Development of a bioprocess for microbial production of 1,3-propanediol from biodiesel derived crude glycerol

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By

Narisetty Vivek

AcSIR Enrolment No: 10BB14A36006

Under the guidance of

Dr. Binod Parameswaran

Microbial Processes and Technology Division
(Formerly Biotechnology Division)
CSIR-National Institute for Interdisciplinary Science and Technology
Thiruvananthapuram – 695019
Kerala, India

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National Institute for Interdisciplinary Science and Technology (NIIST)
Council of Scientific & Industrial Research (CSIR)
(Department of Scientific and Industrial Research, Ministry of Science and Technology, Govt. of India)
Industrial Estate P.O., Trivandrum - 695 019
Kerala, India

Binod Parameswaran, *PhD*
Senior Scientist
Microbial Processes & Technology Division

Tel:+91-471-2515 361
Fax:+91-471-24 91 712
E-mail: binodkannur@niist.res.in
binodkannur@gmail.com

CERTIFICATE

This is to certify that the work incorporated in the Ph.D. thesis entitled " **Development of a bioprocess for microbial production of 1,3-prropanediol from biodiesel derived crude glycerol"** submitted by **Mr.Narisetty Vivek** (Enrolment No. 10BB14A36006) to the Academy of Scientific and Innovative Research (AcSIR) in fullfilment of the requiremnets for the award of the Degree of Doctor of Philosophy in Biological Sciences emodies original research work carried out by him under my supervision. I further certify that this work has not been submitted to any other University or Institution in part or full for the award of any Degree or Diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

NARISETTY VIVEK

Dr. Binod Parameswaran

(Thesis Supervisor)

Thiruvananthapuram

June 2019

डॉ. विनोद परमेश्वरन / Dr. BINOD PARAMESWARAN
वरिष्ठ वैज्ञानिक / Senior Scientist
माइक्रोबियल प्रक्रिया तथा प्रौद्योगिकी प्रभाग
Microbial Processes and Technology Division
सी एस आई आर- राष्ट्रीय अंतर्विषयी विज्ञान एवं प्रौद्योगिकी संस्थान
CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Govt. of India
तिरुवनन्तपुरम/Thiruvananthapuram-695 019, Kerala

Declaration

I, **Narisetty Vivek** (AcSIR Enrolment No. 10BB14A36006) hereby declare that the work presented in this thesis entitled "**Development of a bioprocess for microbial production of 1,3-propanediol from biodiesel derived crude glycerol**" is a bonafied record of research work carried out by me under the guidance and supervision of Dr. Binod Parameswaran, at Microbial Processing and Technology Division, CSIR – National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, Kerala, India. I also declare that all relevant suggestions made by the audience during pre-synopsis presentation, recommendations by the Doctoral Advisory Committee, and those by thesis reviewers have been incorporated in this thesis. I also declare that the work incorporated in this thesis or any part of it has not been submitted for the award of any other degree, diploma, associateship or any other title or recognition.

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List of abbreviations

%	Percent
µ	micron
µg	microgram
µL	microlitre
°C	Degree Celsius
ADP	Adenosine diphosphate
ANN	Artificial neural networks
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
BBD	Box-Behnken Design
BDO	Butanediol
BLAST	Basic Local Alignment Search Tool
bp	Base-pair
CAGR	Compound annual growth rate
CCD	Central Composite Design
cm	Centimetre
CoA	Co-enzyme A
CS	Chitosan
CSIR	Council for Scientific and Industrial Research
CSL	Corn Steep Liquor
DHA	Dihyroxyacetone
DHAP	Dihyroxyacetone phosphate
DNA	Deoxy Ribonucleic Acid
DOE	Design of Experiments
EPS	Exopolysaccharides
FID	Flame ionization detector
Fig.	Figure
FPH	Fish Protein hydrolysate
FTIR	Fourier transform infrared spectroscopy
g	gram
g cm^{-3}	Density
g g^{-1}	gram per gram
g L^{-1}	gram per litre
g L^{-1} h^{-1}	gram per litre per hour

G3P	Glyceraldehyde-3-phosphate
GMO	Genetically modified organisms
h	hour
HPLC	High Performance Liquid Chromatography
HPA	Hydroxy propionaldehyde
L	litre
MEGA	Molecular Evolutionary Genetics Analysis
M	Molar
ME	Meat extract
mg	milligram
min	minute
mL	millilitre
mM	millimolar
mPa	millipascal-second
MRS	DeMan Rogosa and Sharpe
NAD	Nicotinamide Adenine dinucleotide
NAG	N-acetylglucosamine
NG	N-glucosamine
NIIST	National Institute for Interdisciplinary Science and Technology
nm	nanometre
O.D	Optical density
PBD	Plackett-Burman Design
PBT	Polybutylene terephthalate
PCR	Polymerase Chain Reaction
PDO	Propanediol
PEP	Phosphoenol pyruvate
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
Pi	Phosphate
PLA	Poly Lactic acid
PTT	Polytrimethylene terephthalate
RID	Refractive Index Detector
rpm	Rotations per minute
RMSE	Root mean square error
RSM	Response Surface Methodology

rRNA	Ribosomal RNA
SEM	Scanning Electron Microscopy
sp.	Species
TGA	Thermo gravimetric analysis
TLC	Thin layer chromatography
Tm	Melting temperature
UV	Ultravoilet
v/v	Volume per volume
vvm	Volume per volume per minute
WAXD	Wide angle X-ray diffraction
w/v	Weight per volume
YE	Yeast Extract

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Chapter 1

Introduction and Review of Literature

Chapter 1 Introduction and Review of Literature

1.1. Introduction

The dependence of humanity on nature is inevitable. The ENERGY requisite made human to master the environment, and to build the amenities for pleasure and comfortable living. The basic idea which marched the humans to bring changes in the civilization was better living and a goal of producing more output with less human effort. With energy as the primary criteria, the drastic changes in the human life and structure of the society can be classified as pre-industrialization, industrialization and post-industrialization era. The advent of 19th century marked the industrial revolution providing sophisticated machinery and transportation facilities. The quest for reliable and substantial energy sources has become a need, which led to the search for accessible, reliable and dependable energy sources. The pre-industrial society was reliable on biomass derived energy sources like wood, peat or coal. With the advent of industrialization, soon the biomass derived energy sources were replaced by crude oil and petroleum resources, as the inventions in the industrial sector provided machinery for mining the fossil fuel deposits. Similar to the energy needs, there was growing demand for chemicals and materials for the society, till then they were produced using coal gas (Clark et al.,2007; Octave & Thomas, 2009). In 1920's the feedstock for these chemicals and materials was replaced by natural gas and petroleum derivatives, isopropyl alcohol produced at Standard Oil's Bayway was the first petrochemical manufactured at commercial scale. Then the further timeline witnessed many innovations and discoveries, involvement of organic chemists led to the development of complex chemical processes for breaking down the petroleum derivatives into variety of chemicals like hydrocarbons, building blocks or monomers, speciality chemicals, commodity chemicals and bulk chemicals. Whether it is a plastic, synthetic dye, rubber, textiles, minerals and metals the manufacturers were dependent on petroleum or fossil fuel based feedstock. Until 20th century the chemical industries were so effective meeting the unprecedented rate of societal demands (Ragauskas et al.,2006; Clark et al.,2007). During oil crisis in 1973 and disasters like Bhopal gas tragedy, brought a down fall for chemical industries. These events brought public and government concerns over the depleting natural resources and environmental safety. Along with public concerns, exponential growth in legislation regarding the registration and authorization of the chemicals and the synthesis processes is becoming difficult, where the manufacturers facing difficult in reduced supply of feedstock, increased energy costs and disposing of hazardous waste. With the growing energy demand and

population, these finite petroleum resources will be depleted and become extinct for further generations. Along with the limited petroleum reserves, various oxides of sulfur, nitrogen, carbon, lead, hydrocarbons and other greenhouse gases on combustion of these fossil fuels has a great impact on environment and pose a threat for global warming (Clark et al.,2007). To resume these global impacts and ecological disturbances, a thought of alternative sources was evolved. Biological approaches to fuel production using agro-residual biomass have gained impetus in the recent years and are preferred method compared to harsh chemical methods. In the early 21st century the transition of fossil fuel based society into a situation where agriculture sector is the source of food supply and the renewable feedstock for the chemical and material production. The major technology envisioned in the modern society was biotechnology, capable of providing health and wealth for betterment of the society and bio-based economy. The biotechnological processes were comparatively cleaner and environmental friendly compared to petrochemical and thermo chemical processes, as the biotechnological process can be performed with less energy and in adaptable physical conditions (Gavrilescu et al.,2005; Ragauskas et al.,2006). The process can bring change in the market potential of the products, by producing in higher yields; reducing dependence on fossil fuel based feedstocks and reduced emission of greenhouse gases.

The plant biomass constitutes large number of molecules like carbohydrates, lignin, protein and fats representing 95% of total plant volume. This plant biomass is a source for food and non-food fractions. On photosynthesis, plant metabolite reserve consists of easily accessible sugars like starch and sucrose, but the rest 70% of the biomass constitutes lignocelluloses. In the biofuel processing industry based on different pretreatment strategies, lignocelluloses were converted into three streams (i) carbohydrates in the form of starch, cellulose, hemicelluloses and monomeric sugars (ii) aromatic compounds like lignin and (iii) hydrocarbons in the form of triglycerides (oils).With the idea of using the whole plant, except the parts used as food stuffs, led to development of concept of biorefinery, where new technologies can be developed to produce new renewable chemicals with sustainable economy (Bozell et al.,2008; Dale et al.,2003). The first and second generation of biofuels like bioethanol was mostly produced from the monomeric sugars like hexoses and pentoses obtained from the biomass, and the streams of aromatic compounds and hydrocarbons were found as effluents or waste streams in the biofuel industry. If these waste streams can be used as the starting materials for the production of any value added chemicals, the process prevents waste generation, turns out to be cost effective process, it generates economy for the biofuel industry and provides employment in the society. Hence in this

century we can witness the exchange of chemical process with the biological process, actually this handover finds an opportunity for the chemical industries, as the feedstock processing in the biological process is not intense like petroleum feedstocks. In the next generation biorefinery, involvement of chemists, process engineers and biotechnologists, the lignocellulosic feedstocks can be further fractionated or pretreated to produce various other higher value products (Sheldon, 2008).

In the world energy consumption scenario, diesel fuels play a major role in industrial, transport and agricultural sectors in the developing countries. An alternative strategy for this nonrenewable diesel is production of economically and environmentally feasible and acceptable fuel derived from renewable sources like plant derived oils. This fuel derived from biomass sources was termed as biodiesel (Demirbas,2009b; Ayoub & Abdullah,2012; Meher et al.,2006; Johnson et al.,2007). Biodiesel is manufactured from vegetable, plant derived oils or animal fat as feedstock consisting of long chain alkyl esters. Biodiesel is chemically synthesized by transesterification of lipids with an alcohol in the presence of catalyst resulting in a mono alkyl ester. Various methods like batch processing, supercritical, ultrasonic and microwave treatment techniques can be used for the production, but the most likely used is transesterification. Important vegetable feed stocks used as a source of fatttyacids for biodiesel production are rapeseed, soybean, pongamia, jatropha, mustard seed, jojoba, flax, sunflower, palm, coconut, hemp, cotton seed, canola, castor and used vegetable oil (Demirbas, 2009b; Ayoub & Abdullah, 2012). Biodiesel is the only potential fuel source in the current available biofuels, as it can completely replace the petroleum diesel.The biodiesel can be used as such or in the form of blends in the conventional diesel engines without any modifications to the engines or the fuel systems. This potential biofuel has major concern regarding the major by-product formed during the transesterification reaction, i.e., glycerol in crude form. In the production process 10% wt crude glycerol is generated as the by-product. Though glycerol has various applications as additive in the pharmaceutical and food industries, rise in the biodiesel production and cost of refining the crude glycerol to pure is not an attractive strategy. As biodiesel is being produced from cheaper substrates, the surplus glycerol produced cannot meet market potential in terms of purity and cost. Instead the crude glycerol can be directly used as the feedstock or substrate for the production of value added chemicals which increases the economy of biodiesel industries and solve the concern about waste management (Johnson et al., 2007).

Besides the paradigm shift of petroleum derived fuels to sustainable biofuels, the chemical industries realized that the future would be on the chemicals derived from the

renewable raw materials rather than the petroleum hydrocarbons. The bulk chemicals like lactic acid, acrylic acid, glutamic acid, gluconic acid, 1,3-propanediol, succinic acid, few vitamins and amino acids are produced biologically via fermentation through whole cell or enzymatic approach. Glycerol, a simple monomer with highly reduced carbon atoms can be utilized as the substrate for the production of many chemical compounds like dihydroxyacetone, butanol, propionic acid, succinic acid, polyhydroxyalkanoates, vitamin B12, ethanol, citric acid, acetic acid, mannitol, erythritol, fumaric acid, arabitol, and 1,3-propanediol. Among them 1,3-propanediol gained industrial importance and value due to its flexible properties and use as a monomer in the synthesis of polymers like polyurethanes, polyesters by condensation reactions (Johnson et al., 2007).

1,3-propanediol (1,3-PDO) is a specialty chemical monomer gained an economic importance from being a fine chemical to commodity bulk chemical. Though thermo chemical production of 1,3-PDO from oleo chemicals derived from petroleum derivatives is well known, the fermentative mode of synthesis found more environmentally friendly and economical. In 1881 August Freund reported the biological production of 1,3-propanediol using *Clostridium pasteurianum*. This chemical has wide range of applications as monomer in the production of polymers like polyurethane, polyether etc. and also in cosmetic, food, pharmaceutical, textile industries. Hence the biological production of 1,3-propanediol using crude glycerol as the substrate provides an opportunity to capitalize the economic process of biodiesel industries by utilizing the waste stream for value addition. To educate and enlighten the advantages and importance of biological 1,3-propanediol production over traditional chemo approach, different aspects of upstream and downstream processes were reviewed further in this chapter.

1.2. Biodiesel production and efficiency

Biodiesel is manufactured from edible and non-edible oils consisting of long chain alkyl esters through transesterification with an alcohol in the presence of catalyst resulting in a mono alkyl ester. The composition analysis of plant derived oils was found to have free fattyacids, phospholipids, sterols, water and other components, that reduce the usage of oil directly as a fuel. Hence the oil was modified to produce free fatty acyl esters by transesterification reaction. Monoalkyl esters of long chain fattyacids are the principle components of biodiesel derived from waste vegetable oils and plant oils. In the transesterification reaction, oils or fats on reaction with alcohol (ethanol or methanol) in the presence of alkali catalyst (potassium hydroxide or sodium hydroxide) break open to long chain fattyacid groups to form methyl/ethyl esters, separating the glycerol back bone of long chain fatttyacids as crude glycerin. The

obtained methyl or ethyl esters are termed as biodiesel, which is pale yellow in color, medium light combustion fuel (Meher et al., 2006). From the plant to yield of biodiesel comprise multiple tedious steps: crop production, harvesting, seed cleaning and drying, oil and meal production, later transesterification using extracted oil. Investment for a biodiesel production unit requires 78% in the form of price for feedstock, but 93% returns can be expected from biodiesel yield. The time consuming process in the whole production is crop yield and harvesting where it takes 4-7 years for first yield based on different oil crops, for an example, *Jatropha* (4-5 yrs) and *Pongamia* (6-7 yrs) (Sheehan et al., 2000).

As the physical properties of biodiesel are more similar to petroleum derived diesel, it has profound applications as a fossil fuel alternative. It can be either used in pure form B100 or as blends with different concentrations ranging from 5% to 20%, where the remaining concentration will be petroleum derived diesel. Among these blends B20 is most preferable, as such we can use in the present motors without altering the engine make up. On combustion of biodiesel effluents discharged has 41% reduction in greenhouse gases, reduced emission of unburned hydrocarbons, carbon monoxide and particulate matter by 21%, 11% and 10% respectively (Sheehan et al., 2000).

Till today edible vegetable oils either in fresh or used form are using as raw material for biodiesel production, but almost 400 species of non-edible oil producing plant species are available, which can be cultivated to produce enough raw material. Globally waste lands, degraded forests and non-forest lands other than food and fodder cultivable lands can be used for oil based crop production, to establish a cleaner and greener environment with this clean fuel (biodiesel) reducing greenhouse effect, environmental and ecological imbalances due to pollution cause by exhausts from vehicle tail pipes. This clean fuel is non-toxic, biodegradable and suitable for sensitive environments. The schematic representation of production process of biodiesel from vegetable or animal fats is represented in Fig. 1.1.

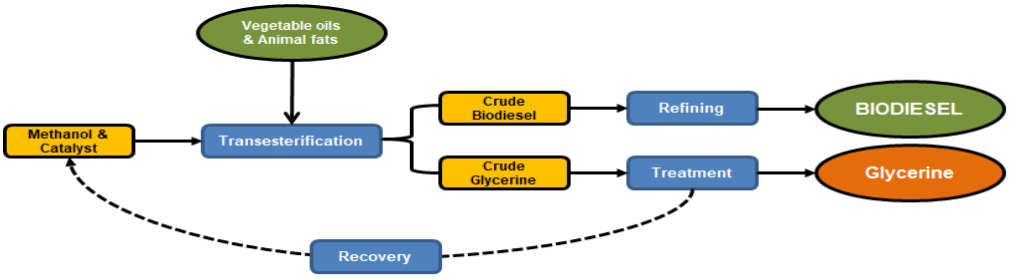


Figure.1.1. A schematic representation of biodiesel production process from vegetable oils or animal fats.

1.2.1. Glycerol as the byproduct and its composition

Glycerol commonly known as glycerin is the major by-product obtained during transesterification reaction for biodiesel production. The ratio of crude glycerol to biodiesel is 1:10, means for every 10kg of biodiesel produced 1 kg of crude glycerin is obtained. The crude glycerol phase get settled at the lower part of the production tank, which can be easily separated from biodiesel. As it is crude form the concentration of glycerol varies from (70-98%) and rest of impurities includes fatty acid methyl esters, fattyacids, methanol, water, soap and ash content. Due to various impurities viscosity of crude glycerin ranges between 15-1213 mpa.s, and density (1.01-1.2 g/cm^3) is found to be less than the pure glycerol (1.31 g/cm^3) (Tan et al., 2013, Yang et al., 2012). The unique physical and chemical properties of glycerol and its non-toxic nature made it as a compatible renewable raw material with various applications. As biodiesel is globally accepted for a better alternative fossil fuel, entrepreneurs and industrial relevance increased and resulted in industrial scale production, which limits the question of waste glycerol management available in surplus amounts. It has become an economical issue for biodiesel industries. As glycerol is mostly used as a commodity chemical in various chemical synthesis, food and pharmaceutical industries, the crude form can be distilled to obtain the pure glycerol, but the cost of distillation and purification is comparatively higher than the market price, which made the process uneconomical (Meher et al., 2006; Demirbas, 2009a).

The structural configuration of glycerol shows three carbon back bone with three hydroxyl groups, responsible for its hygroscopic nature and water solubility. Its highly reduced nature, make glycerol a better raw material for the production of chemicals and value added products. In the commercial scale, glycerol is available in three different forms, (i) crude glycerol (ii) purified/refined glycerol (iii) commercially synthesized glycerol. As biodiesel is being produced from cheaper substrates, the surplus glycerol produced cannot meet market potential in terms of purity and cost. Instead the crude glycerol can be directly used as the feedstock or substrate for the production of value added chemicals which increases the economic value of glycerol in the market as well as due to establishment of new production plants for conversion of glycerol employment can be given, having a societal influence (Hu et al., 2012; Santibáñez et al., 2011).

1.2.2. Applications of glycerol

Functionality of glycerol resembles the reactive behavior of hydrocarbon derived from petrochemical derivatives; hence various chemicals and value added products can be synthesized from glycerol through various chemical reactions. Primarily glycerol is used in manufacturing of cosmetics, soaps, resins, food, drinks, esters, polymers and other products. Later due to high reducing nature found in glycerol by its structural and physical characterization made biorefineries to utilize crude glycerol as the raw material either in oxidation or reduction process to produce many chemicals like dihydroxyacetone, mesoxalic acid, glyceraldehyde, glyceric acid, malonic acid, hydroxypyruvic acid, lactic acid, pyruvic acid, propylene glycol, Propionic acid, glycidol, acrylic acid, propanol, isopropanol, acetone, propylene oxide, propionaldehyde, allyl alcohol, acrolein, acetol, glycerol carbonate etc. (Luo et al., 2016; Zheng et al., 2008; Santibáñez et al., 2011).

In oxidation and reduction process, oxidation is easier conversion where glycerol in the presence of potassium permanganate is converted to tartronic acid and in presence of nitrous acid is converted to glyceric acid. Two important chemicals fuel oxygenate a fuel additive is produced by etherification of glycerol and isobutene and quinoline is produced from glycerol and aniline (Díaz-Álvarez& Cadierno, 2013). These chemicals have an established value in the market and higher price than the crude glycerol which makes this conversion process more efficient than distillation of glycerol to avail pure form of glycerol in the market.

Glycerol as a reduced carbon compound with three functional hydroxyl groups, made it more reactive and used as starting material in biological conversions as the process conditions and catalysts used in the chemical synthesis are tedious and expensive. The microbial cell can easily take up the glycerol molecule through facilitated diffusion, and several microorganisms have metabolic pathways oxidizing and reducing glycerol to produce primary and secondary metabolites.

Various value added fine and bulk chemicals like dihydroxyacetone, propionic acid, succinic acid, citric acid, acetic acid, fumaric acid, like erythritol, arabitol, 1,3-propanediol, 2,3-butanediol (fig. 1.2), polymers like polyhydroxyalkanoates, and gases like hydrogen and methane are produced using biological micro reactors like microorganisms as catalysts resulting in reduced toxic intermediates and undergoing bioconversions in the room temperatures rather in high temperatures and pressures required for chemical processing (Johnson & Taconi, 2007; Yang et al., 2012; Li et al., 2013a; Clomburg & Gonzalez, 2013; Kumar et al., 2015a).

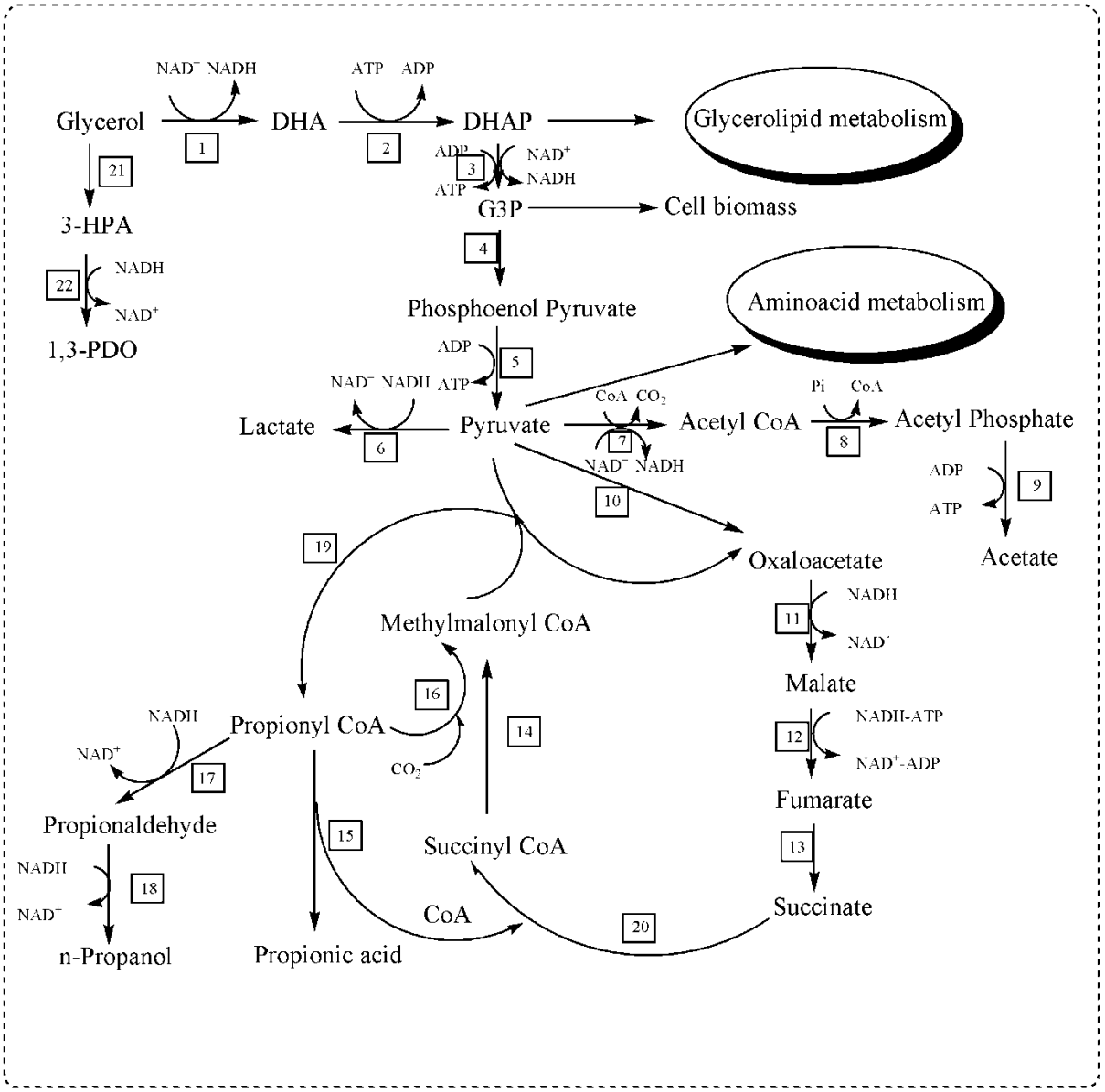


Figure.1.2. Glycerol dissimilation in oxidative and reductive pathways. The numbers represent the enzyme mediated reactions 1. Glycerol dehydrogenase, 2. Dihydroxy acetone (DHA) kinase, 3. Isomerase, 4. G3P dehydrogenase, 5. Pyruvate kinase, 6. Pyruvate kinase, 7. Pyruvate dehydrogenase, 8. Phosphotransacetylase, 9. Acetate kinase, 10. Pyruvate carboxylase, 11. Malate dehydrogenase, 12. Fumarase, 13. Succinic dehydrogenase, 14. Methylmalonyl CoA Isomerase, 15. Acyl CoA synthase, 16. Methylmalonyl CoA carboxylase, 17. Propionaldehyde dehydrogenase, 18. Propanol dehydrogenase, 19. Propionyl CoA carboxylase, 20. Succinyl CoA synthetase, 21. Glycerol dehydratase, 22. 1,3-PDO dehydrogenase.

Various biological products derived from glycerol using different biocatalysts, production titers and yields were represented in table 1.1.

S.No.	Product	Microorganism	Titers (g/L)	Yield (g/g)	References
1	Succinic acid	*Actinobacillus succinogenes* *Anaerobiospirillum succiniciproducens* *Basfia succiniciproducens*	4.9-35	0.6-0.87	Kongruang & Kangsadan, 2015; Lee et al., 2001; Scholten et al., 2009; Blankschien et al., 2010
2	Citric acid	*Yarrowia lipolytica*	86.5-157.5	0.59-0.9	Rywińska & Rymowicz, 2010;
3	Propionic acid	*Propionibacterium acidipropionici* *Propionibacterium acnes* *Clostiridum propionicum* *Propionibacterium jensenii* *P. freudenreichii subsp. shermanii*	11.5-47.28	0.3-0.54	Barbirato et al., 1997; Dishisha et al., 2013;Liu et al., 2012; Wang et al., 2015
4	Lactic acid	*E. coli* *Lactobacillus rhamanosus* *Enterobacter faecalis*	26.53-85.8	0.5-0.9	Hong et al., 2009; Prada-Palomo et al., 2012; Murakami et al., 2016
5	Glyceric acid	*Gluconobacter cerinus* *G. frateurii* *Acetobacter tropicalis*	57-101	0.7-0.9	Habe et al., 2009; Habe et al.,2010
6	1,3-Propanediol	*Clostridium butyricum* *Clostridium diolis* *Klebsiella pneumoniae* *Citrobacter freundii* *Lactobacillus reuteri* *Lactobacillus brevis* *Lactobacillus diolivorans*	20-98	0.5-0.7	Zhong et al., 2014; Guo et al., 2010 Wilkens et al., 2012 Otte et al., 2009 Pflügl et al., 2014 Vivek et al., 2016 Celińska et al., 2015 Vaidyanathan et al., 2011 Tang et al., 2009

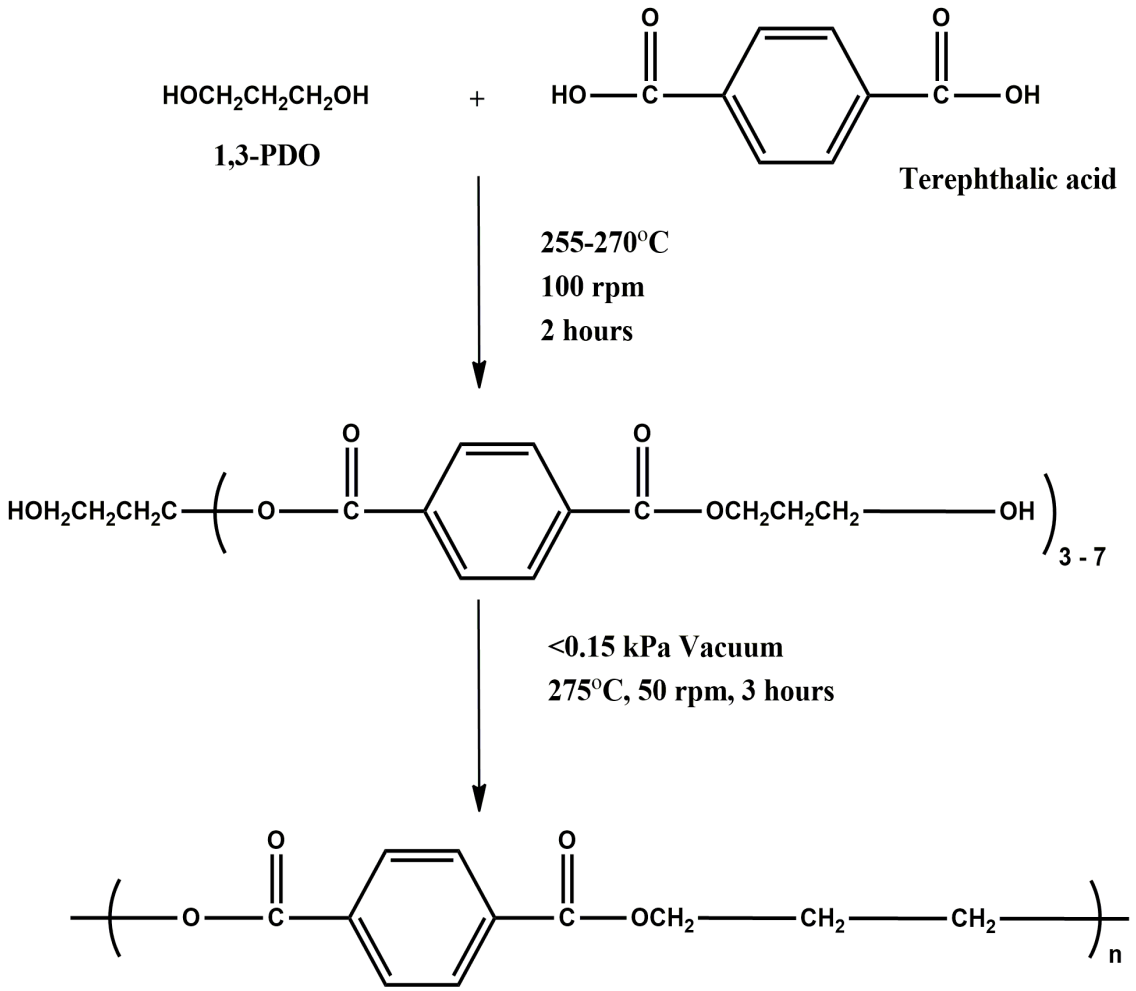
Table.1.1. List of various metabolites produced by various microorganisms using crude glycerol as the substrate.

1.3. 1,3-Propanediol

1,3-PDO ($C_3H_8O_2$), also known as trimethylene glycol, 1,3-dihydroxypropane or propylene glycol, is an organic chemical with two hydroxyl groups at 1st and 3rd carbon atoms. It is a colorless, viscous liquid with slightly sweet in taste and the compound is more soluble in polar solvents like water and ethanol and less soluble in non-polar solvents like benzene.

1,3-propanediol has similar applications as ethylene glycol, 1,3-butanedioland 1,4-butnediol. The two hydroxyl groups on the 1st and 3rd carbon, makes the chemical efficient for polycondensation reaction with the dicarboxylic acids or their ethers to produce polyesters, polyethers and polyurethanes. It is less toxic in nature, but permit was not allowed as a food additive for livestock feed additive. It has wide range of applications in improving nature of resins, adhesives, lubricants, pharmaceuticals, solvents, antifreezes, fuel additives and cosmetics (Kaur et al., 2012).

The most widely known application of 1,3-PDO is as a monomeric building block of plastics. A versatile polymer or polyester synthesized using 1,3-PDO is polytrimethylene terephthalate (PTT), a product of polycondensation reaction between 1,3-PDO and terephthalic acid or dimethyl terephthalate (fig.1.3). The polyester is known for its physical and mechanical properties, linear in structure and having a combination of mechanical properties of PET (polyethylene terephthalate) and permeability characteristics of PBT (polybutylene terephthalate). In 1941 Whinfield and Dickson synthesized these three polyesters PTT, PET and PBT, but PTT was never commercialized due to scarcity and cost of the monomer 1,3-propanediol (Whinfield & Dickson 1941). In 1995 Shell chemicals announced a product named “Corterra^{TM} PTT” later in 2003 DuPont commercialized a bio-based PTT called “Sorona”, in which 1,3-PDO is biologically derived from corn glucose (Bhatia & Kurian, 2008; Kaur et al., 2012).



Polytrimethylene Terephthalate (PTT)

Figure.1.3. Schematic representation of thermoplastic polyester polytrimethylene terephthalate (PTT) Synthesis by direct esterification and polycondensation of 1,3-PDO and terephthalic acid.

The chain confirmation of PTT has considerable difference between the polyesters PET and PBT, PTT has odd number of methylene repeat units adjacent to terephthalate esters in trans-gauche-gauche-trans confirmation, with high contraction and low conformational energy. But high conformational trans arrangement was observed in PET and PBT with even numbered methylene groups. This unique structural arrangement of PTT imparts elastic strength and higher resilience than the other polyesters. PTT with an average molecular mass of 68870 g/mol was synthesized using polycondensation; the differential scanning calorimeter provided the glass transition (T_g) temperature of 45^{o}C and melting temperature of 228^{o}C, both the characteristics lies in between PET and PBT. The glass transition depends on the crystallinity of the polymer, upto 30% crystallinity T_g of PTT was observed to be 45^{o}C,

whereas an increase upto 70^{o}C was observed at 50% crystallinity (Enriquez et al., 2016; Roupakias et al., 2005).

Due to high elasticity, dirt resistance, microbial resistance, texture retention, stain resistance, minimal deformation capability and resistance to UV, chlorination and fouling the polyester can be applicable in production of fiber, films, textile garments, paper making, decoration items, composites, inner lining of automotives and thermosetting foam modules in industries. Fiber made of PTT has many end applications like readymade apparels, and carpet making (Enriquez et al., 2016; Roupakias et al., 2005; Liu et al., 2010a; Li et al., 2017).

1.3.1. Importance and Economy of 1,3-propanediol

1,3-propanediol (1,3-PDO) is known as the monomer for synthesis of PTT. The journey of 1,3-PDO started as the speciality chemical, as mentioned above the application of this valuable monomer in polyester synthesis was in 1941, but the lack of abundance of this chemical, the market potential was not established. This scenario was totally overturned by leading 1,3-PDO makers, Shell and DuPont. The chemical has its growth from speciality to bulk chemical, DuPont Tate & Lyle Bioproducts together commissioned a Bio-PDO plant in Loudon, Tennesse with a production capacity of 140 million pounds per annum. The 1,3-PDO market is comparatively small and newer to ethylene glycol, 1,4 and 1,3-butanediol. But the potential of 1,3-PDO in developing the new applications driving the market statistics. The market value of 1,3-propanediol in various aspects either as monomer or substituent in polymer industries or cosmetics was $310.5 million in 2014. The compound annual growth rate (CAGR) was expected to increase by 19.9 % from 2012-2019 with estimated increase of market value from $157 (2012) to $560 (2019) million (Chemical, 2015),whereas an another report advances the survey until 2021 and says $621.2 million as the estimated market value with increase in CAGR of 10.4 % from 2014-2021. The world market statistics displays America as the leading market consuming about 75% of world's production followed by Asia-Pacific and European countries. As polytrimethylene terephthalate (PTT) production is the major application of 1,3-propanediol as major substituent monomer, 81.2% of 1,3-PDO market is consumed for PTT production in 2012 and statistical estimation was increase in CAGR of 16.3% by 2019. Then polyurethane utilizes PDO more after PTT reaching a CAGR of 15.2 % by 2019. In personal care and detergents, 1,3-PDO has its application, earning about $25.9 million in 2012 (Research, 2014a; Research, 2014b). DuPont Tate & Lyle Bioproducts company, LLC (U.S.), metabolic Explorer SA (France), Zhangijiagang Glory Biomaterial co. Ltd. (China) and ZoupingMingxing Chemical co. Ltd. are current

the leading 1,3-propanediol producing companies. As the market demand of 1,3-PDO was emerging tremendously, as per the report global demand for 1,3-PDO was 60.2 kt in 2012 and estimated was 150 kt by 2019. As the major application of PDO, PTT manufacturers represent the 90% of the 1,3-PDO market. The manufacturing process of 1,3-PDO is patent protected, which restricts the entry of competitive players, until and unless a path breaking technology was introduced into the market, that brings the new players. With the comparative market potential, it can be concluded that the 1,3-PDO journey of economic transformation from speciality chemical to a commodity chemical, provides the importance of the chemical in various applications.

1.3.2. Traditional chemical synthesis of 1,3-propanediol

The chemical manufacturing processes for 1,3-PDO start from ethylene or acrolein through two different process.

1.3.2.1. Degussa process

DuPont developed an alternative process for 1,3-PDO production using acrolein as the starting material. In this process, initially acrolein is hydrated to 3-hydroxy propionaldehyde (3-HPA) by addition of water molecule; later 3-HPA was hydrogenated to PDO (fig. 1.4 A). Acrolein is a simple unsaturated aldehyde with a tendency to polymerize by self-condensation, in Degussa process, hydration reaction should compete for self-condensation reaction, resulting in higher concentrations of 3-HPA. Acrolein is observed to be abundant raw material for 1,3-PDO production compared to ethylene oxide and this is usually produced from propylene, recent reports on chemical catabolism of glycerol was observed to produce acrolein (Liu et al., 2010a; Liu et al., 2010b;Da Silva et al., 2009; Lee et al., 2015a; Vivek et al., 2017a; Vivek et al., 2017b).

1.3.2.2. Shell process

A process using ethylene oxide as the raw material for 1,3-propanediol production was developed by Shell chemicals in 19th century. The process comprises of two stages, first is hydroxylation and later hydrogenation. In Hydroxylation the ethylene oxide reacts with carbon monoxide in the presence of organometallic catalyst resulting in a hydroxyl aldehyde, later resulting aldehyde is hydrogenated to 1,3-PDO (fig. 1.4 B). In this process the catalyst requires a tendency to mediate the ring opening of ethylene oxide, there after the ethylene oxide reacts with carbon monoxide resulting in aldehyde. In the hydrogenation reaction synthetic hydrogen gas was used as

source of hydrogen in the presence of copper chromite catalyst. Hence the process requires two sets of reaction with two different reaction conditions and two different expensive catalysts. Hence Shell chemicals come up with a new approach of one pot synthesis with coupled reaction in the presence of homogenous bimetallic catalyst using cobalt and ruthenium in 1:1 ratio with 1,2-diphospholanoethaneligand. The present reaction occurs in high temperature and pressure in presence of gaseous methyl tert-butyl ether with approximately 90% yield. This one pot synthesis resulted in favorable yield and the process was commercialized with a polymer named Corterra™, the manufacturing unit was established at Geismar, Louisiana with a production capacity of 83000 tons per annum (Da Silva et al., 2009; Lee et al., 2015a; Vivek et al., 2017a; Vivek et al., 2017b).

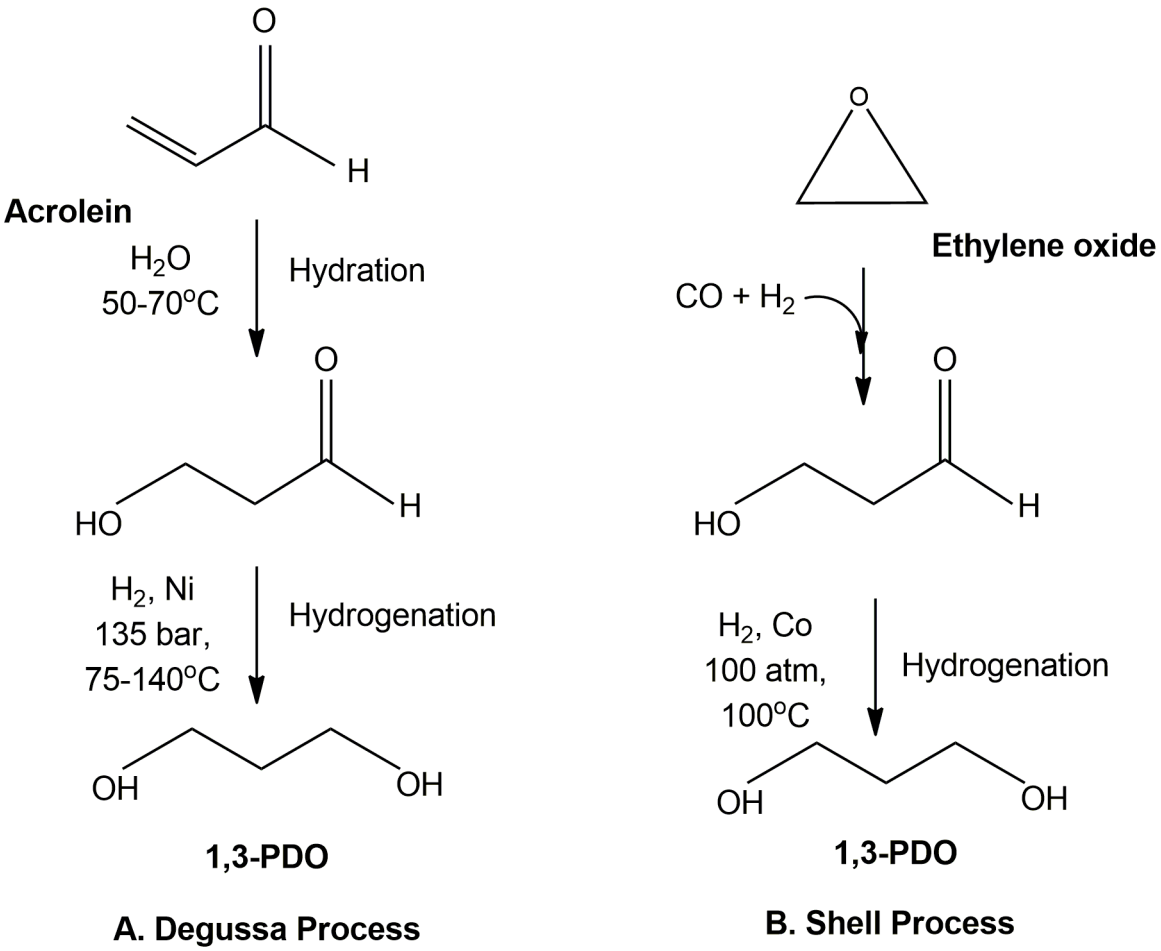


Figure.1.4. Schematic representation of two chemical synthesis processes for 1,3-Propanediol production A. Degussa process developed by DuPont and B. Shell process developed by Shell chemicals.

1.3.2.3. Hydrogenolysis of glycerol

The abundance of glycerol in the traditional markets due to increased production of sustainable fuels like biodiesel and bioethanol has channeled the use of this byproduct as the raw material for the production of value added chemicals. The compounds with higher O/C ratio can be deoxygenated to synthesize value added chemicals; hence the dehydroxylation allows the cleavage of C-O bond with an addition of H-atom, this type of hydrogenation is referred to as hydrogenolysis (fig. 1.5). This process is an efficient method to derive propanediol. Two propanediols, 1,2-PDO and 1,3-PDO can be derived from hydrogenolysis of glycerol, the selectivity of the product depends on the catalyst and reaction conditions. The difficulty in the deoxygenation arises with the accessibility of 2^o alcohol group, as the 1^o alcohol is more reactive (Lee et al., 2015a). Till date several homogenous and heterogeneous catalysts were reported for chemical conversion of glycerol to 1,3-PDO. A Celanese corporation developed a homogenous catalyst Rhodium with 1-methyl pyrolidinone and H2WO4 as the promoter, the reaction was carried out in syngas environment with 32 MPa pressure at 200^oC resulting in 20% 1,3-PDO yield. Shell's homogenous palladium complex catalyst in sulfolane or water as solvent yielded 30.8% 1,3-PDO (Lee et al., 2015a). As the homogenous catalyst is hard to separate from the reaction mixture, the continuous bonding between the substrate and the product formed might be a reason for lower yields of 1,3-PDO. To avoid this limitation, Chaminand et al., (2004) developed a heterogeneous catalyst comprising metal like Cu, Pd, Rh supported on zinc oxide (ZnO) or aluminum oxide (Al_2O_3) using water/sulfolane/dioxane as the reaction medium, operated under 800 bar H_2 pressure and 180^oC. Even the author explained the forwarding the reaction towards aldehyde production by addition of tungstic acid (H_2WO_4) and later the metal ions like Cu/Fe, increases the selective production of 1,3-PDO from 3-hydroxypropanal (Chaminand et al., 2004). During the hydrogenolysis of glycerol the alternate reactions can proceed to produce by-products like ethanol, ethylene glycol, methanol or methane, Kusunoki et al., (2005) developed a process of supporting the metal catalyst on activated charcoal and presence of an activated cation exchange resin namely amberlyst 15, reduced by-product reactions, by controlling the reaction at lower temperatures like 120^oC (Kusunoki et al., 2005).

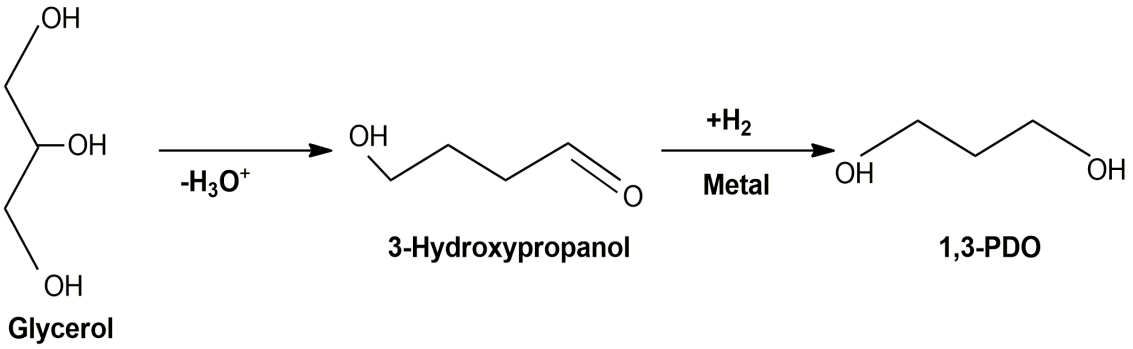


Figure.1.5. Schematic representation of chemical conversion of glycerol to 1,3-propaendiol via hydrogenolysis.

