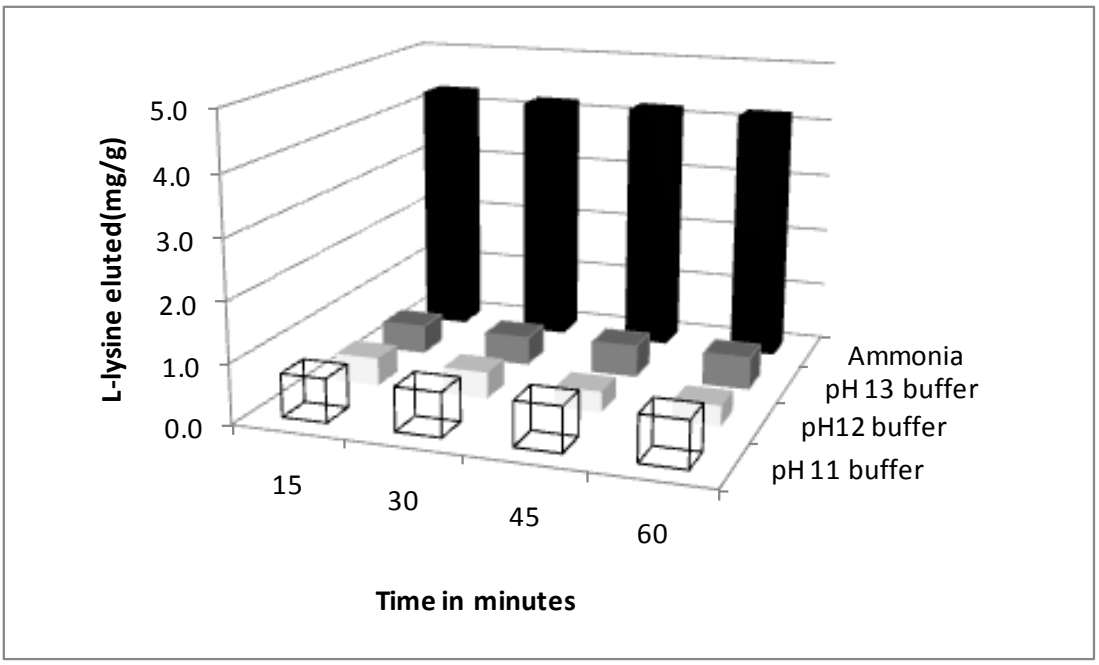


Fig 6.8. Elution of L-lysine at different pH and time intervals by Seralite IR 120

The conditions for L-lysine adsorption and elution were optimized using standard L-lysine and are summarized in **Table 6.4.**

Table 6.4. Conditions of ion exchange purification of L-lysine

Parameter	**SRC 120**	**WRC 50**
Moisture	46.5±2	47±2
Swelling on adsorption	-	-
Swelling on ion exchange	45±2%	40±3%
Percentage of adsorption	86±1%	82±2%
Time for adsorption	20 min	50 min
pH for adsorption	2-9	5
Eluent	2M Ammonia	2M Ammonia
Percentage of recovery	92±2%	86±2%

6.3.5. Purification of L-lysine from fermented broth

The conditions optimized with the standard L-lysine solution were used in purification of L-lysine from crude fermented broth using Seralite SRC 120 and WRC 50. L-lysine was preferentially adsorbed at pH 5 on the resins by displacing the ions. Elution by aqueous ammonia (2M) effectively separated L-lysine from other minor contaminants. The recovery efficiency of L-lysine from fermentation broth by Seralite SRC 120 was 85 ± 2% and from WRC 50 was 80 ± 2%. The purified fractions were visualized by TLC and HPLC (**Fig 6.9 and Fig 6.10.**). The purified fractions were evaporated to remove ammonia and dried to obtain L-lysine powder.

Fig 6.9. Visualization of purified L-lysine from fermented broth by TLC.

Numbers1and 2 represents standard L-lysine at concentration 20µg.
Numbers 3-8 represents purified fractions from L-lysine fermented broth

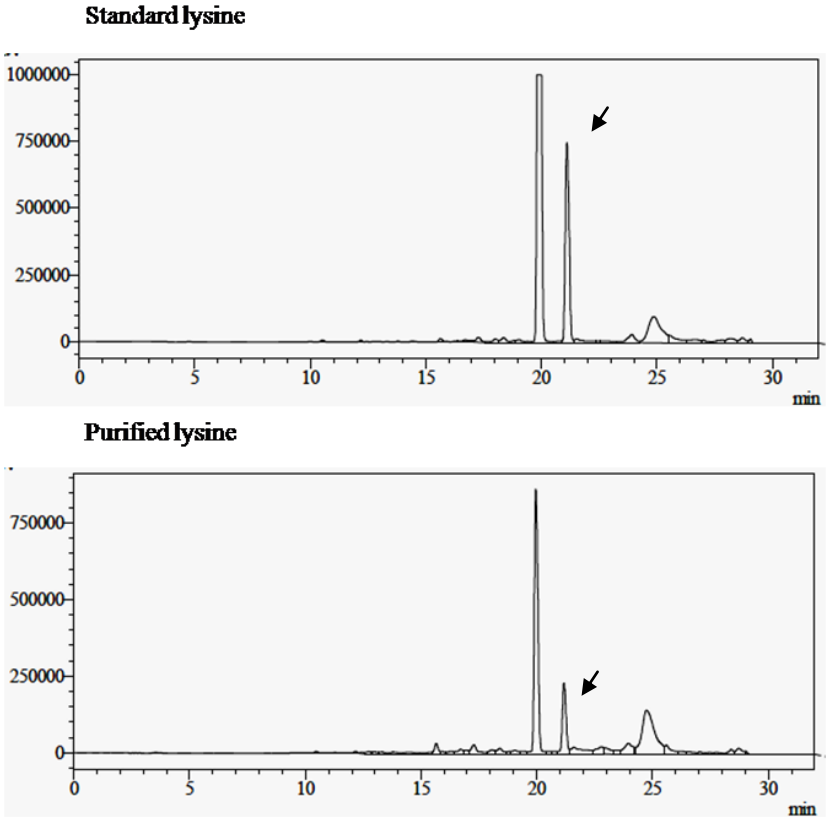


Fig 6.10. HPLC chromatogram of purified L-lysine from fermented broth. The arrows indicate L-lysine peak

A selection of ion exchange chromatography techniques are described by various authors for purification of L-lysine and other amino acids. From the survey of reports, it was evident that majority of the reports on ion exchange chromatography were patents, most of them used cation exchange chromatography and nearly all of them owned by industrial giants. Considering this, it can be assumed that it is main method of amino acid purification in industries. The present study to use ion exchange resins to check the feasibility of obtaining pure L-lysine from jackfruit seed powder hydrolysate can be thus reasonable.

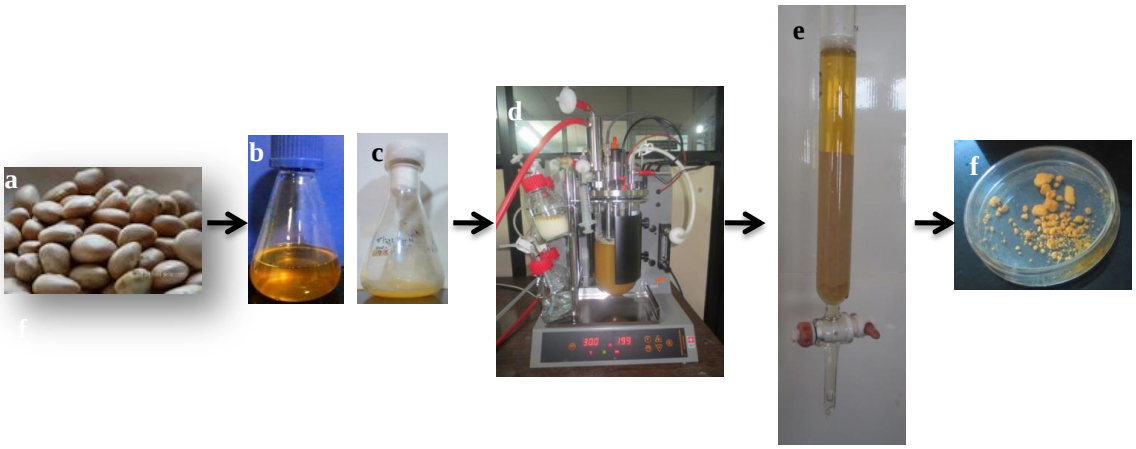


Fig 6.11. Process flow chart of L-lysine fermentation and purification.
a- jackfruit seeds, b-jackfruit seed powder hydrolysate, c-*C. glutamicum* DM1729 inoculum, d-L-lysine production in fermenter, e-purification using ion exchange chromatographic column, f-purified L-lysine .