Although the chemical process was less time consuming and comparatively simpler downstream processing, the processes has their drawbacks in terms of yield, physical conditions, catalysts and etc. In Shell process the yield was only 40%, but Degussa process resulted in 80% yield, even the current hydrogenolysis of glycerol resulted in 30-40% 1,3-PDO yield. Utilization of expensive catalysts, operated at higher temperatures and pressures, makes the process uneconomical and considered as environmental hazard. Hence these limitations of chemical processes and market dependence on this potential chemical raise a need for an alternative green process using renewable feedstocks. The biological process addresses the limitations by providing comparatively higher yields, renewable feedstocks like corn glucose or glycerol, low temperatures (30-37^{o}C) and pressures, no toxic intermediates or by-products.

1.3.3. Biological dissimilation of glycerol to 1,3-propanediol

The biological synthesis of 1,3-PDO was carried out using glycerol or corn starch as the raw material.

1.3.3.1. Microorganisms producing 1,3-propanediol

It has been a decade since the first report on microbial production of 1,3-propanediol was published. Later in 1914 a Bacillus sps., and an Enterobacteriaceae member in 1928 but a final biotechnological route was recognized in late 1990. Since then several microorganisms were isolated and screened for 1,3-PDO production from glycerol as sole carbon source. The natural producers like *Klebsiella pneumoniae* (Ashok et al., 2011; Avci et al., 2014; Cui et al., 2014; Hong et al., 2013; Li et al., 2014; Liu et al., 2007; Oh et al., 2012; Petrov & Stoyanov, 2012; Zhou et al., 2015), *Clostridium butyricum* (Biebl, 1991; Chatzifragkou et al., 2012; Chatzifragkou et al.,

2011; Chatzifragkou et al., 2010; Chatzifragkou et al., 2014; Ferreiraa et al., 2014), *Clostridium diolis* (Kaur et al., 2012a), *Clostridium pasteurianum* (Dabrock et al., 1992; Jensen et al., 2012a; Jensen et al., 2012b),*Clostridium acetobutylicum* (Forsberg, 1987), *Lactobacillus reuteri* (Baeza-Jiménez et al., 2011), *Lactobacillus diolivorans* (Pflügl et al., 2012; Pflügl et al., 2013; Pflügl et al., 2014), *Lactobacillus panis* (Kang et al., 2014a; Kang et al., 2014b), *Citrobacter freundii* (Anand et al., 2010; Celińska et al., 2015; Drożdżyńska et al., 2014; Ferreira et al., 2012), *Citrobacter werkmanii* (Maervoet et al., 2012), *Citrobacter amalonaticus* (Ainala et al., 2013) *Enterobacter aerogenes* (Anggraini, 2014) and a newly isolated thermophilic strain AT1 (Wittlich et al., 2001) can utilize crude and pure form of glycerol as substrate to produce 1,3-PDO. In these vast pool of natural 1,3-PDO producers *Klebsiella sp*., and *Clostridium sp*., are extensively considered and found to be the efficient producers, but these microorganisms are reported to be opportunistic pathogens, henceforth handling these microbes in an industrial scale will pose a risk (Celińska, 2010; Kaur et al., 2012b).Overwhelming genetic engineering strategies and molecular techniques designed genetically modified microorganism by adopting the genes specific for 1,3-propanediol from natives. The GMO's like *E. coli* (Tong et al., 1991; Zhang et al., 2006; Wang et al., 2007; Jin & Lee, 2008; Dabrowski et al., 2012) and *S. cerevisiae* (Rao et al., 2008; Ma et al., 2013) are heterologous hosts constructed by harboring specific genes from *K. pneumoniae*.

1.3.3.2. Mechanism of glycerol dissimilation

Organization of genes in a specific order, expression and regulation of these genes in different environments under specific conditions would be supporting the organisms in pattern of evolution. Metabolism of glycerol in these microbes is a coupled oxido-reductive process (fig. 1.6). In which glycerol acts as the sole carbon source for oxidative as well as reductive pathway. In oxidative pathway, NAD^+ dependent glycerol dehydrogenase enzyme encoded by *dhaD* gene converts glycerol to dihydroxyacetone (DHA), which is phosphorylated to dihydroxyacetone phosphate (DHAP) by transfer of phosphate group from ATP in the presence of kinase enzyme encoded by *dhaK* gene. This DHAP is further metabolized to phosphoenol pyruvate and pyruvate, synthesizing reducing equivalents and energy, for growth and development of microorganism. To maintain equilibrium concentrations of $NAD^+/NADH^+H^+$ inside the micro compartments and cytoplasm, reductive pathway was observed which depends on reducing equivalents generated by oxidative pathway (Celińska, 2010;Kaur et al., 2012b; Nakamura & Whited, 2003)

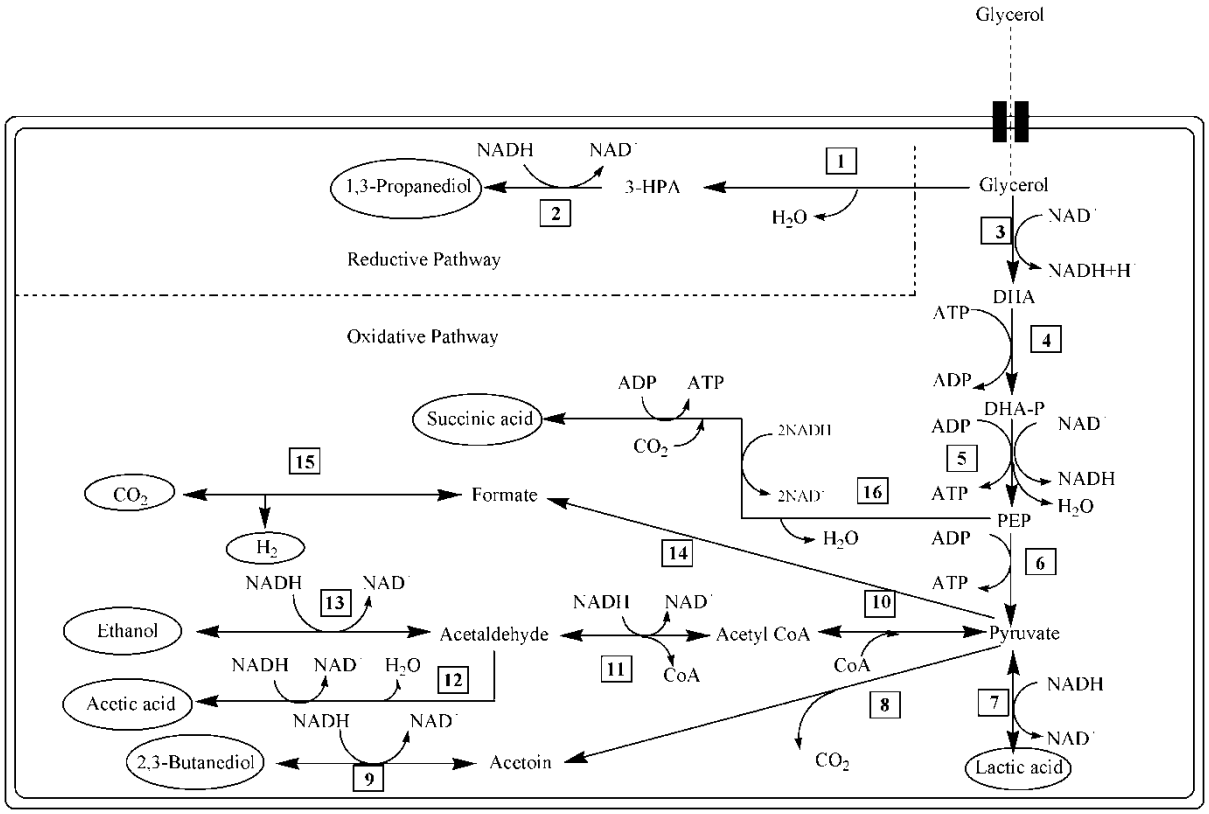


Figure.1.6. Metabolic pathway of glycerol: Ellipsoid figures represent the end products in oxidative and reductive pathway, line with one arrow head represents non reversible reaction and line with double arrows reversible reactions, Numbers represents the enzymes mediating specific reaction. 1. Glycerol dehydratase, 2. 1,3-PDO oxidoreductase/dehydrogenase, 3. Glycerol dehydrogenase 4. Dihydroxyacetone (DHA) kinase 5. 6. Pyruvate kinase 7. Lactate dehydrogenase 8. Pyruvate decarboxylase 9. Acetoin reductase (Forward reaction), BDH dehydrogenase (Reverse reaction) 10. Pyruvate dehydrogenase, 11 and 12. Bifunctional Acetaldehyde dehydrogenase 13. Alcohol dehydrogenase 14. Pyruvate formate lyase, 15. Formate dehydrogenase, 16. NADH Linked malic enzyme (gluconeogenic).

In reductive pathway, glycerol is metabolized in two consecutive enzyme catalyzed reactions to produce 1,3-propanediol as the end product. In first step glycerol dehydratase encoded by *dhaB* gene converts glycerol to 3-hydroxypropanaldehyde (3-HPA), the reactive intermediate reduces to 1,3-propanediol catalyzed by 1,3-PDO oxidoreductase (1,3-PDOR). Glycerol dehydratase is class II coenzyme B_{12} containing enzyme that binds to cobalamin. The mechanism of glycerol dehydratase is radical mediated, binding of coenzyme and apoenzyme activates the Co-C bond, then glycerol initiates the radical reaction by inducing hemolytic cleavage of Co-C bond, as the mechanism is substrate induced. The cleavage of Co-C bond creates two radicals Cob(II)alamin and adenosyl radical. The adenosyl radical is essential for the primary step of catalysis, the radical abstracts the H-atom from the substrate resulting in formation of substrate derived radical and 5'-deoxyadenosine. The susbtrate derived

radical converts to product radical by transfer of a hydroxyl group from 2nd carbon to 1st carbon. Then the product radical gets a H-atom from 5'-deoxyadenosine and forming the final product and also the reactivation of the coenzyme. The radical intermediates formed in this catalysis steps are essential and should maintain their reactivity at the active site of the enzyme, the quenching of these radicals may lead to side reactions or reactivation of the co-enzyme may not be feasible, that leads to inactivation. As the modified co-enzyme is in intact with the enzyme, the exchange with the free co-enzyme B_{12} is not possible, that leads to cessation of the reaction. Hence glycerol dehydratase is a rate limiting enzyme in 1,3-PDO production. With the available genomic data bases and bioinformatics tools, it was found that the enzyme glycerol dehydratase is found in various gram positive and gram negative bacteria, namely *Enterobacteriaceae*, *Propionibacteriaceae*, Solventogenic *Clostridiaceae*, and *Lactobacillaceae* members. The glycerol dehydratase enzyme is cofactor (vitamin B_{12}) dependent in *Klebsiella sp.*, *Citrobacter sp.*, whereas cofactor independent glycerol dehydratase was found in *Clostridium sp.* The mechanism of this enzyme is well known that it is radical mediated, but in clostridium members, adenosylcobalamin is replaced by s-adenosylmethionine (SAM), and requires 2 Fe^{2+} and 1 Fe^{3+} as the co-factors. The enzyme glycerol dehydratase has three subunits dha B, dha C and dha E in genus *Citrobacter*, *dhaB1, dha B2* and *dha B3* in *Klebsiella* sp., and as only one subunit *dha B1* in *Clostridium* strains. Though enzyme glycerol dehydratase metabolizes the glycerol towards reductive pathway, glycerol in higher concentrations than the tolerance range is a suicidal reaction for the enzyme which gets inactivated. The reactivation of glycerol dehydratase is catalyzed by the enzyme glycerol dehydratase reactivase encoded by globular complex polypeptide comprising $\alpha_2\beta_2$ subunits encoded by *dha F* and *dha G* genes. This mechanism of reactivation occurs in two steps ADP dependent cobalamin release and ATP dependent dissociation of apoenzyme factor complex, where ATP hydrolysis renders the conformational change in inactive form of the enzyme to ADP bound active form of the enzyme (Németh& Sevella, 2008; Kaur et al., 2012b; Celińska, 2012).

Intermediate produced in the first step is toxic to growth and metabolic activity of microorganism upon accumulation, which can be overcome by the action of 1,3-PDO oxidoreductase enzyme (E.C.1.1.1.202) which metabolize 3-hydroxypropionaldehyde to 1,3-propanediol (Barbirato et al., 1996). 1,3-PDOR gene is encoded by *dhaT* gene. NADH is the cofactor for 1,3-PDOR which is produced in the oxidative pathway. In general reduction of NAD^+ to $NADH+H^+$ occurs in electron transport mechanism in oxygen sufficient conditions, but in anaerobic and micro-anaerobic conditions the

metabolism should favor reductive pathway for regeneration of these reducing equivalents or should produce more reduced compounds like ethanol, lactate etc via oxidative pathway. Hence in metabolic pathway of glycerol anaerobic conditions will be suitable for organism to depend on reductive pathway for regenerating reducing equivalents, in turn producing 1,3-propanediol. An isoenzyme of 1,3-propanediol oxidoreductase and NADP-dependent dehydrogenase transcribed and translated using yqhd gene was found in *E.coli* strains showing increased activity than 1,3-PDO oxidoreductase in native producers (Cao et al., 2006; Chen et al., 2011).

1.3.3.3. Genetics of dha regulan

The genes responsible for dissimilation of glycerol and assimilation of 1,3-propanediol anaerobically in the bacteria are clustered into the dha operon. Organization, sequence of genes and homology of the enzymes have been investigated in *Clostridium butyricum*, *Clostridium pasteurianum*, *Clostridium acetobutylicum*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Citrobacter werkmanii* and *Citrobacter amalonaticus*. In *Clostridium butyricum,* nucleotide sequence analysis revealed six open reading frames dha S, dha A, dha B_1, dha B_2, dha T, ORF 6 and a truncated ORF 7, which are transcribed in the same direction. The genes dhaB1 and dhaB2 are found upstream to dha T. The transcriptional terminator is found downstream of dha T, which is the master regulator of the operon. The ORF dhaB1, dhaB2 and dha T comprise of 2364, 915 and 1158 base pairs respectively, which translate into enzymatic subunits with molecular mass of 88074 Da, 34149 Da and 41558 Da respectively. Each ORF can be distinguished with a gap bases after a stop codon and start codon of adjacent ORF and has a ribosome binding site upstream to start codon. The two genes dha S and dha A acts as signal transduction system in *Clostridium* sp., where dha S acts as sensor molecule and dha A as regulatory unit. The structural arrangements of these genes in an open reading frame with ribosome binding sites are represented in figure.1.7. Enzyme glycerol dehydratase encoded by dha B1 in *Clostridium sp* was found to be vitamin B_{12} independent and studies added supportive information showing homology with the native 1,3-propanediol producers. The dha T gene encodes 1,3-propanediol dehydrogenase having 76-85% identical to 1,3-PDO dehydrogenase produced by *Clostridium pasteurianum*, *Citrobacter freundii* and *Klebsiella pneumoniae* (Raynaud et al., 2011; Raynaud et al., 2003; Nakamura & Whited, 2003).

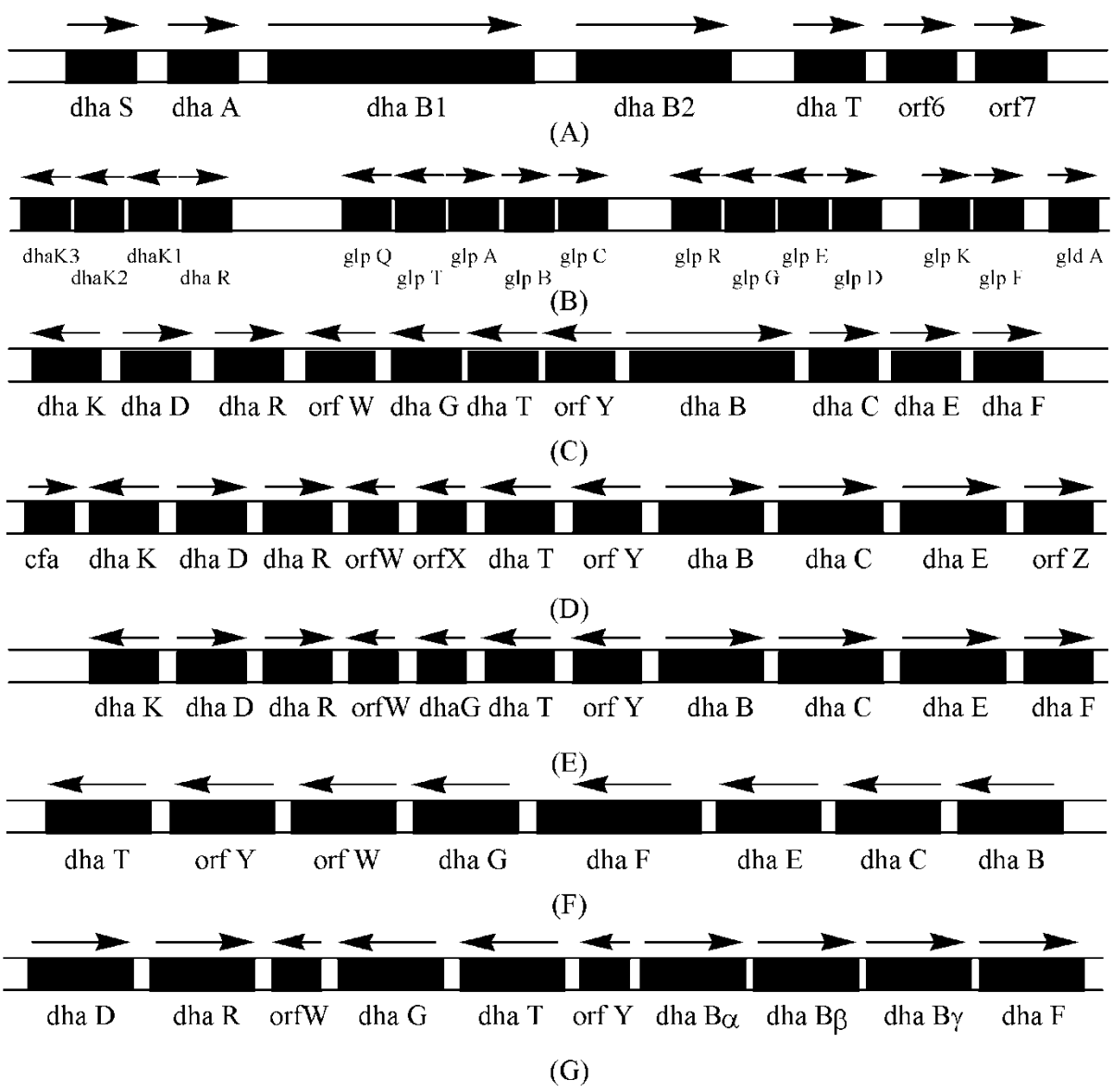


Figure.1.7. Schematic representation of dha regulon genes involved in oxidative and reductive pathway of glycerol in various organisms. (A) *Clostridium butyricum* VPI1718 (B) *Citrobacter amalonaticus* Y19 (C) *Citrobacter freundii* (D) *Citrobacter freundii* (E) *Citrobacter werkmanii* DSM 17579 (F) *Clostridium pasteurianum* DSM 525 (G) *Klebsiella pneumoniae* ATCC 25655

The dha operon mediating 1,3-propanediol production in *Klebsiella pneumoniae* was sequenced and characterized. The availability of genomic data will be helpful for construction of engineered strains. The *K. pneumoniae* gene carrying dha B and dha T genes is sequenced along with the dha B subunits dha B_{α}, dha B_{β}, dha B_{γ} and observed the tandem arrangement, along with the transcribing behavior in different directions, under the control of different promoters (Skraly et al., 1998; Tao et al., 2012; Celińska, 2012).Conserved sequences with identity of 96% were revealed in *Citrobacter werkmanii* strains compared to *Citrobacter freundii* and 87.5% identical to *Klebsiella pneumoniae* dha cluster. Nucleotide sequencing and chromosome walking

revealed a 12,911 nucleotide long dha cluster similar to other native producers. Oxidative pathway of glycerol initiated by glycerol dehydrogenase coded by dha D gene is a hexamer in *Citrobacter freundii* comprising of 365 amino acids in each subunit and in *Citrobacter werkmanii* gene transcribes into 365 amino acid long peptide chain. Genetic makeup and sequence of amino acids in the peptide reveals a 99% homology between *Citrobacter freundii* and *Citrobacter werkmanii.* The second important enzyme is dihydroxyacetone kinase for oxidizing DHA obtained from glycerol to DHAP; the enzyme kinase is encoded by dha K. It is the first gene in the dha cluster in *C. freundii*, *K. pneumoniae* and *C. werkmanii*; whereas *C. butyricum* has two dha K genes in the gene cluster. The transcription of both genes dha D and dha K is in opposite direction in *C. freundii*, *K. pneumoniae* and *C. werkmanii,* but it is in the same direction in *C. butyricum,* which shows the homology between the *enterobacteriaceae* members and organization difference between different genera. In all of these native 1,3-PDO producers the genes responsible for the reductive pathway dha BCE and dha T are found downstream to dha D and dha K. The expression of the dha cluster is regulated by dha R in *Citrobacter sp*., and *Klebsiella sp*., similar to dha S and dha A in *Clostridium sp*. Two unannotated orf sequences, Orf W and Orf Y, are found in *C. werkmanii*. Apparently, their functionality is activating glycerol dehydratase by converting the inactive cobalamine cofactor to the active form of adenosylcobalamine (Maervoet et al., 2014a; Maervoet et al., 2014b).

A new candidate *Citrobacter amalonaticus* was reported for 1,3-propanediol production lacking a dha cluster. However, sequencing analysis revealed glycerol and diol utilizing genes on a pdu like operon as in *Lactobacillus* species. Oxidative assimilation of glycerol is carried out by series of genes – glp K, glp D, glp ABC - found scattered all over the chromosome. *Citrobacter amalonaticus* shares homology with *E. coli* in gene sequence and arrangement. Both lack the genes dha B and dha T required for reductive utilization of glycerol and production of 1,3-propanediol. *Citrobacter amalonaticus* was reported as efficient glycerol assimilator and has a vitamin B_{12} synthesis operon, the major cofactor for glycerol dehydratase. The unknown YdhD or any other oxidoreductase isoenzyme is found to convert 3-HPA to 1,3-propanediol. The fermentative pathway in *C. amalonaticus* was found active when exogenous electron acceptors like formate were supplemented into the fermentation media (Ainala et al., 2013). In *Lactobacillus* species like *L. reuteri*, *L. buchneri* and *L. diolivorans,* the propanediol utilizing Pdu gene cluster plays a role in glycerol dissimilation and 3-HPA synthesis when glycerol and glucose is co-fermented. *Lactobacillus* species lack the enzymes for an oxidative metabolism of glycerol (Stevens et al., 2011; Vaidyanathan et al., 2011) so glycerol can only be used as a co-

substrate for fermentation.1,3-propanediol oxidoreductase, an NAD^+ dependent enzyme having similar properties like the dhaT gene of *K. pneumoniae* and *L. reuteri*, was found in *L. brevis* and *L. buchneri* (Veiga-Da-Cunha & Foster, 1992). In *L.panis* PM1 Pdu CDE codes for glycerol dehydratase of nine gene operon Pdu ABCDEGHKJ genes. A transcriptional repressor PocR with 359 amino acid length was found upstream to pdu operon coding in opposite direction. The repressor upon over expression showed a deleterious effects on diol dehydratases enzyme activity and 1,3-propanediol production, experimentally 22% decrease in activity and 40% decrease in production. The 1,3-propanediol oxidoreductase gene Pdu Q and a putative alcohol dehydrogenase was found in locus not of Pdu C gene locus (Kang et al., 2014b).The genes responsible for glycerol dissimilation, generation and regeneration of reducing equivalents and energy sources by yielding value added products and by-products though differ in each organism will find similarities in one or other extent and few are totally identical and homologous.

1.3.3.4. Bottlenecks for biological 1,3-propanediol production

Biological process is considered as eco-friendly compared to chemical process. Although the processes are carried out at physiological temperature and atmospheric pressure, it suffers from fewer limitations which need to be addressed to overcome and make the process economically feasible. The drawbacks of the process are low yield and productivity, inhibition due to substrate, product and other by-products and downstream process. The literature has provided information regarding the efforts put forth by various research groups and individuals to overcome these limitations and to make an economically and environmentally feasible biological 1,3-PDO production. The solutions outraging these limitations are categorized as process development, optimization of production strategies, metabolic engineering of native and non-native strains.

1.3.3.5. Utilization of biodiesel derived crude glycerol as the substrate for 1,3-PDO production

One of the advantage in bioconversion of 1,3-PDO is that it can use renewable feedstock as substrates (Jin et al., 2011). Though researchers were continuously engaged in developing an efficient and optimized process for higher yields, the cost of the feedstock remains a question for technical feasibility of the process. As glycerol is the only natural substrate for 1,3-PDO production, earlier the cost of glycerol is high, as it was synthesized through chemical route. The cost of

glycerol used to be between 1.10 – 1.25 US $/kg (Anand et al., 2010), but the 1,3-PDO production would be feasible only if the feedstock price is less than 0.3 US $/kg. In these circumstances, utilization of biofuel industry derived crude or waste glycerol can be a cost effective alternative feedstock for 1,3-PDO production (da Silva *et al.*, 2009). Later with the tremendous growth in the biodiesel industry and surplus amount of crude glycerol, various researchers were engaged in the isolation and optimization of microorganisms producing 1,3-PDO from waste glycerol. The strains of *Clostridium*, *Klebsiella*, *Citrobacter* and *Lactobacillus* were observed to be effectively grown in crude glycerol with additional nutrient supply, maximum conversion yields of 0.5-0.7 $g_{1,3\text{-PDO}}/g_{Glycerol}$ was observed.

S.No.	Parameters	Refine Glycerine (Pasand^{TM} GLY200)
1	Colour	Off – White (Yellow tone)
2	Specific gravity	1.24 – 1.25
3	Purity %	85 – 90
4	Moisture	10 – 12
5	Fat & Ester	0.1 – 0.5
6	Chloride (as Cl) PPM	< 1
7	MONG	0 – 1
8	pH	6 – 7
9	Ash %	2 – 3

Table 1.2. Composition of crude glycerol

Even the effect of impurities in the waste glycerol on growth and metabolism was observed to be unaffected. The utilization of non-modified strains and industrial waste effluents for the production of value added chemical would become a sustainable process.

1.3.3.6. Co-fermentation strategies

Glycerol is the sole substrate for 1,3-PDO production, that involves removal of a hydroxyl group (-OH) at 2C-position. A theoretical maximum of 0.83 $g_{1,3\text{-PDO}}/g_{Glycerol}$ can be synthesized. In the group of microbes like *Clostridium sps., Klebsiella, Enterobacter* and *Citrobacter sp*., the glycerol is used as carbon source as well as substrate for 1,3-PDO production resulting in lower yields (0.5-0.6 $g_{1,3\text{-PDO}}/g_{Glycerol}$) (Chatzifragkou et al.,2014; Kaur et al.,2012a). With the concern of market potential of the product, the higher yields are more important, utilization of commercial sugars or

lignocellulosic biomass derived hexose and pentose sugars supplementation along with the glycerol resulted in increased 1,3-PDO yields. Lignocellulosic biomass is widely used in bioethanol production, the process involves the utilization of hexose sugars, but the pentose stream obtained after the acid or alkali pretreatment was observed to be unutilized because of high lignin content and inhibitors like hydroxyl methyl furfural. In a report author described the utilization of the pentose stream obtained after rice straw hydrolysis, as a carbon source for *Klebsiella pneumoniae* strain with higher yields and 1,3-PDO titers (Vivek et al.,2017). In this scenario, the supplemented sugars will meet carbon energy and growth requirements and glycerol will be biotransformed to 1,3-PDO with a maximum of 0.8 $g_{1,3\text{-PDO}}/g_{Glycerol}$. Co-fermentation of glycerol and glucose by *lactobacillus* sp. was reported to increase the biomass and alter the end product profile by suppressing the production of ethanol (Schutz and Radler, 1984) and lactate (Veiga Da Cunha and Foster, 1992). In the production media, glucose is utilized for the microbial growth and energy production, where glycerol is for the utilization of reducing equivalence. Even in the recombinant strains, glycerol glucose co-fermentation shows better yield than using glycerol as sole carbon source (Biebl and Marten, 1995). The yield of 1,3-PDO from glycerol was improved from 0.46 $g_{1,3\text{-PDO}}/g_{glycerol}$ with glycerol alone to 0.63 $g_{1,3\text{-PDO}}/g_{glycerol}$ with glucose co-fermentation and 0.55 $g_{1,3\text{-PDO}}/g_{glycerol}$ with xylose co-fermentation in engineered *E. coli* expressing *K. pneumoniae* dha regulon genes (Tong and Cameron, 1991).

1.3.4. Analytical methods for qualitative and quantitative estimation of 1,3-propanediol

The biological synthesis can be estimated by the rate of substrate utilization and product formation rate. The rate can be estimated only through quantitative data. In the biological dissimilation of glycerol, along with 1,3-propanediol we know that by-products like diols (2,3-butanediol), sugar alcohols (ethanol), organic acids (propionic acid, lactic acid, acetic acid, butyric acid, succinic acid) and some amount of residual glycerol can be observed. Hence an estimation technique is required for simultaneous quantification of these products to determine the 1,3-PDO producers effectively.

From the decades of research on 1,3-PDO it is observed that high performance liquid chromatography (HPLC) is the best quantitative estimation method. Mostly a cation exchange resin based column Aminex HPX-87H or RezexRoA-organic acid column was used with 5 mM H_2SO_4 as the mobile phase and analyzed using refractive index detector (RID). A few reports using gas chromatography equipped with FID detector, 2m x 5mm glass column packed with chromosorb 101 and using He/N_2 as

the carrier gas was reported. However, for the wide range of metabolite profile obtained during glycerol fermentation, it is hard to estimate all the metabolites through GC as single column may not be suited for detection of all these products, but the organic acid column (H^+) is suitable to estimate all the metabolites simultaneously.

Later Anand et al., devised an efficient and simple qualitative method for screening 1,3-PDO producers using thin layer chromatography (TLC). Using chloroform and methanol in 80:20 volume ratio, further development using vanillin reagent, a purple colored spot with a R_f value of 0.62 was observed for 1,3-propanediol (Anand et al.,2011). This test has become one of the basic, economical and reliable 1,3-PDO producer screening technique.

1.4. Process optimization for enhanced 1,3-propanediol production

The process optimization of any biological process is of most important as the physical conditions and media composition significantly affect the product yield and volumetric productivity. The efficiency and market potential of the specialty compound chemicals like 1,3-PDO, citric acid, ethanol, succinic acid and 2,3-butanediol depends on the cost of the production media, operation parameters and the downstream process. Various studies were carried out on optimization of media components and physical parameters to enhance the 1,3-propanediol production using various biocatalysts. In this process optimization objective there are two main strategies followed, that are conventional “one variable at a time” and “statistical designs”.

1.4.1. One variable at a time approach

The advantage of this conventional “One variable at a time” approach is simple and easy to perform. The rationale behind this approach is, keeping the multiple components in a constant value except one variable. The value of this variable is changed accordingly; later the observation concludes the effect of this variable on the performance or production of the biological compound. Similarly the individual components were evaluated in their desired ranges to understand the optimum values of each component that increase the performance of the whole process. Due to its ease and convenience various researchers used this approach to optimize the media components and physical parameters to understand the behavior of the biocatalyst.

Understanding the effect of physical parameters like pH, temperature and agitation rate and nutritional factors like media components, nitrogen sources and metal ion concentrations on 1,3-propanediol production using different native strains was evaluated by several researchers. Even the observations provided the information regarding the critical effect of these variables on growth and 1,3-propanediol

production efficiency. Hence it is desirable to optimize the process conditions to enhance the 1,3-PDO production.

Although this conventional approach provides us the initial understanding on the effect of individual parameters on production of the desired end product, the approach fails in determining the mutual interactions between the variables and their combined effect on the desired outcome. On contrary, statistical experimental designs can provide with a valid experimental design for the optimization and can eliminate the limitations of conventional one-factor-at-a-time approach (Baş and Boyaci, 2007). This Statistical approach involves a limited number of experiments with an ease of observing and visualizing the interactions between several experimental variables simultaneously. Later the experimental data obtained can be used to analyse the predicted data to observe the efficiency of the experiment.

1.4.2. Statistical media optimization

The statistical media optimization can be carried out provided statistical software that can design the experiment based on the input variables we provide. To understand the effect of individual parameters and their mutual interactive effect on desired output can be elucidated through these following design strategies.

1.4.2.1. Plackett - Burman design

Plackett-Burman design (PBD) is a type of screening design where the number of factors in performing the experiment has a potential effect on the desired output, it is critical to understand the effect of each factor individually. Once the critical parameters are screened then we can proceed with the optimization and learning their mutual interactions. PBD was developed in 1946 by statisticians, R. L.Plackett and J. P. Burman as a screening method to identify the effective factors on the performance and output usinga few experimental runs, in early experimental phase by making the process economical (Plackett and Burman, 1946).A maximum of 47 individual factors with their squared and two way interactions can be screened using Plackett-Burman design. In screening of these 47 factors the software can design the experiment with a runs between 12 and 48, and is always as multiple of 4. During the design of PBD experimental runs, each factor is provided in 2 levels, if you add centre points to observe the pattern of the curvature, then the design will be with 3 levels for each factor. This Plackett-Burman design is useful for fitting the first order models (linear models) and can determine the second order models provided with centre point for 3 levels.

1.4.2.2. Steepest ascent

After the performance of the screening design, and obtain a linear regression equation provided with main effective parameters, if we would like to either maximize or minimize the response, one should follow a path of ascent for maximizing and a path of descent to minimize the response. The macro command steepest ascent requires (i) response data obtained from PB design and (ii) columns with uncoded levels of the factors. Then the design will be provided by the software extrapolating the limit of each variable factor that provides us the maximum and minimum limits of the each effective parameter (R. Myers and D. Montgomery 2002).

1.4.2.3. Response surface methodology

Response Surface Methodology (RSM), is a method originally described by Box and Wilson in 1951. RSM is a mathematical and statistical based on the fit of a polynomial equation (Bezerra et al.,2008) which enables to assess the effect of the independent variables, and their interactions to improve, and optimize the experimental process (Bas & Boyaci, 2007). Actually RSM determines the mutual interaction between the important factors obtained through fractional factorial or full factorial designs, where there is a curvature in the response surface. The RSM optimize a performance or quality characteristics of process or also termed as response which is influenced by a set of input values or independent variables. The response obtained after the experimental runs can be represented in the form of three dimensional plots or contour plots that helps in visualizing the response surface. Response surface methodology generates a mathematical model, which can describe the process, for this reason RSM models are applied in different research fields such as, i) Food Technology to study the possibility of use of rice starch, and fructooligosaccharides as substitutes for phosphates, and dextrose in cooked hams (Resconi et al.,2015), or to determine the interactions of processing variables on physicochemical, textural and sensory properties of cooked pork patties (Jung & Joo, 2013), ii) in Chemistry to optimization of analytical methods (Bezerra et al.,2008), iii) in Biorefinery to study the potential of apple pomace for lactic acid production using simultaneous saccharification and fermentation process (Gullón et al.,2007), or to obtain an economic ethanol production from starch-containing rice hulls (López et al.,2011), iv) in Environmental Sciences to optimize operational parameters to nitrogen removal from ammonium-containing wastewater (Guo et al.,2016).

An empirical statistical model can be developed that describes the relationship between the process variables and their maximum values that brings out the desirable values of the response in an economical approach. The quadratic or squared equation

obtained through response surface methodology and linear equation obtained through factorial design differs in squared (or quadratic) terms that allow modelling the curvature in the response. These squared terms are useful in understanding, (Minitab Inc., 2010):

1. Region of a response surface.
2. Affect in the response based on change in variable.
3. Finding the optimum level of a variable factor.
4. Optimizing the process conditions to obtain the maximum desired response or output.

There are two main types of response surface designs

(a) Central Composite design

Central Composite designs (CCD) can fit a full quadratic model. They are often used when the design plan calls for sequential experimentation because these designs can include information from a correctly planned factorial experiment (Minitab Inc., 2010). It has a few steps, viz., initial determination of the optimum region for the variables, behaviour of the response in the optimum region, estimation of the optimal condition and verification (Khuri & Cornell, 1987; Myers & Montgomery, 1995). A CCD is the most commonly used response surface designed experiment. CCDs are a factorial or fractional factorial design with centre points, augmented with a group of axial points (also called star points) that allows estimating the curvature. A central composite design can be used to efficiently estimate first- and second-order terms and model a response variable with curvature by adding center and axial points to a previously-done factorial design. CCDs are especially useful in sequential experiments because it can be built on previous factorial experiments by adding axial and centre points. When possible, CCD has the desired properties of orthogonal blocks and rotatability (Minitab Inc., 2010).

(b) Box-Behnken design

Box-Behnken designs (BBD) usually have fewer design points than CCDs, 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. BBDs always have three levels per factor, unlike CCDs, which can have up to five. Also, unlike CCDs, BBDs never include runs where all factors are at their extreme setting, such as all of the low settings (Minitab Inc., 2010).

Nevertheless, RSM is not the only available tool for modelling and predicting production of compounds of interest.

1.4.2.4. Artificial neural networks

Artificial neural networks (ANNs) offer other attractive possibility for providing non-linear behaviours for response surfaces (Bezerra et al.,2008). For this reason, in the last three decades, ANNs have become one of the most used techniques for modelling and optimization, especially for non-linear problems (Desai et al.,2008). Neural models are computational mathematical methods based on representative cells in biological neural system (neurons) (Bezerra et al.,2008; Bas & Boyaci, 2007) with deep and complex connections, forming a parallel network (Baliyan et al.,2015). ANN learn in the similar way that human brain, and presents benefits in learning process such as; i) can learn from empty data, ii) neural models can use experimental databases with noisy, and iii) can model non-linear behaviours (Bezerra et al.,2008; Bas & Boyaci, 2007; Perpetuo et al.,2012). The most used ANN model is the Multi Layer Perceptron (MLP) (Desai et al.,2008; Meireles et al.,2003; Geyikci et al.,2012) where neurons are arranged in different layers; i) one input layer constituted by input neurons which collect input information (independent variables), ii) one, or more, intermediate layers associate with input neurons and iii), one output layer, associate to the last intermediate layer, to provide the predicted value (Bezerra et al.,2008). MLP models allow modelling complex and highly non-linear behaviour (Csépe et al.,2014; Witek-Krowiak et al.,2014) through the different neural layers.

1.5. Process parameters and conditions influencing 1,3-propanediol production

The process parameters like temperature and physiological pH of the fermentation process has tremendous effect on the growth and metabolite synthesis.

1.5.1. Effect of physiological pH

Metabolites required for growth and development of microorganism were produced in oxidative pathway mediated by various enzymes and co-factors. Activity of these enzymes and mediators are altered by changes in extracellular or intracellular pH, which affect the metabolite profiles. Typically anaerobic fermentation is coupled organic acid production pathways, the release of organic acids like lactic acid, acetic acid and butyric acid, acidifies the fermentation broth which gradually reduce the growth and production efficiency of microorganism. This organic acid inhibition is a well-known phenomenon in the case of 1,3-propanediol producers like *Clostridium*, *Citrobacter*, *Lactobacillus* and *Klebsiella sp*.,.

In the majority of 1,3-PDO producers the optimum pH was observed to be 7.0, like *Klebsiella pneumoniae* GLC 29 produced 20.4 g/L with 40g/L glycerol at pH-7.0 (da Silva et al., 2014). An observation on alkaline conditions favors 1,3-propanediol production by shifting metabolic pathways (Grahame et al., 2013) was supported by various strains like *Klebsiella pneumoniae* Xj-Li produced maximum 12.2 g/L of 1,3-PDO at pH-8.0. *Clostridium* strains like *C. butyricum* and *C. pasteurianum* were observed to have maximum growth and 1,3-propanediol production at a physiological pH in the range of 5.8-6.5 and even lactic acid bacterial memebers were observed to produce 1,3-PDO in the similar range.

The optimum physiological pH of each strain may differs based on the sample location and habitat conditions from which it was isolated. Hence the preliminary examination on the range of pH which provides maximum growth, utilization of substrate and product formation, will provide the necessary data for further validations.

1.5.2. Effect of temperature

The physiological temperature in which the microorganism is incubated for the production of biological molecules is utmost important. The majority of microorganisms reported for the production of 1,3-propanediol are mesophiles growing at moderate temperatures. According to the literature reports the optimum temperature for the production of 1,3-propanediol ranges from 30-37^{o}C. An optimal temperature of 30oC, was reported for the growth and 1,3-PDO production by *Citrobacter freundii* and few *Klebsiella pneumonia* strains, while *Clostridium butyricum* is cultivated and incubated at 35^{o}C optimum temperature.

Mostly lactic acid bacteria (LAB) are grown at 37^{o}C, though the strains can tolerate upto 40^{o}C. The 1,3-PDO producers like *Lactobacillus reuteri* and *Lactobacillus panis* were reported to cultivate at 37^{o}C, whereas *Lactobacillus diolivorans* was incubated at 30^{o}C for 1,3-PDO production.

1.6. Engineering microorganisms for enhanced 1,3-PDO titers and yield

Metabolism of glycerol through oxidative pathway results in secondary metabolites like lactic acid, acetic acid, succinic acid, formic acid, propionic acid, butyric acid and ethanol, that are considered as by-products in 1,3-PDO production. The concentration of these metabolites depends on the flow of electrons and reducing equivalents, metabolic shift of $NADH+H^{+}$ towards reductive pathway increases 1,3-propanediol production. Enhanced NADH dependent 1,3-PDOR expression levels or decreased expression levels of dehydrogenase enzymes in the oxidative pathway can

only mediate the metabolic shift, in order to increase the production titers and understand the mechanism of improved 1,3-PDO production several strain improvement strategies like conventional physical and chemical mutagenesis, pathway engineering, gene knockouts and overexpression of limiting genes are carried to construct recombinant strains.

1.6.1. Traditional random mutagenesis

An entirely random mutation is incorporated into the microorganism by exposing the cells either physical agents (UV/IR) or chemical agents (EMS, NTG and LiCl). Later the mutant strains with desired traits were selected. The selection of mutant strain for 1,3-PDO production follows a criteria the strain should posses (i) tolerance towards high glycerol concentration, (ii) tolerance to 1,3-PDO concentration and (iii) low concentrations of by-product synthesis. The classical mutations tend to increase 1,3-PDO production, but the process is quite slow, Otte et al., (2009) worked on genome shuffling of chemically (NTG) mutated strains of *Clostridium diolis* DSM 15410, where the strains were initially exposed to known concentration of NTG and the desired mutants were selected. Later through protoplast fusion, genome shuffling was carried out between different mutant and wild type strains resulting in modified strains with 80% increased tolerance level to substrate (glycerol) and product (1,3-PDO) with maximum production titers of 80 g/L 1,3-PDO (Otte et al., 2009). Two mutant strains of *Clostridium butyricum* DSM 5431 were screened by inducing a stress mediated response like osmotic pressure and proton suicide method using 1,3-PDO at increased concentrations, the strains obtained have 44% increased glycerol utilization rate and 50% increased 1,3-PDO production titers compared to wild type. This stress mediated response resulted in a metabolic shift towards reductive pathway, the experimental observation was in agreement with the genetics of the strain, where increase in glycerol concentrations increased enzyme activities of glycerol dehydrogenase, glycerol dehydratase and 1,3-propanediol oxidoreductase in mutant strains but enzyme activities shown a steep decline in parent strain. Intracellular NADH, NAD+ and acetyl coA, concentrations remained constant in mutants while increased in wild type strain showing an increase in ratio of acetic acid and butyric acid compared to mutants (Abbad-Andaloussi et al., 1995). A *Clostridium pasteurianum* strain chemically treated with ethyl methane sulphonate (EMS), resulted in mutants with six times increase in cell dry weight compared to wild type and 1,3-propnediol production up to 33% and butanol upto 46% (Jensen et al., 2012b; Kubiak et al., 2012). In glycerol dissimilation, we observed that reductive environment favors 1,3-PDO production, the oxidative reduction potential (ORP) values represents the extent of reduced

environment tolerated by the strains. The *Klebsiella pneumoniae* M5aL wild type strain ORP value, lies between -160 to -190 mV, providing greater reductive potential than the wild type, then the strains withstanding different ORP values were selected. A mutant YMU1 can tolerate -280 mV and has enhanced 1,3-propanediol production of 63.1% compared to parent strain (Du et al., 2007).

As physical and chemical mutagenesis provides random mutations in the genetic material, the increased 1,3-PDO production cannot be experimentally validated except with increased enzyme activities and decreased by-products. Validation compared to genetic aspects was found to be difficult, as the modified mutants have same morphological and genetic characteristics. To observe the genetic behavior of the mutant strains, specific gene over expressions and knock outs were carried out. Those genetic engineering aspects and techniques in different 1,3-PDO producers were discussed in the content below.

1.6.2. Metabolic engineering in *Clostridium sp.*

The best 1,3-PDO producers are *Clostridium butyricum*, *Clostridium pasteurianum*, and *Clostridium diolis*. In this genera, *C. butyricum* is a potent strain with higher 1,3-PDO production titers upto 94 g/L and being an obligate anaerobic bacteria, handling, and maintenance in an industrial scale falls as a limitation, but in favorable conditions wild type strains themselves give better yield and productivity compared to recombinant strains. *C. acetobutylicum* DG1 strain which do not grow on glycerol as the only carbon source is recombined by expressing 1,3-PDO producing pathway from *C. butyricum* VPI 3266 using pSPD5 plasmid. In *C. acetobutylicum*, NADH formed in the oxidative pathway of glycerol cannot be regenerated as genes for reductive pathway are not found. The oxidative pathway of glycerol was also varied from *C. butyricum* and *C. acetobutylicum*, glycerol dehydrogenase and DHA kinase mediates oxidation in *C. butyricum*, whereas glycerol kinase and glycerol 3-phosphate dehydrogenase in *C. acetobutylicum*. The hydrogenase activity is tenfold higher in *C. acetobutylicum* compared to *C. butyricum*. The increase in 4-fold and 3-fold activities of glycerol dehydratase and 1,3-propanediol oxidoreductase was observed compared to the wild-type strain. While acid production also increased along with enzyme activities resulting in the production of 29.6 g/L of 1,3-PDO (González-Pajuelo et al., 2006). As the wild type strain by nature produce higher concentrations of 1,3-PDO and having a cofactor-independent glycerol dehydratase enzyme, instead of homologous or heterologous recombination of *C. butyricum* strains, they are recognized as

heterologous donors of dharegulan genes for cloning and expression in heterologous hosts (Kubiak et al., 2012).

1.6.3. Metabolic engineering in *Klebsiella sp.*

Although *Klebsiella pneumoniae* and other few species of *Klebsiella* were classified as biohazard class II microorganisms, various industrially valuable chemicals are produced by this group of microorganisms. The available genetic information, gene ontology and engineering tools for modification of *K. pneumoniae,* studies were carried out with respect to individual genes. In *Klebsiella* sp., *Klebsiella pneumoniae* and *Klebsiella oxytoca* are well known best producers for 1,3-propanediol. The accumulation of organic acids like lactic acid in the fermented broth alters the pH of the medium, resulting in reduced growth and 1,3-PDO production (Zeng et al., 1993), to understand the behavior of the strain under non-lactate conditions, *Klebsiellaoxytoca* M5aL ∆*ldh* A mutant was constructed by knocking out lactate dehydrogenase gene. Why lactic acid is ?the enzyme lactate dehydrogenase is a competitor for 1,3-PDOR in regeneration of reducing equivalents. If the enzyme lactate dehydrogenase is knocked out the flux of $NADH+H^+$ ions shift towards the reductive pathway. *K. oxytoca* M5aL ∆*ldh* A mutants produced 83.56 g/L, 60.11 g/L of 1,3-propanediol and 2,3-butanediol when sucrose was used as co-substrate for glycerol. The higher titers just resolved the problem of lower yields in wild type strains, But the purification was so tedious as 1,3-PDO and 2,3-BD have relatively similar properties which made them hard to purify from each other (Yang et al., 2007). The limitation was later addressed by Zhang et al. (2012) by constructing a mutant of *Klebsiella oxytoca* by blocking the 2,3-butanediol pathway. *bud* A gene responsible for reducing acetoin to 2,3-butanediol was knocked out. The *bud* A gene knock resolved limitation of the downstream process, not only the DSP another important aspect is that increasing the availability of $NADH+H^+$ ions for 1,3-PDOR, as 2,3-BD dehydrogenase enzyme also regenerates NAD^+ ions. The deleted mutant strain produced 62 % higher 1,3-propanediol and 46% increase in cell dry weight compared to the wild-type strain (Zhang et al., 2012). *Klebsiella pneumoniae* DSM 2026 a native 1,3-propanediol producer was cloned and overexpressed with NAD^+ dependent gamma glutamyl gamma aminobutyraldehyde dehydrogenase (PuuC) for co-production of 3- hydroxypropionic acid (3-HP) and 1,3-PDO and observed a cumulative yield of 51% (Ashok et al., 2011), and overexpressing aldehyde dehydrogenase (KGSADH) gene increased cumulative molar yield upto 80%, which would be efficient to produce two value added chemicals (Kumar et al., 2012). Heterologous expression of pyruvate decarboxylase (*pdc*) and aldehyde dehydrogenase (*ald* B) from *Zymomonas mobilis* into acetolactate (als)

synthesis-deficient *Klebsiella pneumoniae*, to enhance generation of reducing equivalents and conversion of 3-HPA to 1,3-PDO, resulted in increased production of 68.2 g/L, 0.63 g/g yield and 1.42 g/L/h productivity (Lee et al., 2014). An isoenzyme of 1,3-propanediol oxidoreductase (*dha* T gene) i.e., alcohol dehydrogenase (*YqhD* gene), found in *E. coli*, was expressed individually and co-expressed along with native *dha* T gene in *Klebsiella pneumoniae*. The metabolite synthesis during the fermentation showed a 25 % decrease in the accumulation of intermediate inhibitory compound 3-HPA. The overexpression altered the enzymatic activities when *dha* T is overexpressed 1,3-propanediol production is increased, whereas *YqhD* as alcohol dehydrogenase increased the molar yield of ethanol, but on co-expression 1,3-propanediol yield was increased by 11.8% (Zhuge et al., 2010). As an efficient biotransformation glycerol should be utilized as a substrate towards 1,3-propanediol production. Various reports specify that glycerol dehydrogenase (GDH) and 1,3-propanediol oxidoreductase (1,3-PDOR) are the limiting enzymes for glycerol metabolism, any alteration in these enzymes will have the effect on glycerol utilization and 1,3-propanediol production efficiency (Horng et al., 2010). Overexpression of these two enzymes were carried out, and the effect was observed in micro-aerobic conditions, confirming least effect on the concentration of 1,3-propanediol but decreased the production of by-products which would be useful in addressing the major limitation of 1,3-propanediol production (Zhao et al., 2009). During the glycerol fermentation *Klebsiella pneumoniae* produces mostly capsular polysaccharides encoded by *cps* gene, the production not only limits the substrate for end product synthesis but also increases the viscosity of fermentation broth making it complicated in separation and purification of 1,3-propanediol. The mutation was induced in cps gene of *K. pneumoniae* CGMCC1.6366 increasing 1,3-propanediol production from 9.73-10.37 g/L in flask experiments and the final yield of 78.13 g/L in the fed-batch process, which can be considered as best production levels (Guo et al., 2010).

1.6.4. Metabolic engineering in *Lactobacillus sp.*

*Lactobacillus sps.,*a group of heterofermentative microorganisms, are another group as known native 1,3-propanediol producers. These groups of bacteria are non-pathogenic; probiotic can be easily isolated from food and dairy products. Hence, recombinant and genetic engineering techniques are applied on *Lactobacillus sps.,* to develop industrial potent strain for scale-up studies. Four strains of this genera *L. reuteri*, *L. diolivorans, L. brevis* and *L. panis* are reported for 1,3-propanediol production and genetic organization of PDO production genes are unveiled. The

heterologous expression of 1,3-propanediol oxidoreductase (*YqhD* gene) from *E. coli* in *Lactobacillus reuteri* ATCC 55730 resulted in 34% and 13% increase in productivity and molar yield of 1,3-PDO (Vaidyanathan et al., 2011). *Lactobacillus sp*., are known for the production of 3-hydroxypropionaldehyde the intermediate with the anti-microbial property, but due to this heterologous expression the intermediate accumulation reduced to 25%. As 1,3-propanediol oxidoreductase (*YqhD*) is NADPH-dependent, whole cellular NADPH molecules are diverted to the 1,3-propanediol pathway which reduced the accumulation of 3-HPA and increased 1,3-propanediol. But the concentration of lactate and ethanol increased with the decrease in acetate concentration because NADH concentration favored production of ethanol and lactate regenerating NAD^+ molecules. In *Lactobacillus reuteri* DSM 20016 found three putative 1,3-propanediol dehydrogenases annotated on pdu operon, studies were carried out to know the specific dehydrogenase expressed for 1,3-propanediol production. The strain was inoculated into media with glucose as sole carbon source and other with both glucose and glycerol. There is 3-times increase in the enzyme activity when glycerol was used as co-substrate, but in three putative dehydrogenases, two has increased activity, and another is down regulated, confirming that specific dehydrogenase is not involved in reductive pathway of glycerol (Stevens et al., 2011). The Scope of lactic acid bacteria as a potent 1,3-propanediol producer lies in knocking out the acid producing pathway or genes like lactate dehydrogenase (ldh), but few studies explains the deletion of *ldh* genes in lactic acid bacteria has deleterious effect in the growth of microorganism by reducing the peptidoglycan synthesis which is necessary for synthesis of cell wall during propagation.

1.6.5. Metabolic engineering in *Citrobacter sp.*

In Enterobacteriaceae members another known 1,3-propanediol producer is *Citrobacter sp*., which can produce 1,3-PDO in anaerobic and micro-aerophilic conditions. Uncoupling the major pathway using metabolic engineering and maintaining a complete transformation of the substrate to product and feeding with co-substrates to produce energy currency and reducing equivalents will be a practical approach. In *C. werkmanii* DSM 17579 effect of growth, metabolite production, substrate and product inhibition kinetics on supplementation with different hexoses, pentoses and DHA, were carried out (Maervoet et al., 2012). Later *dha* D gene encoding glycerol dehydrogenase enzyme required for oxidative conversion of glycerol was deleted. Growth pattern was altered compared to wild-type due to lack of dha D gene, mutant strain did not grow in glycerol fed medium under anaerobic conditions. Utilization of co-substrates along with the glycerol revealed the effect, L-arabinose, D-

ribose, D-galactose, and D-maltose regulated positively, whereas D-glucose, D-mannose, D-fucose, and D-sorbitol showed an adverse effect on the growth of biomass. All the positive regulators are one in the composition of hemicellulosic hydrolysate derived from agro residual biomass, which would be a cheap co-substrate for 1,3-PDO production. *C. werkmanii* ∆*dha D* mutant strain produced 5.76 g/L of 1,3-PDO when glucose was added as co-substrate with a molar yield of 0.94 mol/mol, 5.39 g/L, 0.87 mol/mol yield when galactose was fed as co-substrate. Though production levels are low a theoretical maximum conversion efficiency of 94% was observed and mutations carried out in the strain are justified (Maervoet et al., 2014a). In *Shimwellia blatte* the expression levels of 1,3-PDO oxidoreductase (*dha* T) was higher compared to *C. freundii* AD970. Hence, *C. freundii* AD970 strain is recombined by expressing dha T gene from *S. blatte* heterologously, resulting in 2-fold higher concentration of 1,3-PDO production than the wild-type. A final concentration of 49.9 g/L 1,3-PDO with a yield of 0.49 $g_{PDO}/g_{glycerol}$ and 0.32 g/L/h productivity was observed. It is the first report on the genetic modification of *Citrobacter freundii* for 1,3-PDO production, resulting in higher production levels (Celińska et al., 2015).

1.6.6. Engineering nonnative microbes for 1,3-PDO production

Escherichia coli harmless gram negative, rod shaped, facultative anaerobic bacteria, habitants of gut microbiome. The microorganism is widely studied, regarded as model prokaryote, having less doubling time and can be easily handled. *E. coli* is an important strain in the field of biotechnology and genetic engineering as host for expression of heterologous and recombinant proteins and also expressing recombinant genes for industrial productions. The fate of glycerol metabolism in *E. coli* is different compared to native 1,3-propanediol producers (Murarka et al., 2008; Martínez-Gómez et al., 2012). In wild type *E. coli* strain glycerol is metabolized in aerobic conditions in the presence of glycerol kinase (GK) and two glycerol 3-phosphate G-3-P dehydrogenases,, later enters central carbon metabolism via DHAP. It was stated that glycerol is not fermented by wild type strain of *E. coli*, but later work of Dharmadi et al., explained fermentative production of 1,2-propanediol in anaerobic conditions (Dharmadi et al., 2006). Genetics of glycerol metabolism in *E. coli* in detail was explained by Przystalowska et al. Hence information regarding the utilization of glycerol in aerobic and anaerobic conditions reveal that wild type strain cannot produce 1,3-PDO due to absence of dha B gene, encoding glycerol dehydratase, the limiting enzyme of reductive pathway. So anaerobic fermentation of glycerol and glucose in wild type strain for production of 1,3-propanediol should be heterologous

pathways expressing the 1,3-PDO producing genes from native producers. In this part detailed explanation regarding engineering of metabolic pathways in *E. coli* to produce 1,3-PDO either from glucose directly or enhancing production from glycerol is discussed.

A microbial strain inoculated into a 1,3-PDO production media requires two enzymes, glycerol dehydratase (dha B) and 1,3-PDO dehydrogenase (dha T) or its isoenzyme 1,3-PDO oxidoreductase (yqhD), proteomic and metabolite profiles showed no activity of glycerol dehydratase in *E. coli* strains, which should be genetically engineered. The heterologous expression of dha operon from *Klebsiella pneumoniae* ATCC 25955 into *E. coli* AG1/pTC1 strain was carried out and resulted in yield of 0.46 mol/mol (Tong et al., 1991). Later a new construct was engineered using dha B genes from *Citrobacter freundii* and *yqhD* from wild type *E. coli* strain into *E.coli* JM109 and resulting in recombinant *E. coli* JM 109 (pHsh-*dha* B-*yqhD*) strain (Zhang et al., 2006). An optimization of physical parameters and media components are carried out using response surface methodology resulting in 43.1 g/L with conversion efficiency of 69.7% during validation. The rate limiting enzyme glycerol dehydratase is vitamin B_{12} dependent except in *Clostridium sps*., to avoid the addition of cofactor to the fermentation media *dha* B1 and *dha* B2 the glycerol dehydratase gene and its reactivating factor are expressed in *E. coli* from *Clostridium butyricum* and co-expressed along with *yqhD* from *E. coli* wild type strain. All the genes are heterologously expressed in a tandem array under the control of temperature sensitive promoter in vector pBV2220. The genes dha B, dha T and yqhD along with shine-dalgarno sequence are PCR amplified and ligated with P_LP_R promoter. The recombinant strain expressing the dha operon is validated using high cell density fermentation under initial aerobic and continued anaerobic conditions resulting in 104.4 g/L 1,3-PDO, 2.61 g/L/h productivity and with a conversion rate of 90.2 % $g_{PDO}/g_{glucose}$. As the glucose is used as the initial substrate, fermentation resulted in by-products accumulation like pyruvate, acetate, formate, lactate and succinate (Tang et al., 2009). The strain *Saccharomyces cerevisiae* W303-1A was genetically modified by *Agrobacterium tumefaciens* LBA 4404 mediated transformation of plasmid pZR4 containing *dha B* and *yqhD* genes. Plasmid pZR4 is constructed from amplified genes products of plasmid pZR1 containing *yqhD*gene and pZR2 containing *dha B* genes. The constructed strain *S. cerevisiae* W303-1A-ZR can synthesize only 5-6 g/L of glycerol and 0.4 g/L of 1,3-propanediol as a final concentration (Rao et al., 2008). The lower yields of 1,3-propanediol production could be due to low glycerol production levels, but the gene construct shown that yeast can be recombined to produce 1,3-

propanediol. Later dha B, dha T, gdr A and gdr b genes from *K. pneumoniae* was recombined individually into *E. coli* JM109 and *Saccharomyces cerevisiae* W303-1A, after the fermentation, the metabolite analysis showed 1,3-propanediol production only in *E. coli,* the enzymatic profile of important enzymes required for 1,3-PDO production shown lower expression levels in *S. cerevisiae* than *E. coli*. In order to enhance the production levels, dha B and dha T genes are over expressed by transforming the yeast cells with plasmid pZ-BT containing both the genes. The obtained recombinant strain *S.cerevisiae*W303-1A-BT has 38 and 41 fold higher dha B and dha T gene expression levels than the parent strain *Saccharomyces cerevisiae* W303-1A (Ma et al., 2010).

In the list of 1,3-propanediol producers either native or non-native producers, the recombinant strain developed by former Genencor and DuPont, *E. coli* with 18 genes modified in the final production strain resulting in 135 g/L at 3.5 g/L/h with 51% yield on glucose, has demonstrated a large scale joint venture between Dupont and Tate & Lyle for producing bio-PDO from corn with a commercial name Sorona.

1.7. Purification of 1,3-propanediol from aqueous solutions and fermented broth

The fermentation process includes biological synthesis considered as the upstream process, separation and purification of end-product from the fermented broth as the downstream process. The market value of the product depends on the purity; hence downstream strategies essentially decide the economical analysis of the whole fermentation process. Approximately 50-70% of total production costs depend on the process of separation and purification of the desired compound. Fermented broth consists of 80-90% water, 1,3-PDO, non-metabolized substrate like glycerol, methanol and salts if crude form of glycerol is used which are obtained during transesterfication of triglycerides to biodiesel and glycerol. Along with the main 1,3-PDO as product, other by-products like 2,3-butanediol, butyric acid, succinic acid, lactic acid, formic acid, acetic acid and ethanol are found. Though all these by-products are not found in common, but depend on the microorganism used as the biocatalyst. For example butyric acid is observed as major by-product with *Clostridium butyricum* (Himmi et al.,1999; Szymanowska-Powalowska et al.,2013; Chatzifragkou et al.,2012; Biebl 1991; Moon et al.,2011; Abbad-Andaloussi et al.,1995; Reimann et al.,1998; Kaur et al.,2012b), 2,3-butanediol and acetic acid are formed when *Klebsiella pneumoniae* (Liu et al.,2007; Durgapal et al.,2014; Oh et al.,2013; Zheng et al.,2008; Ji et al.,2009),

lactic acid with *Lactobacillus sp.,* (Vaidyanathan et al.,2011; Baeza-Jiménez et al.,2011), and acetic acid, Succinic acid, lactic acid and ethanol with *Citrobacter sp.,* (Anand et al.,2010) during glycerol dissimilation. As 1,3-PDO has high boiling point and strong hydrophilic nature, purification of 1,3-propanediol is more tedious and challenging. Various chemical and physical methods are proposed to downstream 1,3-propanediol from the fermentation broth. The post-fermentation or downstream process includes three main steps. The first step is removal of microbial cells generally through flocculation, membrane separation or centrifugation. The second step includes the removal of proteins or other impurities either by distillation, electrodialysis or ion exchange chromatography and final step includes rectification or vacuum distillation for purification.

The first method for separation of 1,3-propanediol was devised by Malinowski using liquid-liquid extraction (Malinowski, 1999), where organic solvents are used to create a soluble and insoluble phase where the product being hydrophilic, transits into the hydrophilic solvent and later it was extracted by evaporating the solvent (organic) phase. Later reactive extraction method was devised using acetaldehyde to react with 1,3-propanediol to form 2-methyl,1,3-dioxane (2-MD). The dioxane formed reacts with organic solvents to purify 1,3-PDO, xylene, ethyl benzene and toluene showed a recovery of 69%, 71% and 74% (Malinowski, 2000; Hao et al., 2005). An aqueous two-phase extraction system was proposed by Rukowicz using hydrophilic solvents and inorganic salts as two phases. Acetone, ethanol, and 2-propanol as solvents, sodium chloride, dipotassium hydrogen phosphate and ammonium sulfate was used as salts for extraction. In solvents ethanol and 2-propanol could form single phases where extraction will be difficult, but acetone formed two phases. Acetone / $(NH_4)_2SO_4$ and acetone / K_2HPO_4 complexes were found to be efficient two solid-liquid phases for extraction of 1,3-propanediol from fermentation broth (Rukowicz et al..2014). The highest purity of 98% and 82% yield was obtained when ethyl acetate was used as the solvent for extraction and later purified on silica resins, a final yield of 70% was noted (Cho et al., 2006). Based on sorption and desorption of 1,3-propanediol various resins are synthesized and modified to increase the adsorption efficiency of the resin specifically towards 1,3-PDO, 001X7H-pretreated cation exchange resin with efficiency of 360 mg/g PDO (Wang et al., 2014), H^+ ion exchange resin (Rukowicz et al., 2014), the XAD-7 and XAD-16 (Luerruk et al., 2009) where adsorption behavior of XAD-7 is higher than XAD-16, but desorption is greater in XAD-16 (Luerruk et al., 2009). Another little efficient but economical and continuous downstream processing technique like pervaporation using Na-ZSM-5 zeolite (Li et al., 2001c) and X-type zeolite membranes (Li et al., 2001a; Li et al., 2001b)was explained. Here the flux of

diffusion rates is altered with ternary and quaternary solvents as each component in the solvent intervene with the diffusion of another component by decreasing the flux of metabolites and also reducing the purification efficiency. A quaternary solution has lower flux compared to pure water, in a solution with 1,3-PDO, glycerol, glucose and water, the selectivity of 1,3-PDO and glycerol depends on adsorption efficiency and diffusion rates whereas selectivity of either 1,3-PDO or glucose only depends on diffusion rate. Hence, the flux of selectivity decreases with the feed concentration of 1,3-PDO, glycerol and glucose (Li et al., 2001c). In the fermented broth as an initial step biomass and soluble proteins should be removed to make the downstream process easier. Two different approaches are put forward for this step one is flocculation (Hao et al., 2006) using polyacrylamide and chitosan, another is microfiltration using hollow fiber cartridge (Anand et al., 2011; Avci et al., 2014). In flocculation absorbance of spent medium decreased to 0.02 and reduced the protein concentration to half. But in the second strategy after microfiltration by using activated charcoal 96% of soluble proteins are removed along with decolorizing the broth. Notably 15% loss in the concentration of 1,3-PDO was observed when treated with activated charcoal. After flocculation, the resultant supernatant was flown through four counter current reactors with butyraldehyde and supernatant in opposite directions. The reaction resulted in the formation of acetals of 1,3-PDO, 2,3-butanediol and glycerol. The mixture contains 98% of 1,3-PD and 2,3-BD acetals and only 10% of glycerol acetals. The recovery of glycerol acetals increased with increase in butyraldehyde volume. Later obtained reaction mixture was hydrolyzed to get a concentration of 407 g/L 1,3-PDO, 252 g/L 2,3-BD, 277 g/L glycerol and 146 g/L glycerol acetal while the feed volume consists of 15 g/L 1,3-PDO. Anand et al., used vacuum distillation to concentrate the sample and remove the minimum concentrations of ethanol and then purified using silica gel chromatography with chloroform and methanol as mobile phase resulting in 75.47 overall yields in the purified manner (Hao et al., 2006, Anand et al., 2011). To separate biomass efficiently from the fermentation broth novel cell modification was done using carboxylated superparamagnetic nanoparticles attached to the surface of *L. reuteri*. On the start of downstream processing, the powerful magnet was used to separate bio-nanocomposites and later can be activated by addition of fresh medium (Chen &Chen, 2013). Based on pH of the aqueous phase and partition among the hydrophobic and hydrophilic phase, organic acids like succinic acid concentration is reduced from 33 g/L to 1 g/L when hydrophobic ionic liquids are used as water repelling phase, coefficient of distribution of succinic acid is higher with this ionic liquid trihexyltetradecyl phosphonium methane

sulfonate at pH 7.0 (Klasson et al., 2004). Recently a novel method of using super critical CO2 micro emulsion system with nonionic dodecyl ether (poly (ethylene-methyl ethylene glycol)) LS-54 as the surfactant was described. In this process, the extraction of 1,3-propanediol depends on the property of solubility. Here the 1,3-PDO in aqueous solution get selectively soluble in the micro emulsion system, which was confirmed by small angle X-ray scattering (SAXS) experiment with ethanol as co-solvent. In this experiment the size of the emulsion formed clearly shows whether 1,3-PDO is solubilized into the quaternary system (Yu et al., 2015).

Although various researchers attempted to separate and purify 1,3-PDO, several limitations in each process was observed, providing a still economical challenge and an obstacle to develop an efficient and economical microbial production. Hence the downstream processing of 1,3-PDO is a worthwhile to be considered in developing a simple and industrially viable process.

1.8. Applications of 1,3-propanediol

Plastics or polyesters have wide range applications in day to day life like packaging, paper coatings, fibers, films, disposable articles and medical applications. The raw materials for the production of these polyesters are petrochemical derivatives, non-degradable, and discharge large amount of greenhouse gases these polyesters cause serious threat to the environmental pollution. In near future the petrochemical derived chemicals and polymers are likely to be replaced with the renewable and bio-based chemicals. One such chemical monomer that attained much interest due to to its applications as polymeric subunit, pharmaceutical formulations, antifreeze agent and plasticizer is 1,3-propanediol (1,3-PDO). Initially 1,3-PDO based polymers did not attain commercial interest as the process is technically and economically not feasible due to low availability and high cost of the monomeric 1,3-propanediol. Over the past few decades production of 1,3-PDO was developed along with the global market potential. A new polyester called Polytrimethylene terephthalate (PTT) was synthesized using 1,3-PDO and terephthalic acid through polycondensation reaction. A major application of 1,3-PDO as the monomer is the production of polyester, Polytrimethylene terephthalate (PTT). In 1995 Shell chemicals announced a product named “Corterra^{TM} PTT” later in 2003 DuPont commercialized a bio-based PTT called “Sorona”, in which 1,3-PDO is biologically derived from corn glucose (Bhatia & Kurian 2008; Kaur et al., 2012). As a monomer, 1,3-propanediol is used as building blocks, chain extender, to increase thermal resistance and mechanical properties. The monomer can improve the physical nature of resins, adhesives, laminates, mouldings, detergents, cosmetics, deodorants and powdered coatings due to its properties like

less toxicity and heat stability it was used in engine coolant formulations (Anand et al., 2010; Da Silva et al., 2009; Kaur et al., 2012). Recently a novel application of 1,3-PDO as plasticizer in preparation of composite films was reported where the monomer provides an additional property of elasticity and mechanical strength to the film (González et. al., 2017).

Objectives of the study

Microbial production of 1,3-propanediol is well known since the first report in 1881 by August Freund. Since then a lot of work has been carried out to understand the biochemical pathways behind the dissimilation of glycerol. But the significance of 1,3-PDO was recognized only after characterization of its role as the platform chemical in synthesis of next generation polymers. In this context, the present investigation was envisaged with an idea to develop a complete bioprocess for 1,3-propanediol production using biodiesel industry generated crude glycerol.

With the above background the objectives of the study are

1. Isolation, screening and optimization of non-pathogenic, facultative anaerobic bacteria for 1,3-propanediol production from environmental samples.
2. Optimization of physical conditions and media components to improve the 1,3-PDO production titers and yields by the selected strain.
3. Development of cost effective media for the production of 1,3-PDO using efficient nitrogen sources and biodiesel derived crude glycerol as the substrate.
4. Separation and purification of 1,3-PDO from the fermented broth.
5. Evaluation of 1,3-PDO as the plasticizer in the preparation of dextran like exopolysaccharides and chitosan blended composite films.

Organization of the thesis

The thesis comprises of nine chapters. Chapter 1 is introduction and review of literature. In chapter 2, an onsite enrichment technique for isolation of an efficient glycerol utilizing and 1,3-PDO producing microorganisms are discussed. The chapter also deals with the identification and characterization of the selected 1,3-PDO producer. Chapter 3 deals with the optimization of physical conditions and media components using one factor at a time approach, statistical techniques and artificial neural networks, and also the validation process in flask level and bioreactor studies. Chapter 4 discusses the development of a cost effective production process using cheaper nitrogen sources and biodiesel derived crude glycerol as the substrate in growth of microorganism and 1,3-PDO production. Chapter 5 discusses the downstream process for separation and purification of 1,3-PDO from the fermented broth. Chapter 6 describes the application of 1,3-PDO as the plasticizer in chitosan and EPS blended composite films. Finally, the chapter 7 summarizes the whole work and conclusions are drawn based on the present study.

Chapter 2

Onsite enrichment, isolation, screening and identification of 1,3-propanediol producing microorganisms

Chapter 2 Onsite enrichment, isolation, screening and identification of 1,3-propanediol producing microorganisms

2.1. Introduction

Microbial production of chemicals is referred as a greener process compared to chemical synthesis, but the microbial process involves various challenges and limitations to be addressed before pilot or industrial scale production. The biosynthesis of chemicals from microbes can be divided into following phases.

1. Selection of potential microbial strain.
2. Strain improvement.
3. Optimization of physical parameters and media composition.
4. Evaluation of upstream strategies for improved production of desired product.
5. Downstream process for separation and purification of desired product.
6. Process evaluation.

1,3-Propanediol (1,3-PDO) has gained much attention and significance from the polymer industries, due to its application as the monomer. Though it was chemically synthesized from acrolein or ethylene oxide, the process has its own limitations and drawbacks that are reviewed in chapter 1 introduction and review of literature. Microbial production of 1,3-PDO has addressed various limitations of chemical process and the screening of microorganisms required for the 1,3-PDO production was going on since 1975. Glycerol is the only natural substrate for 1,3-PDO production by natural producers. Few microorganisms belonging to genera *Clostridium*, *Klebsiella*, *Citrobacter* and *Lactobacillus* were observed to be natural 1,3-PDO producers, in that strains of *Clostridium* and *Klebsiella* sp., were regarded as the best producers in titers and yields. But still there are various technical limitations using these strains in an industrial scale as *Clostridium* is an obligate anaerobe and later is an opportunistic pathogen. However after 43 years of active research on isolation, and genetic engineering of artificial and competitive pathways, a single strain *E. coli*, a non-native 1,3-PDO producer is leading the world's 1,3-PDO market (Nakamura et al., 2000). The strain was developed in 2002 by DuPont which produces approximately 135 g/L and attained commercial scale production in a plant at Tennessee. But the limitation caused due to increased biodiesel production i.e., surplus amount of crude glycerol remained in the biodiesel industries was unaddressed. Realizing the importance of finding new microorganisms with the potential of 1,3-PDO production in high titers utilizing crude glycerol as the substrate is the objective of interest. In the current study isolations were carried out using onsite enrichment technique and later isolates were screened on glycerol containing media to create a selection pressure. Morphological

and genetic characterization was carried out for the potent isolate. Then the isolate was evaluated for media components, optimum pH, and incubation time. In this context, following experiments were performed and investigated.

2.2. Materials and methods

2.2.1. Chemicals and reagents

The different chemicals and solvents used in the present investigation are analytical grade. The standard chemicals like 1,3-propanediol, glycerol, lactic acid, acetic acid, glucose for HPLC quantification was procured from Sigma-Aldrich chemicals. The media components like de Man Rogosa and Sharpe (MRS) broth, meat extract B, yeast extract, peptone, agar powder and vitamin B_{12} were purchased from Himedia labs and other media components, inorganic salts and organic solvents were purchased from Sisco Research laboratories (SRL) Pvt. Ltd. India. Distilled water (dH_2O) was used for the preparation of cultivation media and production media.

2.2.2. Quantitative estimation of glycerol, 1,3-propanediol, and other by-products observed in the fermented broth using high performance liquid chromatography (HPLC)

The quantitative estimation of glycerol, 1,3-propanediol, and other by-products of glycerol dissimilation like lactic acid, acetic acid, and ethanol was carried out using high performance liquid chromatography (HPLC) system (Shimadzu prominence UFLC, Shimadzu Corp., Kyoto, Japan).

HPLC Conditions:

Column: Rezex-ROA Organic acid column 300 × 7.8mm (Phenomenex)

Mobile phase: 0.01N H_2SO_4

Flow rate: 0.6 ml/min

Column temperature: 85^oC

Detector: RID-10A refractive index detector (Shimadzu Corp., Japan)

Loading volume: 20µl

Standard solutions of 1,3-propaendiol, glycerol, glucose, lactic acid, acetic acid, and ethanol were prepared in double distilled water at concentration of 10 mg/ml and analyzed for the retention time individually and later a mixture solution with each standard concentration of 10 mg/ml was analyzed to observe the behaviour of chromatograph in individual solution and in mixture solutions. Once the retention time of each standard is known, standard curve was plotted for each metabolite for quantitative estimation. For the standard curve plotting each metabolite solutions were

prepared at different concentrations within a range of 10-50 mg/ml, later for each concentration the peak area was recorded from HPLC chromatogram and graph was plotted with peak area versus concentration resulting in a linear equation.

2.2.3. Onsite enrichment for glycerol utilizing microorganisms and isolation of 1,3-propanediol producers

In this study prior to enrichment of isolated colonies in glycerol rich medium, novel onsite enrichment was carried out, where glycerol was fed into 20 cm deep pit in corporation dumping yard (8°28'49.1" N, 76°57'07.9" E), Thiruvananthapuram, Kerala, India. Then the glycerol dumped pit was left undisturbed for a week. Then soil samples from the same were transferred to laboratory and stored at 4°C before processing. Later the 1g soil sample was taken for dilutions and plated on MRS agar, incubated at 37°C for 4-7 days for pure cultures.

2.2.4. Screening of isolates for 1,3-propanediol production

2.2.4.1. Pre-inoculum preparation

Isolation of lactic acid bacteria were carried out in commercial MRS medium. Pre-culture inoculum for fermentation experiments was made using MRS broth, incubated at 37°C, 200 rpm in an incubator shaker. Cells were maintained in -20°C in 15% (w/v) glycerol.

2.2.4.2. Production medium for 1,3-PDO production

The production media used in fermentation experiments was modified MRS (mMRS) media containing (Pflügl et al., 2012) (per liter distilled water): 10g bacterial peptone, 10g meat extract, 5g yeast extract, 30g glucose, 5g sodium acetate, 2g K_2HPO_4, 2.6g sodium citrate dehydrate, 1.17 g $(NH_4)_2HPO_4$, 0.2g $MgSO_4.7H_2O$, 0.05g $MnSO_4.H_2O$. Unless stated the initial concentration of pure glycerol was 20 g/L. In the experiments carried out to understand the effect of vitamin B_{12} and $CoCl_2$. H_2O on 1,3-PDO production, 4 mg/L was supplemented to the production media respectively. Trace element solution 0.3% (v/v) was added to the production media (concentration per liter of deionized water): 1 ml HCl (25%, v/v), 70mg $ZnCl_2$, 100mg $MnCl_2 \times 4H_2O$, 60mg H_3BO_3, 20mg $CuCl_2 \times 2H_2O$, 20mg $NiCl_2 \times 6H_2O$, 40mg $Na_2MoO_4 \times 2H_2O$. Initial pH of the media was adjusted with either 1M HCl or 1M NaOH. All the experiments are carried out in completely sealed Duran bottles (Borosil) with working volume of 100 ml under anaerobic conditions.

2.2.4.3. Incubation of culture for 1,3-PDO production

The experiments for 1,3-PDO production was carried out in 150ml Scott Duran bottles (Borosil) with working volume of 100 ml. The production media was inoculated with 2% (v/v) pre-inoculum prepared in MRS medium, grown for 16 h attaining an O.D of 0.6 at 600nm. Later these bottles were incubated at 37^{o}C, 200 rpm in an incubator shaker.

2.2.4.4. Post fermentation analysis for estimation of biomass, 1,3-PDO and other by-products

After the desired period of incubation time, the culture sample (2 ml) was withdrawn from the production flask. Sample volume of 1ml was used for growth analysis by measuring the optical density at 600nm in UV-Visible spectrophotometer (Shimadzu series UV 1601). Another part of the sample (1 ml) was centrifuged at 8000 rpm for 5 mins to pellet out the cells. After the centrifugation supernatant obtained was filtered through 0.2 µm nylon filter membrane into the HPLC vials (Borosil) before loading the sample into column for quantitative detection of 1,3-PDO and other metabolites.

2.3. Characterization and identification of selected 1,3-PDO producer

2.3.1. Morphological characterization

The morphological characterization of microbial cells was observed under a clinical light microscope (Leica DM2000, Wetzlar, Germany) and scanning electron microscope (SEM). For SEM analysis the sample preparation of microbial cells was carried out using modified protocol of S.H. Song et.al. 2005. Initially samples were washed with distilled water and fixed with 2% glutaraldehyde solution for 1 h at room temperature. The fixed samples were washed with 0.1 M phosphate buffer at pH-7.0 to remove the residual glutaraldehyde. Later the samples were treated with ethanol sequentially in gradient concentrations of 30-100% for 15 minutes in laboratory fume hood to remove the traces of buffer. The dried samples were spluttered with gold using SC7620 sputter coater device and further analyzed under SEM at an operating voltage of 30kV (Zeiss Evo-18, Carl Ziess, Germany) (Song et al., 2005).

2.3.2. Isolation of genomic DNA and 16s rDNA sequencing

The microbial strain was inoculated into MRS liquid medium and incubated at 37^{o}C, 200 rpm for 16h. Ten milliliters of the culture broth was centrifuged at 8000 rpm for 5 min at 4^{o}C, the supernatant was decanted and cell pellet was freeze dried at -20^{o}C for 60 min. Resuspend the bacterial cell pellet in 250 µl fresh lysozyme (10mg ml^{-}

[1]) in TE buffer and transfer the suspension into micro-centrifuge tube and incubate at 37^{o}C for 2 h, with an intermediate shaking. Further add 50 µl 50 mM EDTA, 50 µl 10% SDS, 50 µl 5 M NaCl were added, then mix gently, later 10 µl RNase was added with a final concentration of 10 µg ml^{-1}and incubate at 37^{o}C for 60 min. Then to the suspension freshly prepared proteinase K (20 mg/ml) was added and further incubated at 37^{o}C for 60 min. the polysaccharides and proteins are precipitated by adding equal volume of Phenol : Chloroform : Isoamyl alcohol (25:24:1). The clear aqueous fraction obtained after centrifugation at 13000 rpm for 5 min, was transferred with a wide bore tipped pipette into a fresh tube. The DNA was precipitated by adding 1 volume of ice cold isopropanol. The solution was gently mixed by inversion and centrifuged at 15000 rpm for 15 mins at 4^{o}C. The DNA pellet was washed twice with 70% ethanol and was allowed to air dry after which the pellet was suspended in 200 µl nuclease free water and stored at -20^{o}C until further use.

Genomic DNA of strain N1E9.3.3 isolated with the above described procedure was used as template for amplification of 16S rDNA region. Then amplification for sequencing PCR was done using universal primers (Hirkala & Germida, 2004).

27F 5'AGAGTTTGATCMTGGCTCAG3'
1492R 5'TACGGYTACCTTGTTACGACTT3'

The PCR conditions followed were initial denaturation at 94^{o}C for 5 mins, followed by 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 56 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min and a final extension step at 72^{o}C for 8 mins. The 16S rRNA amplicon was sequenced using dye terminator sequencing method (ABI genetic analyzer 3500 series sequencer, Hitachi, Tokyo, Japan). Using the NCBI database, and nucleotide BLAST program, the phylogenetic neighbours were determined. The sequences of the closest hit taxons were selected for pairwise similarity using global alignment algorithm CLUSTALW. Later the evolutionary history was analyzed using Neighbour joining method in MEGA 6 software.

2.3.3. Genetic determination of dha genes responsible for 1,3-PDO production

Microbial dissimilation of glycerol towards 1,3-PDO production is a reductive pathway, which involves two enzymatic steps. First, glycerol is dehydrated to 3-hydroxyproionaldehyde by glycerol dehydratase (*dha B*), followed by reduction of 3-HPA to 1,3-PDO by 1,3-PDO dehydrogenase or oxidoreductase (*dha T*), the detailed explanation of glycerol dissimilation was explained earlier in Chapter 1 under the sub heading 3.2.2 biological dissimilation of glycerol to 1,3-propanediol. In *Lactobacillus*

sp., and other 1,3-PDO producers the enzyme glycerol dehydratase was characterized to have three sub-units ($\alpha_2\beta_2\gamma_2$) diol dehydratase large (α subunit, dha B_1), diol dehydratase medium (β subunit, dha B_2), diol dehydratase small (γ subunit, dha B_3) and a diol dehydratase reactivating factor (dha R). For the identification of our environmental isolate *Lactobacillus brevis* N1E9.3.3 isolate harboring these specific enzymes for 1,3-PDO production, the glycerol dehydratase gene subunits (dha B_1, B_2, B_3 and R) and 1,3-PDO dehydrogenase (dha T) gene specific primers were designed based on the published sequences of *L. brevis* ATCC 367. The gene specific primers designed were provided below in table 2.1. The PCR amplification was carried out using genomic DNA of Lactobacillus brevis N1E9.3.3 strain as the template and with gene specific primers in MyCycler, Thermo cycler (Bio-Rad laboratories, California, United states) with initial degradation at 95^{o}C for 5 min followed by 35 cycles of denaturation (95^{o}C for 45 sec), annealing (58^{o}C for 1.30 sec), Initial extention (72^{o}C for 2 mins) and final extention step at 72^{o}C for 8 mins. For PCR amplification of dha B subunits and dha T, master mix (Taq polymerase, dNTP's) from Origin diagnostics and research, India was used. The PCR products were analysed by electrophoresis using 1% agarose gels and 0.5 X TAE buffer, 1Kb gene ladder (NEB) as the molecular weight marker. The PCR products were gel purified following the manufacturer protocol (Qiagen gel extraction kit) and sequenced using ABI 3600 series sequencer.

The sequences obtained were assembled and compared to the database (http://www.ncbi.nlm.nih.gov/Genbank) using the BLASTN 2.8.1 application with the available gene sequences of *L. brevis* ATCC 367 for percentage similarity.

Primer	Sequences (5'-3')	Length	Tm	GC%
FdhaB1	CCGGTCGTGAAGTTGGTCTG	20	60	60
RdhaB1	TGAGCCACAGTGTTAACCCCC	21	62	57
FdhaB2	AGGGGGTTAACACTGTGGCT	20	61	55
RdhaB2	GGCCTCAGTGCCACCAATTT	20	61	55
FdhaB3	CGTTATGGGGAAACCCGCTG	20	61	60
RdhaB3	TCAACCGCACCTTGATCTGA	20	59	50
F dha R	GCCGACTACTACGAATCCCG	20	60	60
R dha R	TGCCGGCTTATCCATTGTGT	20	60	50
F dha T	ACTTCCGAAAGAAGGCATTTGT	22	58.7	41
R dha T	TCGAAATTCGGCGGCTTTTCAT	22	61.7	45

Table.2.1. List of gene specific primers used for genetic determination of 1,3-propaendiol production genes.

2.3.4. Growth inhibition studies

The studies was carried out to access the effect of substrate (glycerol) and products (1,3-PDO, lactic acid and acetic acid) formed during glycerol fermentation by observing the optical density of the bacterial culture for desired incubation time by sampling at regular intervals. The bacterial culture grown in MRS medium overnight at 37^{o}C was used as pre-inoculum. To measure the bacterial growth, optical density was measured at 600nm with the samples withdrawn at regular intervals. For the inhibition studies, MRS medium was initially supplemented with either or substrate or products respectively and adjusted the pH to 6.5 (Commercial MRS media is maintained at pH-6.5; Control). The experiments were conducted in triplicates and the standard deviation calculated did not exceed 10%.

a. Glycerol inhibition

The effect of glycerol concentration (10-50 g/L) on bacterial growth was measured at O.D 600nm for 48 h at regular intervals.

b. 1,3-propanediol inhibition

The effect of 1,3-propanediol concentration (10-50 g/L) on bacterial growth was measured at O.D 600nm for 48 h at regular intervals.

c. Organic acid inhibition

The effect of lactic and acetic acid concentrations (10-50 g/L) on bacterial growth was measured at O.D 600nm for 48 h at regular intervals.

2.4. Results and discussion

2.4.1. Onsite enrichment, sample collection and isolation of microbial cultures

Microorganisms are pre-eminent and each species in a genus is termed as an ecosystem having its own ecological niche. The soil is the natural habitat for diversified microorganisms, with various roles in ecological and environmental balance. The metabolic behavior of a particular microorganism depends on its ecosystem and its role in it. Hence it is an important objective in choosing an isolation site, but contaminated glycerol sites were not found to isolate microorganism with specific glycerol metabolizing activity. As a prerequisite of glycerol utilizing microorganisms, a novel onsite enrichment technique was carried out, where crude glycerol is dumped into 20 cm deep pit in a Corporation dumping yard ($8^{o}28'49.1''$ N, $76^{o}57'07.9''$ E), Thiruvananthapuram, Kerala, India. Later sampling was conducted in the same site. The samples were transported to the laboratory and stored at 4^{o}C, until further processed. The conventional way of serial dilution technique was followed for isolation of pure cultures. After the incubation seventeen cultures were purified and screened for glycerol utilization efficiency and production of secondary metabolites like 1,3-propanediol. Although various wild type strains were reported for 1,3-propanediol production, a prominent habitat for isolation is not reported, but it was explained that methane fermentation medium (Szymanowska-Powałowska et al., 2012) would be a suitable source and similarly high through put screening of resistant strain at higher substrate and product concentrations was proved (Ringel et al., 2012).

The enriched and purified cultures were screened for 1,3-propanediol production with initial 20 g/L pure glycerol. This study was carried out primarily to evaluate the potent strain; hence the characterization and identification of large number of cultures will be minimized. The production media with 2% pure glycerol and 3% glucose was autoclaved and sparged with sterile nitrogen gas for 10 minutes before inoculation to replace the oxygen in the head space of cultivation flask. An overnight grown pre-inoculum was used in these shake flask experiments and incubation was carried out for 72 h without pH regulation, at 37^{o}C, 200rpm under anaerobic conditions.

Fifteen of seventeen isolates produced comparable amounts of 1,3-propanediol between 1.5-14 g/L (Fig. 2.1A), but five cultures N1E9.1.4, N1E9.1.5, N1E9.3.3,

N1E9.3.4, and N1E9.4.2 shown yield efficiency more than 0.6 $g_{1,3\text{-PDO}}/g_{Glycerol}$ (Fig. 2.1B).

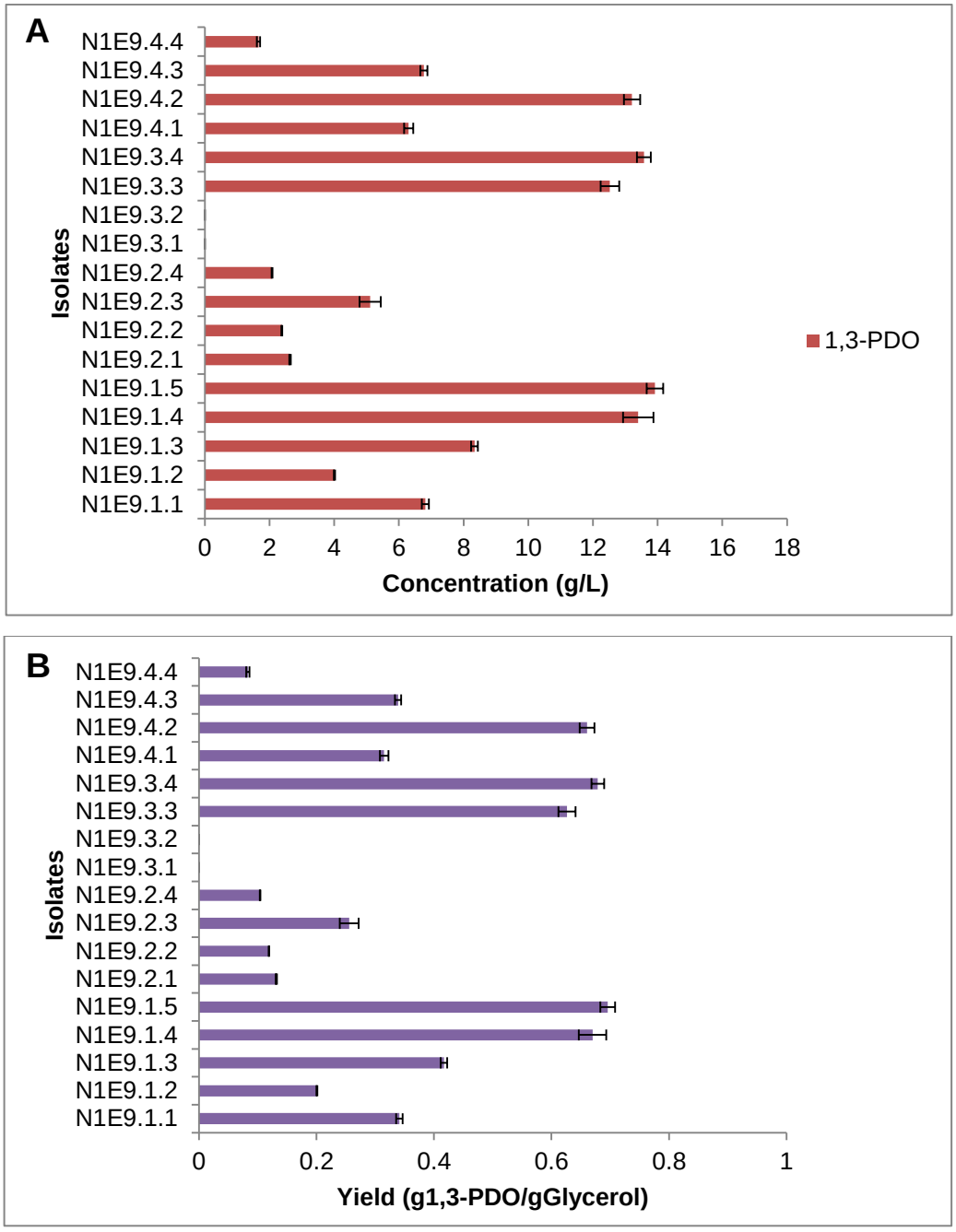
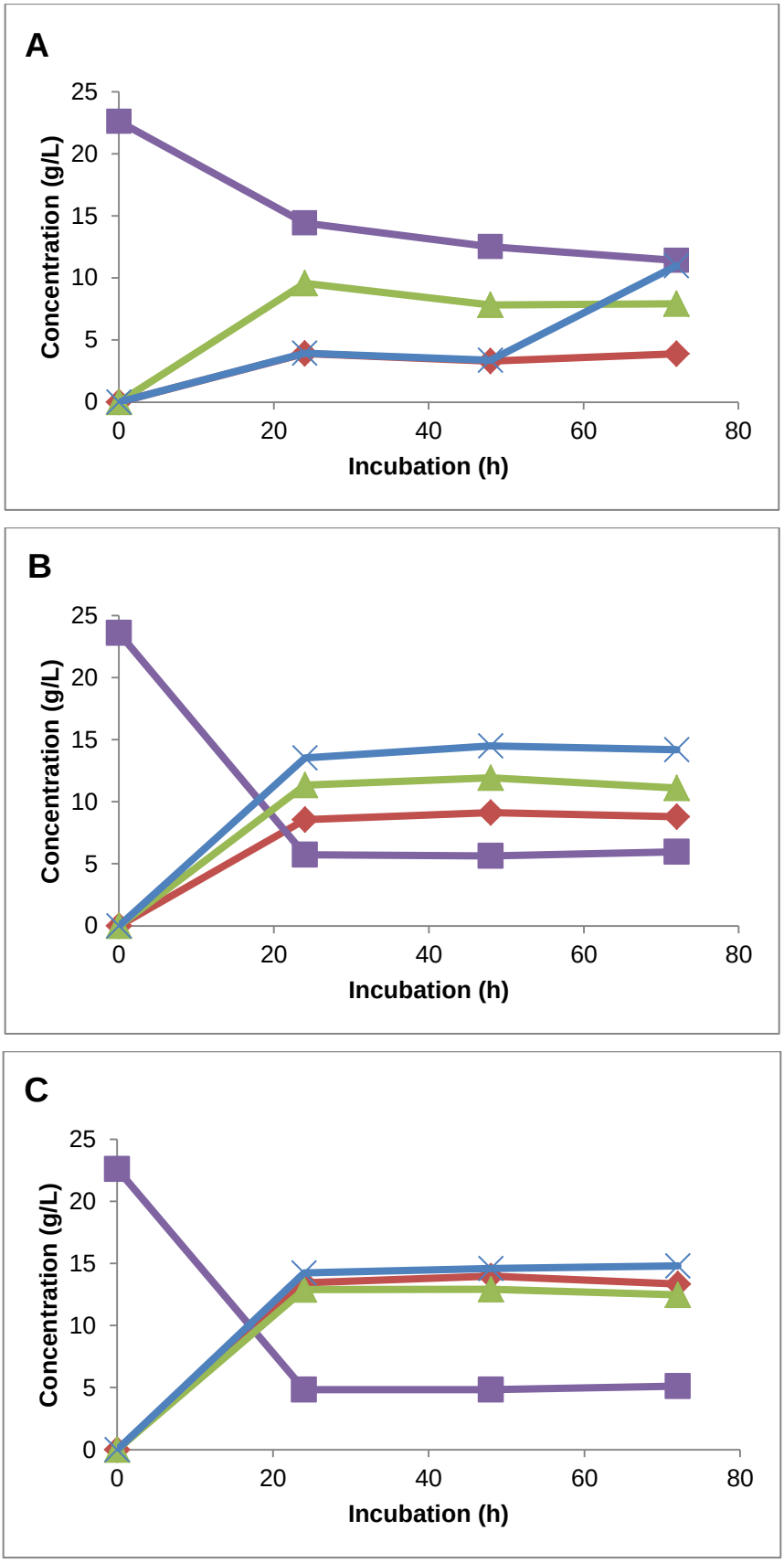
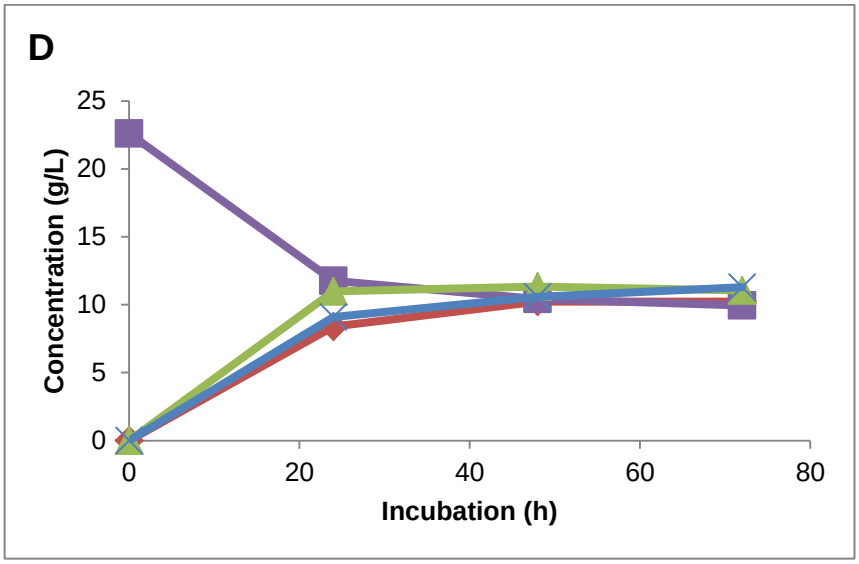


Figure.2.1. Overview of isolates related to growth and 1,3-propanediol production in batch cultivations. (A) 1,3-PDO titers when initial 2% pure glycerol is supplemented as substrate (B) effect of initial glycerol concentration on volumetric yield of 1,3-propanediol of isolates.