According to an invention, ion exchange columns connected in series were alternated to accomplish maximum L-lysine adsorption and elution. With the use of resins like Amberlite I-120, Duolite C-20 or Diaion SK-1B, adsorbed amino acid to 98% and was eluted with ammonium hydroxide up to 97% and purity of 98% (Terutsugu et al., 1971). Alternatively, the pH of culture broth was adjusted to 11, heated and aerated at 1vvm and brought pH back to 5.0 with sulphuric acid. The broth was filtered and passed through Amberlite IR 120 or Amberlite IR 84 and eluted with ammonia (Tanaka et al., 1978).US patent US5268293 A, described use of acidified culture broth adsorbed to Duolite C-20N and consequent elution with ammonia with a recovery of 94% (Oh et al., 1993). For high purity applications, the purified fractions are always concentrated and crystallized (Sifniades, 1976; Sifniades & Tunick, 1975). Otherwise, the purified fractions may be dried using a spray granulator (Binder & Stevens, 2004) or spray dryer, drum dryer, tunnel dryer etc. Direct drying of L-lysine fermentation broths is also practiced which although results in inconsistent concentrations are preferred for animal feeds. Different forms of L-lysine preparations were described (Binder et al., 1997; Binder et al., 1995; Wurster, 1963). The quality of the final product is decided by the end use application and hence there is always continuous quest for cheaper alternatives like Biolys and aqueous lysine preparations. However, for high purity applications, ion exchange chromatography and crystallization remains the industrial favorite.

6.4. Conclusion

Cation exchange chromatography is an efficient method for purification of L-lysine from fermented broth. L-lysine has a high PI value of 9.7.Theoretically, in cation exchange chromatography; L-lysine should bind to the ion exchanger at any pH below the isoelectric pH and for elution the pH should be higher than the PI. At this pH all the minor contaminants and other amino acids should elute before the elution of L-lysine. Thus, cation exchange chromatography was successfully employed in the purification of L-lysine from jackfruit seed starch hydrolysate based fermentation broth.

Chapter 7

CO-EXPRESSION OF ENDOGLUCANASE AND β-GLUCOSIDASE GENES IN *CORYNEBACTERIUM GLUTAMICUM* DM1729 TOWARDS ONE STEP BIOCONVERSION OF CELLULOSE TO L-LYSINE

7.1. Introduction

Lignocellulosic biomass is considered as the cheap, sustainable and renewable feed stock of the decade as it generally avoids competition as food and fodder (Saxena et al., 2009). Concentrated efforts are not given for lignocellulosic biomass generation as it is usually agricultural, industrial or farmyard waste or forest forage. Due to the ease of access and low cost, there was always labored efforts towards utilization of lignocellulosic biomass for production of bio based fuels and chemicals. Most of the bacterial bioproduct producers lack the inherent ability to naturally degrade the cellulose chains to assimilable sugar monomers and hence lignocellulosic biomass is underutilized in this direction. On the other hand, the feed stock has to be extensively pretreated and hydrolysed so as to be accessible for the microbes. To fully capitalize on the available lignocellulosic feed stock for production of products of interest, the target microbes has to be engineered for heterologous expression of cellulose degrading enzymes or cellulases (Lynd et al., 2002).

Cellulose is the water insoluble plant biomaterial which is composed of repeated β-linked D-glucose units and cellulases are enzymes that degrade cellulose. Cellulases are composed of multiple enzymes acting synergistically in cellulose hydrolysis to generate monomer units. Cellulases have higher efficiencies in combined action and higher combined activity than the sum of individual activities. Endoglucanases, or Endo-1, 4-β-glucanase (EG) cleave intramolecular β-1, 4-glucosidic linkages in the amorphous regions of cellulose fiber and produces new chain ends and higher DP (degree of polymerization) or lower DP cello oligosaccharides. β-glucosidases cleave the cello oligosaccharides preferentially cellobiose and form glucose monomer units. It is the key enzyme in relieving product inhibition of endoglucanases. These enzymes form an important step for the development of a lignocellulosic biorefinery.

A lignocellulosic biorefinery is where the lignocellulosic biomass is converted to fuels and chemicals in various steps. An efficient biocatalyst capable of multiple product production will be advantageous to the biorefinery. The supreme microbe for lignocellulosics to bioproduct conversion should have high productivities, co utilization of different sugars for efficient fermentation, broad substrate utilization range, tolerance to inhibitors generated during pretreatment, minimal byproduct formation etc (Sukumaran et al., 2010).

C. glutamicum is versatile in the production of multitude of products including organic acids, fuels, platform chemicals and other bioproducts. *C. glutamicm* is also engineered for substrate broadening to utilize lignocelluloses derived sugars (Gopinath et al., 2012). Under oxygen deprived conditions and high cell densities, *C. glutamicum* has also shown resistance to inhibitors for alcohol fermentation (Inui et al., 2004) which will be an added advantage in a lignocellulosic biorefinery.

As a first step towards the utilization of lignocellulosic biomass, under a DBT-BMBF Indo-German collaborative programme, our group developed *C. glutamicum* mutants capable of utilizing pentose sugars. The strains were able to utilize xylose and arabinose present in the lignocellulosic biomass hydrolysate by over expressing the genes *xylA* and *araBAD* operons from *Escherichia coli* and *xylB* from *Xanthomonas campestris* (Gopinath et al., 2011; Meiswinkel et al., 2013). Subsequently, as an initial step towards direct utilization of lignocellulosic biomass for L-lysine production, cellulose hydrolyzing enzymes such as endoglucanase and β-glucosidase were cloned and expressed in *C. glutamicum* DM1729. The plasmids were constructed under a DST–DAAD collaborative project with the Wendisch lab, University of Bielefeld, Germany.

In this study, we transformed endoglucanase and β-glucosidase containing plasmids in *E. coli* DH5α and *C. glutamicum* DM1729 for the assimilation of carboxymethyl cellulose or cellobiose for L-lysine fermentation. Three recombinant strains were obtained, with secreted endoglucanases, and either secreted or surface displayed β-glucosidase.

7.2. Materials and Methods

7.2.1. Bacterial strains and media

Bacterial strains and plasmids used in the study with their relevant characteristics are listed in **Table 7.1**. *E.coli* DH5α (Hanahan, 1983) was used as the host for DNA manipulation. *Corynebacterium glutamicum* DM1729 (Georgi et al., 2005) and derived strains were used for recombinant protein expression and L-lysine fermentation.

E.coli was grown in LB medium at 37 ˚C and *C. glutamicum* was grown in LB medium at 30 ˚C. For recombinant selection, LB agar medium with either kanamycin (25-50 µg/mL) or spectinomycin (100 µg/mL) or in combination was used.

For L-lysine fermentation studies CGXII mineral medium was used with either glucose, cellobiose, carboxymethyl cellulose as carbon sources or their blends. The inoculum was prepared as described in section 2.3.1. When appropriate, kanamycin (25 - 50 µg/mL) and spectinomycin (100 µg/mL) was used. IPTG was added in a concentration up to1 mM for induction. All L-lysine fermentations were carried out in 250 mL Erlenmeyer flasks with a volume of 25 mL at 30 ˚C and 200 rpm, unless stated otherwise.

Table 7.1. Bacterial strains and plasmids used in the study with their relevant characteristics

Microorganism	***Description***	***Reference***
E.coli DH5α	*Fthi-1 endA1 hsdr17(r-, m-) supE44 _lacU169 (ϕ80lacZ_M15) recA1gyrA96 relA1*	(Hanahan, 1983)
C. glutamicum DM1729	*lysC P458S, homV59A, pyc T311I*	(Georgi et al., 2005)
Corynebacterium variabile		DSM
Xanthomonas campestris		ATCC
Saccharophagus degradans		ATCC
DM1729(pVWEx1-*TAT-XCC2387*)	DM 1729 harbouring (pVWEx1-*TAT-XCC2387*)	This study
DM1729(pVWEx1-*TAT-XCC3521*)	DM 1729 harbouring (pVWEx1-*TAT XCC3521*)	This study
DM1729(pEKEx3-*TAT-Sde1394*)	DM 1729 harbouring (pEKEx3-*TAT-*	This study

	Sde1394)	
DM1729(pEKEx3-*porC-Sde1394*)	DM 1729 harbouring (pEKEx3-*PorC-Sde1394*)	This study
Plasmids		
pVWEx1	*KanR, Ptac, lacIq*	(Peters-Wendisch et al., 1998)
pEKEx3	*SpecR, Ptac, lacIq*	(Stansen et al., 2005)
pVWEx1-*TAT-XCC2387*	Vector for endoglucancase expression*(XCC2387* from *Xanthomonas campestris)*	This study
pVWEx1-*TAT-XCC3521*	Vector for endoglucancase expression*(XCC3521* from *Xanthomonas campestris)*	This study
pEKEx3-*TAT-Sde1394*	Vector for β-glucosidase expression*(Sde 1394* from *Saccharophagus degradans)*	This study
pEKEx3-*porC-Sde1394*	Vector for β-glucosidase expression*(Sde 1394 f*rom *Saccharophagus degradans)*using *porC* anchor protein	This study

7.2.2. Cultivation of C. glutamicum strains in a microbioreactor (BioLector®)

The BioLector® (m2p- labs GmbH, Germany)(**Fig 7.1.**) is a bench top Microbio reactor system performing high-throughput fermentations clubbed with online monitoring of the most common fermentation parameters like biomass, pH and dissolved oxygen.