2.4.2. Evaluation of N1E9 isolates for substrate utilization rate during 1,3-PDO production

The productivity of potent isolate relies on rate of substrate utilization and product formation. All the five N1E9 isolates were investigated for their efficiency in batch cultivation when 3% glucose and 2.5% pure glycerol was supplemented into the mMRS medium and incubation was carried out at 37^{o}C, 200rpm under anaerobic conditions. During the batch cultivations samplings were carried out in regular intervals of time and analyzed for the metabolite profile. The glucose as carbon source converted to lactic acid, acetic acid as major byproducts and ethanol in minute concentrations, glycerol is reduced to 1,3-propanediol. The active exponential phase was observed in all the five isolates for first 24 h, where the maximum substrates were utilized, but isolate N1E9.3.3, has shown 78.75% utilization rate of substrate, which is comparatively best figures in all the five isolates, resulting in 14.23 g/L 1,3-propanediol, with yield of 0.62 $g_{1,3\text{-PDO}}/g_{Glycerol}$ conversion rate (Fig. 2.2 C). Along with 1,3-PDO as the end product, 1.3% lactic acid and 1.2% acetic acid were produced as byproducts. But to observe the maximum production, the batch cultivation was prolonged to 72 hours. The final titers of 1,3-PDO was 14.8 g/L and concentration of lactic acid and acetic acid remains the same. The other isolates N1E9.1.4, N1E9.1.5, N1E9.3.4, and N1E9.4.2 produced 10.95, 14.17, 11.3 and 11.14 g/L 1,3-PDO respectively, which are slightly lower compared to N1E9.3.3 isolate. In specific, the metabolism shown a shunt after 24 h, as glucose supplemented to the media was completely utilized. In the graphical representation of metabolite profiles of all the five isolates reached plane, which explains the reduced substrate utilization and product formation. The reduction of glycerol towards 1,3-propanediol requires reducing equivalents in its pathway where intermediate 3-hydroxypropionaldehyde is converted to 1,3-propanediol, which flows from the oxidative pathway of glucose. Hence depletion in the glucose concentration reduced the final titers of 1,3-propanediol. The experimental results resembles *L. reuteri* ATCC23272, where author observed, reduced substrate utilization and 1,3-PDO production after 24 h due to accumulation of secondary metabolites (Vieira et al., 2015). In secondary metabolites the effect of lactic acid and acetic acid as inhibitors of growth and metabolic activity is well investigated (Chen et al., 2015).


A
Concentration (g/L)
25
20
15
10
5
0
0
20
40
60
80
Incubation (h)
B
Concentration (g/L)
25
20
15
10
5
0
0
20
40
60
80
Incubation (h)
C
Concentration (g/L)
25
20
15
10
5
0
0
20
40
60
80
Incubation (h)



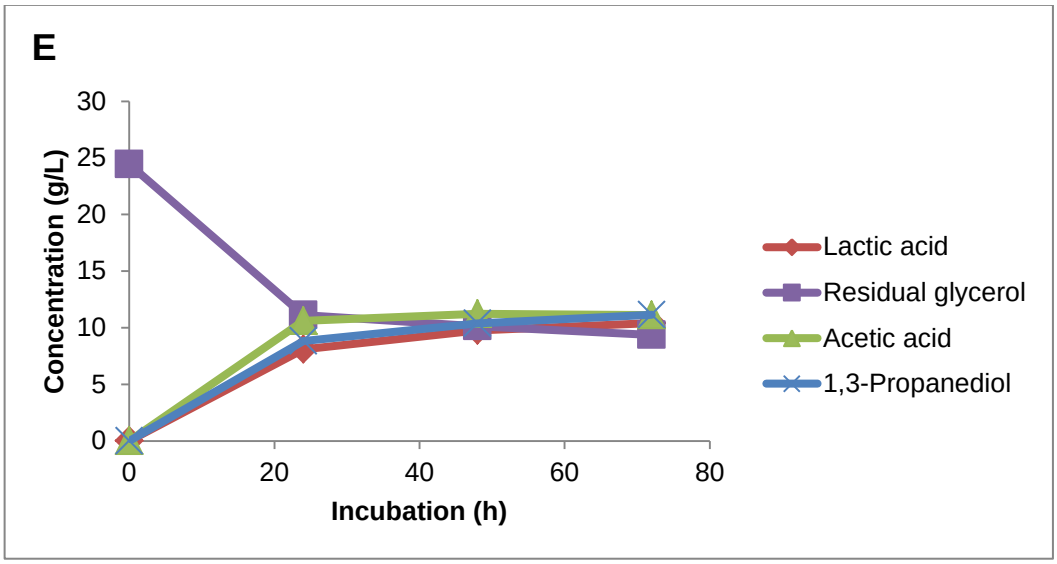


Figure. 2.2. Evaluation of glycerol utilization rate and product formation rate in five isolates: (A) N1E9.1.4, (B) N1E9.1.5, (C) N1E9.3.3, (D) N1E9.3.4, and (E) N1E9.4.2.

2.4.3. Microscopical studies

The Figure 2.3A, represent the pure cultures of N1E9.3.3 isolate grown on MRS agar plate. Under the microscopic observation after gram staining the isolate N1E9.3.3 was phenotypically identified as Gram positive (Fig.2.3B), rod (Fig. 2.4 A and 2.4 B) shaped in nature.

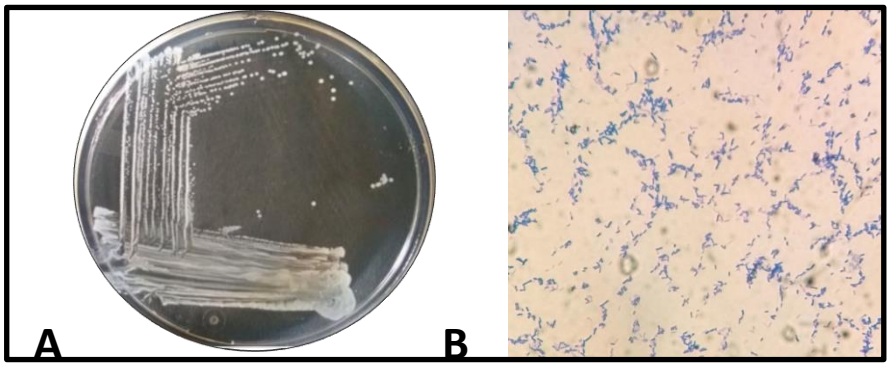


Figure.2.3. (A) N1E9.3.3 strain grown on MRS agar plate (B) Microscopic characterization of gram stained N1E9.3.3

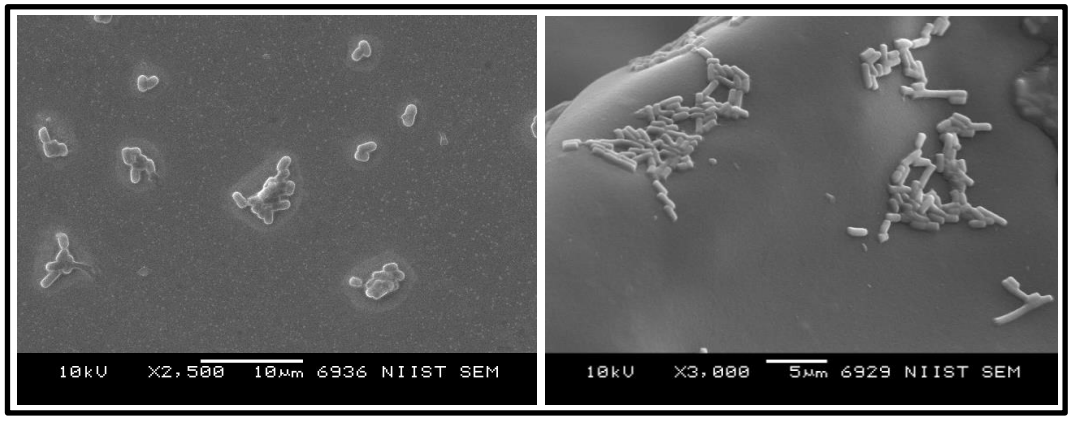


Figure.2.4. SEM characterization of N1E9.3.3 (A) Suspended cells (B) Immobilized onto polyurethane foam

2.4.4. Identification of isolate by 16S rDNA analysis

The isolate N1E9.3.3 was identified as *Lactobacillus brevis* bearing highest homology to the type strain *Lactobacillus brevis* ATCC 14869 as confirmed by 16S rDNA analysis using NCBI-BLAST and sequence alignment data obtained from EzTaxon server. Fig.2.5 displays the phylogenetic tree generated based on 16S rDNA sequences of *Lactobacillus* strains, which were found to be similar to the present isolated strain N1E9.3.3.

Sequencing result of 16S rDNA region (1431bp)

TGCAAGTCGAACGAGCTTCCGTTGAATGACGTGCTTGCACTGATTTCAACAATGAAGCGAGTGGCGA
ACTGGTGAGTAACACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGGTGCTAATA
CCGTATAACAACAAAATCCGCATGGATTTTGTTTGAAAGGTGGCTTCGGCTATCACTTCTGGATGATC
CCGCGGCGTATTAGTTAGTTGGTGAGGTAAAGGCCCACCAAGACGATGATACGTAGCCGACCTGAG
AGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAA
ACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTG
GGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAAGT
GCATCGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAATG
CGTAGATATATGGAAAGAACACCAGTGGCGAAGGCGGCTGTCTAGTCTGTAACTGACGCTGAGGCT
CGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAG
TGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGA
CCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGCCAATCTTAGAGATAAGACGTTCCCT
TCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTCAGTTGGGCACTCTGGTGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGACGGTACAACGAGTTGCGAAGTCGTGAGGCTAAGCTAATCTCTTAAAGCCGTTC
TCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCA
TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACC
CAAAGCCGGTGAGATAACCTTCGGGAGTCA

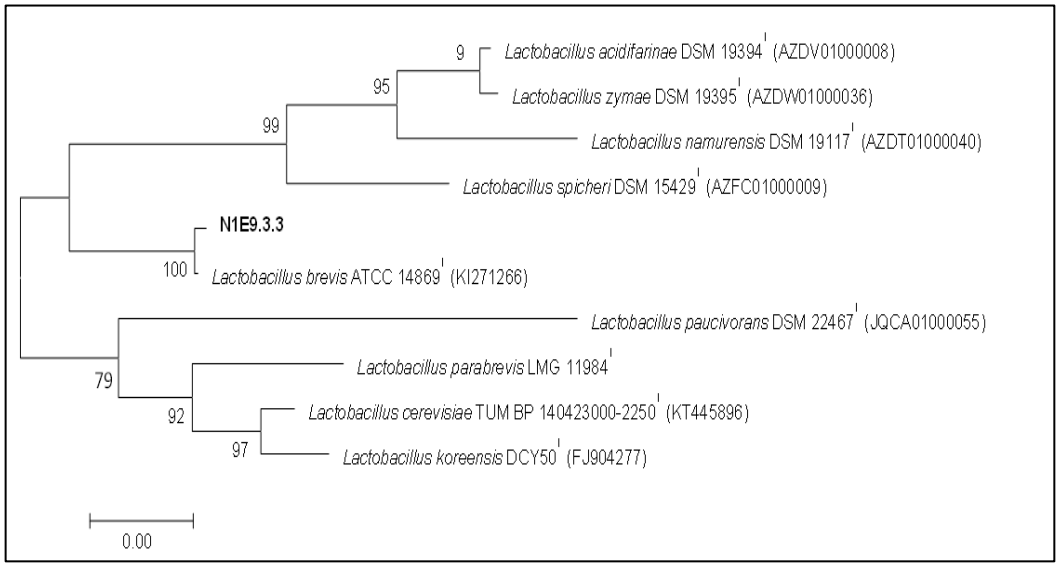


Figure.2.5. Maximum likelihood tree constructed using the 16s rDNA gene nucleotide sequences displaying the phylogenetic position of N1E9.3.3 strain in *Lactobacillus* genus

2.4.5. Genetic determination of dha genes responsible for 1,3-PDO production

The metabolic dissimilation of glycerol to 1,3-propanediol, is mediated by two enzymes, glycerol dehydratase and 1,3-PDO oxidoreductase. The former glycerol dehydratase is a tetrameric protein, dha B_1, dha B_2, dha B_3, are catalytic subunits and a reactivase (dha R) unit, and later 1,3-PDO oxidoreductase (dha T) is a single unit enzyme. The sequences coding for these enzymes were amplified using genomic DNA of isolate *L. brevis* N1E9.3.3 as the template and with the gene specific forward and

reverse primers (Table 2.1), fragments of around 1677 (dha B_1), 720 (dha B_2), 528 (dha B_3), 1851 (dha R) and 1173 (dha T) base pairs (bp) were obtained (Fig.2.6). The sequences obtained using forward and reverse primers were merged using EMBOSS merger (http://emboss.bioinformatics.nl/cgi-bin/emboss/merger) and similarity search was carried out using the gene sequences available in the database. The highest level of nucleotide similarity and identity with the gene sequences of *Lactobacillus brevis* ATCC 367 (Table.2.2).

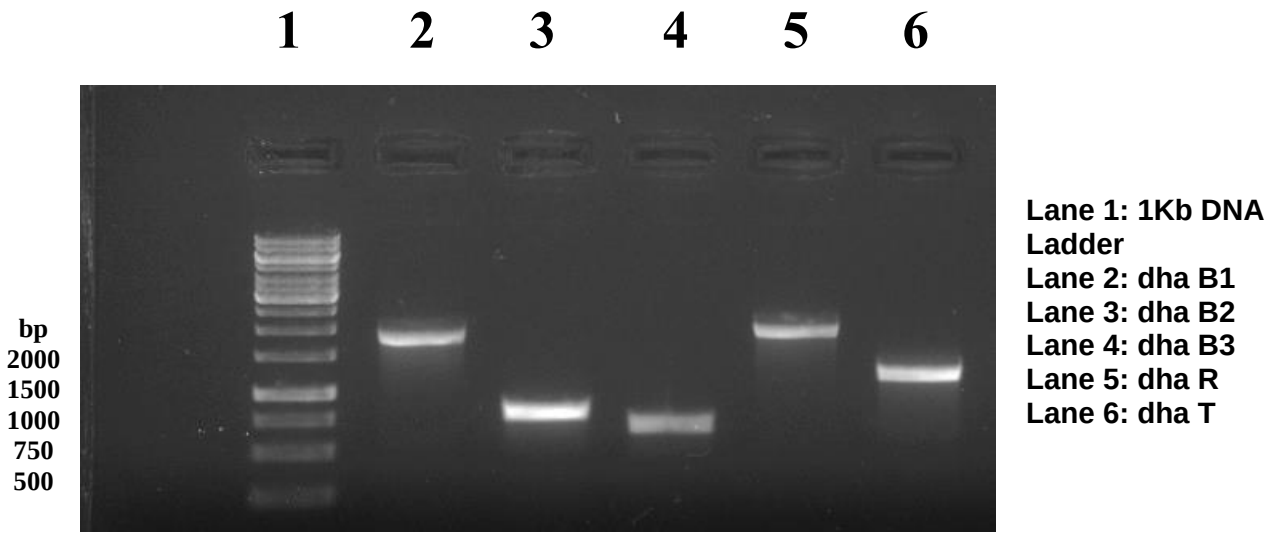


Figure.2.6. PCR amplification of genes glycerol dehydrogenase subunits dha B_1 (Lane 2), dha B_2 (Lane 3), dha B_3 (Lane 4), dha R (Lane 5) and 1,3-PDO oxidoreductase dha T (Lane 6) from *Lactobacillus brevis* N1E9.3.3.

Gene	Read length	Actual length	BLASTn (%)	Identity (%)	tBLASTx (%)
Dha B1	1678	1676	100	99	100
Dha B2	775	719	100	99	99
Dha B3	659	527	100	99	100
Dha R	1690	1850	100	99	100
Dha T	1130	1172	100	99	99

Table.2.2. Analysis of similarity and prediction of glycerol dehydrogenase gene subunits and 1,3-propanediol dehydrogenase gene between *Lactobacillus brevis* N1E9.3.3 and *Lactobacillus brevis* ATCC367.

2.4.6. Evaluation of the performance of *Lactobacillus brevis* N1E9.3.3 isolate in glycerol fermentation

The common limitations observed in biological processes are poor performance of the biocatalysts in terms of titers, yields, productivities, lower tolerance to substrates, products and physiological conditions. Hence the isolate *L. brevis* N1E9.3.3 was evaluated to address these limitations with the following experiments.

a. Growth inhibition studies

In Lactic acid bacteria, glycerol dissimilation occurs only through reductive pathway and results in 1,3-propaendiol production, where the external carbon source supplied get metabolized through oxidative pathway and produce end products like lactic acid, acetic acid and ethanol. These end products and substrates may have inhibitory effect on biomass formation at a critical concentrations, Tobajas et al., 2009 reported that 1,3-PDO production is in proportionality with biomass formation. Hence it is of great interest to understand the growth hindrance of *L. brevis* N1E9.3.3 strain towards these products at different concentrations.

i. Effect of glycerol concentration

Glycerol is the only natural substrate for 1,3-propanediol production, it is evident that to improve the final titers of 1,3-PDO in the fermented broth, the bacterial culture should withstand higher concentrations of glycerol. Each genera and species may have their own threshold limit to the increasing glycerol concentrations, which may leads to cessation of growth and metabolic arrest once the limit is exceeded. Hence it is very important to understand the threshold of each strain before declaring the potentiality in production titers.

In this study, it is clearly evident that addition of glycerol into the growth medium enhanced the bacterial growth, because the addition of glycerol in the growth medium, shifts the metabolic flux towards reductive pathway, where NADH molecules produced form the oxidative pathway can be utilized to regenerate NAD^+ molecules, which is a competitive step for lactic acid and ethanol production. As lactic acid and ethanol production were decreased, the pyruvate molecules were shifted towards acetic acid production along with 1 mol ATP. Hence energy packets and reducing equivalents required for the growth and development of bacteria was synthesized without any inhibitory effect. From the fig.2.7, we can observe that, increased glycerol concentrations from 1% to 4% has positive effect with the growth attaining a maximum O.D of 6.6 with 4% glycerol and exponential phase remained until 36h of incubation. With an increase of glycerol concentration from 4 – 5%, there is a slight decrease in

the growth compared to other concentrations, but a comparable optical density was attained at 36h of incubation, with which we can conclude that *L. brevis* N1E9.3.3 strain can tolerate concentrations more than 5% glycerol, the results observed is in acceptance with the potent *Lactobacillus* strain *L. diolivorans*, having a tolerance up to 5-6%, but have more tolerance level than strains like *Clostridium diolis*, *Citrobacter freundii*, and *Lactobacillus reuteri*. Impact of high glycerol loadings caused reduced protein expressions required for metabolism resulting in decreased growth and 1,3-propanediol production (Gungormusler-Yilmaz et al., 2014).

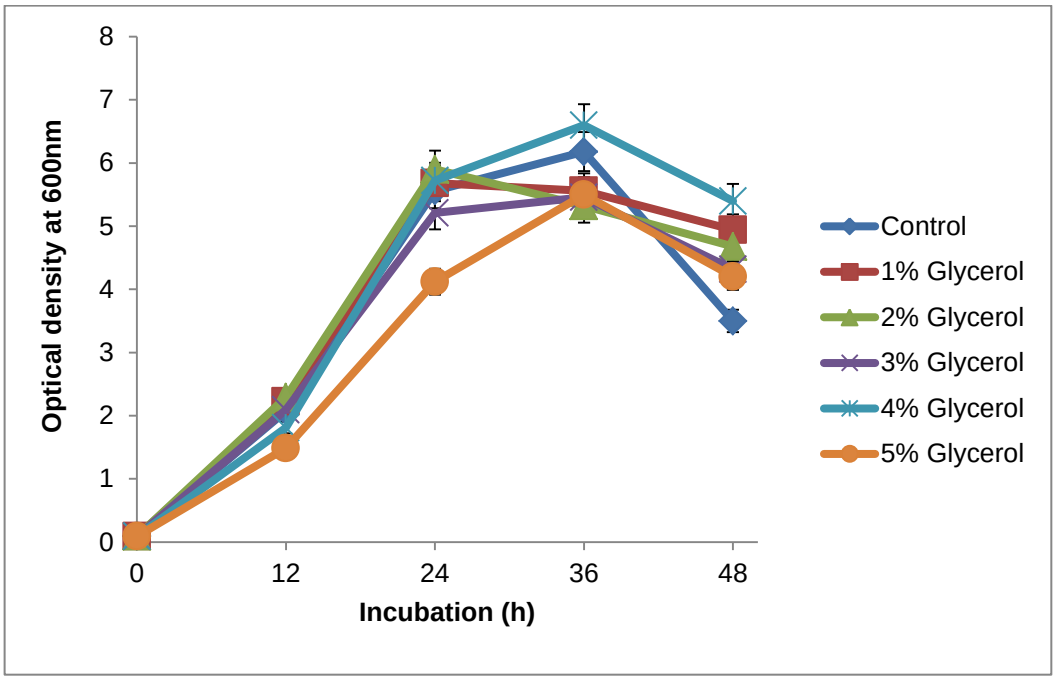


Figure.2.7. Graphical representation of effect of different concentrations of glycerol (1%-5%) on growth of *Lactobacillus brevis* N1E9.3.3 isolate, optical density measured at 600nm.

ii. Effect of 1,3-PDO concentration

In order to develop a commercially viable process, along with tolerance towards high substrate concentrations, the strains should be resistant to the products formed during the fermentation. 1,3-propanediol may have a product mediated inhibition on the growth and 1,3-PDO production. The mechanism involved in the inhibition is still unknown, but it was hypothesized that, the mechanism may be similar to alcohol mediated inhibition of growth where the membrane organization was altered resulting in cellular death. In the tolerance test with increased 1,3-PDO in the fermented broth, the isolate did not show any hindrance in the growth, the growth pattern was in comparison with the control, where 1,3-PDO was not supplemented. The maximum optical density of 7.12 was observed with 3% 1,3-PDO at 36h of incubation (fig.2.8). In

the literature, high resistant strains reported were *Clostridium butyricum* VPI 1718 having a tolerance level of 3% of 1,3-PDO and found to have longer lag phase and fermentation duration when concentration increased above 5% (Chatzifragkou et al., 2012). Another report specifies 6.5%1,3-propanediol is inhibitory for growth of *Clostridium butyricum* (Colin et al., 2000). In *Clostridium butyricum* CNCM 1211 produced and initially added 1,3-propanediol has shown similar results of inhibitory concentrations of 6.5% and tolerance levels of 8.3%, which is more and efficient compared to other *Clostridia* strains reported (Colin et al., 2000). In *Citrobacter* species like *C. freundii*, *C. werkmanii*, *C. brakii* inhibitory concentration in the range of 4% and found to be totally inhibiting the growth and productivity at 8% (Maervoet et al., 2012). In a report Menzel et al., 1997 explained concentrations of substrate and product, where observations shown maximum productivity and growth rate, upon exceeding showed a residual substrate and decreased production efficiency (Menzel et al., 1997).

With regards to substrate and product tolerance the strain *L. brevis* N1E9.3.3 displayed a higher potential in comparison to the potent strains reported for 1,3-PDO production.

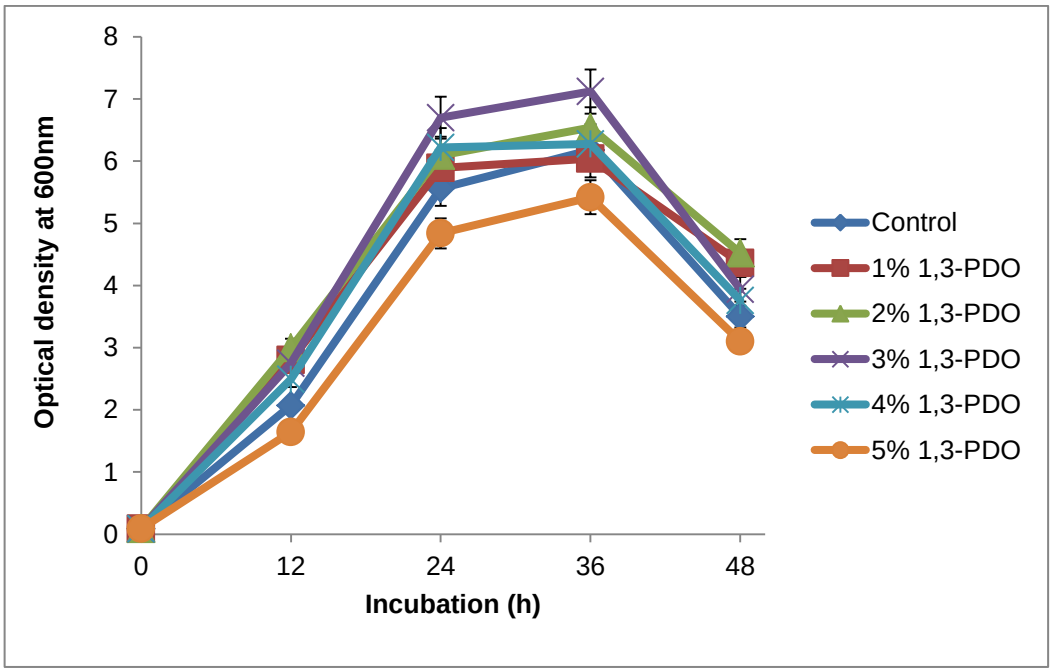
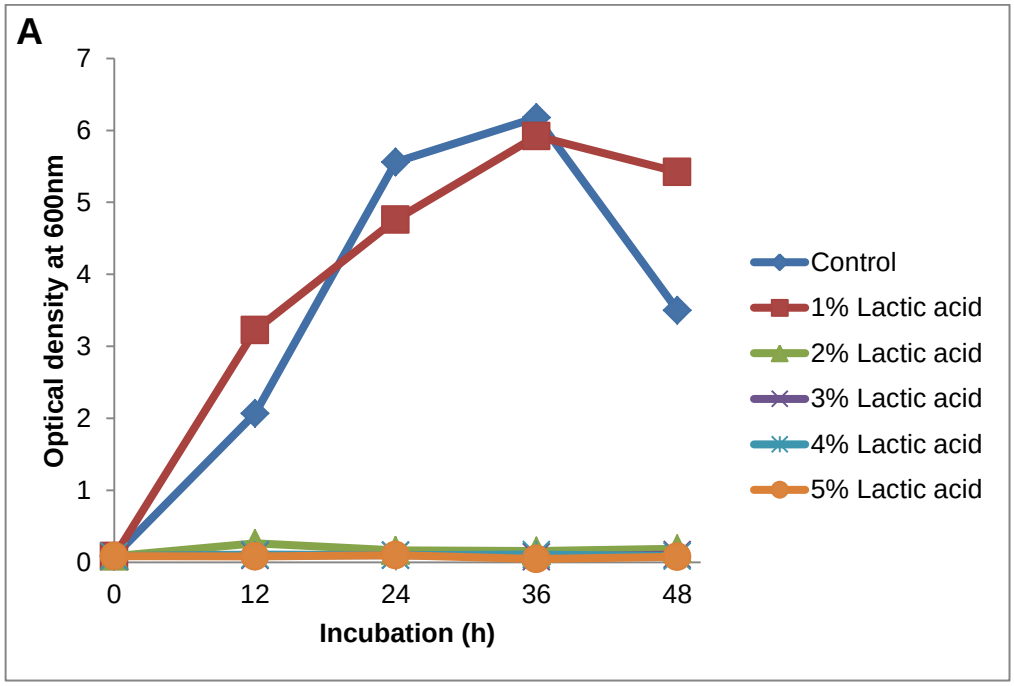


Figure.2.8. Graphical representation of effect of different concentrations of 1,3-propanediol (1%-5%) on growth of *Lactobacillus brevis* N1E9.3.3 isolate, optical density measured at 600nm.

iii. Effect of organic acid concentration

Lactobacillus brevis, a heterofermentative LAB produce lactic and acetic acid as the major organic acids via 6-phosphogluconate/phosphoketolase pathway, in which 1 mol of glucose results in 1 mol lactic acid and 1 mol acetic acid along with 1 mol ATP and 1 mol CO_2 (Zalán et al., 2010). As lactic and acetic acid are the major organic acids produced, inhibitory concentrations of these metabolites on *L. brevis* N1E9.3.3 strain was evaluated. It was observed that *L. brevis* strain displayed higher tolerance levels compared to various LAB strains published in the literature. The strain has tolerance to 1% lactic acid (fig.2.9A) and 3% acetic acid (fig.2.9B). Initial supplementation of acetic acid up to 2% increased the growth compared to control flask, that might be due to change of pyruvate flux towards ethanol production, which can regenerate 2 NADH molecules compared to 1 NADH in lactate biosynthesis pathway. The maximum optical density of 6.98 was observed after 36h of incubation at 600nm, and lower O.D of 0.06 was observed with 5% acetic acid, where at this concentration the growth of microorganism was totally inhibited. The inhibitory effects of these organic acids on growth and development might be due to (i) acidification of the environment and cytosol, (ii) dissipation of membrane potential, and (iii) increased accumulation of dissociated anions in the cytoplasm (Pieterse et al. 2005; Axe & Bailey, 1995; Hutkins & Nannen, 1993). In LAB strains the toxicity of lactic acid is more effective than acetic acid (Chen et. al. 2015), as lactic acid accumulation leads to disturbance in flux of reducing equivalents, where the NAD^+ regeneration mechanism is disrupted, which is required to maintain the equilibrium between the intracellular and extracellular environment (Kashket, 1987). The mechanism of organic acid inhibition was well investigated in *E. coli, Streptomyces* sp., *Lactobacillus plantarum* and *Lactobacillus acidophilus*, it involves the F_1-F_0-ATPase pump, the organic acids acts as protonophores, then perturb the membrane phospholipids resulting in dissipating the membrane integrity, as the LAB intracellular environment is alkaline, inward flux of H^+ ions increases and F_1-F_0-ATPase pump cannot maintain the efflux of H^+ ions resulting in acidic intracellular environment (Pieterse et al. 2005). Because of this ΔpH between the extracellular and intracellular environment, hindrance to metabolic activities like glucose utilization, glycerol consumption, that in turn inhibit the growth of microorganism due to reduced sugar uptake.



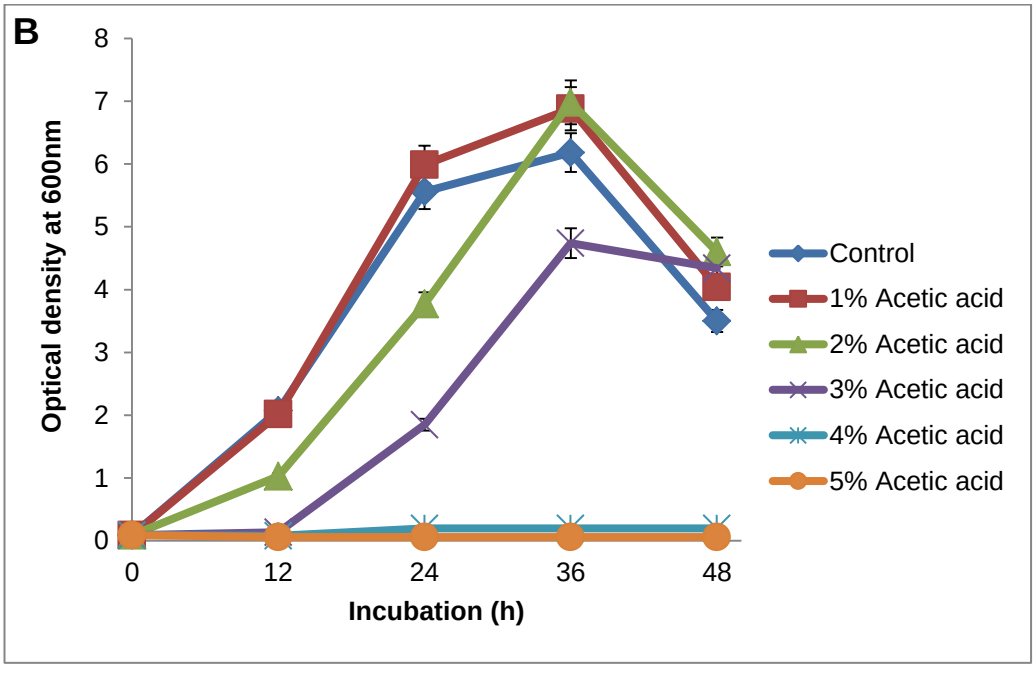


Figure.2.9. Graphical representation of effect of different concentrations of (A) Lactic acid (1% - 5%) (B) Acetic acid (1% - 5%) on growth of *Lactobacillus brevis* N1E9.3.3 isolate, optical density measured at 600nm.

b. Effect of different carbon sources as co-substrates on growth and 1,3-PDO production

Glycerol is the sole natural substrate for 1,3-propanediol production in wild type strains, while engineered strains can utilize corn starch and glucose as the substrates. *Enterobacteriaceae* and *Clostridiaceae* have enzyme glycerol dehydrogenase (dha D),

which metabolize glycerol via oxidative pathway, and these organisms are devoid of extra carbon sources other than glycerol. But lactic acid bacteria lacks the dha D gene that encodes for glycerol dehydrogenase, hence these strains recognize glycerol as the substrate instead of carbon source, so there is a requirement of carbon sources for growth, generation of energy and reducing equivalents which support the primary and secondary metabolic activities in the microorganism. The theoretical yield of 1,3-propanediol from glycerol is 72%, hence a chance awaits to increase the molar yield of 1,3-propanediol using lactic acid bacteria as the biocatalyst by supplementing the cheaper carbon sources like hemicellulosic hydrolysates (Jin et al., 2011). In this study ten different sugars like glucose, fructose, lactose, sucrose, galactose, arabinose, xylose, cellobiose, mannitol and maltose as co-substrates along with glycerol in 1:1 (wt %) ratio was investigated on the biomass formation, substrate utilization and 1,3-propanediol production. In the control experiment (without any extra carbon source added to the medium) the glycerol is unutilized but 100% utilization was observed when glucose is supplemented which resulted in 0.71 $g_{1,3\text{-PDO}}/g_{Glycerol}$ yield (Tab.2.3). Approximate 0.5 - 0.7 $g_{1,3\text{-PDO}}/g_{Glycerol}$ yield was observed with mannitol, fructose and maltose. Pentose sugars like xylose and arabinose supplemented in the medium has no effect either on glycerol utilization or 1,3-propanediol production. The carbohydrate metabolism pathway varies among different strains and generation of energy molecules and reducing equivalents will be different based on the carbohydrate present in the medium. Increased accumulation of $NADH+H^+$ ions will increase the 1,3-PDO yield as bioconversion of intermediate 3-hydroxypropionaldehyde to 1,3-propanediol requires one mole of $NADH+H^+$, as a cofactor for 1,3-propanediol dehydrogenase (Kang et al., 2013).

In lactic acid bacteria, glucose is transported into the strain by monosaccharide transporter (GlcS, GluU/GluT, GluV), and enters glycolysis or pentose phosphate pathway as α-D-glucose-6-phosphate, where results in pyruvate as end product, along with ATP and NADH+H+. In similar way entry of other sugars like mannitol, fructose or maltose may be mediated by either PTS permease or ABC transporters, but later enter the central carbon metabolism via glycolysis, mannitol is converted to fructose-1-phosphate and maltose to β-D-glucose-6-phosphate by two step reaction mediated by maltose phosphorylase [EC: 2.4.1.8] and β-phosphoglucomutase [EC: 5.4.2.6]. Although galactose supplementation did not show higher production titers, but 6.45 g/L 1,3-PDO production was shown with 7.3 g/L biomass, comparatively higher cell dry weight. The metabolism of galactose occurs through Leloir pathway, showing α-D-glucose-1-phosphate as end product mediated by aldose-1-epimerase [EC: 5.1.3.3], galactokinae [EC: 2.7.1.6] and galactose-1-phosphate uridyl transferase [EC:

2.7.7.12]. As 1,3-propanediol production depends on biomass growth, probably the concentration of glucose could be reduced by supplementing galactose for an increase in biomass concentration. The isolate *L. brevis* has shown a similar behavior in xylose mediated growth compared to *L. brevis* strain (Kim et al., 2009; Kim et al., 2010), which has shown better xylose utilization and this heterofermentative bacteria found to lack carbon catabolite repression, which marks profound effect on utilizing mixed sugar substrates like hemicellulosic hydrolysates.

Sugars	Biomass ($O.D_{600}$ nm)	Residual glycerol (g/L)	1,3-PDO (g/L)	Yield $g_{1,3\text{-PDO}}/g_{Glycerol}$	Productivity $g_{1,3\text{-PDO}}$/L/h
Control	3.7	18.15	1.57	0.07	0.065
Glucose	6.7	0.0	14.2	0.71	0.59
Fructose	4.2	3.5	11.52	0.57	0.48
Lactose	6.9	13.8	1.24	0.06	0.051
Sucrose	4.5	15.03	1.04	0.05	0.043
Galactose	7.3	9.36	6.44	0.32	0.26
Arabinose	3.9	16.24	0.88	0.04	0.036
Xylose	7.1	18.76	0.0	0.0	0.0
Cellobiose	3.6	12.71	1.67	0.08	0.069
Mannitol	5.2	2.99	11.45	0.57	0.47
Maltose	7.8	3.24	14.1	0.7	0.58

Table.2.3. A study on effect of different sugars as carbon source on biomass and 1,3-PDO production using the strain *L. brevis* N1E9.3.3 on modified MRS medium with 2% pure glycerol as the substrate. The incubation was carried out for 24 hours with an initial pH-7.0, 37^{o}C, under anaerobic conditions.

c. Effect of vitamin B_{12} and $CoCl_2.6H_2O$ supplementation on growth and 1,3-PDO production

The evaluation of genetic organization of dha operon found in *Enterobacteriaceae* and *Clostridiaceae* revealed two different glycerol dehydratase (dha B) genes where previous members has vitamin B_{12} dependent dha B gene and later has vitamin B_{12} independent gene, in lactic acid bacteria, pathway is regulated by another pdu operon, but it has similar sequences with vitamin B_{12} dependent dha B genes, hence other than *Clostridiaceae* members all natural 1,3-PDO producers are vitamin B_{12} dependent strains (Vaidyanathan et al., 2011; Stevens et al., 2011). Hence

an investigation was conducted in mMRS medium with supplementing Vitamin B_{12}, $CoCl_2.6H_2O$, individually and together with control flask avoiding both these components in the media. The incubation was carried out for 24 hours at 37°C, 200 rpm under anaerobic conditions. Here $CoCl_2$ is considered because, it is the central metal ion in the biochemical structure of vitamin B_{12}, and lactic acid bacteria were reported for the production of vitamin B_{12} hence, $CoCl_2$ is supplemented to observe the changes in yield and productivity. In the experiment other than the control flasks 4mg/L of vitamin B_{12} and 4mg/L of $CoCl_2.6H_2O$, is supplemented to the medium. In the study exponential increase in the titers of 1,3-PDO from 5.88 to 10.97 g/L with $CoCl_2.6H_2O$, 16.66 g/L with vitamin B_{12} and 16.3 g/L was observed when both the components were supplemented together (Fig.2.10). Along with 1,3-propanediol, increase in concentrations of lactic acid from 14 to 18 g/L and acetic acid from 8.6 to 11.8 g/L was observed.

The supplementation of $CoCl_2$ and vitamin B_{12}, has no profound effect on biomass concentration, 6.5 over 5.5 g/L in control flask (Fig.2.10), when vitamin B_{12} and $CoCl_2.6H_2O$ was supplemented into the fermentation medium. In general, the addition of cofactors and vitamin B_{12} resulted in an increase of product formation and substrate utilization rate. The unexpected inference was observed with $CoCl_2$ supplementation, with the agreement to *L. reuteri* $CoCl_2$ didn't affect the growth rate, but increased the 1,3-PDO titers. When compared to $CoCl_2$ and vitamin B_{12} supplementation, the results are in concurrence with previous reports of *L. reuteri* and *L. diolivorans*, suggesting that glycerol dehydratase of *L. brevis* isolate is vitamin B_{12} dependent (Pflügl et al., 2012; Maria Antonietta et al., 2015).

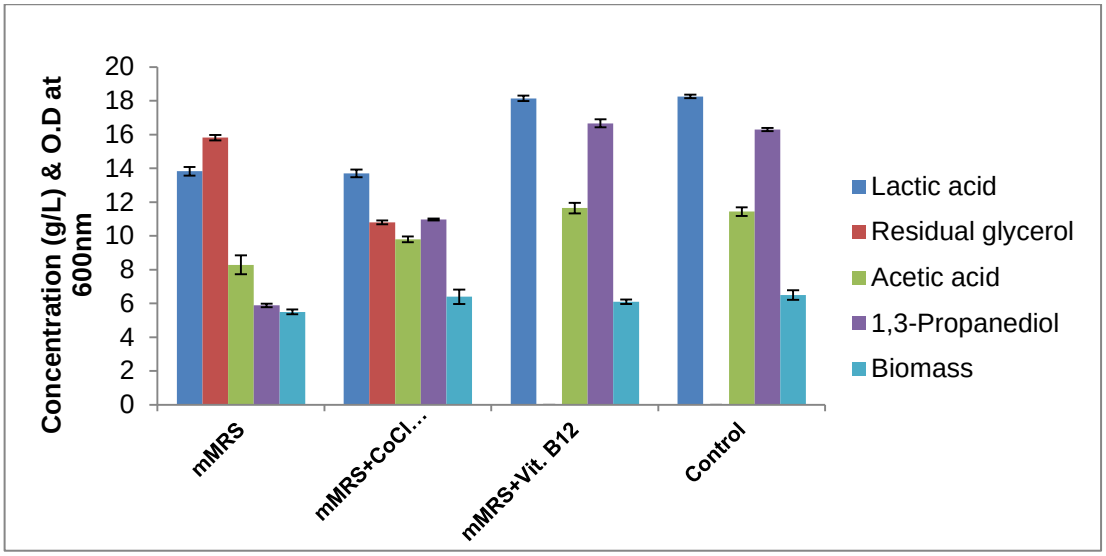


Figure.2.10. Effect of Co^{+2} and vitamin B_{12} on biomass, 1,3-propanediol and byproduct production using *L. brevis* N1E9.3.3 strain.

d. Effect of glucose : glycerol ratio on *L. brevis* N1E9.3.3 growth and 1,3-PDO production

The strain was evaluated for best-suited carbon source in batch cultivation with 3% carbohydrate and 2% glycerol as the substrate, in which glucose gave maximum production. A study was carried out to confirm with the optimal concentration of glucose in the production medium with pure and crude glycerol in individual experiments. An increasing concentration of glucose from 0.5 % to 3.0% along with 2% glycerol was supplemented to the medium. The glucose supplemented in the medium was completely utilized within 24 hours and concentration of lactic acid was found to be increasing with increase in sugar concentration, comparatively equal titers of acetic acid was produced. Increase in concentrations of glucose also observed to have a direct relation with the substrate consumption and 1,3-propanediol production, titers of 16.8 g/L was observed with 2.5% glucose and 2% pure glycerol (Tab.2.4 A). When crude glycerol was used, maximum titers of 15.38 g/L was observed with 3% initial glucose concentrations (Tab.2.4 B). The accumulation of acetic acid is increased with increase in initial glucose concentration, which shows a positive correlation in 1,3-propanediol production. The biomass concentration was also increased which relates the enhanced growth rate and metabolic behavior in different pathways. An increased ATP and NADH molecules may result in a shift of pathway towards 1,3-propanediol production for regeneration of NAD^+ molecules (Pflügl et al., 2012; Baeza-Jiménez et al., 2011). The maximum 1,3-PDO yield was 0.84 $g_{1,3\text{-PDO}}/g_{\text{pure glycerol}}$ and 0.76 $g_{1,3\text{-PDO}}/g_{\text{crude glycerol}}$, with volumetric productivity of 0.7 and 0.64 $g_{1,3\text{-PDO}}$/l/h.