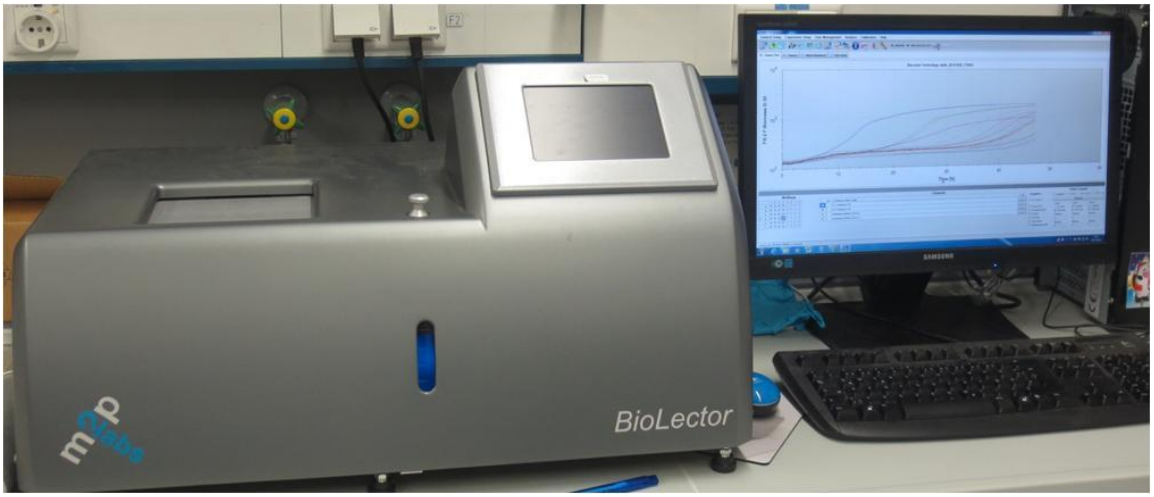


Fig 7.1. BioLector®

The BioLector® microbioreactor is used for cultivations in microtiter plate called Flower Plates ® and operates with non-invasive, optical sensors. The plates are shaken rigorously during the optical measurements and the whole experimental time making it well suited for aerobic cultivations.

Fig 7.2. BioLector Flower Plate®

The Flower Plate® is the microtiter plate used with BioLector® which combines unique mass transfer performance with online measurements of fermentation parameters. The geometry of the plate is very advantageous for aerobic fermentations. The flower shape of the individual wells acts like baffles in shake flasks and noticeably increases

mixing and gas/liquid mass transfer. This unique feature outshines all common microtiter plate formats providing a distinct solution for conducting 48 biocatalytic reactions simultaneously.

The recombinant *C. glutamicum* DM1729 showing positive selection on antibiotic plates were screened for growth in the BioLector®. The pre-culture and inoculum was prepared as in section 2.3.1. Selected recombinant strains were cultivated in CGXII minimal medium supplemented with appropriate carbon source (20 g/L cellobiose and 20 g/L Carboxymethyl cellulose) at 30 °C and a shaking speed of 1100 rpm. The scattered light from the BioLector® was detected with optodes in the Flower Plate ®with a filter set appropriate to measure high cell densities (gain of 20), which implies that low optical densities (below OD600=1) are measured with decreased accuracy. The biomass (OD600) was calculated automatically by the instrument.

7.2.3. Plasmid construction and general recombination methods

Molecular biology reactions including PCR, restriction digestion and ligation, were executed according to standard protocol (Sambrook & Russell David, 1989). Gibson assembly in the isothermal reaction buffer had been applied for the construction of plasmids, in addition to the ligation reaction (Gibson et al., 2009). PCR products were purified using PCR purification kit or Min Elute PCR purification kit (QIAGEN, Hilden, Germany).

E.coli - C. glutamicum shuttle vectors, pEKEx3 and pVWEx1were used as the vector backbone for plasmid construction in the study (Peters-Wendisch et al., 1998; Stansen et al., 2005). The β- glucosidase and endoglucanase genes were amplified from the respective genomic DNA sequences by KOD PCR (KOD, Novagen, Darmstadt, Germany). For the present study, six PCR amplified products identified as betaglucosidase genes [*Corynebacterium variabile* DSM 44702 *–Cvar 2943* (*bglB*), *Xanthomonas campestris pv. campestris* str. ATCC 33913 -*XCC1090* (*bglX*) with the secretory signal, *XCC1090* (*bglX*), *XCC1250* (*bglX*), *XCC1404* (*bglS*), *XCC3814* (*bglX*) and *Saccharophagus degradans str.* ATCC 43961 *–Sde1394* (*bglB*)] in Wendisch lab were used for cloning in to the vector pEKEx3.

Similarly, five PCR amplified products identified as endoglucanase genes [*Xanthomonas campestris* pv. *campestris* str. ATCC 33913 i.e. *XCC0026, XCC0027*,

XCC0028, XCC2387 and XCC3521] were cloned in the vector pVWEx1. The amplified fragments along with RBS and TAT secretion signal, in both cases were cloned in the respective vector using Gibson assembly.

Of the endoglucanase and β- glucosidase genes described above, the plasmids constructed with *Sde 1394(*β- glucosidase*), XCC2387 & XCC3521* (endoglucanase) were selected for further studies based on growth in BioLector ® and enzyme activities. Hence, the plasmids for endoglucanase expression were named pVWEx1-*TAT-XCC2387* and pVWEx1-*TAT-XCC3521*. The plasmid for β- glucosidase expression on the cell surface with the *porC* anchor was constructed, and the resultant plasmid was named pEKEx3-*porC-Sde1394* and the secreted form was pEKEx3-*TAT-Sde1394*.

7.2.4. Preparation of competent cells

7.2.4.i. E. coli DH5α

E. coli competent cells were prepared by RbCl method (Hanahan, 1983). A pre-culture of *E.coli* DH5α cells was prepared by inoculating a single colony in 5 mL LB medium. This was incubated in a tube roller at 37 ˚C for 18 hours. One mL of overnight culture was used to inoculate 50 mL LB medium and incubated at 37 ˚C and 200 rpm till OD_{600} of 0.6. The cells were harvested by centrifugation at 4000 rpm for 10 minutes at 4 ˚C. The harvested cells were washed three times with 25 mL RF1 buffer (4000 rpm for 10 minutes at 4 ˚C) and re suspended in fresh RF1buffer. The RF1 buffer was composed of 12 g/L rubidium chloride, 9.9 g/L $MgCl_2$ x 4 H_2O, 2.9 g/L potassium acetate, 1.5 g/L $CaCl_2$ x 2 H_2O, and 150 g/L glycerol (86 %). The pH was adjusted to 5.8 with 0.2 N acetic acid. In between every washing step, cells were and stored on ice for 10 minutes. The cell pellet harvested after final wash was re suspended in 2 mL RF2 buffer and 150 µL aliquots were transferred to ice cold microfuge tubes stored at -80 ˚C. The RF2 solution contains 2.1 g/L MOPS, 1.2 g/L rubidium chloride, 11 g/L$CaCl_2$ x2 H2O, and 15 g/L glycerol (86 %). The pH was adjusted to 6.8 with 0.4 M NaOH. Both buffers were filter sterilized and stored at 4 ˚C. All the steps after harvesting cells were carried out at 4 ˚C or in ice.

7.2.4.ii. C. glutamicum DM1729

C. glutamicum competent cells were prepared according to Eggeling and Reyes (2005). An overnight inoculum of *C. glutamicum* DM 1729 was prepared by inoculating

cells in 5 mL LB medium and culturing at 30 ˚C and 200 rpm for 18 hours. Two mL of the culture was used to inoculate 100 mL LB medium and incubated at 30 ˚C and 200 rpm till the OD reaches OD_{600} of 0.6.At this point 30 µL of ampicillin (5 mg/mL stock) was added and incubated further for 90 minutes. Cells were harvested by centrifugation at 4000 rpm for 10 minutes at 4 ˚C and suspended in 25 mL of ice cold EPB1 buffer. EPB1 buffer contained 20 mL of 0.5 M HEPES, 28.74 mL of glycerol (86%) and the volume made up to 500 mL with deionized water. The centrifugation and resuspension steps were repeated three times. The final cell pellet was suspended in 1.5 mL ice cold EPB2 and 100 µL aliquots were transferred to pre cooled microfuge tubes. The cells were stored in -80 ˚C. EPB2 buffer contained 2 mL of 0.5 M HEPES, 34.48 mL glycerol (86%) and made up to 200 mL with deionized water. Both buffers were autoclaved and stored at 4 ˚C.

7.2.5. Transformation of bacterial cells with plasmid DNA

7.2.5.i. E. coli DH5α

E. coli transformation was performed using RbCl method (Hanahan, 1983). The thawed competent cells (150 µL) of *E.coli* were mixed with 300 ng to 1µg of plasmid DNA. The tubes were incubated for 15 minutes on ice. The cells were subjected to a heat shock at 42 ˚C for 90 seconds. Cells were then mixed with 1 mL of LB medium and incubated for 60 minutes at 37 ˚C and 500 rpm in a thermomixer (Eppendorf, Hamburg). After incubation, cells were pelleted, resuspended in 100 µL LB medium and plated on appropriate antibiotic selection agar plates.

7.2.5.ii. C. glutamicum DM1729

C. glutamicum was transformed via electroporation (Van der Rest et al., 1999). For the transformation, 100 µL of competent cells were thawed in ice was mixed gently with 300 ng to 1µg of plasmid DNA and transferred to pre cooled electroporation cuvettes. The mixture was incubated in ice for 15 minutes. Transformation was performed in BioRad Gene Pulser Xcell (Hercules, USA) at a voltage of 2500 V, resistance of 200 Ω, and capacitance of 25 µF. All cloned DNA fragments were confirmed by sequencing for integrity.

7.2.6. Isolation of plasmid DNA from E. coli

Plasmid DNA was isolated according to manufacturer's protocol by Qiagen miniprep plasmid isolation kit (QIAGEN, Hilden, Germany). An overnight culture of *E.coli* cells was prepared in 5mL LB medium. The cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4 ˚C. The supernatant was poured off and the pelleted bacterial cells were resuspended in 250 µL Buffer I. To the suspension 250 µL Buffer II was added and gently inverted the tube 4-6 times followed by the addition of 350 µL Buffer II, I inverted the tube immediately 4-6 times. The resulting white precipitate was centrifuged for 10 minutes at 13,000 rpm on a table-top microcentrifuge and the supernatant was added to mini prep spin column by pipetting. This was centrifuged for 30-60 seconds and the flow-through was discarded. The column was washed with 750 µl of wash buffer by centrifuging for 30-60 seconds. The flow-through was discarded and column was centrifuged for an additional 1 minute to remove residual wash buffer. The column was placed in a clean 1.5 mL microcentrifuge tube and the plasmid DNA was eluted by adding 50 µL elution buffer and centrifuging for 1 minute. Buffer I (Resuspension buffer) consisted of 50 mM Tris-Cl, pH 8.0, 10 mM EDTA and 100 µg/mL RNase A. Buffer II (Lysis buffer) contained 200 mM NaOH and 1% SDS (w/v). Buffer III (Neutralization buffer) was composed of 3M potassium acetate, pH 5.5; Wash buffer contained 1M NaCl and 50 mM Tris-Cl, pH 7.0,Elution buffer was10 mM Tris-Cl, pH 8.0.

7.2.7. Determination of nucleic acid concentration

The plasmid DNA concentration was determined with a Nanodrop Spectrophotometer ND-1000 (Peqlab Biotechnologie GmbH, Erlangen,Germany) .The extinction was measured spectrophotometrically at 260 nm by blanking against the wash buffer (10 mM Tris-Cl, pH 8.0) and expressed as ng/µL. Protein impurities were determined by the factor of E260/E280 (1.8 - 2.0) , while contaminations of carbohydrates were determined with the factor of E260/E230 (> 2.0) (Sambrook & Russell David, 1989)

7.2.8. Enzyme Expression

The cloned genes coding for betaglucosidase /endoglucanae enzymes were under the control of IPTG inducible promoter. The cells were cultured in the CGXII minimal medium with cellobiose or carboxymethyl cellulose as carbon sources for 96 hours and

sampled at 24 hour intervals. Induction was done at the time of inoculation, with IPTG up to 1mM concentration. Either the cell free supernatant or cell pellet was used for further analysis of enzyme expression.

7.2.9. Enzyme Assay

7.2.9.i. Qualitative Assay

For qualitative determination of endoglucanase and β-glucosidase activities, either CMC or cellobiose agar plates were streaked with the transformants and incubated at 30 ˚C for 48 hours. Qualitative determination of endoglucanase expression was done on CMC agar plates showing clearance zone of hydrolysed polysaccharide when flooded with 0.1% congo red followed by 1M NaCl (Teather & Wood, 1982) ; or trypan blue (Tsuchidate et al., 2011). β-glucosidase activity was determined by formation of zones with iodine reagent on cellobiose agar plates. Endoglucanase activity was also determined by CMC agar plates flooded with iodine (Kasana et al., 2008).

7.2.9.ii. Quantitative Assay

For β-glucosidase activity measurement, the *C. glutamicum* DM1729 cells were separated by centrifugation at 6000 rpm for 10 minutes at 4 ˚C. The cell pellet was washed with 1X PBS twice and resuspended in 50 mM citrate buffer. The cell pellet fraction and the cell free supernatant were used to determine β-glucosidase activity. Endoglucanase activity was determined by measuring the glucose yield from carboxymethylcellulose (CMC) using a modified protocol (Xiao et al.,2005). The enzyme assay was explained in detail in section 2.4.4.ii. and 2.4.4.iii.

7.2.10. Pretreatment of biomass and enzymatic hydrolysis

Rice straw and sugarcane tops, obtained locally were used for pretreatment. Rice straw is the aerial vegetative part of the rice plant. After harvesting, it is either burned or left in the field or used as livestock feed. Sugarcane tops are the leaves of sugarcane plant and are usually burned after harvesting sugarcane.

The milled and processed biomass was subjected to dilute acid pretreatment with 4% w/w sulphuric acid at 121 ˚C for 60 minutes. The biomass so obtained was neutralized after cooling with 10 N sodium hydroxide and air dried for hydrolysis. The recombinant *C. glutamicum* strains were incubated in CGXII medium at 30 ˚C for 48 hours and the supernatant was obtained by centrifugation at 6000rpm for 10 minutes at 4 ˚C. The

supernatant thus obtained was used as the enzyme mixture for biomass hydrolysis. The reaction mixture consisted of 2% w/v pretreated biomass in the enzyme solution and hydrolysis continued for 72 hours at 30 ˚C.The reducing sugars were analysed by DNS assay at 540 nm after obtaining the supernatant by centrifuging at 6000 rpm for 10 minutes.

7.2.11. Fermentation

Pure polysaccharides like caboxymethyl cellulose and cellobiose individually or in combination at a concentration of 2% w/v were used as carbon source for fermentation in CGXII medium. For direct conversion of the polysaccharides, fermentation was carried out in CGXII medium at 30 ˚C for 72 hours in both cases.

7.3. Results and Discussion

Engineering of microbes for direct substrate utilization by heterologous expression of enzymes so as to broaden the substrate spectrum is crucial in the development of a microbial biorefinery. In this direction, several enzymes has been successfully expressed in *C. glutamicum* (Adachi et al., 2013; Seibold et al., 2006; Tateno et al., 2007b).Thus it proves, *C. glutamicum* is an excellent host for heterologous protein expression considering the limited extracellular protease activity and efficient secretion mechanisms for proteins (Geisseler & Horwath, 2008) .

7.3.1. Recombinant C. glutamicum DM1729 expressing endoglucanase and β-glucosidase genes

Endoglucanase and *β-glucosidase* containing plasmids were transformed in *E. coli* DH5α (for hassle free isolation of desired quantity of plasmid DNA for electroporation) and then electroporated in *C. glutamicum* DM1729. Of the six betaglucosidases, *Sde1394* gave detectable heterologous expression in *C. glutamicum* DM 1729 and among the endoglucanases *XCC2387* and *XCC3521* showed heterologous enzyme expression. Thus in this study, the recombinant strains selected for further studies were, (1) *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*)(pEKEx3-*PorC-Sde1394*) (2) *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*)(pEKEx3-*TAT-Sde1394*) and (3) *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-TAT-*Sde1394*). The vector map for plasmid encoding representative endoglucanase (a) and betaglucosidase (b) is depicted in **Fig7.3. a & b** respectively.