Initial glucose (% w/v)	CDW (O.D $_{600nm}$)	Residual Glucose (g/L)	Residual glycerol (g/L)	1,3-Propanediol (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0.5	4.9	0.0	9.93 ± 0.004	9.01 ± 0.3	0.89 ± 0.02	8.34 ± 0.27
1.0	4.45	0.0	12.43 ± 0.05	7.72 ± 0.004	5.05 ± 0.19	8.79 ± 0.96
1.5	4.75	0.0	10.93 ± 0.05	11.09 ± 0.29	7.84 ± 0.37	10.26 ± 0.05
2.0	5.4	0.0	4.17 ± 0.05	14.68 ± 0.25	10.54 ± 0.32	11.19 ± 0.07
2.5	6.0	0.0	0.0	16.80 ± 0.18	15.27 ± 0.17	11.79 ± 0.07
3.0	5.8	0.0	0.0	16.3 ± 0.03	17.47 ± 0.04	11.42 ± 0.28

Table.2.4A. A study on biomass and metabolites observed at the end of batch cultivation of strain *L. brevis* N1E9.3.3 on modified MRS medium with 2% pure glycerol as the substrate and increasing concentrations of initial glucose.

Initial glucose (% w/v)	CDW (O.D $_{600nm}$)	Residual Glucose (g/L)	Residual glycerol (g/L)	1,3-Propanediol (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
0.5	5.5	0.0	13.1 ± 0.03	4.31 ± 0.07	3.27 ± 0.11	8.53 ± 0.51
1.0	4.4	0.0	15.95 ± 0.14	3.26 ± 0.34	2.57 ± 0.09	8.32 ± 0.25
1.5	4.65	0.0	7.03 ± 0.005	10.87 ± 0.18	7.81 ± 0.19	10.4 ± 0.00
2.0	5.45	0.0	0.0	14.28 ± 0.2	13.5 ± 0.12	11.2 ± 0.13
2.5	6.55	0.0	0.0	12.23 ± 0.06	15.05 ± 0.28	10.4 ± 0.37
3.0	6.05	0.0	0.0	15.38 ± 0.28	17.83 ± 0.18	11.2 ± 0.12

Table.2.4B. A study on biomass and metabolites observed at the end of batch cultivation of strain *L. brevis* N1E9.3.3 on modified MRS medium with 2% crude glycerol as the substrate and increasing concentrations of initial glucose.

2.5. Summary and Conclusion

An onsite enrichment technique was found to be a potent method for the isolation of microbes from soil with specific functionalities. The method resulted in an isolate N1E9.3.3, which was later identified as *Lactobacillus brevis.* In an initial screening experiments, the isolate N1E9.3.3 was observed to produce 14.7 g/L, with a volumetric yield of 0.6 $g_{1,3\text{-PDO}}/g_{Glycerol}$. Further confirmation of 1,3-PDO production by N1E9.3.3 isolate was carried out using genetic determination of glycerol dehydratase (dha B) and 1,3-PDO oxidoreductase (dha T) enzymes.

In the glycerol – glucose co – fermentation strategy, with experimental observations we can assume that:

1. Glucose is the limiting substrate for growth and metabolism of the strain.
2. Bioconversion of glycerol by *L. brevis* depends on concentration of glucose in the fermentation medium.
3. Growth attained stationary phase when glucose is completely utilized, which showed decrease in glycerol utilization, as energy and reducing equivalent supply is inhibited.

With an initial glucose: glycerol concentrations in 1: 1 (w/w) ratio maximum 1,3-PDO titers of 14.68 and 14.28 g/L with a volumetric yields of yield of 0.73 $g_{1,3\text{-PDO}}/g_{Pure\ Glycerol}$ and 0.71 $g_{1,3\text{-PDO}}/g_{Crude\ Glycerol}$ was observed respectively.

Chapter 3

Optimization of physical parameters and media components for improved 1,3-PDO production using *Lactobacillus brevis* N1E9.3.3 isolate

Chapter 3: Optimization of physical parameters and media components for improved 1,3-PDO production using *Lactobacillus brevis* N1E9.3.3 isolate

3.1. Introduction

The members of genus *Lactobacillus* were well-known probiotic strains with industrial relevance. Few strains like *Lactobacillus reuteri* (Chen et al., 2015), *Lactobacillus diolivorans* (Pflügl et al., 2014), *Lactobacillus panis* (Grahame et al., 2013), and *Lactobacillus brevis* (Veiga-Da-Cunha and Foster, 1992) were found to produce 1,3-propanediol from glycerol. Though *L. brevis* was investigated to produce higher yields, only one report was observed which describes the presence of 1,3-propanediol oxidoreductase gene and the heterologous expression studies of that particular gene (Veiga-Da-Cunha and Foster, 1992). Hence, detailed study on optimization of media components to increase the product concentration and yield would add relevance for utilization of the strain in an industrial scale production. The classical model to optimize experimental procedure is one-variable-at-a-time to monitor the influence of one factor at a time on an experimental response (Bezerra et al., 2008). This method involves a large time of work, does not allow determine cross effects of the parameters in process investigation (Bas and Boyaci, 2007; Astray et al., 2016a), and increase the number of necessary experiments to conduct the research (Bezerra et al., 2008). These conditions involves a great disadvantage, to solve these, different analytical methods have been carried out by using multivariate statistic techniques (Bezerra et al., 2008), one of the most used option is the response surface methodology (RSM). Later the experimental results obtained through response surface methodology, was modelled using Multi-Layer Perceptron (MLP) model of neural networks. Therefore, it seems clear that response surface models, and neural models have a large scope of applications; however, its use as mathematical method to model 1,3-propanediol production has not been extensively studied.

The aim of this study was to optimize the culture condition of *Lactobacillus* strain *L. brevis* N1E9.3.3 for 1,3-PDO production. The strain was isolated from soil samples in a municipal dumping yard through onsite enrichment technique which has shown significant 1,3-propanediol yields with the pure and crude form of glycerol under alkaline conditions. 1,3-PDO titers were optimized using uniform design and response surface methodology. Then, with the data obtained from various experimental runs, we compared the RSM model, and the ANN model to improve fermentation media composition.

3.2. Production media and cultivation conditions

The production media was prepared by using the chemicals and media components as mentioned in Chapter 2, section 2.2.1 and the pre-inoculum and incubation conditions were as mentioned in section 2.2.4.1 and 2.2.4.3.

3.3. Effect of pH

As effective physical parameters, pH has its role in the growth and metabolism of the strain in the fermentation medium. In a biological process the conversion of substrates towards products are catalyzed by enzymes and effect of cofactors are utmost important, these enzymes and cofactors are active only at their specific pH range, any intolerable pH changes the mode of enzyme action. In order to understand the effect of pH on 1,3-propanediol production by *Lactobacillus brevis* N1E9.3.3 isolate, experiments were performed in wide range of initial pH from pH 5.0 – 9.0. The experiments were conducted in triplicates and average values were graphically represented with standard deviation <10%.

3.4. Screening of effective media components using Plackett-Burman statistical design

In preliminary study physical parameters were optimized using one factor at a time approach as described earlier. With these conserved factors, the effect of each component in the production media on 1,3-propanediol production was analysed using Plackett-Burman design (PB). The experimental design is a factorial design; screening n variables, in this study eleven factors, coded between -1 and+1 for real values (Table.3.1), along with 20 g/L glucose and glycerol respectively.

S.No	Parameters	Low level (-1) g l^{-1}	High level (-1) g l^{-1}
1	Meat extract B	3	10
2	Yeast extract	2	5
3	Peptone	3	10
4	Sodium acetate	2	5
5	K_2HPO_4	1	3
6	Sodium citrate	1	3
7	Ammonium hydrogen orthophosphate	0.5	1.5
8	$MgSO_4.7H_2O$	0.05	0.2
9	$MnSO_4.H_2O$	0.02	0.05
10	$CoCl_2.H_2O$	0.001	0.004
11	Vitamin B_{12}	0.001	0.004

Table.3.1. Values for the Plackett-Burman experimental design for 1,3-propanediol production

The PB design is based on first order polynomial Eq. (3.1).

$$Y = \beta_0 + \sum_{i=1}^{N} \beta_i X_i \qquad (3.1)$$

In the equation; Y is the response (1,3-PDO concentration g/L), β_o is the model constant, β_i is the linear coefficient, X_i is the level of independent variable (Anusree and Nampoothiri, 2015). The effect of each individual variable on 1,3-propanediol production was determined using the Eq. (3.2).

$$E(X_i) = \frac{\sum_{i=1}^{N} M^{i+} - \sum_{i=1}^{N} M^{i-}}{N} \qquad (3.2)$$

Where $E(X_i)$ is the calculated response of variable either in negative or positive, $\sum M^{i+}$ corresponds with the sum of high level responses, $\sum M^{i-}$ corresponds with the sum of

low level responses obtained for each individual factor, finally, N is the total number of experimental run (Anusree and Nampoothiri, 2015).

3.5. Determining the higher limit and lower limit of the effective media components using steepest ascent method

The path of steepest ascent directs in a quick fashion to maximize the response in limited number of experimental runs based on the observations from initial factorial design (Plackett-Burman design). Hence using the effective parameters predicted from PB design, based on the independent factors and the response i.e, 1,3-propanediol production, the concentrations of these factors were extrapolated in the path of steepest ascent to maximize the response. This path resulted in new eleven experimental runs with five factors: meat extract B, yeast extract, $MgSO_4.7H_2O$, $MnSO_4.H_2O$ and vitamin B_{12}. The experimental design was automatically created by using steepest ascent code based on PB design in Minitab 17 software.

3.6. Optimization of significant parameters using response surface methodology

Response surface methodology (RSM) was used to optimize the potential production of 1,3-PDO using *L. brevis* N1E9.3.3. The second order experimental equation was developed to fit the experimental data and predict the effect of different independent variables in terms of linear, quadratic and cross correlations terms. An example of RSM model is presented in Eq. (3.3), where y_{pred} corresponds with the predicted value for 1,3-PDO, *etc.,* corresponds with the constant, the independent variables are represented with x_1, x_2 andx_3, the regression coefficients are defined as β_1, β_2 andβ_3, the regression coefficients for cross terms areβ_{12}, β_{13} andβ_{23}, finally,β_{11}, β_{22} andβ_{33}corresponds with the quadratic coefficients (Astray et al., 2016a).

$$y_{pred} = cte + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_3 x_3^2 \quad (3.3)$$

The parameters or variables given as inputs were coded between -1 and+1 for real values (Table.3.2).

S.No	Parameters	Low level (-1) g l^{-1}	High level (-1) g l^{-1}
1	Meat extract B	18.5	20.5
2	Yeast extract	10.37	11.35
3	$MgSO_4.7H_2O$	0.42	0.46
4	$MnSO_4.H_2O$	0.07	0.08
5	Vitamin B_{12}	0.00673	0.00734
6	Glycerol	40	60
7	Glucose	30	50

Table.3.2. Values for the Response Surface Methodology design for 1,3-propaendiol production

3.6.1. Neural networks

Neural networks can be developed using all the experimental cases available. In this work, a model with all data cases was developed; nevertheless, the correct procedure is when the database is divided into different groups, training phase and validation phase. Validation phase is composed with untrained data to ensure the correct validation procedure. Once, training and validation phases were established, neural network models were developed based on trial and error technique.

The training phase begins when training cases are presented to the first neural network layer, called input layer (Figure 3.1).The input information is introduced as a vector following Eq.(3.4). The information is propagated to the first intermediate layer using the propagation function; Eq. (3.5). This equation is implemented in intermediate and output neurons. In Eq.3.5 all inputs variables (x_i) were processed using the importance value (called weights, w_{ih}) and the bias value (b_h). The weight value corresponds to the connection strength between neurons, in this case, the previous neuron (i) and the intermediate neuron (h). The data obtained (S_h) is treated by other function, called activation function, Eq.(3.6). In the same way that propagation functions, activation function is implemented in each intermediate and output neuron. Activation function determines an output value (y_{pred}) for neuron. There are different activation functions, nevertheless one of the most used activation function is the sigmoidal function (Bezerra et al., 2008), Eq. (3.6), showing good results, even in large dimensional problems (Banakar and Azeem, 2008).This function was used in this research and in other studies (Astray et al., 2016a; Suárez et al., 2015; Astray et al., 2016b). In the output layer, the last layer in the network, the final predicted value is obtained. The weight values (w) and the bias

value (*b*), changes its value during the learning process (training cycles), this change allows that the neural model learn from training cases (Bezerra et al., 2008; Venkatasubramanian et al., 2003).

$$x = (x_1, x_2, x_3, x_4, ..., x_n) \tag{3.4}$$

$$S_h = \sum_{i=1}^{N} w_{ih} x_i + b_h \tag{3.5}$$

$$y_{pred} = \frac{1}{1 + e^{-Sh}} \tag{3.6}$$

As mentioned above, typical ANN model presents a topology, with three different types of layers: i) the first layer is the input layer where input information is presented, ii) the last layer of the neural is the output layer where the predicted value for 1,3-PDO is generated, between the input and the output layer, iii) there is one, or more, hidden layers (even called intermediate layer). The intermediate layers were developed with different numbers of neurons following the expression 2*n*+1, where *n* corresponds with the number of input variables.

Due the huge number of neuronal models implemented it is necessary to name them using specific terminology to facilitate their identification. Following terminology were used to identify neural model; N_{in}-(N_1 - N_2 - N_3)-N_{out} where N_{in} corresponds to the neurons in the input layer, N_1, N_2 and N_3 correspond to the first, second and third intermediate layers and N_{out} corresponds to the number of neurons in the output layer. Fig. 3.1 shows a typical neural model topology with seven input neurons in the input layer, three neurons in the intermediate layer and one neuron in the output layer to provide the 1,3-PDO value predicted.

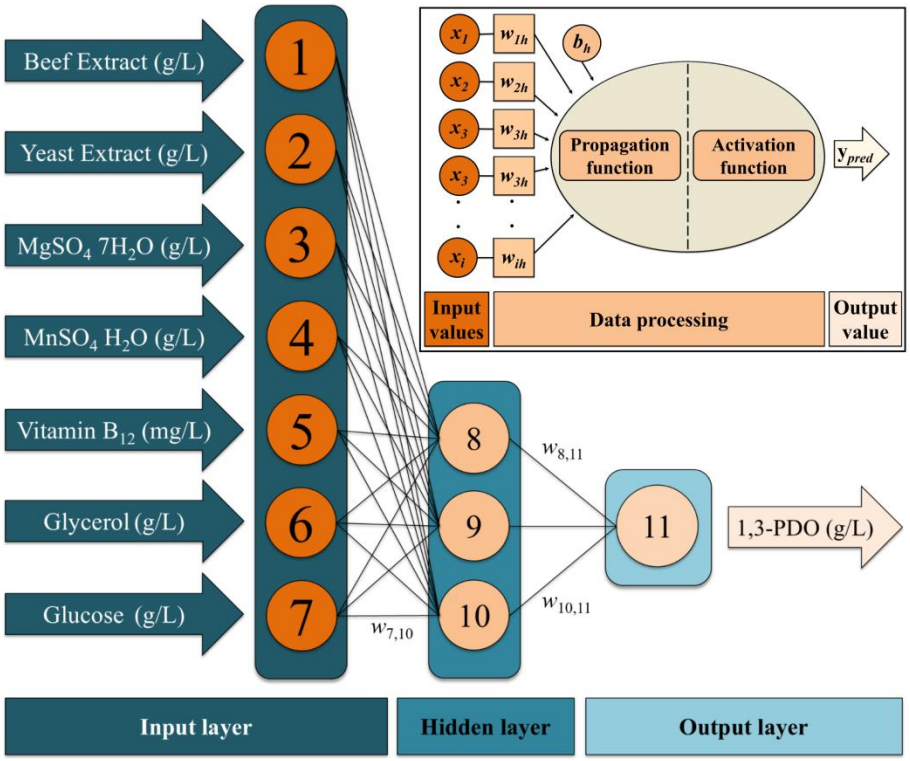


Fig 3.1. Diagram of a neural network topology 7-(3)-1 and operation procedure of an intermediate artificial neuron.

In order to verify the real power prediction of neural networks, the neural models were developed on the same way of RSM models, that is, using the same training cases, validation cases, input variables and output variable. All of that to obtain a homogeneous comparison to determine the advantage of the neural models against the RSM models. Input and output variables, training cases, topologies, cycles and fitting analysis have been explain in the following sections.

3.6.2. Fitting of Data and Modelling

Different parameters were used to check the power prediction of RSM and ANN models. Determination coefficient (R^2) was used to express the model significance, Eq.(3.7).A high R^2 value indicated a good fit for the predicted model (Rakshit et al., 2015; Sharma et al., 2015). Subscript$_{pred}$ corresponds with the predicted value; subscript $_{real}$ is the real value and$\bar{y}$ is the mean value.

$$R^2 = \frac{\sum_{i=1}^{N}\left(y_{i\ pred} - \bar{y}\right)^2}{\sum_{i=1}^{N}\left(y_{i\ real} - \bar{y}\right)^2} \quad (3.7)$$

A small error between experimental data and predicted data is required. In this research, Root Mean Square Error (RMSE), Eq. (3.8), and Average Percentage Deviations (APD), Eq. (3.9), were calculated to check the model error.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{N}\left(y_{i\ pred} - y_{i\ real}\right)^2}{N}} \quad (3.8)$$

$$APD = \frac{\sum_{i=1}^{N}\left(\left|\frac{y_{i\ pred} - y_{i\ real}}{y_{i\ real}}\right|\right)\cdot 100}{N} \quad (3.9)$$

3.6.3. Validation of optimized physical parameters and media components in shake flask experiments.

Batch co-fermentation runs of glycerol-glucose were carried out in shake flasks with the optimum physical parameters like initial pH-8.5, 37^{o}C, 200 rpm and media composition (Table.3.3).

S.No.	Media Component	Composition (g/L)
1	Meat extract B	22.9
2	Yeast extract	12.5
3	Peptone	3
4	Sodium acetate	5
5	Sodium citrate	3
6	Ammonium dihydrogen ortho phosphate	0.5
7	K_2HPO_4	1
8	$MgSO_4.7H_2O$	0.37
9	$MnSO_4.H_2O$	0.058
10	Vitamin B_{12}	0.006
11	$CoCl_2.H_2O$	0.004
12	Glucose : Glycerol	1:1 (Ratio)

Table.3.3. Optimized media composition (mMRS)

3.7. Bioreactor studies

Bioreactor studies were carried out in a parallel bioreactor (Multifors, Infors HT, Switzerland) and stirred tank bioreactor (Minifors, Infors HT, Switzerland) under the controlled physical parameters like pH-8.5, 37^{o}C, 200 rpm and optimized media composition (Table.3.3).

3.7.1. Batch glycerol-glucose co-fermentations

The batch fermentation experiments were carried out in parallel bioreactor with 300 ml working volume. Individual experiments were carried out with different glycerol-glucose concentrations (40-100 g/L). Throughout the desired incubation time, constant pH 8.5±0.5, 37^{o}C, and stirrer speed at 200 rpm was maintained. High purity nitrogen was sparged into the fermenter at a flow rate of 0.2 vvm, to maintain anaerobic conditions. Batch fermentations were started by inoculating 5% of 16 hr old pre-inoculum grown in MRS broth.

3.7.2. Self-cycling (Repeated batch fermentation)

In the repeated batch mode, the bioreactor was initially run in the batch mode; later a 50% of the fermented broth was withdrawn and replaced with fresh sterile production media. The remaining 50% of the broth in the reactor was used as inoculum for the next batch process. The repetitions were carried out until the glycerol utilization efficiency of the microorganism was reduced.

3.8. Software equipment

Statistical software package Minitab 17.1.0 Trial, Minitab Inc., Pennsylvania, USA was used to create the experimental designs and analyse the results observed. RSM models were developed using PAST 3.13 (Hammer et al., 2001). ANN models were developed using EasyNN plus v14.0d from Neural Planner Software Ltd. Experimental data were fitted using commercial software Microsoft Excel 2013 from Microsoft, USA. Neural networks have been implemented in a Server with an Intel® Core™ i7 processor with 16 GB of RAM memory. Figure 3.1 was developed with Microsoft PowerPoint 2013 from Microsoft, USA; the other figures were developed with Sigma plot 13 from Systat Software Inc.

3.9. Results and discussion

3.9.1. Effect of pH

In this study the metabolite profile of *L. brevis* was observed in the pH range of 5.0 – 9.0. The initial alkaline conditions were found to be effective on final 1,3-PDO titers, a maximum 1,3-PDO of 18.85 g/L, with yield of 0.8 $g_{1,3\text{-PDO}}/g_{Glycerol}$ and 0.78 $g_{1,3\text{-PDO}}$/L/h was observed at initial pH-8.5 in batch cultivation. According to the literature survey and my knowledge we think this would be the first report of wild type *Lactobacillus* isolate with 0.8 $g_{1,3\text{-PDO}}/g_{Glycerol}$ yield, as genetically engineered *E. coli* has shown similar figures with glycerol as the sole carbon source in a two-stage fermentation process (Tang et al., 2009). With respect to 1,3-propanediol production, 100% glycerol utilization was observed at alkaline pH (Fig. 3.2). The explanation was also supported by the correlation between the yield and pH (Fig. 3.2). The unexpected observation was that approximately constant titers of acetic acid were found throughout the pH range, but the accumulation of lactic acid increased. It can speculate that initial acidic environment was not favorable for the production of acids, which reduces the glucose metabolism and reducing equivalents production, unlike acidic environment, alkaline environment increased production of lactic acid, which is the common behavior of *L. brevis*. The increase in pH beyond 8.5 decreased the metabolic behavior of *L. brevis*, but maximum production of *L. panis* was observed at pH-10 (Grahame et al., 2013). In order to improve the production titers, experiments were carried out on strategies like controlled pH behavior (Sattayasamitsathit et al., 2011) and pH fluctuations (Ji et al., 2009; Petrov & Stoyanov, 2012).

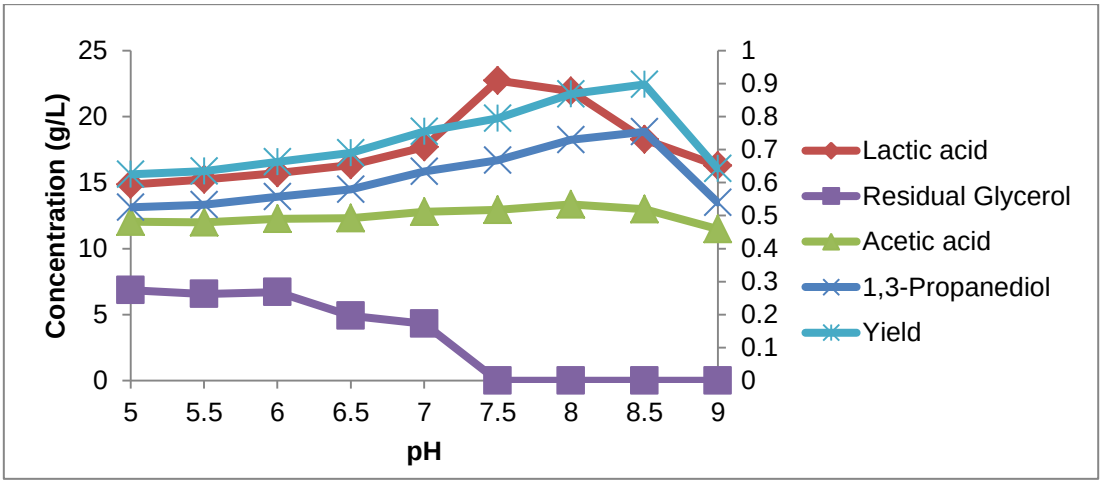


Figure.3.2. Influence of pH on *L. brevis* N1E9.3.3 strain producing 1,3-propanediol and byproducts during glucose/glycerol co-fermentation.

3.9.2. Screening of effective media components using Plackett-Burman statistical design

The production of the desirable product depends on chemical composition of the production media and the physical parameters. Hence the optimization of production media components would add beneficial effect in increasing the yields of final desired product. Effect of individual media components and the interactive response of them on microbial physiology would be effective in determining the end product concentrations. Hence, it is advantageous to observe the significant process variables and then optimize those using conventional methods. However, the complex media with several variables would be tedious to determine significant among them using one-factor-at-a-time experiments. Developed statistical tools that are fast and reliable like PB designs will be beneficial to optimize complex media compositions. The experimental design generated by Minitab software for the high and low values of 11 variables given as an input is shown in Table.3.4, along with 1,3-PDO produced with the different media combinations. Fig. 3.3 shows the coefficient of the significance of each variable on 1,3-PDO production obtained using Eq. 3.2. From the 11 variables, it is evident that meat extract B, yeast extract, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$ and vitamin B_{12} have a positive correlation with the response and peptone has a considerable negative effect. Meat extract B and yeast extract were known to supplement the production media with large amounts of carbon, nitrogen and mineral salts which tends to increase the 1,3-PDO production (Himmi et al., 1999; Olivieri et al., 2016) . In Lactic acid bacteria (LAB)*, Klebsiella* and *Citrobacter* genus the glycerol dehydratase enzyme assimilating glycerol to 3-HPA is vitamin B_{12} dependent while it was independent in *Clostridium* genus. This catalytic conversion of glycerol is known to precede by a radical mechanism involving Vitamin B_{12}as an essential cofactor. Hence the change in Vitamin B_{12} concentration varied the assimilatory behaviour of glycerol dehydratase. The phosphate source in the production media was explained to be positively affecting the response by favouring the growth of microorganism, energy supplements and reducing equivalents of the cell (Himmi et al., 1999; Olivieri et al., 2016), but in this study a negative effect of dipotassium hydrogen phosphate and ammonium hydrogen orthophosphate on the response were observed. The maximum 1,3-propanediol concentration was observed when meat extract B, yeast extract, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$ and vitamin B_{12} present higher concentrations. But the limits of these individual variables are unknown to estimate the maximum response and interactions between these variables.

StdOrder	RunOrder	Peptone	Meat extract B (g/L)	Yeast extract (g/L)	Sodium acetate (g/L)	K_2HPO_4 (g/L)	Sodium Citrate (g/L)	Amm. Dihy. Phosphate (g/L)	MgSO4. $7H_2O$ (g/L)	MnSO4. H_2O (g/L)	$CoCl_2$ (mg/L)	Vitamin B_{12} (mg/L)	1,3-PDO (g/L)
13	1	10	3	5	2	1	1	1.5	0.2	0.05	1	4	13.08
1	2	10	3	5	2	1	1	1.5	0.2	0.05	1	4	13.30
10	3	10	3	2	2	3	3	1.5	0.05	0.05	4	1	5.89
7	4	3	10	5	5	1	3	1.5	0.05	0.05	1	1	13.28
15	5	3	10	5	2	3	1	0.5	0.05	0.05	4	4	15.43
22	6	10	3	2	2	3	3	1.5	0.05	0.05	4	1	8.61
11	7	3	10	2	2	1	3	1.5	0.2	0.02	4	4	13.38
20	8	3	3	5	5	3	1	1.5	0.2	0.02	4	1	14.03
23	9	3	10	2	2	1	3	1.5	0.2	0.02	4	4	13.50
17	10	10	10	2	5	3	1	1.5	0.05	0.02	1	4	10.84
12	11	3	3	2	2	1	1	0.5	0.05	0.02	1	1	9.25
24	12	3	3	2	2	1	1	0.5	0.05	0.02	1	1	8.05
6	13	10	10	5	2	3	3	0.5	0.2	0.02	1	1	11.05
16	14	10	3	5	5	1	3	0.5	0.05	0.02	4	4	12.69
19	15	3	10	5	5	1	3	1.5	0.05	0.05	1	1	14.02
2	16	10	10	2	5	1	1	0.5	0.2	0.05	4	1	13.84
14	17	10	10	2	5	1	1	0.5	0.2	0.05	4	1	11.02
9	18	3	3	2	5	3	3	0.5	0.2	0.05	1	4	13.02
5	19	10	10	2	5	3	1	1.5	0.05	0.02	1	4	7.93
4	20	10	3	5	5	1	3	0.5	0.05	0.02	4	4	10.52
18	21	10	10	5	2	3	3	0.5	0.2	0.02	1	1	13.97
3	22	3	10	5	2	3	1	0.5	0.05	0.05	4	4	13.35
8	23	3	3	5	5	3	1	1.5	0.2	0.02	4	1	10.04
21	24	3	3	2	5	3	3	0.5	0.2	0.05	1	4	12.58

Table.3.4. Plackett-Burman experimental design for 1,3-Propanediol production.

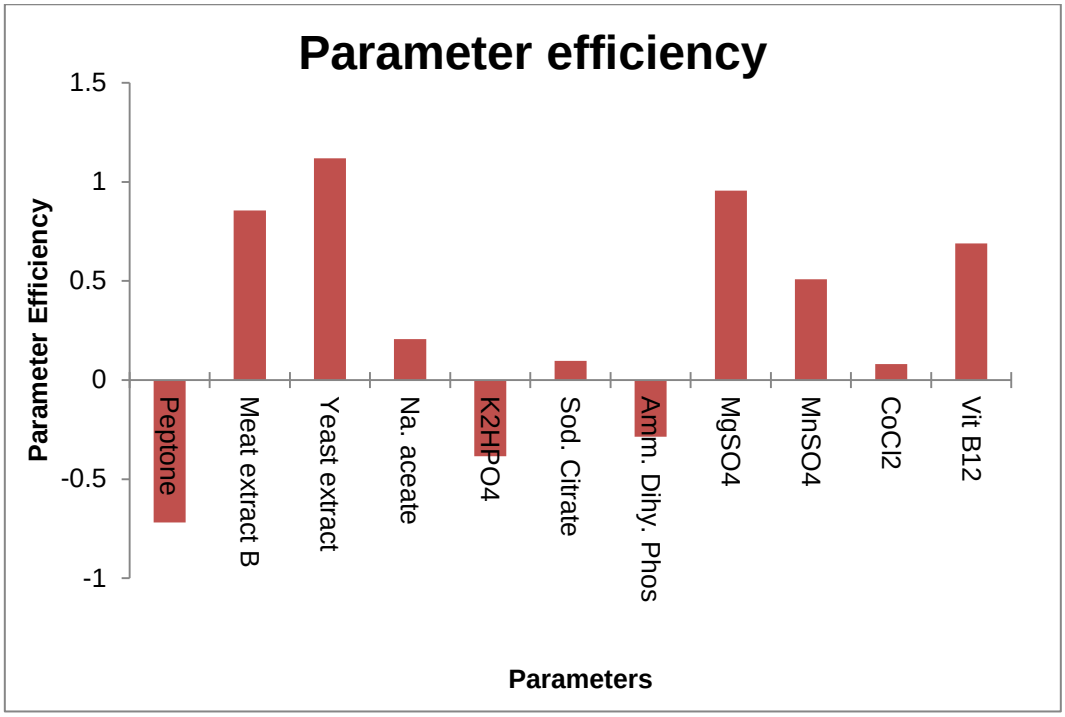


Figure.3.3. Effect of Coefficients of individual variables observed using Plackett-Burman design.

3.9.3. Determining the higher limit and lower limit of the effective media components using steepest ascent method

The steepest ascent path determines the direction of individual variables to be altered towards higher responses. The design was created based on the previous factorial screening experiments, where the limits of each variable were designed with equal spaces in the path. With a limited number of runs, a path of steepest ascent was operated to observe the maximum concentration of these independent variables to maximize the response. Thus, the design represented a path to increase the concentration of meat extract B, yeast extract, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$ and vitamin $B_{12}$to increase 1,3-PDO production. The remaining factors of production media sodium acetate, K_2HPO_4, sodium citrate and ammonium dihydrogen orthophosphate were maintained at lower limits taken in PB design. The response i.e., 1,3-PDO production obtained with steepest ascent design runs were summarized in Table 3.5. In a path of steepest ascent the maximum response was obtained at following concentrations 18.75 g/L yeast extract: 10.37 g/L, $MgSO_4.7H_2O$: 0.42 g/L, $MnSO_4.H_2O$: 0.07 g/L and vitamin B_{12}: 6.73 mg/L i.e., at 8^{th} run of

the design the response shows maximum concentration. Few variables were observed to have positive correlation with the response to a particular level, later on increasing the concentration the same variable has negative effect on the response. An optimum level should be investigated to have a conclusive final concentration of each independent variable in the production media.

Runs	Meat extract B (g/L)	Yeast Extract (g/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	$MnSO_4 \cdot H_2O$ (g/L)	Vitamin B_{12} (mg/L)	1,3-PDO (g/L)
1	6.50	3.50	0.13	0.04	2.50	12.46
2	8.25	4.48	0.17	0.04	3.10	12.46
3	10	5.46	0.21	0.04	3.71	13.06
4	11.75	6.44	0.25	0.05	4.31	13.54
5	13.50	7.43	0.29	0.05	4.92	13.56
6	15.25	8.41	0.33	0.06	5.52	13.80
7	17	9.39	0.38	0.06	6.13	14.03
8	18.75	10.37	0.42	0.07	6.73	14.91
9	20.50	11.35	0.46	0.07	7.34	14.58
10	22.25	12.33	0.50	0.08	7.94	14.33
11	24	13.32	0.54	0.08	8.55	13.93

Table.3.5. Steepest ascent design for 1,3-PDO production

3.9.4. Optimization of significant parameters using response surface methodology

With the earlier experiments the path of ascent evaluated the optimum concentrations of individual variables. The levels of these five effective variables along with additional two variables glycerol and glucose were further optimized using central composite design. As glycerol is known as the sole source for 1,3-PDO production and LAB strains require glucose for growth and glycerol assimilation, these two factors were included in optimization.

3.9.4.1. RSM and ANN models development

a. RSM and ANN type one

The central composite experimental design was evaluated to understand the interactions between the individual factors contributing to the production of 1,3-propanediol. Two factors glucose concentration and glycerol concentration along with the significant factors obtained through PB design were used, that is, RSM and ANN models

were developed using; i) meat extract B (g/L), ii) yeast extract (g/L), iii) $MgSO_4 \cdot 7H_2O$ (g/L), iv) $MnSO_4 \cdot H_2O$ (g/L), v) vitamin B_{12} (mg/L), vi) glycerol (g/L) and vii) glucose (g/L), to study the interactions in 160 experimental runs (Annexure I). In this first approach (models type one), the 160 experimental cases were used to develop the models without validation cases to check the real prediction power. Table 3.7 shows the fit statistics for RSM_1 and ANN_1 models developed in this first approach. The results obtained for 1,3-PDO production show that the ANN model can predict with accuracy the experimental values. Table 3.6 shows the good fits obtained by ANN_1model (determination coefficient of 0.917) and small average percentage error, 1.37%. Fit statistics for RSM_1 model presents worse fit than ANN_1 model, both in determination coefficient and RMSE.

Model	Cycles	Topology	R^2	RMSE	APD
RSM_1			0.607	1.13	4.48
ANN_1	64,000	35-26-1	0.917	0.52	1.37

Table.3.6. Fit statistics for RSM and ANN models (Type one (subscript 1)) developed. R^2 is the determination co-efficient, RMSE corresponds to root mean square error (g/L) and APD is the average percentage deviations (%).

Experimental 1,3-PDO concentration increased rapidly to contents of glycerol equal to 50 g/L where it reaches a maximum of 24.37 g/L, after this point, 1,3-PDO concentration decreased slowly with glycerol concentration. Glucose concentration presents a similar influence in experimental 1,3-PDO concentration, when glucose concentration increases, 1,3-PDO reaches the maximum at 40 g/L and gradually descending from this point. Response surface model reaches a maximum value of 22.66 g/L (under glycerol= 50 g/L and glucose= 40 g/L). RSM_1 model can predict the optimum operational conditions, nevertheless, surface model cannot predict with accuracy the maximum 1,3-PDO concentration, in fact, the error is around 7.04%. On the other hand, neural model can predict the optimum operational conditions (glycerol= 50 g/L and glucose= 40 g/L) with more accuracy than RSM_1 model (24.07 g/L, which it corresponds to an error of 1.24%). Taking into account these results, it can be concluded that the neural model predicts with good accuracy (R^2=0.917 and RMSE=0.52 g/L) the 1,3-PDO production.

b. RSM and ANN type two

Nevertheless, a correct prediction model needs to be checked with different data that were used to implement the model. In this sense, two new models (models type two) based on response surface methodology and artificial neural network were developed. In this case, previous database were divided in two different groups, one group to develop the models (formed by 125 experimental cases) and other group, validation group (31 experimental cases) to check the power prediction model. Four cases in this database were deleted as they have been considered anomalous data. The input variables were the same variables used in models type one.

All networks models were implemented with different topologies and training cycles, to find the best neural model to predict 1,3-PDO concentration. More than seven hundred models were developed, using trial and error method, to find the best ANN model type two. All neural models were developed with the same input variables used for RSM model. With this procedure, the predictive power of RSM and ANN models can be compared. The best neural network to predict 1,3-PDO was chosen based on the APD for validation phase.

Table 3.8 shows the fit statistics for the new RSM (RSM_2) and the best implemented neural model (ANN_2) developed in this research. RSM_2 model presents better determination coefficient (0.656) than the previous developed model RSM_1 (0.607). In terms of root mean square error for training phase, the new RSM_2 model obtain a value of 1.07 g/L, lower than that $RMSE_1$ obtained in the previous model (1.13g/L). In this case, ANN_2 model presents worse results than previous developed model (ANN_1) and worse fit statistics than RSM_2. Neural model obtain for the training phase a good determination coefficient of 0.830, with a $RMSE_T$ of 1.24 g/L. In this phase, RSM_2 model presents better adjust in terms of RMSE and APD than ANN_2 model, but worse in terms of determination coefficient (Table 3.7). In validation phase, where the real power prediction is tested, an opposite behaviour is observed. In this phase, ANN_2 present better adjust in terms of determination coefficient (0.684) than RSM_2 model (0.067). This behaviour also happens for root mean square error where ANN_2 presents lower APD value (1.46 g/L) than RSM_2 model (1.66 g/L).The results obtained for 1,3-PDO show that neural model can predict with more accuracy the experimental values used in validation cases.

Fig. 3.4 shows the dependence of glycerol and glucose for all cases assayed on experimental 1,3-PDO yield (Fig.3.4A), for RSM (Fig.3.4B) and ANN (Fig.3.4C) models. As mentioned above, 1,3-PDO concentration behaviour increased rapidly with contents of

glycerol equal to 50 g/L and reaches a maximum of 24.37 g/L.Glucose concentration presents a similar influence in experimental 1,3-PDO concentration, reached the maximum at 40 g/L.

Model	Cycles	Topology	R^2_T	$RMSE_T$	APD_T	R^2_V	$RMSE_V$	APD_V
RSM_2			0.656	1.07	4.25	0.067	1.66	6.18
ANN_2	4,000	35-33-1	0.831	1.24	4.72	0.684	1.46	5.47

Table.3.7. Fit statistics for RSM and ANN models (Type two (subscript 2)) developed. R^2 is the determination co-efficient, RMSE corresponds to root mean square error (g/L) and APD is the average percentage deviations (%). Subscript T corresponds with the training phase and V corresponds to validation phase.

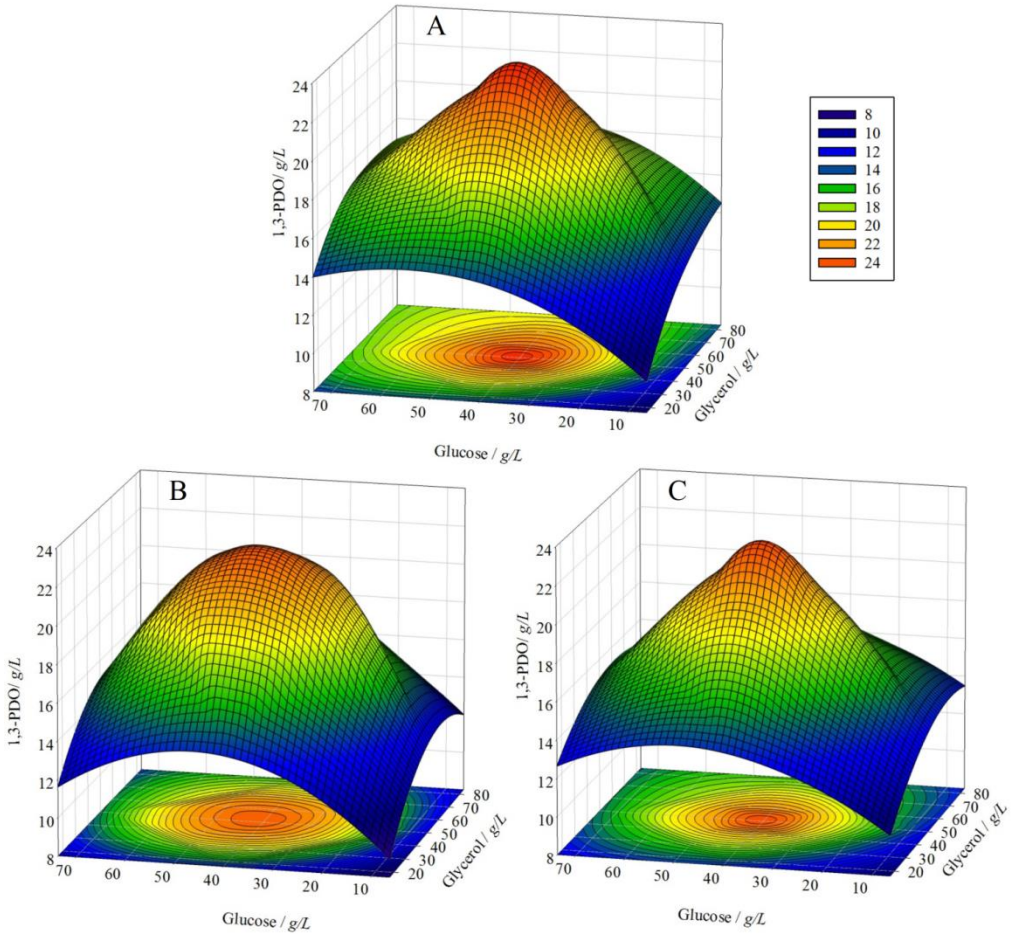


Fig. 3.4. 3D mesh plot showing the influence of independent variables glycerol (g/L) and glucose (g/L), into all experimental 1,3-PDO production (Figure 3.4A), predicted values by RSM_2 model (Figure 3.4B) and predicted values by ANN_2 model (Figure 3.4C).

Fig.3.4 B-C illustrates significant difference between RSM and ANN model against experimental data (Fig. 3.4A). Fig. 3.4B shows a similar behaviour that presents the RSM_1 model, but in this case, surface model reaches a maximum value of 22.79 g/L (under glycerol= 50 g/L and glucose= 40 g/L) that corresponds with an individual percentage deviation of 6.51% compared with the experimental value. In this new RSM model (RSM_2) the error in optimum conduction has descended compared with the first model (RSM_1, 7.04 %). Surface model cannot predict with accuracy the maximum 1,3-PDO concentration. On the other hand, ANN_2 model presents a slightly different contour graph than real distribution (oppositely to the previous model ANN_1, where was very similar). Neural model (Fig. 3.4C) can predict the maximum 1,3-PDO concentration in optimum conditions with more accuracy than RSM_2 model (23.41 g/L, which corresponds to an error of 3.94% compared with experimental value). ANN_2 model presents better fits for validation phase with a $RMSE_V$ smaller than the $RMSE_V$ obtained for RSM_2 model (1.46 g/L vs. 1.66 g/L) with an improvement of 12.19%.

The importance for independent variables depends on the weights of each input neuron with the neurons in the intermediate layer (Astray et al., 2016a) and the sum of absolute weight values determines the importance for each variable. The most important original variables for selected ANN were the $MgSO_4 \cdot 7H_2O$ concentration (22.47%), followed by vitamin B_{12} concentration (16.38%) and $MnSO_4 \cdot H_2O$ (13.83%).

Both ANN models (type one and two) have improved RSM model. Average percentage deviation in training phase and in validation phase was between 1.37% and 5.5%. The APD values were low and allow the use of the model to determine the 1,3-PDO production by *L. brevis* N1E9.3.3. The use of seven independent variables to predict 1,3-PDO production may be sufficient, but it would be interesting to check the influence of other different independent variables (cultivation time, temperature, pH, etc…)like other studies reported in literature (Himmi et al., 1999). Li *et al.* (2014) developed a RSM model, with all date used in training phase, that can predict with accuracy the 1,3-PDO production, R^2 of 0.983 (Li et al., 2014). It is possible to find in literature a couple of papers relative to 1,3-propanediol. Kirilova *et al.* (2014) develop an ANN model used to biotransformation process of crude glycerol to formation of 1,3-propanediol using bacteria *Pseudomonas denitrificans* 1625 and also developed ANN models to predict the biotransformation of crude glycerol to 1,3-propanediol with RMSE ≈ 0.78 g/L, for production ranging between values zero, and ≈ 4 g/L (Kirilova et al., 2014), this can imply an average relative error around 10% (value calculated for this research). Other interesting study was developed by

Hongwen *et al.* (2005) where a prediction model developed to optimize the process for key enzymes accumulation of 1,3-propanediol production from *Klebsiella pneumonia* (Hongwen et al., 2005). The obtained model presents good results with and average relative error of 9.43%, and a maximum relative error of 14.0% (Hongwen et al., 2005). These two studies are characterized by the good power prediction of neural models.

The low correlation level for models developed in this research, especially for RSM_2 model, may be due to three reasons: i) the low range of experimental data, ii) possible missed input variables (variable that have not been taking into account), or iii) the model is not able to generalize the relationships between variables learned in the training phase and extrapolate the knowledge to the validation phase. It was observed that pH value of 8.5 is optimum for 1,3-PDO production, using one time – one factor approach, in this sense, a new group of experiments were developed to study the change in metabolite profile and 1,3-PDO production on maintenance of pH-8.5 throughout the incubation time.
The Response surface design (CCD) indicated glucose and glycerol at specific concentrations were limiting substrates and have comparative positive effect on the response i.e., 1,3-PDO production. Hence with the physical parameters optimized earlier and the media components optimized using PB design, steepest ascent and RSM, experiments were carried out in triplicates to investigate the effect on response. The validation experiments carried out in shake flasks with optimized media components and 1:1 ratio of glucose and glycerol using different concentrations (20-100 g/L) at an initial pH-8.5, 200 rpm and 37^{o}C as optimal physical parameters, the results indicated that the optimal 1,3-PDO production of 0.42 $g_{1,3\text{-PDO}}/g_{Glycerol}$ and 0.55 $g_{1,3\text{-PDO}}/g_{Glycerol}$ with pure glycerol and crude glycerol respectively was observed at 40 g/L concentration respectively, (Figure 3.5 A-B). Similarly, using CCD design optimized production media increased 19.78 % 1,3-PDO productivity with *Klebsiella pneumoniae* AC 15 strain (Zheng et al., 2008b).