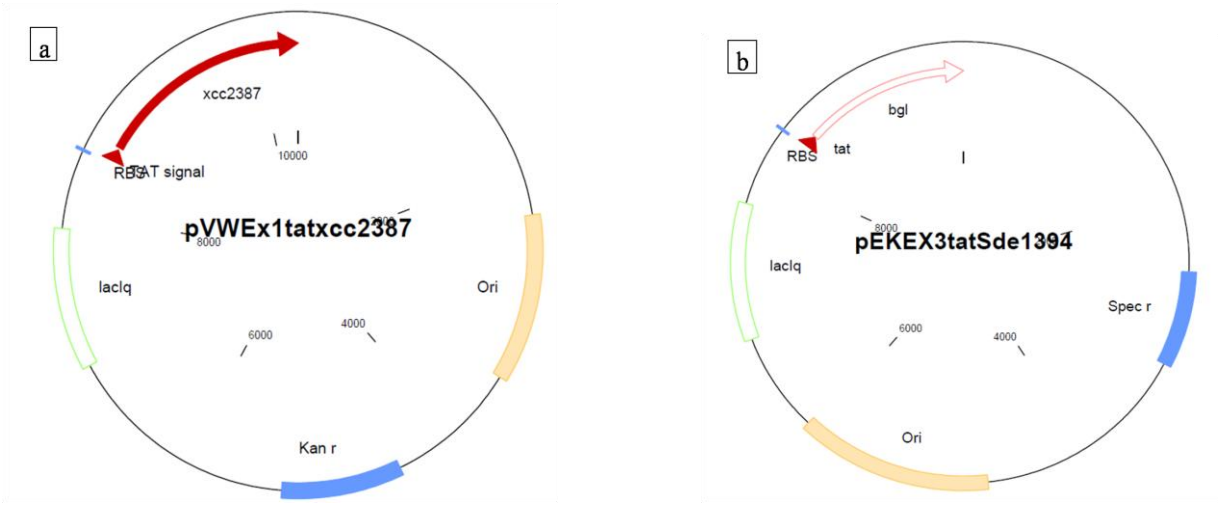


Fig 7.3. (a). The vector pVWEx1 harbouring the endoglucanase gene *XCC2387* **(b).** The vector pEKEx3 harbouring β-glucosidase gene *Sde1394*

All the three recombinant *C. glutamicum DM1729* strains were evaluated for endoglucanase and β-glucosidase expression and L-lysine fermentation. Two of the recombinant strains namely *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*)(pEKEx3-*TAT-Sde1394*) and *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*) had both the enzymes in secreted form and *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*) secreted endoglucanase and expressed β-glucosidase on the cell surface using the *porC* anchor. Porin for cell surface expression of heterologous proteins has been successfully demonstrated earlier (Tateno et al., 2009). *C. glutamicum* has TAT and Sec secretion systems.TAT secretion system used in the study for the expression of proteins in the secreted form, transports fully folded proteins in the cytoplasm (Dilks et al., 2003) and is reported to be industrially efficient. Moreover, the feasibility of choosing the TAT system is corroborated by the earlier reports stating that *Corynebacteria* transports more proteins through the TAT system than other related genera like *Mycobacterium* and *Streptomyces* (Schaerlaekens et al., 2004) .

The rate limiting enzyme in cellulose hydrolysis is β-glucosidase which holds a very important role in cellulose hydrolysis. The substrate of β-glucosidase is the major inhibitor of endoglucanase and cellobiohydrolase at concentrations of 0.3% w/v where hydrolysis efficiencies decrease up to 50% (White & Hindle, 2000). Commercially available cellulases have low levels of β-glucosidases which necessitate the addition of high enzyme loads for increased saccharification efficiency (Sukumaran et al., 2010). Cell surface display of proteins is reported (Lee et al., 2003; Tanaka et al., 2012) to enhance their activity than in secreted form. β-glucosidases were hence displayed on the cell surface in addition to the secreted form. *porC* anchor protein was used as the anchor for cell surface display and has been successfully employed earlier in *C. glutamicum*. Porins are cell wall associated proteins in *C. glutamicum* due to the presence of mycolic acids (Bayan et al., 2003). Cell surface display of proteins were employed previously in gram positive and gram negative bacteria for complex substrate utilization (Adachi et al., 2013; Narita et al., 2006; Tateno et al., 2007a; Yao et al., 2009) .

The expressed cellulases were able to degrade the polysaccharides and produce the reducing sugars for efficient utilization by the recombinant *C. glutamicum* DM1729. This shows that the enzymes were transported out of the cells efficiently.

7.3.2. Cultivation of recombinant C. glutamicum DM1729 in the BioLector

Growth studies of the *C. glutamicum* DM1729 in the BioLector showed the recombinant *C. glutamicum* DM1729 strains were able to grow in 20g/L cellobiose and 20g/L CMC mixture. The initial increase in absorbance may be contributed by the monosaccharides or disaccharides formed from cellobiose or CMC during autoclaving coupled with enzymatic break down. *C. glutamicum* DM1729 recombinant strain III grew better (OD_{600} of 4.8) than strain II (OD_{600} of 4.4) and strain I (OD_{600} of 3.8).This showed that all the three strains were able to assimilate the respective carbon sources for growth. All the three strains were further checked for enzyme activity and L-lysine fermentation in independent experiments.

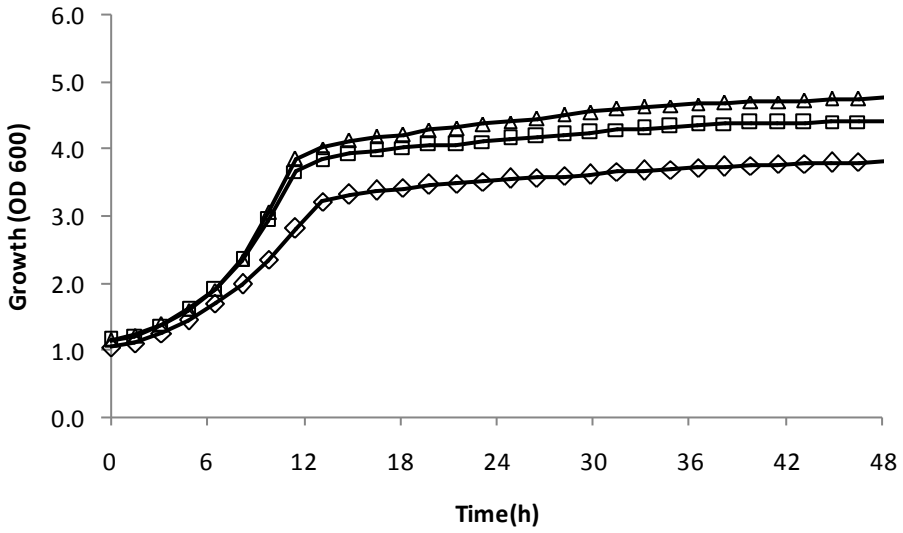


Fig 7.4. Growth of *C. glutamicum* DM1729 recombinants in the BioLector.
Open diamond represent growth at OD $_{600}$ of strain I [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*)]; Open square represent growth at OD $_{600}$ of strain II [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*)]; Open triangle represent growth at OD $_{600}$ of strain III [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*)]

7.3.3. Enzyme activities of recombinant endoglucanases and β-glucosidases

The enzyme activities were qualitatively determined using chromogenic reaction to iodine, trypan blue and congo red. All the recombinant strains were able to grow on the respective substrates, viz, carboxymethyl cellulose and cellobiose. The positive chromogenic reaction was shown by clear zone of polysaccharide hydrolysis around the colonies whereas the parent strain was unable to produce clearance zones. Endoglucanase activity was shown by clearance zones with congo red, trypan blue and iodine whereas positive β-glucosidase was shown by reaction with iodine.

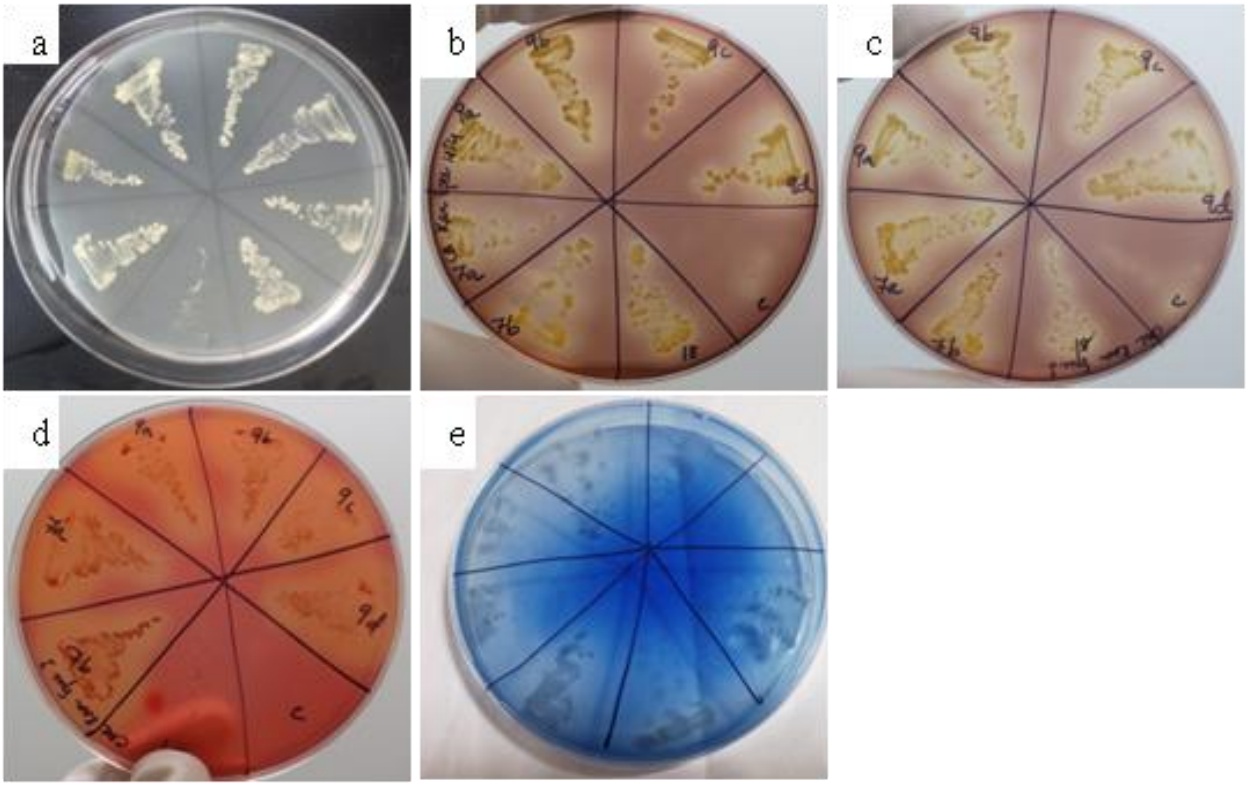


Fig 7.5. Growth and chromogenic reactions of recombinant *C. glutamicum* DM 1729 strains (**a).** Growth of recombinant strains on CMC agar plate. Chromogenic reactions of recombinant enzymes against **(b &c)** iodine indicating endoglucanase and β-glucosidase activities **(d)** congo red and **(e)** trypan blue indicating endoglucanase activity.

The endoglucanase and β-glucosidase activities of the recombinant *C. glutamicum* strains were quantitatively evaluated using standard assay protocols. The endoglucanase and β-glucosidase activities of all the recombinant strains and the parent strain are depicted in **Fig7.6.**

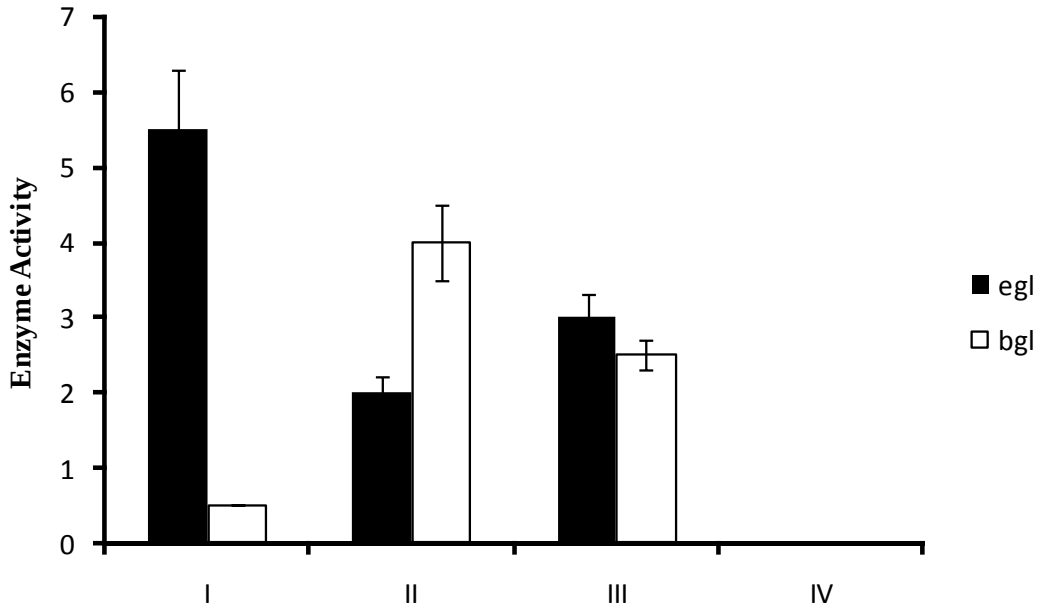


Fig 7.6. Enzyme activities of the *C. glutamicum* recombinants in CGXII medium with 20 g/L CMC and 20 g/L cellobiose. The solid column represents the endoglucanase activity and open column represent the β-glucosidase activity. The strains I-IV represents *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*), *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*), *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*)(pEKEx3-*TAT-Sde1394*) and empty vector control respectively. The enzymes activities are represented in U except for strain I, where the β-glucosidase activity is represented as U/OD_{600} of 1.

Generally, *C. glutamicum*13032 and derived strains lack the ability to assimilate cello oligosaccharides like cellobiose. Unlike the wild type strain, *C. glutamicum* R is reported to utilize salicilin, arbutin and methyl β-glucoside by direct uptake through the PEP- dependant phosphotransferase system (PTS $_{bglF1}$ & PTS $_{bglF2}$) but was unable to utilize cellobiose. Point mutations in the *bglF1* gene in the PTS extended the substrate utilization spectrum of *C. glutamicum* R to cellobiose (Kotrba et al., 2003) and simulataneous utilization of cellobiose, glucose and xylose (Sasaki et al., 2008). We have followed heterologous expression of β-glucosidase genes for cellobiose utilization . The highest β-glucosidase activity of 9 ± 0.5 U/OD_{600} of 1was observed in *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*) in medium containing

cellobiose (20 g/L) as the carbon source and in the medium with both carboxymethyl cellulose and cellobiose in combination, the β-glucosidase activity dropped to 0.5 ± 0.2 U/OD_{600} of 1. Considering the other two strains with secreted β-glucosidases, the activities were higher in the cell surface expressed form. *C. glutamicum* has been engineered for endoglucanase expression and glucan hydrolysis using *Clostridium thermocellum* endoglucanase (Tsuchidate et al., 2011). In the present study, endoglucanase activities were highest in *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*) having 5.5 ± 0.8 U and for the other two strains highest activities were obtained in carboxymethyl cellulose based medium. In all the cases, the parental strain did not show endoglucanase or β-glucosidase activities. The enzyme activities in media where either cellobiose or carboxymethyl cellulose alone was used as carbon source are described in **Table 7.2.**

Table 7.2. Enzyme activities of recombinant strains and L-lysine titre in the media supplemented with either 20 g/L CMC or 20 g/L cellobiose.

Bacterial strains	**Strain I**	**Strain II**	**Strain III**
Fermentation in CGXII supplemented with 20 g/L CMC			
β-glucosidase activity(U or U /$OD_{600}$of 1)	8±0.4	0±0.5	5±0.3
Endoglucanase activity(U)	2.5±0.5	4.5±0.2	3.2±0.8
L-lysine titre (mM)	2.1±0.12	1.7±0.09	2.1±0.12
Fermentation in CGXII supplemented with 20 g/L Cellobiose			
β-glucosidase activity(U or U/$OD_{600}$of 1)	9±0.5	0±0.5	3±0.5
Endoglucanase activity(U)	1.5±0.4	1.5±0.1	2±0.13
L-ysine titre (mM)	2.8±0.12	1.4±0.1	4.8±0.2

Strain I [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*)];
Strain II [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*)];
Strain III [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*)]

7.3.4. Biomass hydrolysis by recombinant C. glutamicum DM1729 strains

The hydrolysis of pretreated biomass with the secreted enzymes of *C. glutamicum* recombinants yielded reducing sugars. Sugarcane tops was more assessable to the recombinant enzymes than rice straw which was evident from the reducing sugars

produced. The recombinant strain I [DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*PorC-Sde1394*)] and strain III [DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*)] showed similar reducing sugar concentrations (5.2±0.26 mg/g substrate and 5.4±0.27 mg/g substrate) with sugarcane tops as the substrate and with rice straw the highest reducing sugar concentration obtained was 4.7±0.23 mg/g substrate. The results are described in **Fig 7.7.**

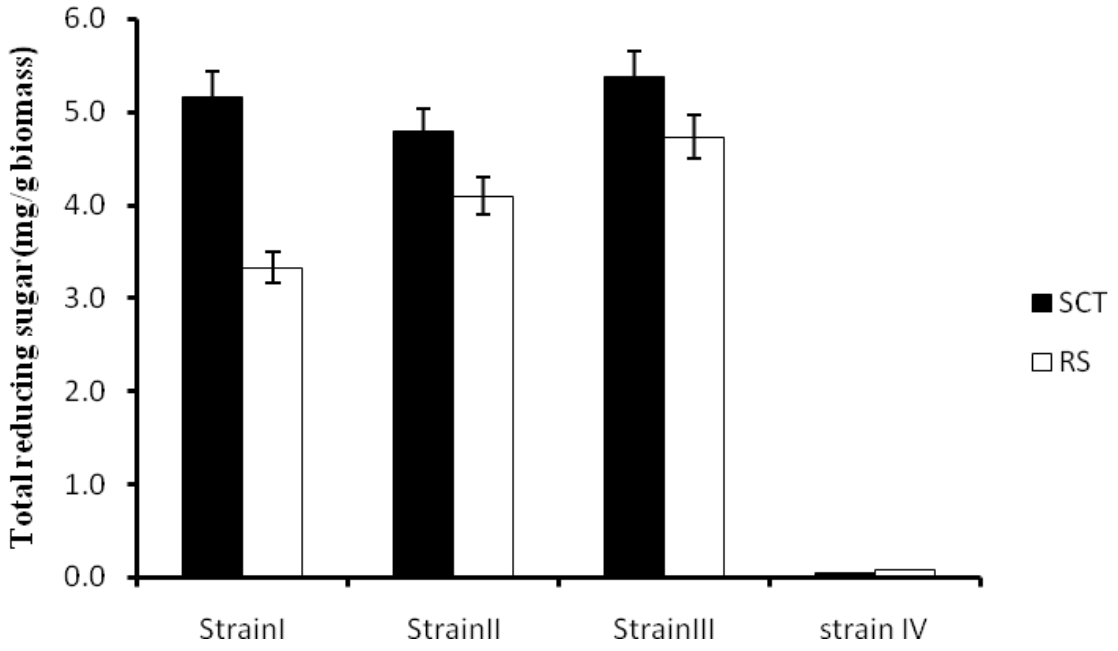


Fig 7.7. Reducing sugars produced from the biomass hydrolysis of pretreated sugarcane tops and rice straw by recombinant *C. glutamicum* DM1729.