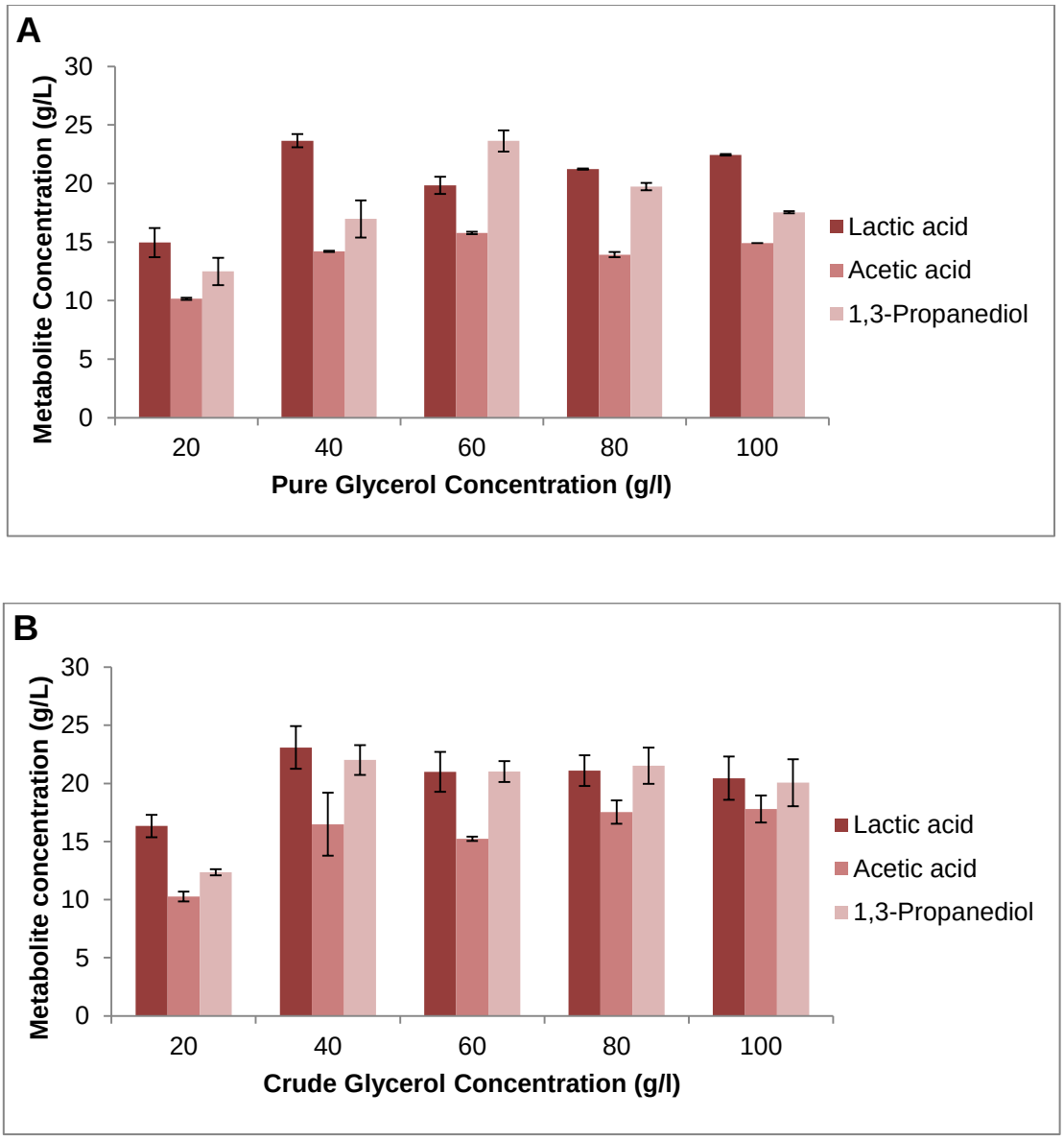


Figure.3.5. The study of metabolite concentrations in glucose, glycerol co-fermentations in 1:1 ratios without pH maintenance in shake flask experiments.

3.9.6. Bioreactor studies

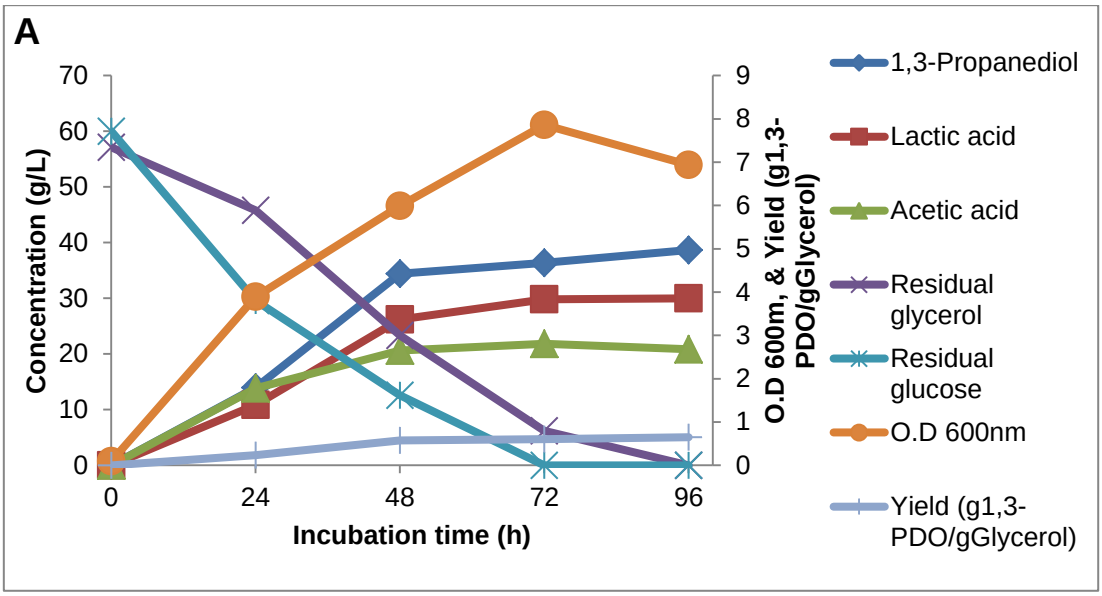
3.9.6.1. Batch glycerol-glucose co-fermentations

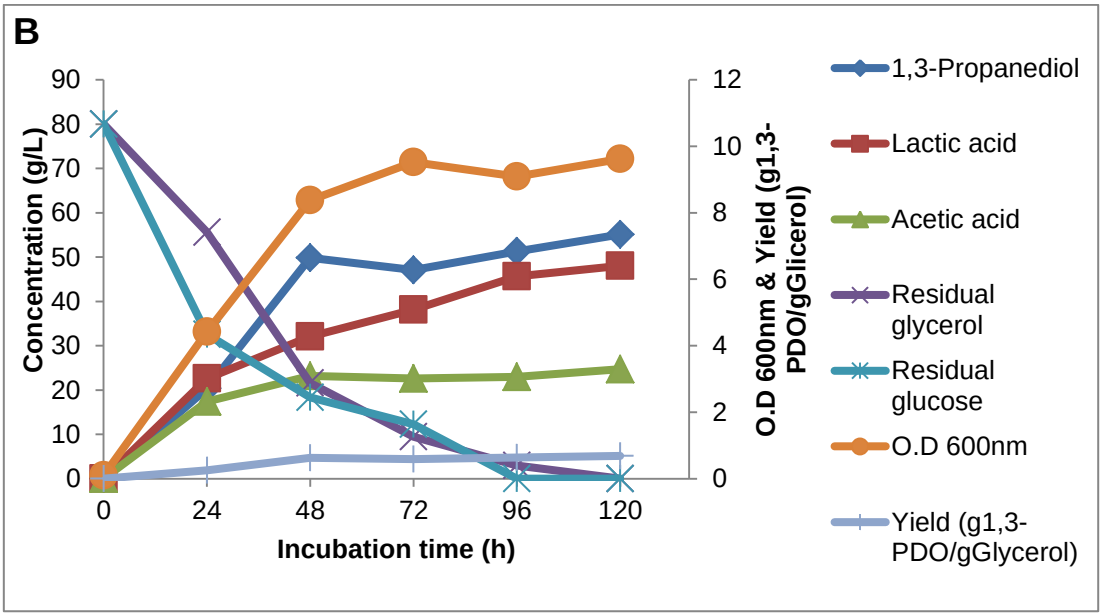
In *Lactobacillus brevis* the uptake of glucose by sugar H^+ transporter tends to vary on physiological pH of the surroundings (Ye et al., 1994). Hence pH of the production medium is one of the major aspects which affect the metabolic flux between oxidative and reductive pathways (Jung et al., 2012). The enzymes and cofactors involved in the metabolic pathways functions at specific pH. Due to the shift of metabolic pathway from

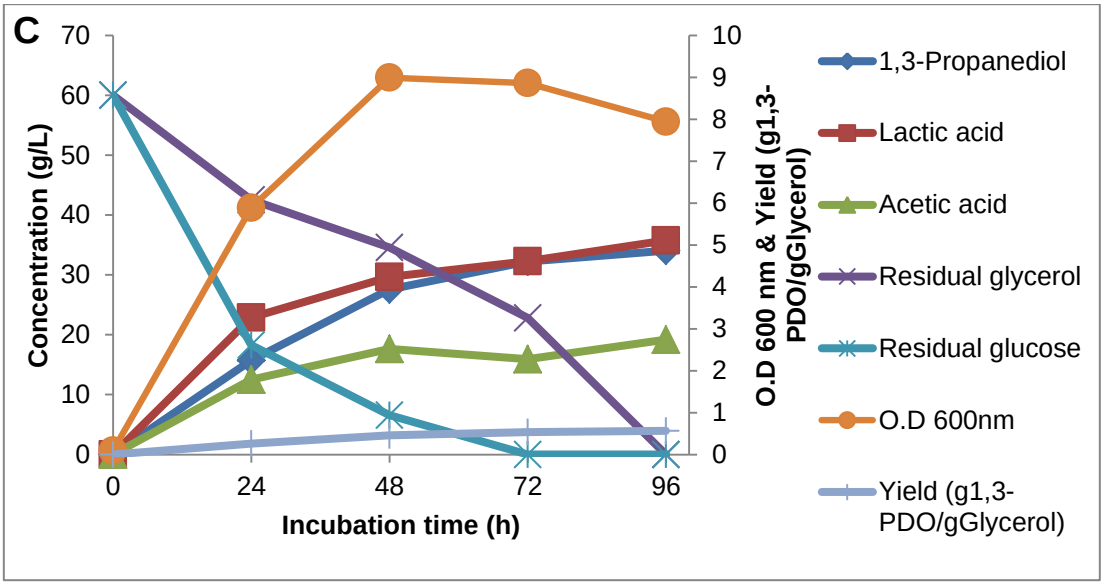
reduction to oxidation of glycerol, inhibitory metabolites are produced. The inhibitory metabolites such as lactic acid and acetic acid could decrease the pH during fermentation which leads to growth inhibition and decreased titers of 1,3-PDO. Glycerol gets converted to 2,3-BDO instead of 1,3-PDO in acidic pH with high concentration of hydrogen ions due to the oxygen sensitivity of glycerol dehydratase and 1,3-PDO oxidoreductase in 1,3-PDO synthesis pathway (Zheng et al., 2008).

In this study batch fermentations were carried out in parallel bioreactors (Multifors, Infors HT) using optimized media components with initial 6% glycerol and 8% glycerol respectively. An alkaline pH of 8.5 was maintained during the fermentation under anaerobic conditions resulting in maximum titers of 38.66 g/L and 34.6 g/L 1,3-PDO with a volumetric yield of 0.5-0.6 $g_{1.3\text{-PDO}}/g_{\text{Glycerol}}$ with 60 g/L pure and crude glycerol respectively (Table.3.8 A). A significant increase in the titers of 1,3-PDO was observed when the substrate concentration was increased to 80 g/L resulting in 55.12 and 51.51 g/L 1,3-PDO in 120 h with a yield of 0.64 $g_{1.3\text{-PDO}}/g_{\text{Glycerol}}$ (Table 3.8 B). In the bioreactor, under the controlled conditions, the 100% of glycerol was consumed at a rate of 0.83 g/L/h, compared to shake flask experiments, where production and growth was affected due to accumulated glycerol. The pattern of metabolite synthesis, glycerol and glucose utilization was shown in figure 3.6 A-D. Along with 1,3-PDO titers, significant amounts of lactic and acetic acid were observed during batch fermentations, maximum titers of 48 g/L lactic acid and 27.8 g/L acetic acid was produced with 8 % glycerol-glucose with a volumetric yield of 0.6 $g_{\text{Lactic acid}}/g_{\text{Glucose}}$ and 0.34 $g_{\text{Acetic acid}}/g_{\text{Glucose}}$ respectively (Table.3.8 B). In the physiological behavior of LAB strains, lactic acid is a competitor for 1,3-PDO in regenerating the NAD^+ molecules from $NADH+H^+$, when the flux of these reducing equivalents directed towards the oxidative pathway we can observe the increase in the lactic acid concentration. These results suggest that pH is a key factor for production of 1,3-PDO using fermentation technology. This fact is due to different acids, like acetic, or lactic, which reduce the medium pH and suppress the metabolic route of 1,3-propanediol (Rodriguez et al., 2016; Tabah et al., 2016). Depending on the microorganism, different strategies can be assessed for pH control with the objective of maximum production of 1,3-PDO; for example, in the case of *Shimwellia blattae*, a new glycerol-consuming biocatalyst, this strategy includes a temporary program with intervals in which pH is allowed to freely fluctuate and others in which it is maintained at fixed value (Tabah et al., 2016). As the concentration of substrate and product increase, biomass formation, productivity, and enzyme activities decline, but intercellular NADH and acetyl Co-A

accumulate. The mechanism of inhibition was suggested to be diol mediated. However, diol tolerance varies with different genus and species. Many *Lactobacilli*, are not inhibited by diols until they reach very high concentrations exceeding 120 g/L. It has furthermore been shown, that the glycerol concentration acts on the rate limiting steps of the reductive metabolic pathway (Mori et al., 1999). In 1996, Reimann et al., suggested that in *Clostridium butyricum* production depends on inhibition caused by glycerol and 1,3-PDO concentration in the fermentation media, availability of $NADH+H^+$ for the reductive pathway and accumulation of by-products. Methyl viologen an artificial electron acceptor was used in iron and phosphate-limiting conditions, where the metabolic shift was observed towards 1,3-PDO production by diverting electron mobility from H_2 to NADH + H^+ production. The initial glycerol concentration has also affected the fatty acid profile in *Clostridium butyricum* strains by increasing saturated vs unsaturated fattyacids (Reimann et al., 1996). The native 1,3-PDO producer *Clostridium diolis* has proven to be affected by a decrease in the specific growth when the glycerol concentration increases to more than 40 g/L (Kaur et al., 2012) . The change in proteomic profile of 1,3-PDO producer cultivated on high glycerol loadings was observed to be the reason for poor yields and productivity (Gungormusler et al., 2014). A substrate inhibition at initial crude glycerol concentration higher than 70 g/L has been reported in *C. butyricum* (Szymanowska-Powałowska & Leja, 2014).During 1,3-PDO fermentation under anaerobic condition, acetic acid was the most inhibitory byproduct with lactic acid and ethanol for *K. pneumoniae*(Cheng et al., 2005). Moon et al.(2010) reported that effect of raw and pretreated glycerol derived from various substrates like soybean and waste vegetable oil, the *Klebsiella* strains has minimum inhibition in the growth rate and 1,3-PDO production efficiency compared to *Clostridium* strains (Moon et al., 2010).


A
Concentration (g/L)
O.D 600m, & Yield (g1,3-PDO/gGlycerol)
Incubation time (h)
0
24
48
72
96
1,3-Propanediol
Lactic acid
Acetic acid
Residual glycerol
Residual glucose
O.D 600nm
Yield (g1,3-PDO/gGlycerol)


B
Concentration (g/L)
O.D 600nm & Yield (g1,3-PDO/gGlicerol)
Incubation time (h)
0
24
48
72
96
120
1,3-Propanediol
Lactic acid
Acetic acid
Residual glycerol
Residual glucose
O.D 600nm
Yield (g1,3-PDO/gGlycerol)



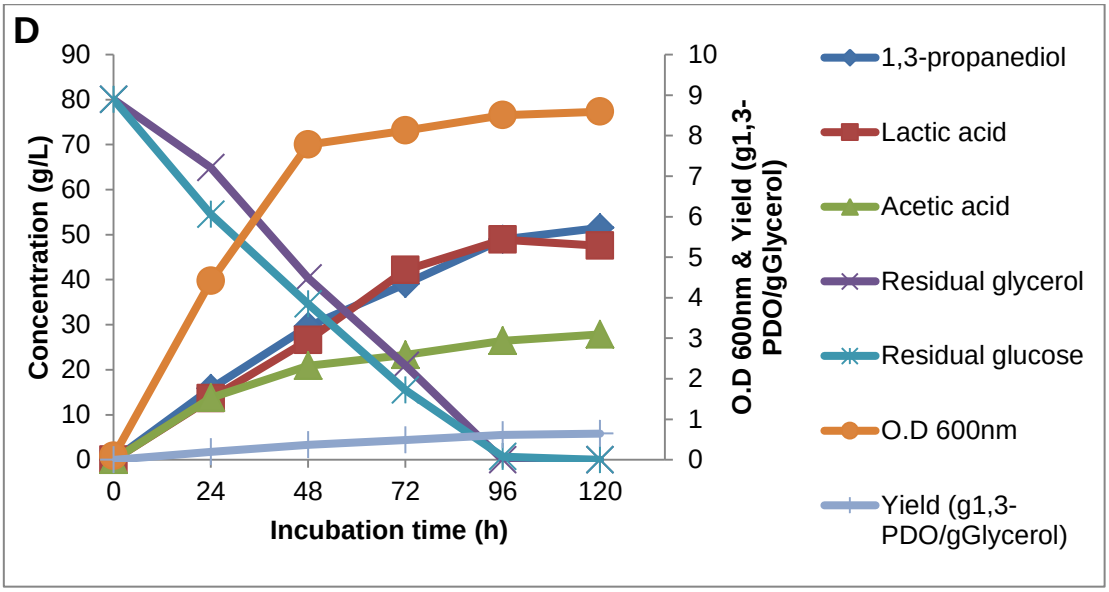


Figure.3.6. Fermentation profile of the strain *Lactobacillus brevis* N1E9.3.3 in glycerol-glucose anaerobic co-fermentation under a constant pH-8.5, temp-37^{o}C and a stirrer speed of 200 RPM. The profiling was carried out at various glycerol and glucose concentrations. (A) 6% Pure glycerol (B) 8% Pure glycerol (C) 6% Crude glycerol and (D) 8% Crude glycerol.

A

Glycerol concentration (g/L)	Glucose concentration (g/L)	Cell density ($O.D_{600nm}$)	1,3-PDO (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Yield $g_{1,3\text{-}PDO}/g_{Glycerol}$
60 Pure	60	6.94	38.66	30.00	20.86	0.64
60 Crude	60	7.94	34.60	35.38	19.54	0.56

B

Glycerol concentration (g/L)	Glucose concentration (g/L)	Cell density ($O.D_{600nm}$)	1,3-PDO (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Yield $g_{1,3\text{-}PDO}/g_{Glycerol}$
80 Pure	80	9.62	55.12	48.07	24.69	0.68
80 Crude	80	8.59	51.51	47.54	27.81	0.64

Table.3.8. Overview of the biomass, metabolites and yield of 1,3-propanediol produced at the end of glycerol-glucose co-fermentation with 6% and 8% pure (A) and crude (B) glycerol respectively.

3.9.6.2. Repeated batch or Self-cycling fermentation in a stirred tank bioreactor

The repeated batch experiments were carried out by replacing the spent production medium with fresh and sterile medium at regular intervals once the concentration of carbon source gets depleted. Compared to the batch, fed batch and chemostat modes of fermentation, repeated batch was observed to increase the productivity and titers of 1,3-PDO (Yang et al., 2016). In a recent report Vieira et.al., described the efficiency and economical operational stability of repeated batch process using *Lactobacillus reuteri* ATCC23272 as a biocatalyst (Vieira et al., 2015). In this study initial batch experiment was conducted using 40 g/L glucose and glycerol concentration respectively. The feed comprising glucose/glycerol in the ratio of 1:1 was fed into the system every 24 hours by replacing 50% of the fermented broth. The repeated batch was carried out in 5 cycles with 24 hour regular intervals.

With the observations made the biomass growth had increased to an O.D of 5.76 until the 3rd cycle, later decreased to 2.34 by the end of 5th cycle (Fig.3.7). 1,3-PDO production increased in a significant pattern after each cycle and at the end 5th cycle 78.33 g/L was observed. The performance of the strain throughout the fermentation considering both

reductive and oxidative phase was significant as the major usual metabolites of oxidative pathway lactate and acetate was produced in a considerable titers upto 45.2 and 53.24 g/L respectively at the end of 5th cycle. The accumulation of these by-products and depletion of nutrients might be the cause of decreased biomass growth. After the end of batch phase the specific substrate utilization rate was constant till the 3rd cycle with increased yield (Fig.3.7). 1,3-PDO productivity was in similar dynamics throughout the fermentation with the maximum range in between 0.5-0.65 $g_{1,3\text{-}PDO}$/L/h. The reduced growth O.D may represent the cell autolysis indicating the termination of substrate utilization (Johnson & Rehmann, 2016). In previous reports repeated batch fermentation with *Clostridium butyricum* DSM15410 strain achieved concentrations upto 67.8 g/L (Kaur et al., 2012) and *Clostridium butyricum* VPI1718 strain 65.5 g/L (Chatzifragkou et al., 2014) 1,3-propanediol. It is worth mentioning that this is the first report on efficient 1,3-PDO production from *Lactobacillus brevis* strain utilizing biodiesel derived crude glycerol as the substrate.

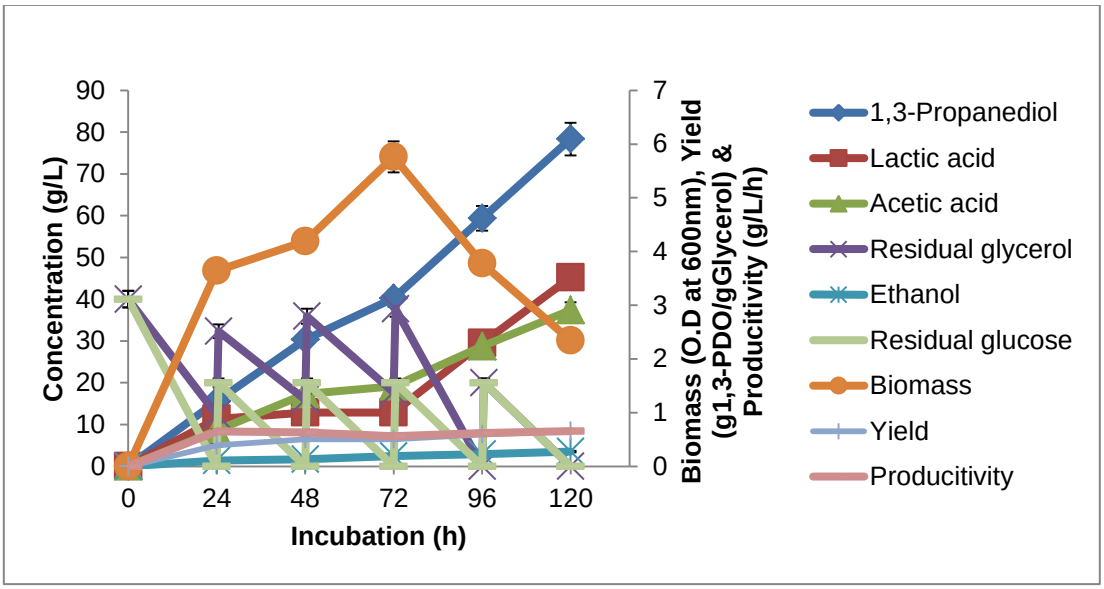


Figure.3.7. Fermentation profile of the strain *Lactobacillus brevis* N1E9.3.3 in glycerol-glucose anaerobic repeated batch or self-cycling fermentation under a constant pH-8.5, temp-37^{o}C and a stirrer speed of 200 RPM.

The 1,3-propanediol titers obtained is higher than the literature reports for lactic acid bacteria in batch mode of cultivation (Table.3.9).

S.No.	Microorganism	Substrate	1,3-PDO (g/L)	Yield ($g_{1,3\text{-PDO}}$ /$g_{Glycerol}$)	Mode of fermentation	References
1	*Lactobacillus diolivorans* DSM 14421	CG	85	0.49	Fed-batch	Pflügl et al.,2014
2	*Lactobacillus panis* PM 1	CG	16.23	0.72	Batch	Kang et al., 2014
3	*Lactobacillus reuteri* ATCC 23272	PG	28.69	0.91	Batch	Baeza-Jiménez et al., 2011
4	*Lactobacillus diolivorans* DSM 14421	PG	41.7	0.59	Batch	Pflügl et al.,2012
5	*Lactobacillus reuteri ATCC 55730*	PG	37.4	0.43	Batch	Jolly et al., 2014
6	*Lactobacillus reuteri DSM 20016*	PG	46	0.55	Batch	Ricci et al., 2015
7	*Lactobacillus brevis* N1E9.3.3	PG	55.12	0.68	Batch	This study
8	*Lactobacillus brevis* N1E9.3.3	CG	51.51	0.64	Batch	This study
9	*Lactobacillus brevis* N1E9.3.3	CG	78.33	0.65	Repeated batch	This study

Table.3.9. Overview of various Lactobacillus strains reported for 1,3-PDO production with an emphasis on 1,3-PDO titers, yield ($g_{1,3\text{-PDO}}/g_{Glycerol}$) in batch and fed-batch mode of cultivation.

3.10. Summary and conclusions

In this research, the optimization of 1,3-PDO production by *L.brevis* N1E9.3.3 was investigated. The optimization of initial physiological pH of the production media using one factor at a time approach, resulted in a volumetric yields of 0.8 $g_{1,3\text{-}PDO}/g_{Glycerol}$ under alkaline (pH-8.5) conditions. Later using Placket-Burman design, the most effective parameters for 1,3-PDO production was observed, whose concentrations in higher and lower limits was observed using steepest ascent method. Different RSM and ANN models were developed to model the 1,3-PDO behaviour as a function of seven independent variables; i) meat extract B (g/L), ii) yeast extract (g/L), iii) $MgSO_4 \cdot 7H_2O$ (g/L), iv) $MnSO_4 \cdot H_2O$ (g/L), v) vitamin B12 (mg/L), vi) glycerol (g/L) and vii) glucose (g/L).

The best neural model, ANN_2 presents a topology with thirty-three neurons in the hidden layer and four thousand training cycles to predict 1,3-PDO production with accuracy. Both ANN models (type one and two) improved the RSM models between a 54.08% in terms of RSM for models type one and 12.19% for RMSE in validation phase for models type two.

Taking into account of the optimized physical parameters and media components the validation experiments conducted proves the efficiency of *L. brevis* N1E9.3.3 strain to tolerance to higher concentrations of glycerol and capable of producing higher concentrations of end products like lactic acid acetic acid and 1,3-propaendiol.

Chapter 4

Development of an efficient and cost effective production media for 1,3-propanediol production using cheaper nitrogen sources and biodiesel derived crude glycerol

Chapter 4: Development of an efficient and cost effective production media for 1,3-propanediol production using cheaper nitrogen sources and biodiesel derived crude glycerol

4.1. Introduction

In fermentation, product formation mainly depends on the availability of the carbon source, nitrogen, energy and hydrogen concentration (Drozdzynska *et al.*, 2011). The microbial processes have certain disadvantages such as high cost, low yields, productivity and tedious downstream processing due to by-products. The cost of the process depends on the cost of the organic and inorganic supplements in the production media, cost of operation etc. Selection of the cost effective raw material and its efficient utilization were the critical factors in economization of the product (Milessi *et al.*, 2013). Lactic acid bacteria are known for their nutrient rich habitats, similarly nutrient rich growth medium that includes amino acids, peptides, fattyacids, vitamins and nucleic acids is required to replicate the habitat conditions. DeMan, Rogosa and Sharpe (MRS) medium is the classical growth medium used in laboratories for growth and maintenance of lactic acid bacteria. In this complex media, nitrogen supplementation along with other essential nutrients were supplemented by meat extract, peptone and yeast extract, which makes the medium costly in commercial scale. Replacing these nitrogen sources in the production media by cost effective raw materials can improve the commercial viability of the product. Peptone, yeast extract and beef extract are mostly used in fermentation studies. But the high price hinders the large scale application of these nitrogen sources, similarly as these nitrogen sources are either bovine or porcine derived, replacing with other cost effective nitrogen sources will benefit the health issues (Yoo *et al.,* 1997). Berg *et al*. (1981) found that the presence of peptides in yeast extract enhanced the growth of *Lactobacillus*. Hujanen and Linko (1996) studied the effect of low cost nitrogen sources along with commercially available yeast extract, peptone and malt sprouts in lactic acid production. Altaf *et al.* (2005) investigated the economic advantages of using low cost nitrogen sources in the production media. It showed that cost of red lentil, an organic nitrogen source, was 40 fold lesser than that of commercial peptone.

Corn steep liquor is a good source of nitrogen as it contains high amounts of amino acids, polypeptides and vitamin B complex (Cardinal and Hedrick, 1948). In a study by Safari *et al.* 2012 reported that fish protein hydrolysate containing growth media have better growth rates of *Lactobacillus* than in commercial MRS medium. Protein hydrolysate is an effective nitrogen source in microbial growth media as it contains free amino acids (Guerard*et al*., 2001). According to Kwon *et al.* 2000, an enzyme-

hydrolysate of soybean meal, Soytone, replaced 15 g/L yeast extract with 19.3 g/L Soytone along with the vitamins as nitrogen source in the production media which resulted in a production of 125 g/L lactic acid from 150 g/L glucose by *L. rhamnosus*. A work done by De Vyust and Vandamme, 1994 on the nisin yield improvement by optimization of nitrogen sources shows that cotton seed can improve the nisin production. Groundnut oil cake produced a good quantity of phytase enzyme by *Mucorracemosus* (Roopesh *et al.*, 2006). Groundnut oil cake contains 40-50% of crude protein. They generally have less than 2-3 % fat (Swick, 1999). To use low cost nitrogen sources, acid hydrolysis of these substrates should be carried out. Dilute acid pretreatment is applicable for a variety of biomass namely hardwoods, softwoods, agricultural residues and municipal solid wastes (Zheng *et al.*, 2009).

In this study, various raw materials like soybean meal, ground nut oil cake, cotton seed cake, corn steep liquor and fish protein hydrolysate were evaluated as nitrogen sources in growth and production medium for the native strain *Lactobacillus brevis* N1E9.3.3.

4.2. Materials and methods

4.2.1. Media composition and cultivation conditions

The production media for the control experiments was prepared by using the chemicals and media components as mentioned in Chapter 3, table.3.3 and the pre-inoculum and incubation conditions were as mentioned in section 2.2.4.1 and 2.2.4.3.

4.2.2. Selection of nitrogen sources

Vermicompost, soybean meal, ground nut oil cake, cotton seed cake, corn steep liquor, fish protein hydrolysate were selected for the purpose of using it as a cheap nitrogen source. Dilute acid pretreatment was done to all the other cheap raw materials except vermicompost, corn steep liquor and fish protein hydrolysate. 20 g of the biomass samples were weighed and 400 ml of 0.25 N HCl (v/v) was added in a screw cap bottle. It was then autoclaved at 121^0 C for 30 minutes. The supernatant was taken and neutralized with 10 N NaOH. Batch fermentations were carried out in Scott bottles (Borosil) to analyze the production of 1,3-PDO.

4.2.3. Estimation of N-content in production media and other nitrogen sources using Kjeldhal apparatus

Kjeldhal digestion is a method used for the quantitative estimation of nitrogen present in a substance. This method was developed by Johan Kjeldahl in 1883. It includes a digestion, distillation and titration process to quantify the nitrogen present in

the biomass sample. The amount of nitrogen and protein in vermicompost, soyabean meal, ground nut oil cake, cotton seed cake, corn steep liquor and fish protein hydrolysate were analyzed. 5ml of slurry of the samples were taken and 0.365 g $CuSO_4$, 6.7g K_2SO_4 and 6.7 ml H_2SO_4 were added for digestion. The process was carried out in kjelTRON^{TM} (kjelTRON-KDIGB8M, Tulin equipments). Excess NaOH was added to the acid digestion mixture. Boiling and condensation were done. The receiving solution was titrated against 25 ml of boric acid. Color change from blue to green was taken as the end point. From the following eq.4.1, the concentrations of nitrogen and protein in the samples were calculated.

$$N = \frac{(V_A - V_B) \times N_A \times 1.4007}{W_g} \qquad (4.1)$$

N: Percentage of Nitrogen
V_A: Volume of standard acid
V_B: Volume of blank
N_A: Normality of acid
W_g: Weight of the sample in grams

4.2.4. Screening of different raw materials as nitrogen supplements in the production media for 1,3-PDO production

Batch fermentations were carried out to investigate the effect of different raw materials as nitrogen supplements for 1,3-propanediol production under anaerobic conditions in 150 ml Scott Duran bottles with a working volume of 100 ml. Pre-culture inoculum was prepared by inoculating an isolated colony from a freshly sub cultured plate into a sterile MRS broth and incubated at 37^0 C for 16 hours. After autoclaving the production media with different concentrations (2-10% w/v or v/v) of each raw material respectively, 5% of the pre-culture was added. Nitrogen gas was purged into the production media to replace the oxygen in the headspace with nitrogen. Incubation was done at 37^0 C, 200 rpm for 48 h.

4.2.5. Evaluation of different concentrations of fish protein hydrolysate (FPH) as sole N-source supplementation for 1,3-PDO production

The fish protein hydrolysate (FPH) was selected as the effective and efficient nitrogen source for 1,3-PDO production. Later the effect of FPH concentration with respect to glycerol concentrations were optimized in shake flask experiments in a batch mode. The varying concentrations of FPH (2-10% w/v) were used to analyze the 1,3-propanediol production. Batch fermentations were carried out in 40, 60, 80, 100 g/L concentrations of pure and crude glycerol respectively. 5% of the pre-culture was

added. Nitrogen gas was purged into the production media to replace the oxygen in the headspace with nitrogen. Incubation was done at 37^0 C, 200 rpm for 48 h. The pH was maintained at 8.5 manually using 5N NaOH at regular intervals.

4.2.6. Comparative determination of fish protein hydrolysate (FPH) efficiency with yeast extract (Y.E), meat extract (M.E) and peptone

Lactic acid bacteria are fastidious and nutritionally dependent on varied amino acids vitamins and other growth factors. And the most expensive components of the growth media being the nitrogen sources, lactic acid growth media has expensive nitrogen sources like yeast extract, meat extract and peptone. With the industrial significance of LAB cultures, a cost effective growth and development medium will be beneficial. In this study, research was focused on finding new and effective nutritional sources, where fish protein hydrolysate was observed to be beneficial. Hence to have knowledge on microbial growth, comparative studies on commercial media and the media developed in this research, following experiments were carried out.

4.2.6.1. Growth analysis

The commercially available DeMan Rogosa and Sharpe (MRS) medium was used as the control medium consisting of (grams per litre) 10g proteose peptone; 10g meat extract B; 5g yeast extract; 20g glucose; 1g tween 80; 2g ammonium citrate; 5g sodium acetate; 0.1g magnesium sulphate; 0.05g manganese sulphate; and 2g dipotassium hydrogen phosphate.

Test medium 1: MRS medium with the above composition along with 2% pure glycerol.

Test medium 2: MRS medium with the above composition along with 2% crude glycerol.

Test medium 3: FPHMRS medium with following composition (grams per litre) 40g fish protein hydrolysate; 20g glucose; 1g tween 80; 2g ammonium citrate; 5g sodium acetate; 0.1g magnesium sulphate; 0.05g manganese sulphate; and 2g dipotassium hydrogen phosphate.

Test medium 4: FPHMRS medium with the above composition along with 2% pure glycerol.

Test medium 5: FPHMRS medium with the above composition along with 2% crude glycerol.

Test medium 6: FPH medium with the following composition (grams per litre) 40g fish protein hydrolysate; 20g glucose; 5g sodium acetate; 3g sodium citrate; 0.05g ammonium dihydrogen ortho phosphate; 0.37g magnesium sulphate; 0.058g

manganese sulphate; 6mg vitamin B_{12}; 4mg cobalt chloride; 1g dipotassium hydrogen phosphate.

Test medium 7: FPH medium with the above composition along with 2% pure glycerol.

Test medium 8: FPH medium with the above composition along with 2% crude glycerol.

Later specific growth rate of the microorganism was measured using the following equation 4.2.

$$\text{Specific growth rate } (\mu) = \frac{\ln N_f - \ln N_i}{t_{f-i}} \quad (4.2)$$

4.2.7. Fermenter studies

The bioreactor studies were carried out in 3 L Infors Minifors bioreactor using FPH medium with crude and pure glycerol respectively. The production media was inoculated using 5% pre-inoculum, conditions were maintained at 37^0 C, 200 rpm and pH was automatically controlled at 8.5 with 5N NaOH. Samplings were taken at regular intervals for HPLC analysis.

4.3. Results and discussion

4.3.1. Estimation of N-content in production media and other nitrogen sources using Kjeldhal apparatus

After the acid pretreatment, the nitrogen content in various nitrogen sources were analyzed using Kjeldhal's method as described in the methodology section. Different cheap raw materials that considered for using as nitrogen source in production media were vermicompost, soya bean meal, ground nut oil cake, cotton seed cake, neem seed cake, corn steep liquor and fish protein hydrolysate. The nitrogen/protein content of different biomass is illustrated in Table 4.1.

Nitrogen source	Amount of nitrogen/protein
Vermicompost	130.2 mg/L
Soya bean meal	350 mg/L
Cotton seed cake	298.8 mg/L
Neem seed cake	93.8 mg/L
Ground nut oil cake	315 mg/L
Fish meal	60 mg/L
Fish protein hydrolysate	70% hydrolysed protein
Corn steep liquor	2-3 g/L

Table 4.1. Nitrogen/protein content of various low cost nitrogen substrates

After analyzing the nitrogen content in each of the sources determined, hydrolysates of soybean meal, ground nut oil cake, cotton seed cake, corn steep liquor and fish protein hydrolysate were selected. These different raw materials were then supplemented to the production media as nitrogen source individually. The production of 1,3-PDO and other by-products along with residual glycerol concentrations were analyzed.

4.3.2. Screening of different raw materials as nitrogen supplements in the production media for 1,3-PDO production

To analyze the production of 1,3-PDO using different nitrogen sources, shake flask batch experiments were carried out. The production media composition was altered in the case of nitrogen sources where soybean meal hydrolysate, ground nut oil cake hydrolysate, cotton seed cake hydrolysate, corn steep liquor and fish protein hydrolysate with varying concentrations were used. The concentrations of each nitrogen sources in the production media were 2-10%. The soybean meal hydrolysate, ground nut oil cake hydrolysate, cotton seed cake hydrolysate, corn steep liquor were taken in volume by volume. The FPH was taken in weight by volume. The glycerol and glucose concentration in the production media was 40 g/L respectively. Batch fermentations were done in 150 ml Scott Duran bottles with 100 ml working volume under anaerobic condition by sparging nitrogen in the head space. After the addition of 5% pre-inoculum, incubation was carried out at 37^0 C, 200 rpm for 48 hours. The samples were taken after 48 hours. The concentration of metabolites present in the production media were analyzed using HPLC.

It was well known that oil seed cakes are rich in crude protein, which makes it as best available sources for amino acids. In these studies, the glycerol glucose co-fermentation experiments supplemented with hydrolysates of ground oil cake (fig.4.1), soybean (fig.4.2), cotton seed cake (fig.4.3) and corn steep liquor (fig.4.4), clearly represents that, these nitrogen sources are not efficient as the peptones and meat hydrolysates utilized in the commercial production media used for lactic acid bacteria. Using ground nut cake hydrolysate, a maximum titers of 11.52 g/L 1,3-PDO, 10.39 g/L lactic acid, and 9.58 g/L acetic acid was produced with a volumetric yield of 0.28 $g_{1,3\text{-}PDO}/g_{Glycerol}$, at 10% v/v of hydrolysate supplementation to the production media (fig.4.1). Similar observations were also observed with soybean meal and cotton seed cake hydrolysates, 14.33 g/L (fig.4.2) and 11.9 g/L (fig.4.3), 1,3-propanediol with a volumetric yields of 0.35 $g_{1,3\text{-}PDO}/g_{Glycerol}$, 0.29 $g_{1,3\text{-}PDO}/g_{Glycerol}$ respectively.

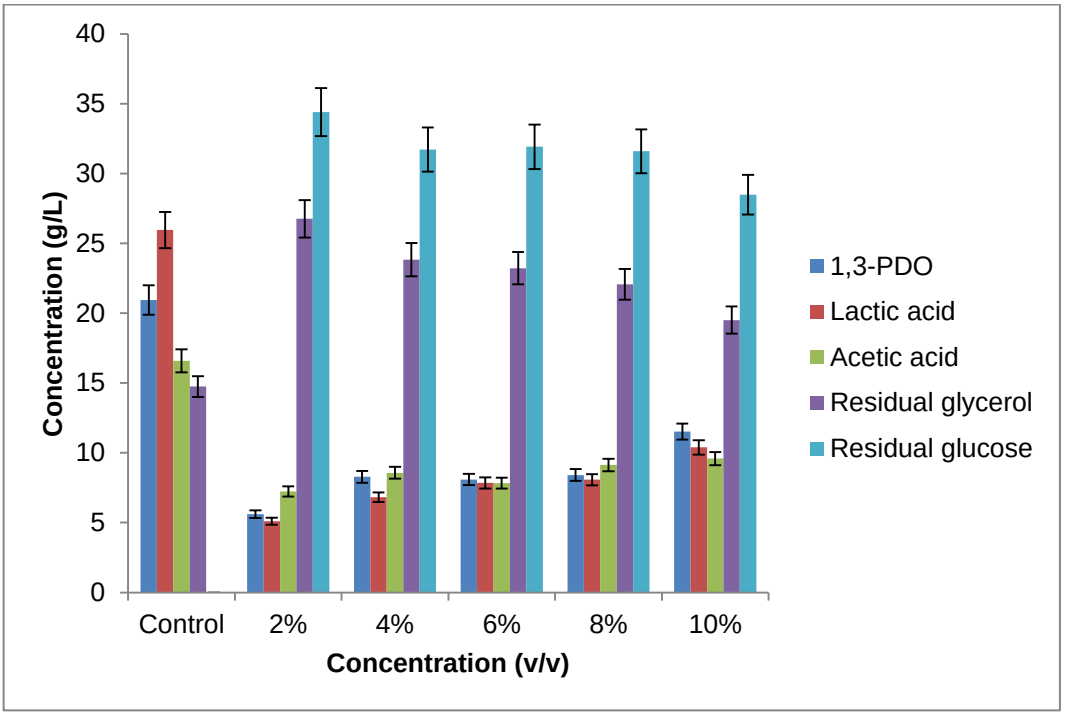


Figure.4.1. Overview of metabolite profiles of glycerol-glucose co-fermentation using ground nut seed hydrolysate as nitrogen source at varying concentrations (2-10% v/v), where control represents the optimized medium with yeast extract, peptone and meat extract B as nitrogen sources.

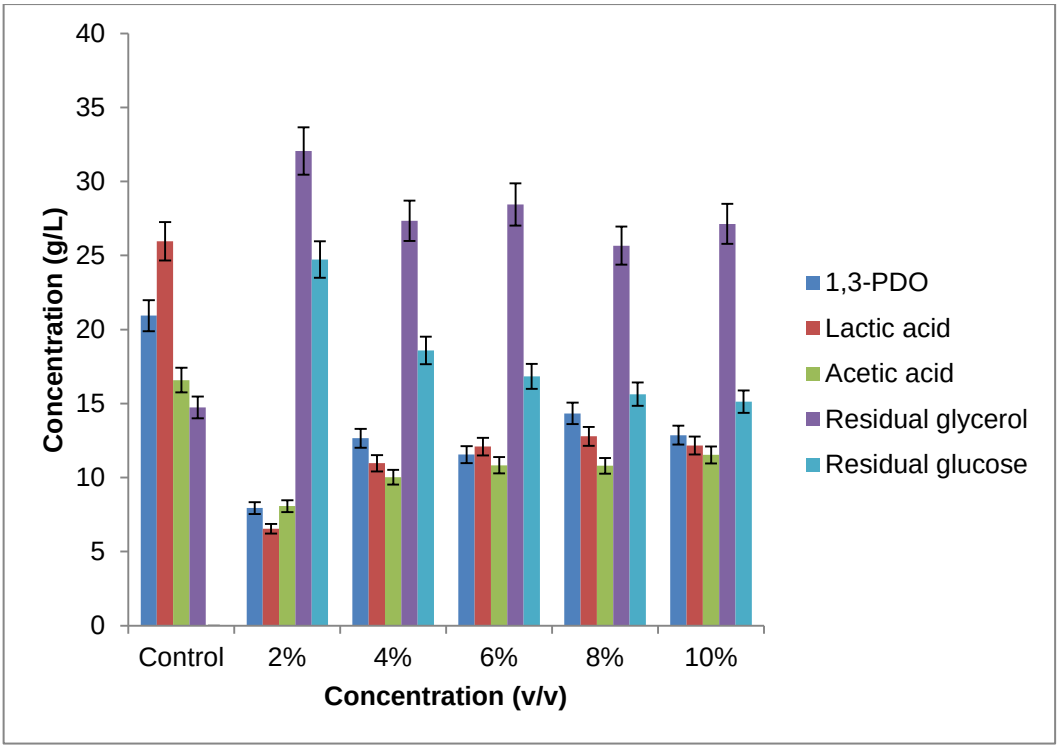


Figure.4.2. Overview of metabolite profiles of glycerol-glucose co-fermentation using soybean hydrolysate as nitrogen source at varying concentrations (2-10% v/v), where control represents the optimized medium with yeast extract, peptone and meat extract B as nitrogen sources.

Certain studies states that groundnut oil cake has been used as the nitrogen source for L-asparaginase production (Baskar and Ranganathan, 2009, Amena *et al*., 2010). In fermentations, cotton seed meal was also used as the nitrogen source to produce xylanase (Liu *et al*., 2008) and endotoxins (Salama *et al.*,1983) whereas soybean meal was used for the production of lactic acid (Kwon *et al*., 2000) and acetoin (Xiao *et al*., 2007). But in this scenario, the results were not according to the literature, where the substrate utilization rate was drastically low in comparison with the control flasks, the fact might be due to deposition of heavy metal ions during the acid hydrolysis of oil seed cakes (Kurbanoglu et al., 2003), or due to unavailability of required nutrients, amino acids and other growth factors, which are usually available in the peptone, yeast extract and meat extracts.

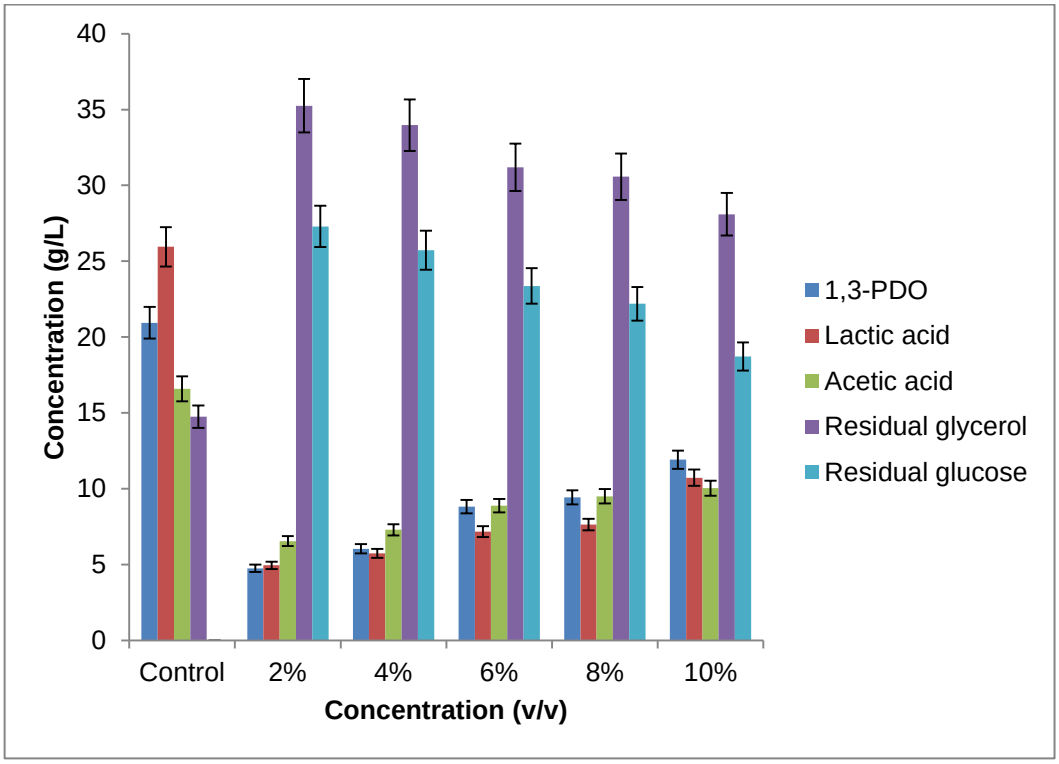


Figure.4.3. Overview of metabolite profiles of glycerol-glucose co-fermentation using cotton seed hydrolysate as nitrogen source at varying concentrations (2-10% v/v), where control represents the optimized medium with yeast extract, peptone and meat extract B as nitrogen sources.