The closed column represent the reducing sugars produced per gram of acid pretreated sugarcane tops and open column represent reducing sugars produced per gram of acid pretreated rice straw after hydrolysis at 30 ˚C for 72 hours. The strain I, II, III & IV were
C. glutamicum DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*),
C. glutamicum DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*),
C. glutamicum DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*)
and empty vector control

7.3.5. L-lysine fermentation by recombinant C. glutamicum DM1729 expressing *endoglucanase and ß-glucosidase*

L-lysine fermentation was carried out in CGXII medium with cellobiose and carboxymethyl cellulose either in combination or alone. The L-lysine titres and growth of the recombinants were depicted in **Fig 7.8**. There was a general decline in the L-lysine titre after 48 hours, except in *C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*TAT-Sde1394*), where the maximum production was in 96 hours of fermentation reaching 5.9±0.5mM.The highest L-lysine titres for the other two strains were 2.8±0.5mM under the same fermentation conditions. L-lysine production by the other strains in the media supplemented with either carboxymethyl cellulose or cellobiose alone is shown in **Table 7.2.**

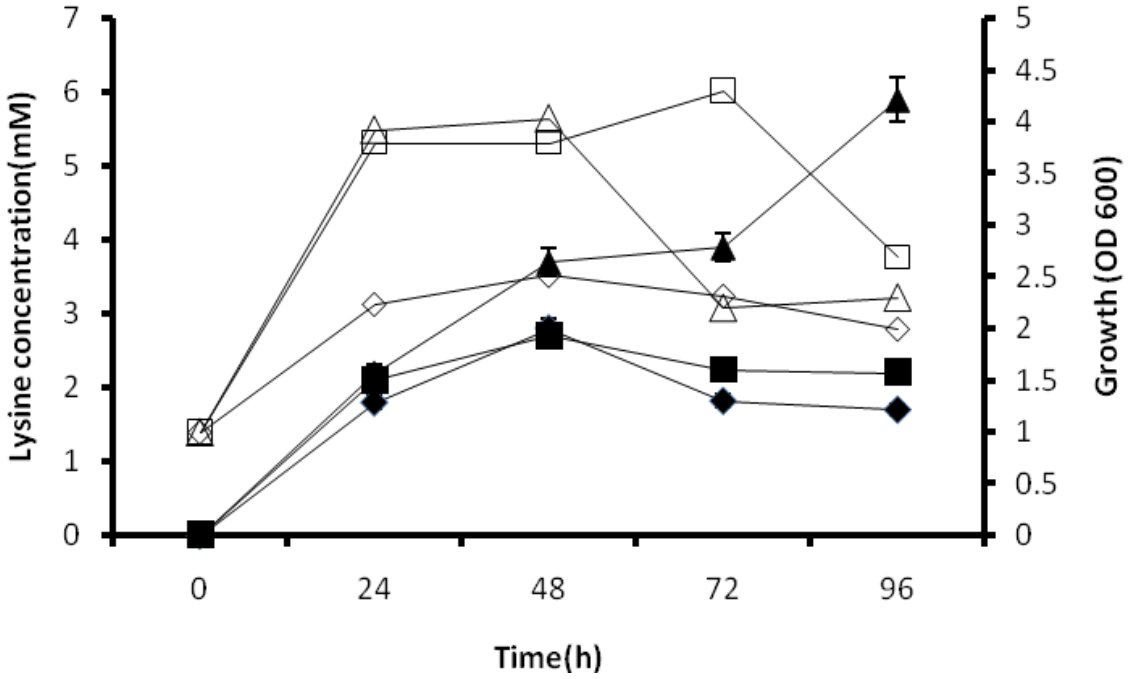


Fig 7.8. L-lysine fermentation and growth of *C. glutamicum* mutants in 20 g/L cellobiose and 20 g/L carboxymethyl cellulose. Closed diamond represent L-lysine produced and open diamond represent growth at OD_{600} of strain I [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*) (pEKEx3-*porC-Sde1394*)];Closed square represent L-lysine produced and open square represent growth at OD_{600} of strain II[*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC3521*) (pEKEx3-*TAT-Sde1394*)]; Closed triangle represent L-lysine produced and open triangle represent growth at OD_{600} of strain III [*C. glutamicum* DM 1729 (pVWEx1-TAT-*XCC2387*)(pEKEx3-*TAT-Sde1394*)]

7.4. Conclusion

C. glutamicum strains co-expressing cellulases were constructed and were capable of converting directly the cellulosic substrates to L-lysine during fermentation. The strains also efficiently saccharified pretreated biomass like sugarcane tops and rice straw to reducing sugars depicting that the heterologous cellulases functioned in synergy. For better utilization of cellulose for high yields of bio products, cellulases like cellobiohydrolases need to be co expressed. Since the hemicelluloses fractions in the lignocellulosic biomass contains pentose sugars as the monomeric units, introduction of these cellulolytic enzymes into pentose utilizing constructs that we already developed may lead to the complete utilization of lignocellulosic biomass leading towards *Corynebacterium glutamicum* based biorefinery for L-lysine production and those efforts are in progress.

Chapter 8

SUMMARY AND CONCLUSION

Amino acids are the basic biomolecules and cellular protein building blocks. They have major commercial applications in dietary supplements, feeds, therapeutics, cosmetics, precursor for industrial chemicals, etc. L-lysine is an essential alpha amino acid in humans and animals. Based on the product type, L-lysine occupies the second position in the amino acid production industry with its largest application in animal feeds. It is used as a supplement or mixed with low protein diets to enhance the available amino acid concentration. It is the limiting amino acid while optimizing the growth and production of lean meat in cattle and poultry. L-lysine also finds uses in pharmaceutical industries as component of infusion solution and in the treatment of cold sore.

In amino acid fermentation, research interests are in lowering the production cost, increasing the productivity, decreasing co-product and byproduct formation using wider range of inexpensive and unexplored C and N sources obtained from agro residual /ligno cellulosic biomass. Use of cheap and easily available substrates which has low competing value as food can provide an alternative carbon source. For industrial fermentations, the substrates are selected based on the geographical location and availability of raw materials. The complex substrates like cane molasses, beet molasses, or starch hydrolysates of corn, wheat or cassava are also in use (Kelle et al., 2005). Microbial fermentation is the most favored means of L-lysine production as it gives higher yields than chemical and enzymatic methods (Hermann, 2003). Moreover, it produces biologically active form which has food and feed applications.

Even though the industrial production process is well established and proprietary to the manufacturing giants, the academic interest still remains for better strain development and on process improvement. There were continuous studies on each unit operation in the view of economic feasibility, technical data generation, better process understanding, novel production processes, increased productivity, etc. Thus there will always be

unfailing efforts from the academics for a superior production process or a superior production strain.

In the present study, the potential of *Corynebacterium glutamicum* DM 1729 was evaluated for L-lysine production from agro residual biomass with focus on jackfruit seed. The versatility of multi substrate utilization and multiple product range has made *C. glutamicum* a favorite microbe in the commodity chemical industry. On the other hand, the starch and amylose content of jackfruit seeds make it an exceptional raw material for amino acid fermentation. Certain varieties of jackfruit seeds were reported to have starch content higher than maize and the carbohydrate content of isolated starch higher than potato starch and corn starch which are used in commercial amino acid fermentation. Jackfruit seeds are usually wasted during fruit processing and consumption which makes it a preferred raw material that adds to the cost effectiveness of the overall production process.