Corn steep liquor (CSL) is a well-known cheap nitrogen source in fermentation media composition for various microorganisms, including fungi and bacteria. The CSL was used as the nitrogen source for the production of PHB (Gouda *et al.*, 2001), L-lactic acid (Yu *et al*., 2008) and succinic acid (Lee *et al.,* 2003). For *Lactobacillus brevis* N1E9.3.3 isolate, CSL as nitrogen supplement has no advantage in glucose or glycerol consumption, even in comparison with the oil seed hydrolysates the titers of 1,3-PDO are very low as 5.85 g/L with 10% v/v CSL supplementation (fig.4.4).

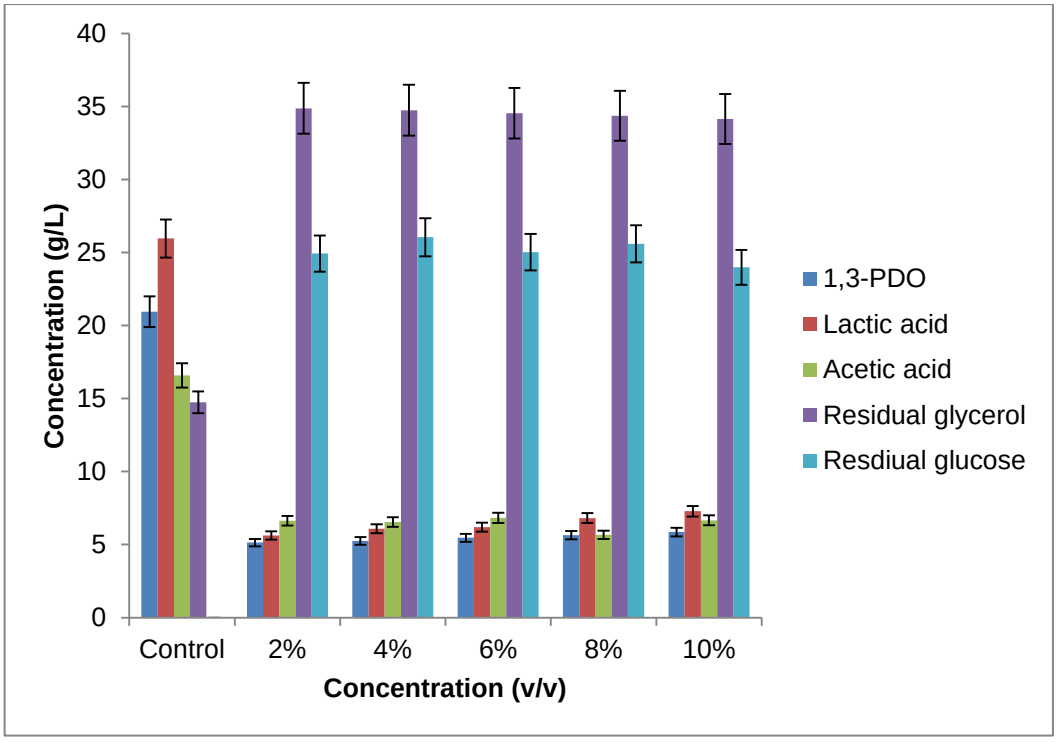


Figure.4.4. Overview of metabolite profiles of glycerol-glucose co-fermentation using corn steep liquor as nitrogen source at varying concentrations (2-10% v/v), where control represents the optimized medium with yeast extract, peptone and meat extract B as nitrogen sources.

Usually the peptones used in the commercial media are either bovine or porcine origin, as these products are of animal origin, the composition of peptides, amino acids, vitamins and minerals for growth and development of microorganism might be abundant. Eventually fish derived peptone was also considered as nitrogen supplement in growth related media for microorganisms since 1949. Fish peptone or fish protein hydrolysate is derived from hydrolysis of fish waste with endogenous enzymes at lower pH favouring the pepsin activity by hydrolysis using exogenous enzymes. The hydrolysis of fish viscera (Aspmo et al., 2005; Horn et al., 2005) may be carried out using acids, organic acids, endogenous or exogenous enzymes and combination of these techniques. Fish protein hydrolysate was used for the production of lactic acid (Gao *et al*., 2006). The idea of fish peptone as nitrogen supplement for the growth and development of lactic acid bacteria was rarely explored (Horn et al., 2005), hence there lies an important factor of improving the media composition for growth of *L. brevis* N1E9.3.3 isolate and develop a cost effective media composition for 1,3-propanediol production.

Among the selected ones, fish protein hydrolysate was found to be the best nitrogen source for the production of 1,3-PDO. At 4% w/v FPH, 23.55 g/L 1,3-PDO, 25.34 g/L lactic acid and 16.51 g/L acetic acid was observed with 0.58 $g_{1,3\text{-PDO}}/g_{Glycerol}$ volumetric yield (fig.4.5), and an increase in FPH supplementation has increased in

glycerol uptake rate and 1,3-PDO production rate. A maximum 1,3-PDO concentrations of 27.44 g/L with a volumetric yield of 0.68 $g_{1,3\text{-PDO}}/g_{Glycerol}$ was observed at 100 g/L w/v FPH in the production media, where the product yield of control experiments was 0.52 $g_{1,3\text{-PDO}}/g_{Glycerol}$.

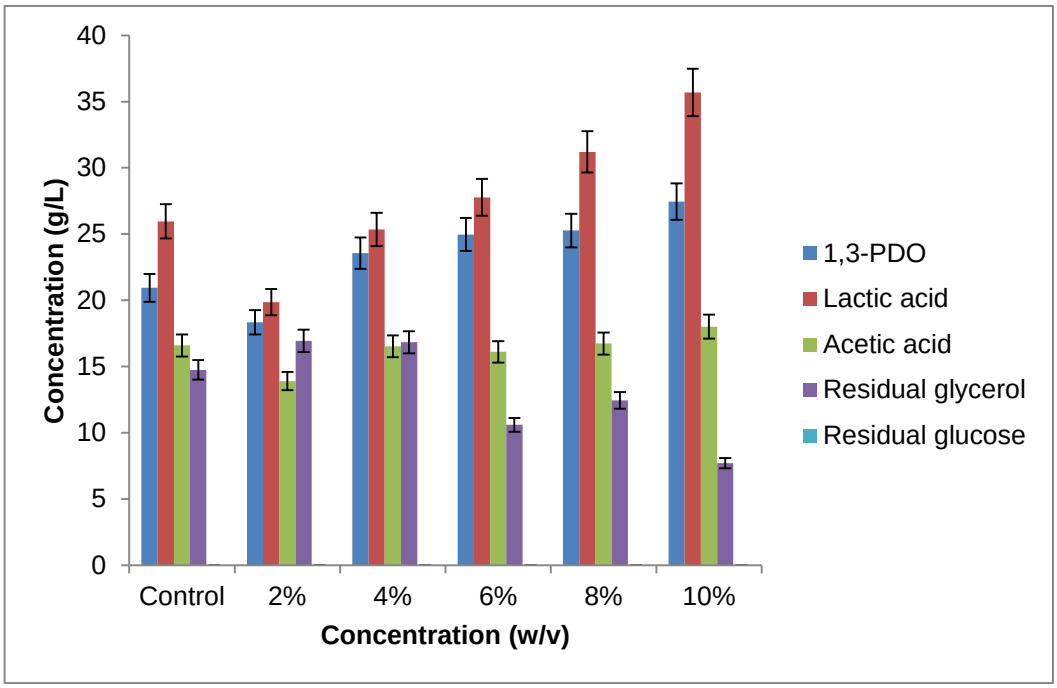


Figure.4.5. Overview of metabolite profiles of glycerol-glucose co-fermentation using fish protein hydrolysate as nitrogen source at varying concentrations (2-10% v/v), where control represents the optimized medium with yeast extract, peptone and meat extract B as nitrogen sources.

Fish peptone has ample amount of peptides, with balanced amino acid compositions with higher side of branched chain amino acids like valine, leucine, and isoleucine. It was also reported that vitamins like B_2 (Riboflavin), B_3 (Nicotinic acid) and B_5 (Pantothenic acid) was available in sufficient quantities, that plays a crucial role in growth of lactic acid bacteria (Aspmo et al., 2005).

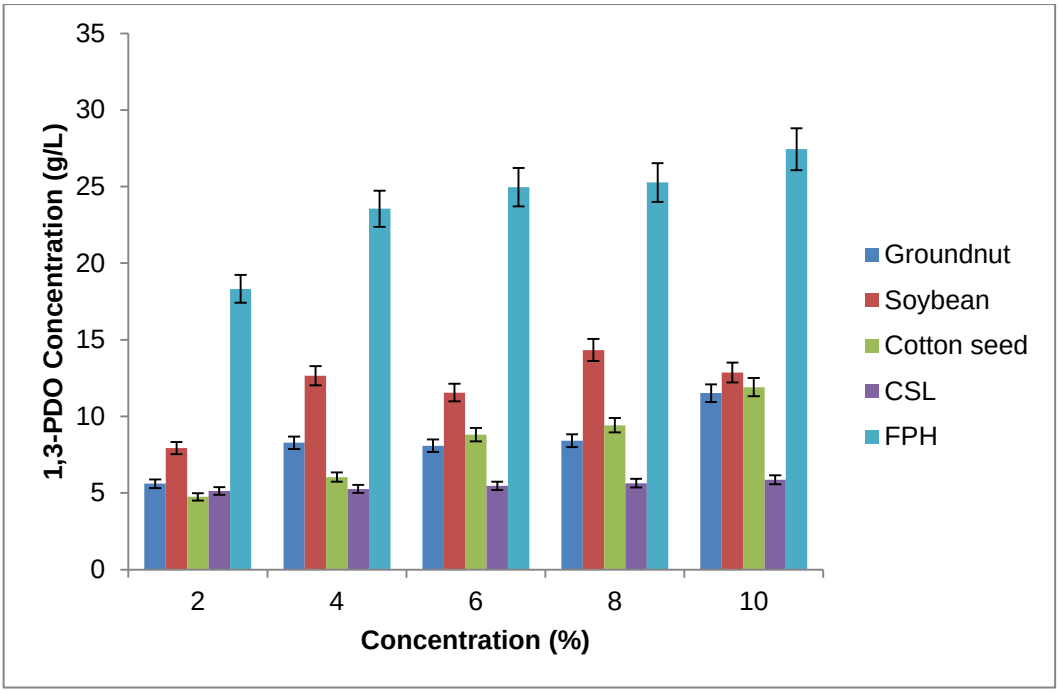


Figure.4.6. Effect of various raw materials and its varying concentrations as nitrogen sources on 1,3-PDO production.

From the results (fig.4.6), FPH was selected as the efficient nitrogen source for the growth and production of 1,3-PDO using *Lactobacillus brevis* N1E9.3.3, replacing yeast extract, peptone and meat extract. Further studies were carried out using FPH as the sole nitrogen source.

4.3.3. Evaluation of different concentrations of fish protein hydrolysate (FPH) as sole N-source supplementation for 1,3-PDO production

To determine the optimum concentration of fish protein hydrolysate by strain *L. brevis* N1E9.3.3, different concentrations (2-14% w/v) was added along with different concentrations of glycerol (60-100 g/L) and glucose (60-100 g/L) respectively. The fermentation process was carried out at 37^{o}C, 200 rpm under anaerobic conditions by sparging nitrogen and by adjusting the pH at regular intervals up to 96 hours.

In the batch experiments using 60 g/L glucose, 60 g/L pure glycerol (fig. 4.7) and 60 g/L crude glycerol (fig. 4.8), it was evident that, effect of FPH concentration has an effect on rate of glycerol utilization and metabolite production. With an increase in FPH concentration from 2-14% w/v, the metabolite flux has changed from reductive pathway towards oxidative pathway, which was evident from lactic acid (58.2 g/L) accumulation, residual pure glycerol (20.36 g/L) (fig. 4.7) and crude glycerol (23.95 g/L) (fig. 4.8) accumulation at 14 % w/v FPH, but a maximum 1,3-PDO production titers of 45.32 g/L and 43.36 g/L was observed at 4% FPH, with a volumetric yield of 0.75 $g_{1,3\text{-PDO}}/g_{\text{Pure Glycerol}}$ 0.72 $g_{1,3\text{-PDO}}/g_{\text{Crude Glycerol}}$ respectively and the by-products lactic

acid (40-50 g/L) and acetic acid (24-25 g/L) was also observed during this glycerol – glucose co-fermentation.

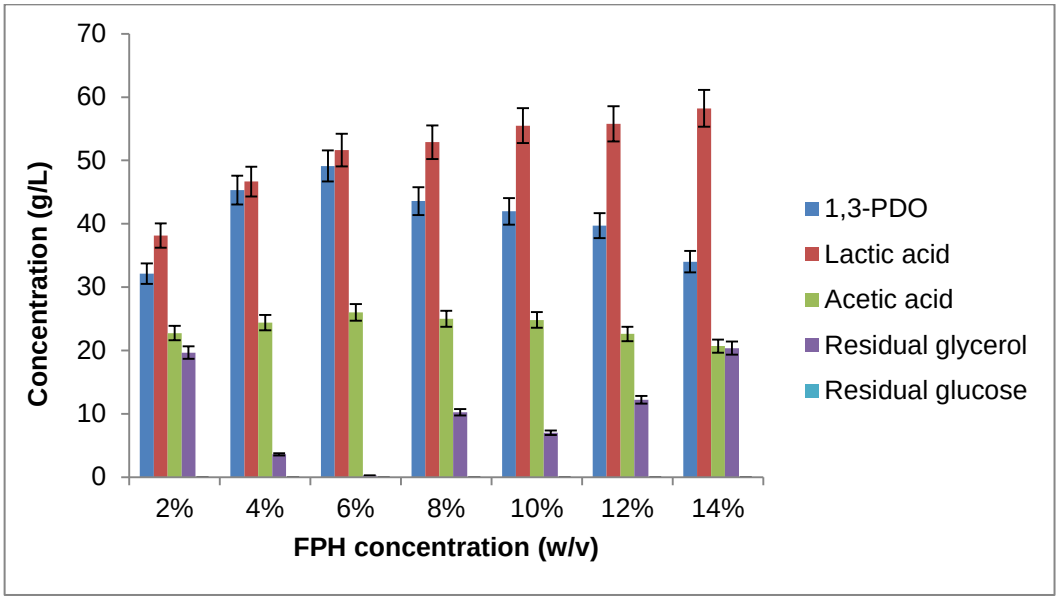


Figure.4.7. Overview of metabolite profiles of co-fermentation of 60 g/L pure glycerol and 60 g/L glucose using fish protein hydrolysate as nitrogen source at varying concentrations (2-14% v/v)

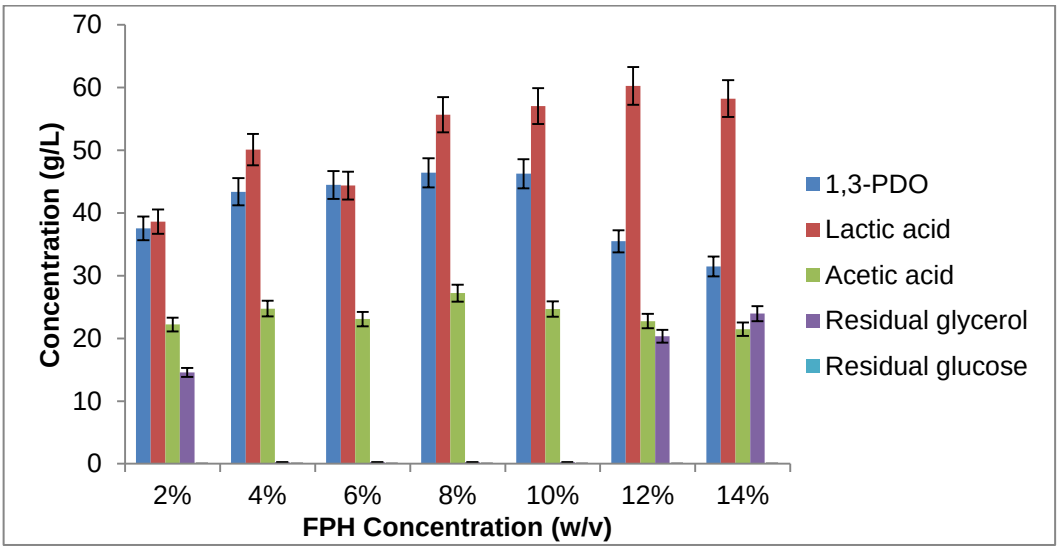


Figure.4.8. Overview of metabolite profiles of co-fermentation of 60 g/L crude glycerol and 60 g/L glucose using fish protein hydrolysate as nitrogen source at varying concentrations (2-14% v/v).

Similar volumetric yield of 0.74 $g_{1,3\text{-PDO}}/g_{\text{Pure Glycerol}}$ was observed with 4% FPH when the initial glucose – glycerol concentrations was increased to 80 g/L (fig. 4.9), where the concentration of 59.75 g/L (fig. 4.9) and 45.87 g/L (fig. 4.10) 1,3-PDO was observed with pure and crude glycerol respectively. In comparison with the

experiments carried out at 60 g/L glycerol, rate of glycerol utilization increased with increased in FPH concentration until 8%, where increase in 1,3-PDO until 63 g/L was observed at 6 % FPH, but this positive relation of nitrogen source to glycerol concentration was existed until 8 % FPH, later increase resulted in increase of residual glycerol concentration. The explanation would be the negative effect on cell growth, induced by concentration of peptides and amino acids in the production media, that influence the rate of hydrolysis of peptides by LAB, which provides free nitrogen to be transported across the membrane by ATP, linked permeases (Hsieh et al.,1999). Hence determining the appropriate concentration of nitrogen source to the carbon and substrate concentrations would be ideal to understand the cell growth and production phenomenon.

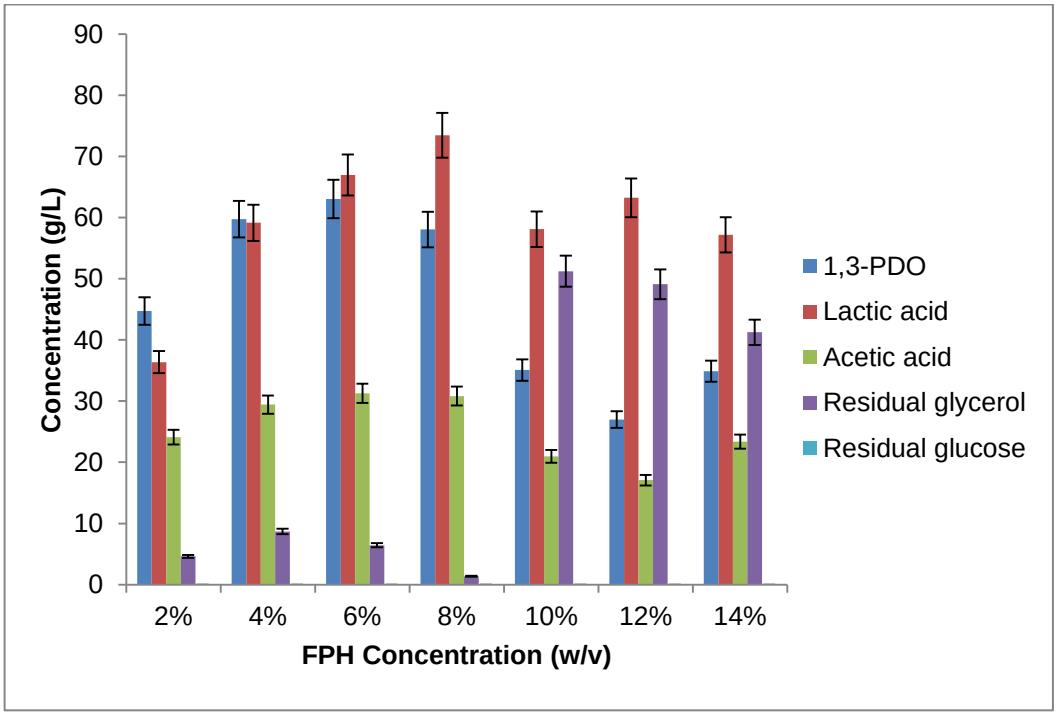


Figure.4.9. Overview of metabolite profiles of co-fermentation of 80 g/L pure glycerol and 80 g/L glucose using fish protein hydrolysate as nitrogen source at varying concentrations (2-14% v/v).

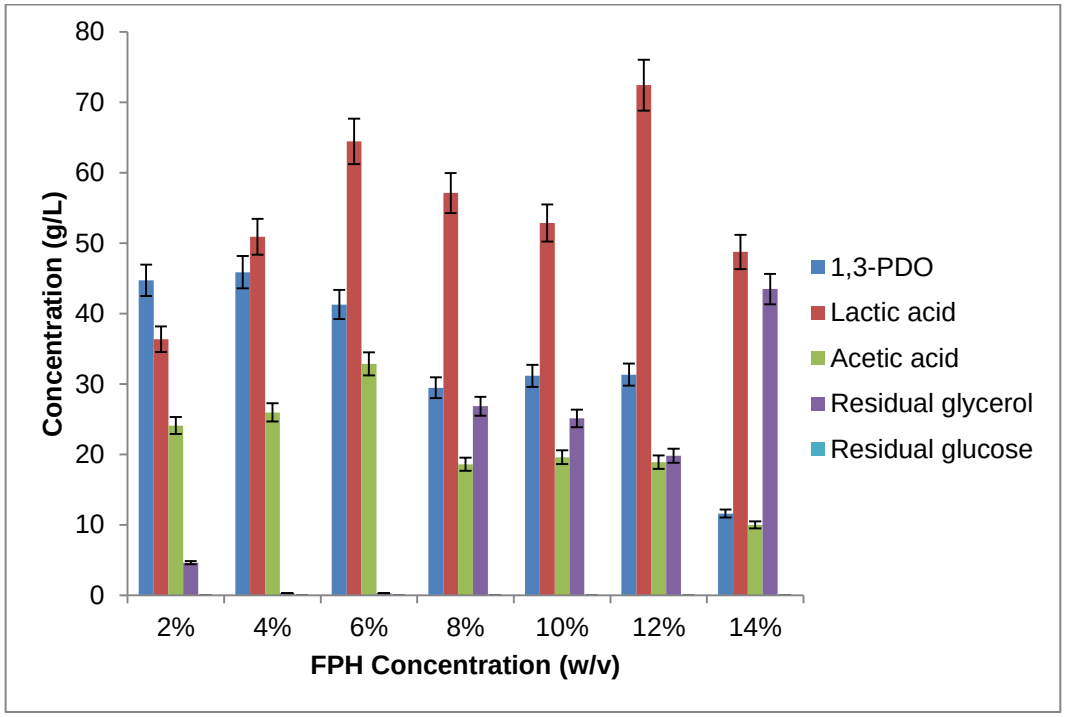


Figure.4.10. Overview of metabolite profiles of co-fermentation of 80 g/L crude glycerol and 80 g/L glucose using fish protein hydrolysate as nitrogen source at varying concentrations (2-14% v/v).

In Chapter 3, it was observed that with the optimized media concentration, at 100 g/L initial glycerol, maximum 1,3-PDO concentration of 17.54 and 20 g/L was observed with pure and crude glycerol respectively (fig. 3.5). But supplementation of FPH, as nitrogen source increased the glycerol utilization rate and 1,3-PDO production rate, hence to observe whether the phenomenon also follows, when glycerol concentration is increased to 100 g/L, an independent experiments with 100 g/L pure and crude glycerol were carried out. Incomparable metabolite profile was observed with FPH as nitrogen source, maximum 1,3-PDO titers of 53.8 g/L (fig.4.11) and 56.41 g/L (fig.4.12) with a volumetric yield of 0.53 $g_{1,3\text{-PDO}}/g_{\text{Pure Glycerol}}$ and 0.56 $g_{1,3\text{-PDO}}/g_{\text{Crude Glycerol}}$ was observed with 8 % and 6 % fish protein hydrolysate respectively.

In comparison with different glycerol – glucose concentrations in co-fermentation strategy, optimal concentration of 4 % fish protein hydrolysate provided higher volumetric yield of 0.72 – 0.75 $g_{1,3\text{-PDO}}/g_{\text{Glycerol}}$, hence further experiments in bioreactors were carried out using 4% w/v FPH as nitrogen source.

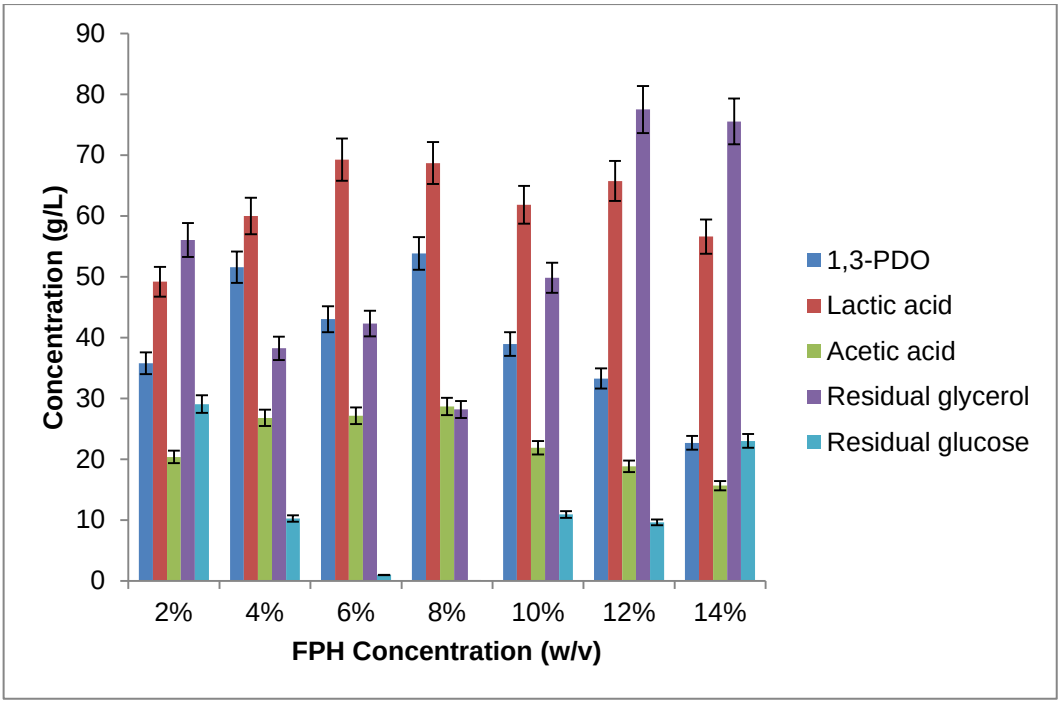


Figure.4.11. Overview of metabolite profiles of co-fermentation of 100 g/L pure glycerol and 100 g/L glucose using fish protein hydrolysate as nitrogen source at varying concentrations (2-14% v/v).

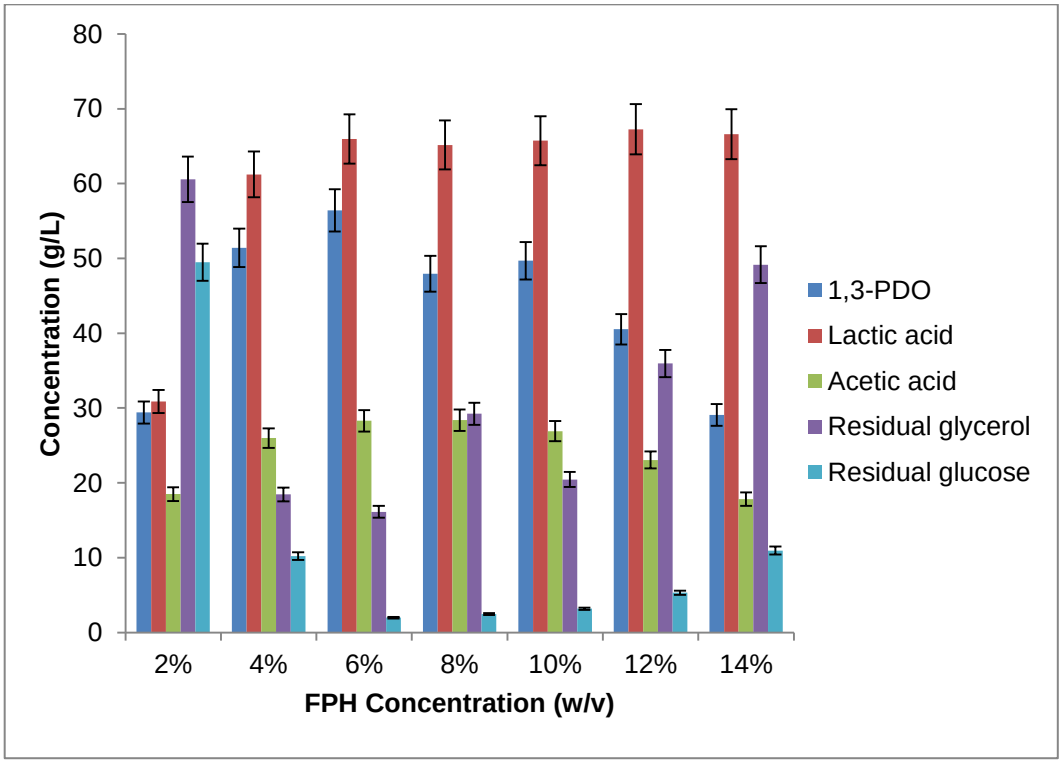


Figure.4.12. Overview of metabolite profiles of co-fermentation of 100 g/L crude glycerol and 100 g/L glucose using fish protein hydrolysate as nitrogen source at varying concentrations (2-14% v/v).

In glycerol-glucose co-fermentations using fish protein hydrolysate as sole nitrogen source, increased titers of 1,3-propanediol, and we can also observe the higher concentrations of other secondary metabolites like lactic acid (50 – 60 g/L) and acetic acid (20 – 30 g/L). There are many reports on optimization of nitrogen sources and development of cost effective medium for lactic acid production using *Lactobacillus sp.,* (John et al., 2007) with the experimental data obtained here, it is evident that FPH could be an efficient nitrogen source for lactic acid bacteria in production of various metabolites. In the literature we can find different reports using corn steep liquor, cotton seed cake, rice bran, wheat bran and other raw materials as nitrogen sources using lactic acid bacteria for D/L lactic acid production (Li et al., 2013b; Ramachandran et al., 2007). But these raw materials are primary sources of fodder for cattle, so usage of costly yeast extract and the fodder nitrogen sources can be effectively replaced by fish protein hydrolysate as nitrogen source in growth and production medium for lactic acid bacteria.

4.3.4. Comparative determination of fish protein hydrolysate (FPH) efficiency with Y.E, M.E and peptone

Synthesis of metabolites during fermentation is growth associated, and the growth depends on carbon source, macro and micronutrients. The type of nitrogen source used in the growth and production medium has a strong effect on cell growth and the concentration of nitrogen source may have either positive or negative effect on the same. Hence to understand the effect of fish protein hydrolysate in comparison with yeast extract, meat extract and peptone, following studies were carried out.

4.3.4.1. Growth analysis

To understand the effect of fish protein hydrolysate on growth of *Lactobacillus brevis* N1E9.3.3 isolate, nine different media compositions were observed along with commercial MRS medium containing 25 g/L complex nitrogen source (10 g/L Meat extract B; 10 g/L yeast extract and 5 g/L peptone). Growth on MRS yielded a maximum O.D of 6.18 (fig.4.13) with a maximum specific growth rate (μ_{max}) of 0.15 (fig.4.14) at 36 hours of incubation. The maximum specific growth rate was in range 0.13-0.15, when commercial MRS was supplemented along with pure glycerol and crude glycerol respectively. In FPHMRS media, the composition similar to commercial MRS except the complex nitrogen sources were replaced by 4% FPH, a maximum O.D obtained was 8.25 (fig.4.13) with a maximum specific growth rate (μ_{max}) of 0.16 (fig.4.14), even a higher optical density of 8.9 (fig.4.13) with a maximum specific

growth rate (μ_{max}) of 0.16 (fig.4.14) was obtained with new media composition FPH+PG, where 20 g/L glucose and 20 g/L pure glycerol was added along with 4% FPH.

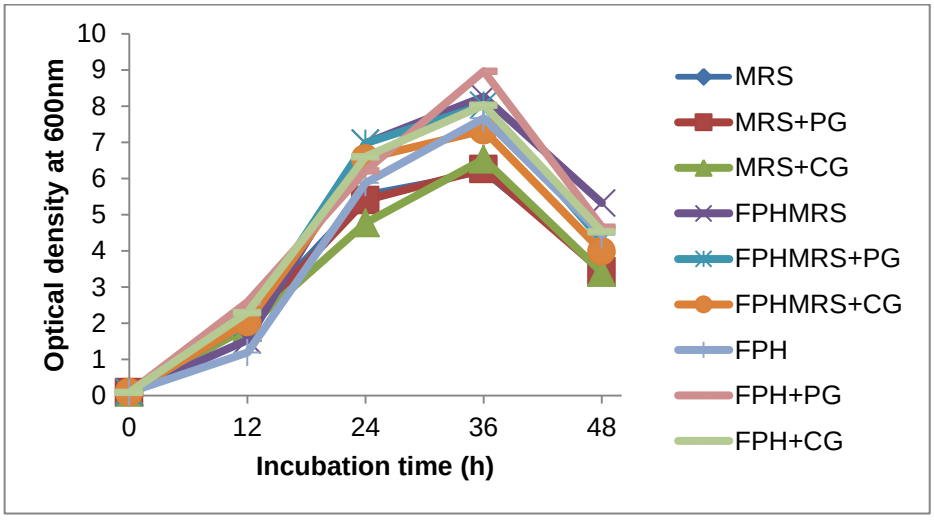


Figure.4.13. Measurement of growth analysis of *Lactobacillus brevis* N1E9.3.3 isolate using different media compositions by observing the optical density (O.D 600 nm) at regular intervals.

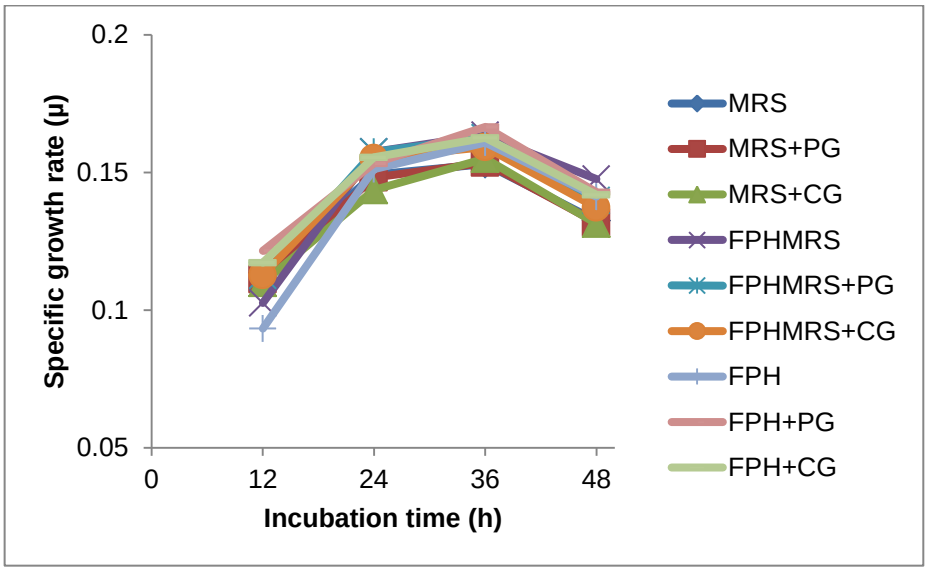


Figure.4.14. Measurement of specific growth rate (μ) of *Lactobacillus brevis* N1E9.3.3 isolate using different media.

4.3.5. Fermenter studies

With the optimum concentration of fish protein hydrolysate, i.e., 4% w/v, 80 g/L glucose, 80 g/L pure glycerol or crude glycerol and along with other micro and macro nutrients, batch experiments were carried out in bioreactors, after the successive trails in shake flask experiments. The fermentation was carried out in 3 L Infors Minifors bioreactor with a working volume of 1.5 L, and incubation was carried out at 37^{o}C, 200

rpm, pH – 8.5±0.5, for 120 hours and sampling was carried out at every 24 hour intervals to analyze the secondary metabolites and observe the biomass growth by measuring the optical density at 600nm. To ensure the incubation is carried out in anaerobic conditions, high purity nitrogen was sparged into production media at 0.2 vvm for 1 hour before and after inoculation and later sparging was continued for 1 hour for every 24 hours.

Fig.4.15. represents the data obtained from 80 g/L glucose, 80 g/L pure glycerol, co-fermentation supplemented with 4% w/v FPH as the sole nitrogen source. It was evident that the 1,3-PDO production is growth dependent, the maximum biomass O.D of 9.0, was observed at 96 hours and then maintained until 120 hours, as the glucose concentration was depleted in the production media, the scenario was also in agreement with the concentrations of secondary metabolites like lactic acid, acetic acid and 1,3-PDO, where there is no sharp increase in the concentrations in comparison with the profile obtained from 0^{th} hour to 96^{th} hour. A maximum 1,3-PDO concentration of 57.9 g/L with a volumetric yield of 0.72 $g_{1,3\text{-}PDO}/g_{Pure\ glycerol}$ and productivity of 0.48 g/L/h was observed. Similarly, the batch co-fermentations with similar concentrations using crude glycerol, maximum titers of 57.84 g/L, with a volumetric yield of 0.72 $g_{1,3\text{-}PDO}/g_{Pure\ glycerol}$ and productivity of 0.60 g/L/h was observed (fig.4.16). In comparison, the growth of *L.brevis* N1E9.3.3 strain in production media supplemented with crude glycerol and glucose, observed decline in growth from 72^{nd} to 120^{th} hr as glucose and glycerol was completely utilized, whereas production media with pure glycerol, the substrate and carbon uptake rate was slightly on lower side due to, effect of crude glycerol composition, which is not causing the cessation of microbial growth. Along with 1,3-PDO, 50-58 g/L lactic acid and 25-28 g/L acetic acid was observed in the production media with pure and crude glycerol respectively. As per the literature report by Pflugl et al., 2012; concentration of acetic acid and 1,3-PDO was in positive correlation, where 0.1 mol acetic acid corresponds to 0.2 mol 1,3-PDO. During 1,3-PDO fermentation under anaerobic condition, acetic acid was the most inhibitory by product along with lactic acid and ethanol for *K. pneumoniae* (Cheng *et al*., 2005). But in this scenario, maximum 58 g/L lactic acid and 28 g/L acetic acid, has no profound inhibitory effect on growth and 1,3-PDO production.

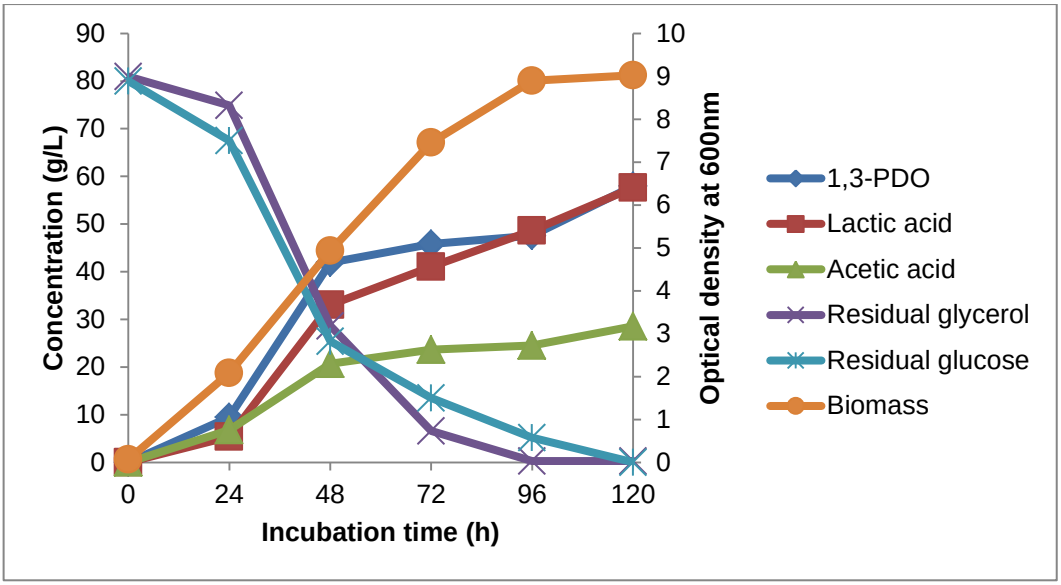


Figure.4.15. Metabolite profile and biomass analysis of *Lactobacillus brevis* N1E9.3.3 strain, in production media with 4% w/v fish protein hydrolysate, 80 g/L glucose and 80 g/L pure glycerol.

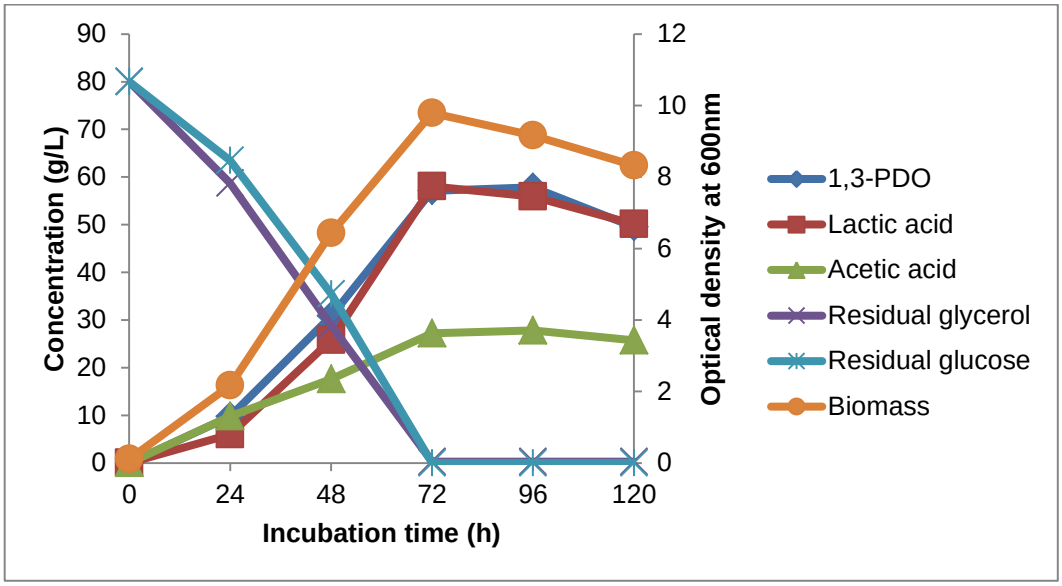


Figure.4.16. Metabolite profile and biomass analysis of *Lactobacillus brevis* N1E9.3.3 strain, in production media with 4% w/v fish protein hydrolysate, 80 g/L glucose and 80 g/L crude glycerol.

4.3.6. Evaluation of 1,3-Propanediol production efficiency of *Lactobacillus brevis* N1E9.3.3 in mMRS and FPH medium.

Depending on the biocatalyst and the culture conditions, different secondary metabolites like, lactate, formate, succinate and ethanol, were generated in variable amounts (Dabrock *et al*., 1992). There was a positive correlation between the acetic acid and 1,3-PDO production, whereas negative between lactic and 1,3-PDO, as these

two metabolic pathways compete for reducing equivalents. The observations noticed in mMRS production media (Chapter 3, fig. 3.4 and fig. 3.5) where the lactic acid concentration increased with the decrease in 1,3-PDO production, claim that change of metabolic shift of reducing equivalents from reductive pathway towards oxidative pathway. During glycerol – glucose co – fermentation, glucose is consumed more rapidly than glycerol during the early log phase and was exhausted before glycerol and 1,3-PDO synthesis is observed only when the carbon source is available (Vaidyanathan *et al*., 2011), this phenomenon is only applicable in lactic acid bacteria. But in the FPH substituted production media, it is clear that a balanced redox potential is maintained between the oxidative and reductive pathway of glycerol metabolism (fig.4.15 and fig.4.16).

As new production media using fish protein hydrolysate was developed for 1,3-PDO production by *Lactobacillus brevis* N1E9.3.3 isolate, a comparison made with the production titers of 1,3-PDO and the volumetric yields between modified MRS and FPH production media (Table. 4.2 and Table. 4.3). With a theoretical maximum of 0.826 $g_{1,3\text{-PDO}}/g_{Glycerol}$, *L.brevis* N1E9.3.3 strain, achieved maximum yield of 0.81 at an initial glycerol concentration of 60 g/L, though further improvement resulted in decreased yield to 0.72 $g_{1,3\text{-PDO}}/g_{Glycerol}$, maximum titers was observed using pure and crude glycerol.

Pure Glycerol concentration (g/L)	1,3-PDO (g/L)		Yield ($g_{1,3\text{-PDO}}/g_{Glycerol}$)	
	FPH production media	mMRS	FPH production media	mMRS
40	28.20	23.43	0.705	0.585
60	49.11	38.66	0.818	0.644
80	57.9	55.12	0.723	0.689
100	53.80	28.54	0.672	0.285

Table.4.2. Comparative study of 1,3-PDO titers and yield with different concentrations of pure glycerol (40-100 g/L) using FPH as sole nitrogen source and mMRS with yeast extract, meat extract B and peptone as nitrogen sources respectively. The values represented in the table are average values of three independent experiments, with the standard deviations < 5%.

Crude Glycerol concentration (g/L)	1,3-PDO (g/L)		Yield ($g_{1,3\text{-PDO}}/g_{Glycerol}$)	
	FPH production media	mMRS	FPH production media	mMRS
40	27.74	21.24	0.693	0.531
60	43.36	34.6	0.722	0.576
80	57.84	51.51	0.723	0.643
100	56.41	39.26	0.564	0.392

Table.4.3. Comparative study of 1,3-PDO titers and yield with different concentrations of crude glycerol (40-100 g/L) using FPH as sole nitrogen source and mMRS with yeast extract, meat extract B and peptone as nitrogen sources respectively. The values represented in the table are average values of three independent experiments, with the standard deviations < 5%.

Various strains of lactic acid bacteria like *Lactobacillus reuteri*, *L. panis*, and *L. diolivorans*, were reported for 1,3-PDO production, a maximum titers of 41.7 and 46 g/L was observed with a volumetric yield of 0.59 and 0.55 $g_{1,3\text{-PDO}}/g_{Glycerol}$ using *L. diolivorans* and *L. reuteri* respectively. Unlike other strains of LAB, *L. brevis* N1E9.3.3 strain has displayed a potential in conversion of pure and crude glycerol to 1,3-propanediol and with significant amount of by-products like lactic and acetic acid. With this cost-effective and efficient production media, our strain *L. brevis* N1E9.3.3 can be further evaluated and considered for industrial significance.

4.4. Summary and conclusions

The current study on 'Evaluation of cost effective raw materials as an alternative nitrogen supplement for 1,3-propanediol production' was carried out by realizing the economical requirements of microbial processes for industrialization. To attain the economic feasibility, a variety of cost effective raw materials were analyzed as the nitrogen supplements in the production media. Commercially available peptone, yeast extract and beef extract causes the upstream processing economically unfeasible. From this study, fish protein hydrolysate (FPH) was observed to be an efficient alternative nitrogen source in the fermentation media for 1,3-PDO production. The fish protein hydrolysates are promising alternative nitrogen sources for lactic acid bacteria and other food grade bacteria, because they support the growth and metabolite synthesis. The nitrogen/protein content analysis proved that the fish protein hydrolysate has higher concentrations in comparison to other raw materials. The experiments conducted proved the efficiency of *L. brevis* N1E9.3.3 strain to withstand higher concentrations of glycerol and capable of tolerance to higher concentrations of end products like lactic acid and 1,3-propaendiol when supplemented with FPH. The increased yield and production of 1,3-PDO and other metabolites was observed in the FPH substituted media than that of mMRS media. The 1,3-PDO concentration and maximum yield obtained in this study were 57.9 g/L and 0.72 g1,3-PDO/gGlycerol respectively. Our preliminary data indicate that using fish protein hydrolysate as the nitrogen source, a growth medium can be formulated for lactic acid and other food grade bacteria.