Different modes of fermentation were employed for L-lysine production. In submerged fermentation, a refined sugar in the fermentation medium was replaced by starch hydrolysate of jackfruit seeds. *Corynebacterium glutamicum* DM 1729 was able to grow and utilize the hydrolysate of jackfruit seed for L-lysine production. The fermentation conditions optimized by OFAT (one factor at a time) in shake flask resulted in a L-lysine yield of 0.14g $_{lysine}$ /g $_{reducing\ sugar.}$ The optimum conditions were checked in a parallel fermenter and the production levels were sustained. Parametric evaluation of physical and biological factors was carried out, and the significant variables affecting L-lysine production was identified with statistical tools like Plackett-Burman design and Response Surface Methodology. Employment of these techniques increased the yield of L-lysine from 0.14g $_{lysine}$/g $_{reducing\ sugar}$ to 0.3 $g_{lysine}/g_{reducing\ sugar}$ in submerged fermentation. The optimum conditions were scaled up to 1L in a fermenter and the production levels were sustained. Jackfruit seed powder and starch saccharifying enzymes were supplemented with the production medium in an attempt for a one step bioconversion of jackfruit seed powder to L-lysine. The production bioprocess was defined as simultaneous saccharification and fermentation and L-lysine production was realized from the production process. The variables affecting the bioprocess were screened and combined in different permutation and combinations to check the influence of the individual and interacting variables in L-lysine production. Thus, in simultaneous

saccharification and fermentation, the maximum L-lysine yield of 0.1g $_{lysine}$/g $_{substrate}$ was obtained.

Solid -State Fermentation (SSF) is the growth of micro organisms on solid material in the absence or near absence of free water (Pandey et al., 2008). Even though SSF process is usually used for fungal fermentation, bacterial fermentations has also been successful (Baysal et al., 2003; John et al., 2007; Nampoothiri & Pandey, 1996; Rojan et al., 2005) for the production of organic acids, amino acids and enzymes. In SSF a solid substrate impregnated with production medium act as inert support .In the present study, the prospective substrates as inert support for SSF was evaluated and sugarcane bagasse was chosen for further experiments. CGXII medium enriched with the starch hydrolysate of jackfruit seed powder (JFS) was used to moisten the solid support matrix. Sugarcane bagasse was used as the inert solid support matrix for the growth and amino acid production. Operational parameters affecting the fermentation were studied coupled with process level optimization. Statistical tools, such as, Plackett-Burman design (PBD) and central composite design (CCD) under response surface methodology (RSM) were employed to evaluate the factors affecting L-lysine production. The maximum L-lysine production was 16 mg/gds after 96 hours of fermentation with an initial sugar concentration of 7.5% w/v and initial moisture content of 70%.

Product purification and recovery is an inevitable part of any industrial production process. Fermentation products can be recovered from the fermentation broth with varying degrees of purity depending on the end use. There has been extensive research on L-lysine recovery processes as it is one of the steps that reflect on the overall production cost of the product. Even though a variety of recovery methods are known, ion exchange chromatography is the industry favorite for L-lysine recovery. The adsorption and elution conditions of cation exchange chromatography were chosen based on the experimental results with standard L-lysine. Two resins- Seralite SRC 120 and Seralite WRC 50 were used in the experiments. Recovery of the amino acid from the fermented broth was accomplished by ion exchange chromatography using acidic cation exchange resins and homogeneity verified by TLC and HPLC. Using standard L-lysine solution, under optimized conditions, the recovery using Seralite SRC 120 was 92±2% and using Seralite WRC 50 was 86±2%. The recovery percentages from fermented broth were 85±2% and 80±2% respectively.

The substrates for L-lysine fermentation has evolved over the decades since the commencement of commercial amino acid fermentation. The substrates ranged from pure sugars and molasses to first generation starchy biomass and second generation lignocellulosic biomass. The biorefinery concept for *Corynebacterium glutamicum* has been hardly studied, despite being the industrial producer of amino acids since its discovery. In the present study, the development of a consolidated proof of concept bioprocess for production of L-lysine with recombinant *C. glutamicum* DM1729 strains expressing endoglucanase and betaglucosidase genes was attempted. The constructs so obtained were able to grow and utilize cellobiose and carboxymethyl cellulose and produce L-lysine giving a maximum L-lysine titre of 5.9±0.5 mM (equivalent to 0.9 mg/mL L-lysine). This could be an initial step towards *C. glutamicum* based biorefinery for L-lysine production from cellulosic substrates.

In conclusion, the demand for amino acids is ever increasing and for L-lysine the increase in demand is quite significant, currently at the rate of 7%. The study exemplifies that agro residual biomass can be used as raw materials for amino acid fermentation and can influence the overall production process. The study started with the utilization of starch hydrolysate of first generation biomass and moved through the various possibilities of its utilization by different fermentation methods and culminated in the consolidated bioprocess towards direct utilization of the second generation biomass for L-lysine production. Recombinant *C. glutamicum* strains with ability to use waste liquor from biomass pretreatment in bioethanol production, will significantly add value to the whole process which in turn may reduce the overall production cost and helps waste management. With the estimated growth of L-lysine in the animal feed industry and the acceptance of *C. glutamicum* in the feeds, advances in metabolic engineering making the wild type strains able to utilize second generation biomass derived sugars or direct utilization of biomass, there will always be a focused research and labored developments in the L-lysine production process and the use of newer agro residual substrates as starting material could be of high use.

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ANNEXURE I

MEDIA COMPOSITION

1. LB Medium (Luria-Bertani Medium g/L)

Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0
pH	7.2

For solid media preparation, 15 g/L agar is added before autoclaving

2. LBG Medium (Luria-Bertani Glucose Medium g/L)

Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0
Glucose	5.0
pH	7.2

For solid media preparation, 15 g/L agar is added before autoclaving

3. M2 medium (g/L)

Glucose	40
K_2HPO_4	0.5
KH_2PO_4	0.5
$(NH_4)_2SO_4$	30
$MgSO_4.7H_2O$	0.025
$FeSO_4.7H_2O$	0.01
$MnSO_4.4H_20$	0.01
Nz-amine	1

Biotin	50µg/L
Thiamine	0.01
$CaCO_3$	20
pH	7.0

4. CGXII medium (g/ L)

Prepare solutions A, B, C, D & E

A. $CaCl_2$ 1 g/100 ml dist. water

B. Biotin 20 mg/100 ml dist. water, dissolve by heating

C. Trace elements

1 g $FeSO_4 \times 7\ H_2O$

1 g $MnSO_4 \times H_2O$

0.1 g $ZnSO_4 \times 7\ H_2O$

0.02 g $CuSO_4$

0.002 g $NiCl_2 \times 6\ H_2O$

Add 90 ml dist. water and dissolve by addition of concentrated

HCl (final pH should be about 1). Sterilize by filtration.

D. Protocatechuate 300 mg in 8 ml dist. water. Dissolve by addition of about 1 ml10 N NaOH. Sterilize by filtration and store at 4 °C.Protocatechuic acid is the same as 3,4-dihydroxybenzoic acid.

E. CGXII salts (g/L)

$(NH_4)_2SO_4$	20
Urea	5
K_2HPO_4	1
KH_2PO_4	1
MgSO4. $7H_2O$	0.25
MOPS (3-N-Morpholinopropane sulphonic acid)	42

Preparation

Add 1ml $CaCl_2$ solution, Adjust pH 7.0 with 10N NaOH, make up to 920mL and autoclave. Add 1mL trace element solution, 1mL protocatechuate and 50 µg biotin. Seperately autoclave 50%w/v glucose and add to the media to get desired sugar concentrations.

Annexure II

LIST OF MAJOR FACILITIES / INSTRUMENTATION

Instruments	**Model**
Autoclave	HVE-50, Hirayama, NewYork
Amino acid analyzer	Shimadzu, Japan
Centrifuge	Eppendorf, Germany; Kuboto, Japan
Deep freezer (-86^{0}C)	Operon, Korea
Fermenter	Infors HT (Switzerland), Infors Minifors, (Switzerland),
Hot air Oven	Kemi Instruments, India
Incubating water bath	Julabo, Germany
Incubator Shaker	Infors HT, Ecotron, Switzerland, Infors HT, Multitron, Switzerland Sanyo MCO-2OAIC- SANYO Electric Co Ltd, JAPAN
Laminar air flow chamber	Clean air System, India
Lyophilizer	Operon hDB-5503, Korea
PCR machine	Mastercycler EP Gradient, Eppendorf, Germany
pH meter	Systronics, India
Phase Contrast Microscope	Leica DM2000, Leica Microsystems, CMS GmbH
Scanning Electron Microscope	JEOL JSM 5600LV, 115 Japan
Sonicator	VCX-750, Sonics, USA
Thermomixer	Thermomixer, Eppendorf, Germany
TLC scanner	CAMAG,Switzerland
UV-VIS Spectrophotometer	UV-160A, Shimadzu, Japan
Vacuum concentrator	Eppendorf 5301, Germany
Weighing balance	Mettler Toledo, Mumbai, India

ANNEXURE III

LIST OF PUBLICATIONS

Research papers

Anusree, M., Wendisch, V.F., Nampoothiri, K.M. 2016. Co-expression of endoglucanase and β-glucosidase in *Corynebacterium glutamicum* DM1729 towards direct lysine fermentation from cellulose. *Bioresource Technology*. doi:10.1016/j.biortech.2016.03.019.

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