Chapter 5

Separation and purification of 1,3-propanediol from the fermented broth using aqueous two phase extraction using dual inorganic electrolytes

Chapter 5: Separation and purification of 1,3-propanediol from the fermented broth using aqueous two phase extraction using dual inorganic electrolytes

5.1. Introduction

Polytrimethylene terephthalate (PTT) is a potential bio-based polymer with excellent physical and mechanical properties compared to commercially available polyethylene terephthalate (PET) and Polybutylene terephthalate (PBT). This polymer has various applications in carpet making, textile industries, and polymer & coating materials. PTT is synthesized through polycondensation of monomeric 1,3-propanediol (1,3-PDO) and a dicarboxylic acid i.e., terephthalic acid. The characteristic of the polymer depends on the purity of the raw materials and the commercialization of the polymer depends on the availability of the raw materials (Bhatia & Kurian, 2008; Kaur et al., 2012b).

Biological process for 1,3-propanediol production address the limitations of chemical synthesis like usage of toxic raw materials, expensive catalysts and unfavorable process conditions. One of the major limitations in the biological process for the production of 1,3-PDO is downstream processing. Low titers of 1,3-propanediol in the fermentation broth, high boiling point, and strong hydrophilic nature makes the process relatively difficult in separation and purification of 1,3-PDO from the fermented broth. The downstream processing of 1,3-PDO from the fermented broth involves following steps

(i) Removal of microbial cells, mostly by centrifugation, membrane filtration, or flocculation.

(ii) Removal of impurities like proteins, nucleic acids, polysaccharides, salts, residual glycerol, by-products like lactic acid, acetic acid and separation of 1,3-PDO.

(iii) Final step is purification of 1,3-PDO by vacuum distillation or preparative chromatographic techniques (Anand et al., 2011).

Several strategies evaluated for separation of 1,3-PDO from the fermented broth were liquid-liquid extraction (Malinowski, 1999), reactive extraction (Malinowski, 2000; Hao et al., 2005), ion exchange resins (Rukowicz et al., 2014), pervaporation (Kaur et al., 2015), ionic liquids (Lee et al., 2015; Müller & Górak, 2012), molecular distillation (Wang et al., 2013) and electro dialysis (Wu et al., 2011). Each of the strategy mentioned above has its own pros and cons and none of the process was mentioned as the effective and efficient downstream process except liquid-liquid extraction. The liquid-liquid extraction has several advantages as the process with ease can be

scaled-up with less energy requirements, but the technique is not applicable for bio-derived chemicals, because these chemicals are highly hydrophilic and couldn't find a suitable solvents for extraction and recovery. Hence designing a novel process or performing improvements or combinations to the known conventional process in regard to increase the end product yield, purity with lower energy consumption would be desirable.

Since 1956 an aqueous two phase system (ATPS) was mentioned as the effective system for separation of biomolecules. In ATPS method, an inorganic electrolyte was used as salting out agent and an organic solvent as an extractant targeting the hydrophilic molecule in the aqueous solution (Li et al., 2013c). The separation of 1,3-PDO was carried out in three different ATPS systems hydrophilic solvent/inorganic salt, hydrophobic solvent/inorganic salt or amphipathic chemicals/inorganic salts based extraction (Li et al., 2015; Aydoğan et al., 2010). The salting out extraction leads to phase separation with the aid of organic solvents. Inorganic salts added to the mixed solution lead to decrease in the solubility of 1,3-propanediol in the aqueous solution and then the solute attains the solubility in the organic phase (Li et al., 2015). The hydration efficiency of organic solvents and cationic behaviour or acidity of inorganic electrolytes greatly affects the formation of two phases in ATP system. The top phase of the ATPS extraction is rich in diols and bottom phase with sugars, salts, acids and residual glycerol. The process scheme representing the salting out extraction of 1,3-PDO is showed in fig.5.1.

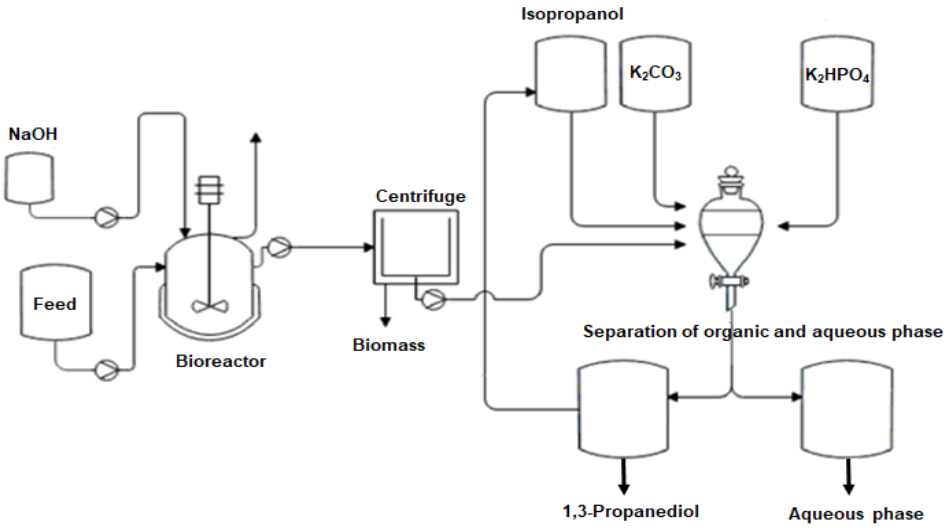


Figure.5.1. Schematic representation of aqueous two phase extraction of 1,3-propanediol from the fermented broth.

The ATPS extraction is more environmental friendly as no energy is required for heating the broth which is required in distillation, however to make the process more economical the inorganic salts can be precipitated by evaporating the water, that requires energy. It is well known that usage of K_2CO_3 was efficient in enrichment of tertiary butanol aqueous solutions, similarly potassium salts used to attain liquid – liquid equilibrium. Hence two different salts K_2CO_3 and K_2HPO_4 were selected.

In the present study the broth obtained after the fermentation of *Lactobacillus brevis* N1E9.3.3 for 1,3-PDO production was evaluated for downstream processing. The major end products in the broth were lactic acid and acetic acid in addition to 1,3-PDO. This work aimed at optimization of potassium carbonate concentration, evaluation of dual inorganic electrolyte combinations for salting out in regard to partition co-efficient (K) of 1,3-PDO and recovery percentage (Y). Effect of physical conditions were evaluated in order to promote the efficacy of ATPS system in separation and purification of 1,3-PDO.

5.2. Materials and methods

5.2.1. Chemicals and solvents

The chemicals and solvents used in this study like potassium carbonate (K_2CO_3), dipotassium hydrogen phosphate (K_2HPO_4) and isopropanol were analytical grade and procured from SRL chemicals (India).

5.2.2. Removal of biomass from fermented broth

The production media components and process operation was as explained in the Chapters 2 and 3. The spent media or fermented broth was collected and stored at 4^oC for further use after each batch operations in the bioreactor. Later the fermented broth was centrifuged at 8000 rpm for 5 minutes to remove the cells. The supernatant obtained was used for further separation and purification studies.

5.2.3. Salting out extraction of 1,3-propanediol

To the culture supernatant obtained after centrifugation, inorganic salts were added and then isopropanol was added to equal volume. Then the mixture was vertexed for 5 mins on vortex machine and incubated for separation at 4^oC for 12 hours to achieve equilibrium between the top and bottom phase. It is evident that the concentration of mono or dual inorganic salt concentrations varied the partition co-efficient of 1,3-PDO, hence different salt concentrations were investigated. Later the effect of pH on clarified broth and incubation temperature on partition behavior of 1,3-

PDO was evaluated using response surface methodology (Central Composite Design). In the fermentation broth according to the literature 1,3-PDO concentration varies from 20-150 g/l, hence the salting out effect of optimized conditions on respective concentrations were investigated. All the experiments carried out in this study were performed in triplicates and the average values were presented, the standard error observed was $p<0.05$.

5.2.4. Statistical optimization of effect of pH and temperature on ATPS extraction using central composite design

RSM-Central Composite Design (CCD) was employed in this study to investigate the effect of physical parameters like pH and temperature on partition and recovery of 1,3-PDO in K_2CO_3+K_2HPO_4/Isopropanol ATPS system. Each factor pH and temperature was studied in three levels (-1, 0, +1), i.e., pH (3, 7.5, 12) and temperature (25, 42.5, 60) in a set of 14 experiments that consists of 4 axial points, 3 center points in the axial, 4 cube points and 3 center points in the cube. The experiments were conducted in duplicates and the average value of partition co-efficient and recovery yield was taken as the response to evaluate the CCD design. The regression equation obtained resulted in an empirical model relating the partition co-efficient and recovery yield responses to the independent variables like pH and temperature. The optimum values of the parameters including pH and temperature were obtained by substituting the required values in the regression equation and by analyzing the response surface plots. The RSM experiments were designed using Minitab 17 software.

5.2.5. Parameters involved in salting out extraction

In the ATP system the optimized concentration of inorganic electrolyte and organic solvent was conditioned based on different parameters like partition/distribution co-efficient (K), recovery percentage (Y), selectivity co-efficient of 1,3-PDO to lactic acid, selectivity co-efficient of 1,3-PDO to acetic acid, removal of lactic acid (R_{la}), removal of acetic acid (R_{aa}), yield ($Y_{1,3\text{-}PDO}$)and purity of 1,3-PDO.

$$K = \frac{C_t}{C_b} \qquad (5.1)$$

$$Y = \frac{C_t}{C_{fb}} \times 100\% \qquad (5.2)$$

$$R_{la} = \frac{C_b}{C_{fb}} \qquad (5.3)$$

$$R_{aa} = \frac{C_b}{C_{fb}} \quad (5.4)$$

$$S_{1,3-PDO} = \frac{1,3-PDO_{(g)}}{by-products_{(g)}} \quad (5.5)$$

The terms mentioned in the above formulae are; C_t: Concentration in top phase; C_b: Concentration in bottom phase; C_{fb}: Concentration in the fermented broth; $S_{1,3\text{-PDO}}$: Selectivity of 1,3-propanediol; Byproducts (g): Total amount of lactic acid and acetic acid.

5.2.6. Final purification of 1,3-propanediol by gradient column chromatography

The purification of ATPS extracted crude 1,3-PDO was carried out by employing silica gel column chromatography with gradient elution. For the purification step, a glass column of size 120 cm length x 5 cm inner diameter was packed with a silica gel (Mesh size) for 90cm with a packed volume of 900ml dissolved in hexane and later column was stabilized using mobile phase (90:10; chloroform : methanol). Mobile phase with increasing polarity (90:10, 85:15, 80:20, 70:30, 75:25, and 50:50) was eluted out of column at a flow rate of 15 ml/min and 100 ml of the fractions was collected at regular intervals of time and later the samples are distilled to separate mobile phase and analyzed by HPLC.

5.2.7. Characterization of purified 1,3-propanediol

After the purification of the crude 1,3-propanediol using silica gel column chromatography, the sample was analyzed on HPLC and the sample which provided a single peak after elution was subjected to ^{13}C NMR (125MHz, $CDCl_3$) and ^{1}H NMR (500 MHz, $CDCl_3$) studies in Bruker NMR spectrophotometer.

5.3. Results and discussion

5.3.1. Effect of organic solvent on two phase formation and 1,3-PDO partition

1,3-propanediol is hydrophilic and readily soluble in water, due to its higher boiling point it is hard to separate and purify. In aqueous two phase systems, 1,3-PDO partitions into organic phase, but the partition depends on the polarity of the organic solvent used. A report evaluated the use of aldehydes and hydroxyl group containing solvents for extraction, but the aldehydes were not cost effective to be scaled up in an

industrial scale. The partition co-efficient K depends on the type of hydrophilic alcohol, Zhiyuan Song reported the efficiency of various organic solvents like methanol > ethanol > isopropanol in extraction of 1,3-propanediol (Song et al., 2013). Usage of methanol in larger quantities is not recommended, because it is explosive and commercial ethanol production does not meet the industrial requirement for ATPS extraction. The experimental result indicated that the phase formation is appropriate when K_2CO_3 and isopropanol (fig.5.2 C) were used, but methanol did not form two phases (fig.5.2 A). 1,3-PDO has higher partition co-efficient (K=1.4) in isopropanol, which is higher and not in comparison with the result published by Wu et.al., (2012). Hence an ATPS system using K_2CO_3/Isopropanol was investigated further.

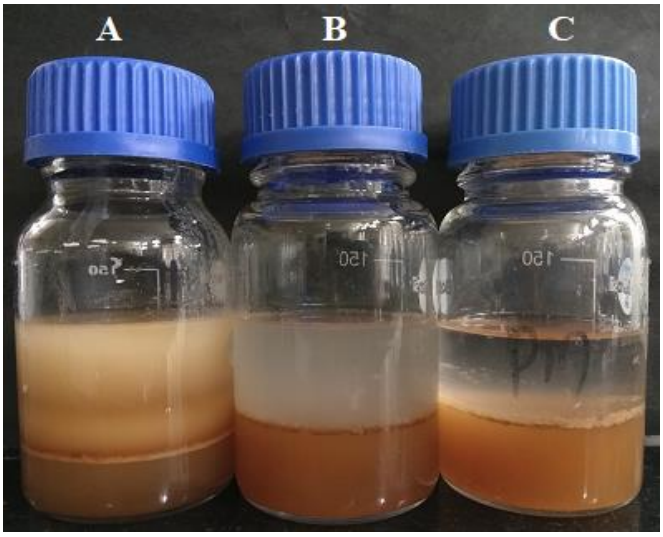


Figure. 5.2. Selection of the organic solvent (Methanol (A); Ethanol (B); Isopropanol (C)) for phase separation using K2CO3 as inorganic salt.

5.3.2. Optimization of K_2CO_3 concentration for ATPS extraction

The partition behavior of organic acids and 1,3-propanediol varies with different inorganic salts and organic solvents used for salting out extraction (Bensch et al., 2007). Hence this difference in partition behavior provides an opportunity to evaluate the combination of inorganic/organic salts and solvents to separate 1,3-propanediol from the fermented broth. When the potassium carbonate (K_2CO_3) and 2-propanol (Isopropanol) was used for salting out extraction (SOE), lactic acid and acetic acid tends to distribute into bottom phase or the salt phase and 1,3-PDO into top organic phase. The electrolytes and their concentration has strong influence on the partition behavior of 1,3-PDO, the electrolyte hydrates with the water molecules, due to the ionic strength and the concentration of the salt salting out and phase formation takes

place resulting in partition of hydrophilic molecules either into organic phase or in the aqueous phase (Gu et al., 2014; Dai et al., 2014).

The distribution of lactic acid, acetic acid and 1,3-propanediol between the top organic phase and bottom salt phase with increasing K_2CO_3 concentration was investigated with regards to partition co-efficient (K) and recovery yield (Y). The K and Y values of 1,3-PDO was observed to increase with the increase in K_2CO_3 concentration upto 60% with a K value 39.19 (fig.5.3 A) and 97.5 (fig.5.3 B) recovery percentage followed by decline in the K and Y values.

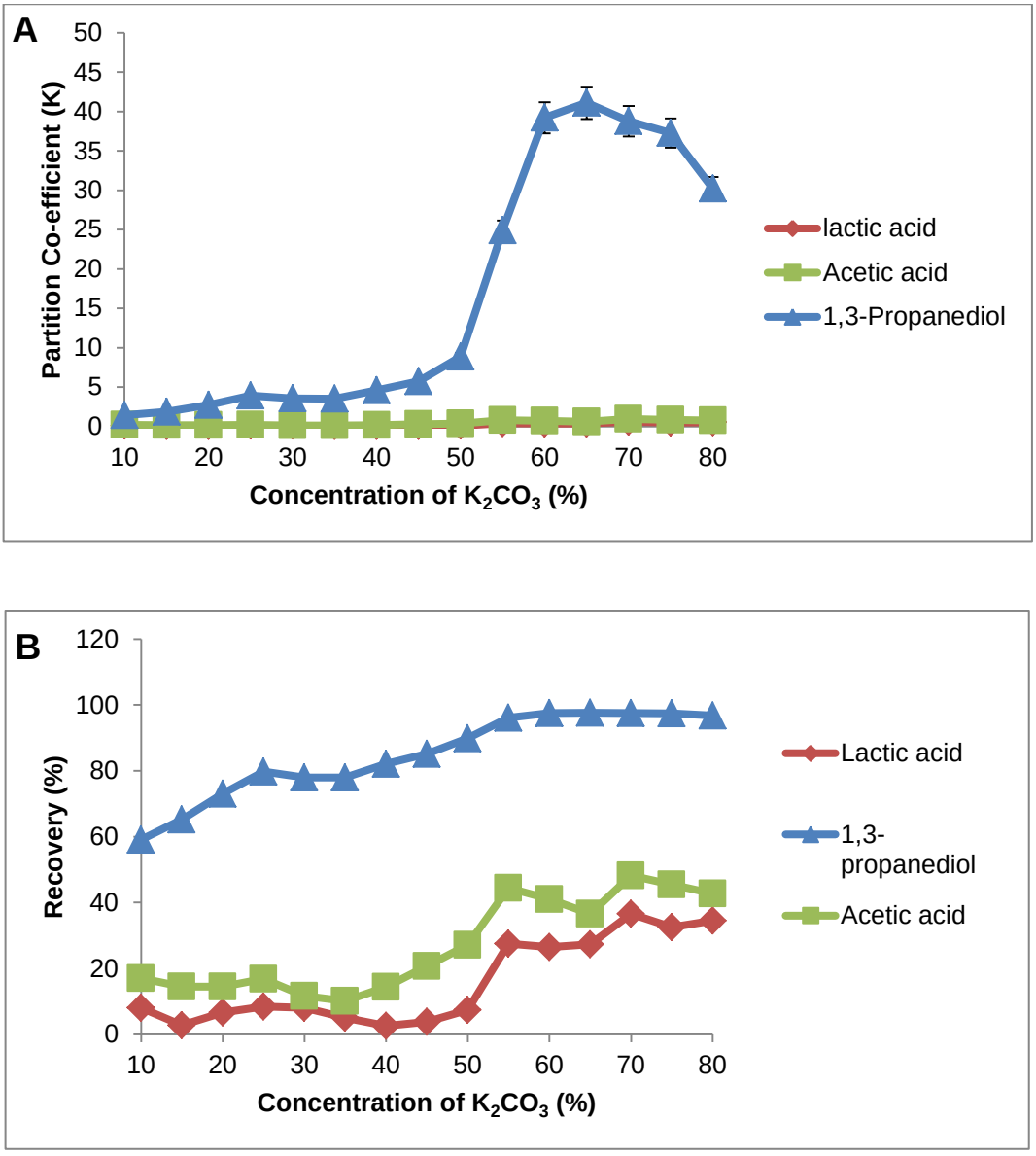


Figure.5.3. Effect of K_2CO_3 concentration on partition co-efficient (A) and recovery percentage (B) of 1,3-propanediol, lactic acid and acetic acid from the fermented broth. K2CO3 concentrations: 10-80% (w/v)

The partition co-efficient and recovery yield units observed in these experiments were comparatively higher than the other ATPS systems like ethanol/sodium carbonate with K value of 8 and recovery yield of 92 % (Li et al., 2013c), and pentanol/sodium phosphate system with K value of 3.72 and recovery yield of 72% (Wu & Wang, 2012). On contrary distribution co-efficient of lactic acid and acetic acid (fig.5.3 A) was observed to be 0.35 and 0.69 with recovery yield (fig.5.3 B) of 26.4 % and 41 % respectively. The selectivity co-efficient of 1,3-PDO over lactic acid and acetic acid tends to increase with increased salt concentration. More than 50% of the organic acids present in the fermented broth was retained, that adds an advantage for an effective separation of 1,3-PDO. K_2CO_3 as the salting out inorganic electrolyte was reported earlier by many groups with similar (Song et al., 2013) and different (Wu & Wang, 2012) solvent systems that K_2CO_3 has low efficiency in salting out, but the results observed were not consistent with those published earlier due to the concentration of inorganic salts used in this experiments. With 1:1 v/v ratio of 2-propanol and fermented broth, 60% K_2CO_3 as effective concentration further experiments were carried out.

5.3.3. Evaluation of dual inorganic electrolytes for ATPS extraction

Wu & Wang (2012) reported on ATPS system for 1,3-PDO purification has mentioned on effect of ionic strength of the inorganic electrolytes on partition of 1,3-PDO, sulphates, carbonates has higher ionic strength compared to phosphates, hence the same with the partition of 1,3-PDO. Because the ionic strength explains the charge distribution on the surface of the molecule which in turn explains the hydration efficiency with the water molecules, lower the charge distribution, and lower the hydration efficiency (Fu et al., 2015). Although we can explain the various parameters that provides an explanation for the ATPS system, the exact mechanism underlying the partition of 1,3-PDO from aqueous phase to organic phase is unknown. K_2HPO_4 as a single salt system in ATPS is reported for recovery of 1,3-PDO (Xie et al., 2016), In order to increase the distribution behavior of 1,3-PDO, dual inorganic electrolytes were added into the fermentation broth for salting out extraction. Wu & Wang (2012) experimental observation elucidates the comparison of distribution co-efficient between various mono and dual inorganic electrolytes, in which K_2HPO_4 increased the distribution co-efficient of 1,3-PDO with K_2CO_3 at an equal concentrations of 1:1.

In this experiment with optimal salt concentration of 60%, individual ratios of K_2CO_3 and K_2HPO_4 were varied (55+5%, 50+10%, 45+15%, 40+20%, 35+25%, 30+30%). The results observed were in consistency with Wu & Wang (2012) that dual inorganic electrolytes increase the partition behavior of 1,3-PDO, increased K and Y values of

98.9 (Fig. 5.4 A) and 99.89 % (Fig. 5.4 B) respectively at a concentration of 45% K_2CO_3 and 15% K_2HPO_4 was observed. The explanation provided by Wu & Wang (2012), that combination of K_2CO_3 and K_2HPO_4 decreases the partition of 1,3-PDO is not consistent with the present observation, but the highest recovery mentioned after different combinations of dual salts was only 92.5% but the better yield of 99.89% was observed with a optimum concentrations of 45% K_2CO_3 and 15% K_2HPO_4. The current finding provided a simple and efficient strategy of ATPS system to separate and purify 1,3-PDO from the fermented broth.

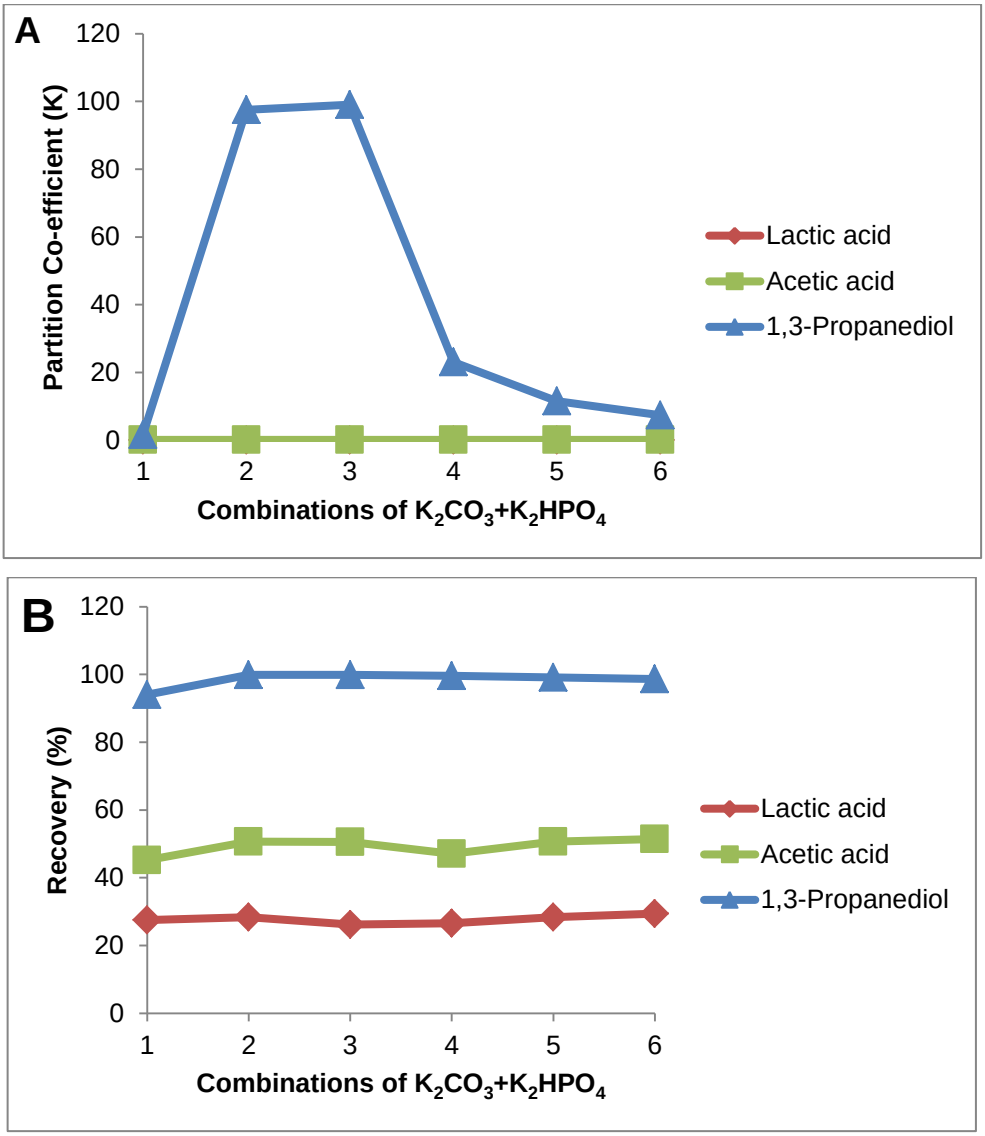


Figure.5.4. Effect of K_2CO_3 + K_2HPO_4 concentration on partition co-efficient and recovery percentage of 1,3-propanediol lactic acid and acetic acid from the fermented broth. K_2CO_3 + K_2HPO_4 concentrations represented in the figure are 55+5% (1), 50+10% (2), 45+15% (3), 40+20% (4), 35+25% (5), 30+30% (6) (w/v).

5.3.4. Statistical optimization of effect of pH and temperature on ATPS extraction using central composite design

The responses (partition co-efficient and recovery yield) obtained at different combinations of the independent parameters under similar operational conditions were listed in Table 5.1. The regression equation obtained by analyzing the responses provides an empirical relationship between the responses i.e., partition co-efficient and the recovery yield of 1,3-PDO in the ATPS system at different pH and temperature.

Run order	x_1 (pH)	x_2 (Temperature ^oC)	Partition Co-efficient (K)	Recovery (%)
1	7.5	42.5	20.07	95.25
2	7.5	42.5	29.54	96.72
3	7.5	60.0	15.59	93.97
4	7.5	25.0	34.91	97.21
5	12.0	42.5	32.62	97.02
6	7.5	42.5	26.21	96.32
7	3.0	42.5	20.14	95.27
8	7.5	42.5	19.21	95.05
9	3.0	25.0	19.3	95.07
10	12.0	60.0	20.07	95.25
11	7.5	42.5	29.54	96.72
12	7.5	42.5	15.59	93.97
13	3.0	60.0	34.91	97.21
14	12.0	25.0	32.62	97.02

Table.5.1. Central composite design matrix with two variables and observed responses, partition co-efficient (K) and recovery yield (R).

$$Y_1 \text{(Partition Co-efficient (K))} = 37.50 + 1.53\, x_1 - 1.493\, x_2 - 0.0678\, x_1^2 + 0.02262\, x_2^2 - 0.0076\, x_1 x_2 \quad (5.6)$$

$$Y_2 \text{ (Recovery yield (R))} = 97.76 + 0.340\, x_1 - 0.2444\, x_2 - 0.0179\, x_1^2 + 0.003549\, x_2^2 - 0.00029\, x_1 x_2 \quad (5.7)$$

In the equation Y_1 (Eq. 5.6), and Y_2 (Eq. 5.7), represents the response partition co-efficient and recovery yield, x_1, and x_2 are the independent variables as the coded values of pH and temperature respectively. The analysis of variance for the partition co-efficient response is given in table. 5.2 A and recovery yield in table. 5.2 B.

A

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	401.355	66.892	17.91	0.001
Blocks	1	0.630	0.630	0.17	0.694
Linear	2	260.374	130.187	34.85	0.000
pH	1	4.267	4.267	1.14	0.321
Temperature	1	256.107	256.107	68.55	0.000
Square	2	135.038	67.519	18.07	0.002
pH*pH	1	5.134	5.134	1.37	0.279
Temperature*Temperature	1	130.901	130.901	35.04	0.001
2-way Interaction	1	1.428	1.428	0.38	0.556
pH*Temperature	1	1.428	1.428	0.38	0.556
Error	7	26.151	3.736		
Lack-of-fit	3	26.151	8.717		
Pure error	4	0.000	0.000		
Total	13	427.506			

R^2: 0.938, R^2 (adjusted): 0.886

B

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	9.5308	1.58847	11.78	0.002
Blocks	1	0.0213	0.0213	0.16	0.703
Linear	2	6.0087	3.00435	22.27	0.001
pH	1	0.4214	0.4214	3.12	0.121
Temperature	1	5.5874	5.5874	41.42	0.000
Square	2	3.2257	1.6128	11.96	0.006
pH*pH	1	0.3594	0.3594	2.66	0.147
Temperature*Temperature	1	3.2225	3.2225	23.89	0.002
2-way Interaction	1	0.002	0.002	0.02	0.906
pH*Temperature	1	0.002	0.002	0.02	0.906
Error	7	0.9443	0.1349		
Lack-of-fit	3	0.9443	0.31477		
Pure error	4	0.000	0.000		
Total	13	10.4751			

R^2: 0.909, R^2 (adjusted): 0.832

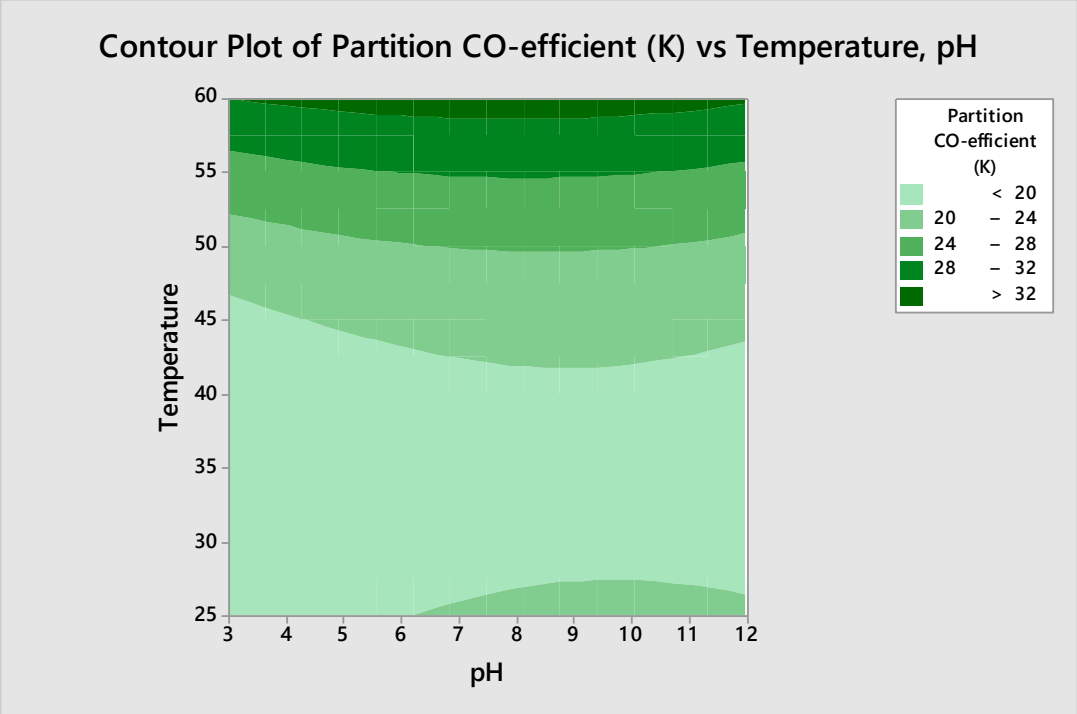
Table 5.2: Analysis of variance (ANOVA) for the linear model using partition co-efficient (K) as the response (A) and for recovery yield (B).

The values of independent variables in terms of $p<0.05$ indicates the significant variables. In this observation the response i.e., partition co-efficient and recovery yield has shown temperature as the only parameter in significant model terms and the other terms with p value greater than 0.1 is regarded as not significant.

These results demonstrate that the temperature has the significant effect on the partition co-efficient and the recovery yield of 1,3-PDO from the fermented broth using ATPS system than the pH of the culture broth. The observations are comparable to the

statement of Sadeghi & Jamehbozorg (2008), that the extraction temperature is key factor in increasing the partition co-efficient. The explanation between the independent variables can also be explained through contour plots (fig. 5.5 A and 5.5 B). Where the relation between pH and temperature for partition co-efficient and recovery yield (%), the K value found to be increased with increase in temperature but pH has no significance in this increase of K value, as the temperature increased the ionic strength of the inorganic salt increased which increased hydration efficiency of water mediating partition of 1,3-propanediol (Fu et al., 2015).

A



B

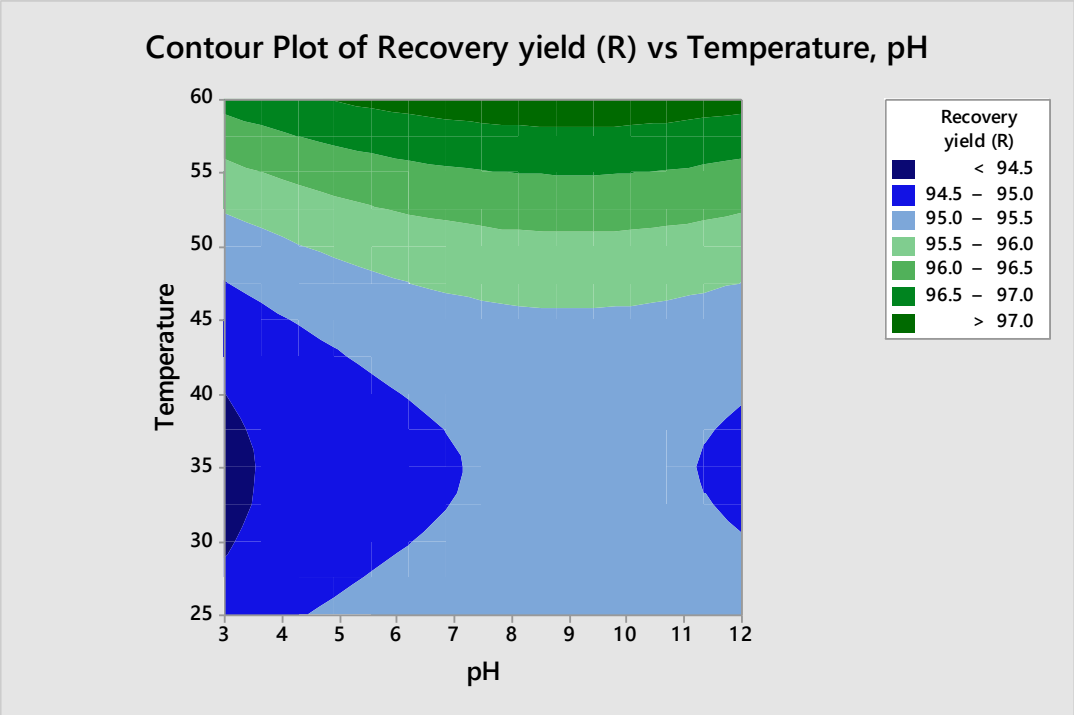


Figure.5.5. Response Contour plots for 1,3-propanediol extraction using ATPS system at different physiological pH and temperature. Contour plot Partition co-efficient (A), and Contour plot for recovery yield (B).

As of the statistical design the optimum conditions to perform the ATPS extraction of 1,3-propanediol is 60^{o}C. In order to validate the observations, the experiments were carried out with synthetic solutions of 1,3-propandiol with varying concentrations from 30 – 180 g/L and also the fermented broth with concentrations adjusted between 30 – 180 g/L.

5.3.5. Evaluation of behavior of 1,3-PDO in aqueous phase and organic phase based on different concentrations in fermented broth and synthetic solutions.

Validation of optimized inorganic salt/solvent concentrations, as well as the physical parameters was carried out in synthetic 1,3-propanediol solution and the fermented broth with concentration adjusted between 30-180 g/L. The partition co-efficient and recovery yield of 1,3-PDO was observed to be higher at 40 g/L concentration with K value of 42.4 and recovery yield of 97.69 % (fig.5.6A). As the diol concentration increased further with constant salt and isopropanol concentration, the partition co-efficient decreased but the recovery yield of average 95 % was maintained

(fig.5.6A and 5.6B), which is most important of the ATPS extraction. The similar behavior was observed with the synthetic solution (fig. 5.6A) and fermented broth (fig. 5.6B). Though the partition co-efficient of 1,3-PDO was decreased through increase in the concentration, the concentration of 1,3-PDO in organic phase increased, with the highest 1,3-PDO concentration of 180 g/L, an average 171 g/L was observed in the top phase with a partition co-efficient of 19.4 and 95% recovery yield.

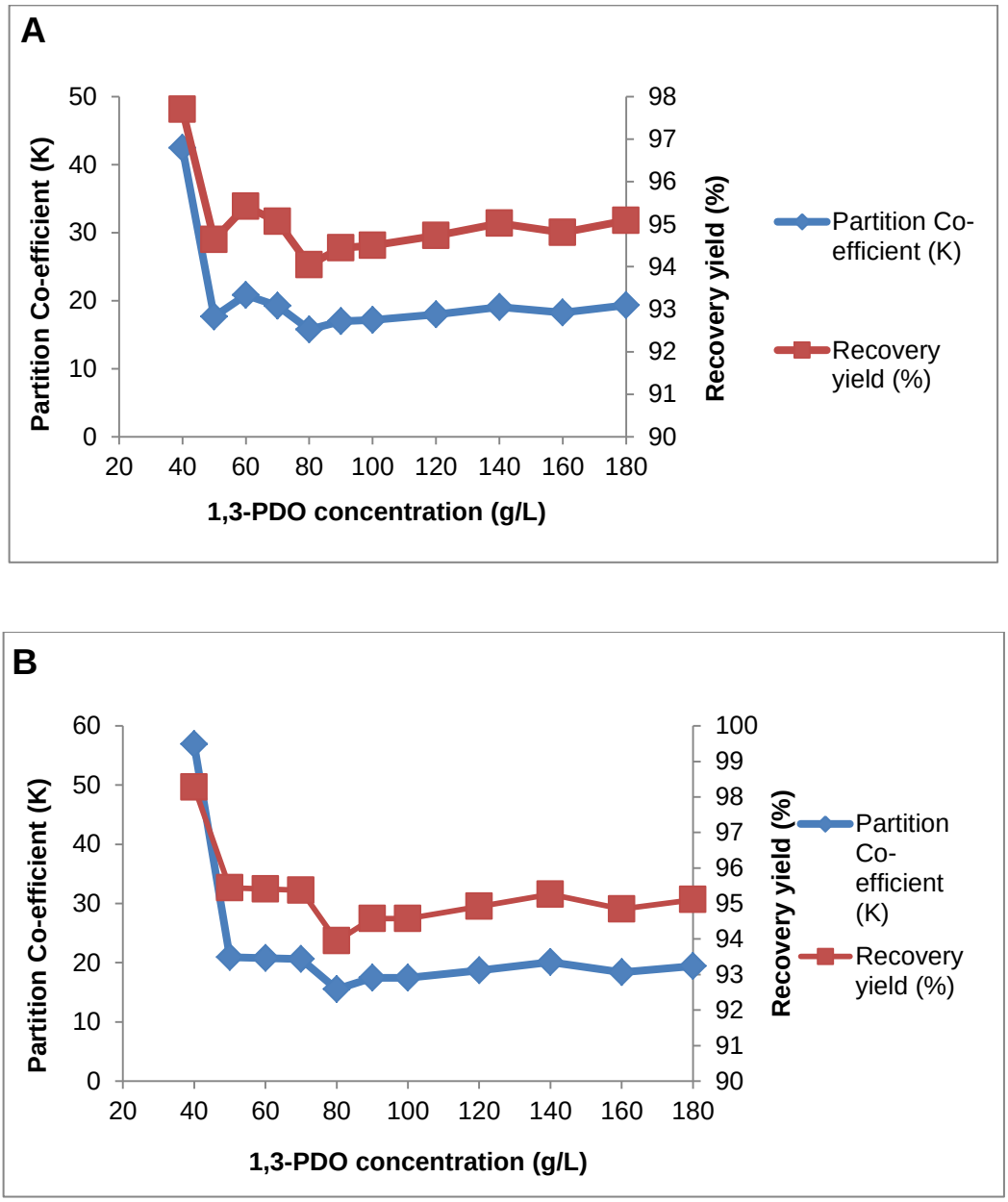


Figure.5.6. Effect of K_2CO_3 + K_2HPO_4 concentration on partition co-efficient and recovery percentage of 1,3-propanediol from the synthetic solution (A) and fermented broth (B) at different concentrations under optimized conditions of 60^{o}C.

5.3.6. Removal of cells and proteins using ATPS extraction

In order to prove the process to be more efficient than the known processes, either it should be cost effective or simple and less time consuming. In the fermented broth along with the desired product like 1,3-propanediol, byproducts, bacterial cells, macromolecules and extracellular proteins are present that might be separated in the initial steps of downstream processing (DSP). Hence in the downstream processing the primary step is removal of biomass i.e., microbial cells either through centrifugation or membrane filtration, proceeding with the removal of proteins by adsorption onto resins or activated charcoal. Through ATPS extraction, these two preliminary steps of DSP can be eliminated, because in ATPS extraction, the microbial cells, macromolecules and proteins remain in the aqueous fraction. In this study fermented broth was directly used without removal of cells, an important observation was the microbial cells and insoluble fraction of macromolecules formed a third phase as the phase of transition between the organic phase and aqueous phase. The removal of cells and proteins in the organic phase is 96 and 92%. Even higher selectivity was found towards 1,3-propanediol than lactic acid and acetic acid. The fermented broth with 25.78 g/L 1,3-PDO, 31.65 g/L lactic acid, and 15.74 g/L acetic acid, after aqueous phase extraction, the organic phase comprised 25.76 g/L 1,3-PDO, 8.28 g/L lactic acid and 7.95 g/L acetic acid, which can be expressed as the removal percentage of lactic acid and acetic acid as 73.83 ± 1.29 and 49.47 ± 0.35% respectively. The K_2CO_3 salt in the aqueous phase can be precipitated by sparging CO_2 gas constantly under optimized physical conditions, and K_2HPO_4 can be recovered by crystallization.

5.3.7. Final purification of 1,3-PDO using gradient column chromatography

The organic phase, the result of aqueous two phase extraction has 60-65% 1,3-propanediol, 15-20% lactic acid and 15-20% acetic acid, for the final purification of 1,3-propanediol, we have adopted a method developed by Anand et al., using silica gel gradient column chromatography. For this study, a glass column of size 120 cm length x 5 cm inner diameter was used and packing was done with silica gel (Mesh size) for 90cm with a packed bed volume of 900ml (fig. 5.7A). Initially Hexane was used to dissolve silica and later column was stabilized using chloroform: methanol (90:10). Here, 150 ml of a concentrated organic phase extract with the concentration of 315 g/L (mg/ml) (47.25 g 1,3-PDO) 1,3-propanediol was loaded into the column. Mobile phase with increasing polarity (90:10, 85:15, 80:20, 70:30, 75:25, and 50:50) was eluted out of column at a flow rate of 15 ml/min and 100 ml of the fractions was collected at

regular intervals of time and later the samples are distilled to separate mobile phase and analyzed by HPLC. The elution profile of 1,3-propanediol, glycerol, lactic acid and acetic acid was presented in figure.5.7B.

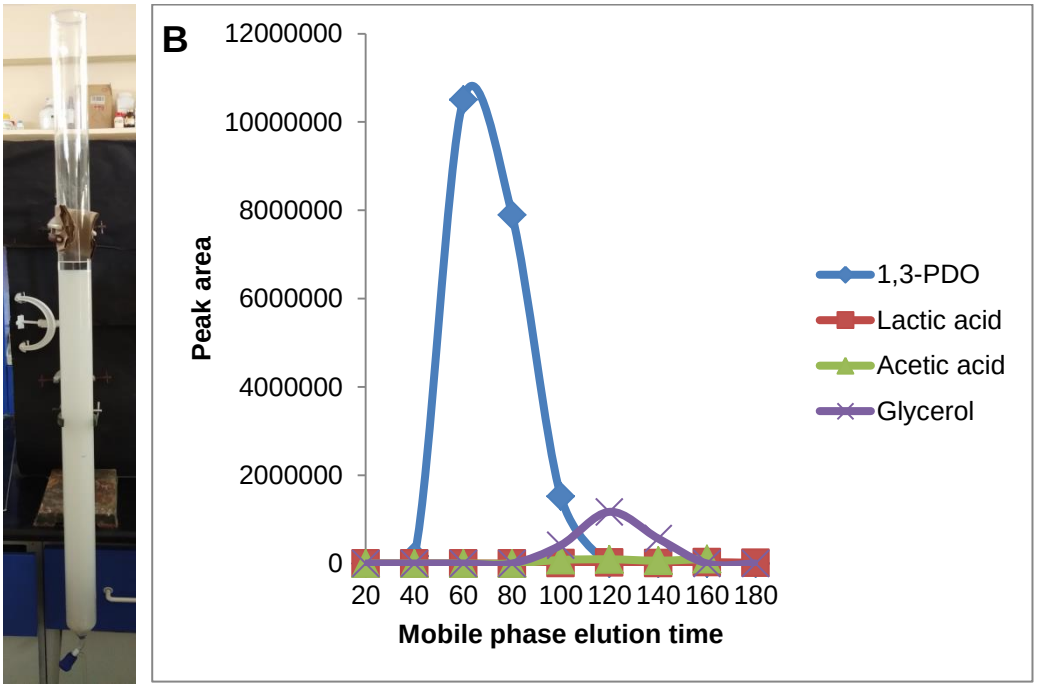


Figure.5.7. (A) Glass column packed with silica gel for purification of 1,3-propanediol from the organic phase extract. (B) Elution profile of 1,3-PDO, lactic acid, acetic acid and glycerol from the silica gel gradient column chromatography using chloroform and methanol as mobile phase with increased polarity.

The fig.5.7B clearly shows the efficiency and ease of separating 1,3-proapendiol from residual glycerol and by-products like lactic and acetic acid. This gradient elution resulted in purification of 46.16 g 1,3-propanediol with a recovery percentage of 97.7%. Later the purified 1,3-PDO was characterized using HPLC and NMR studies.

Total recovery yield of 1,3-propanediol using aqueous two phase extraction:

The following were the steps followed in separation of 1,3-propanediol from the fermented broth:

1. Aqueous two phase extraction : 95.4 % recovery of 1,3-PDO
2. Organic phase separation: No loss of 1,3-PDO
3. Silica gel chromatography: 97.7% recovery of 1,3-PDO
4. Organic phase separation: No loss of 1,3-PDO

Overall recovery yield of 1,3-propaendiol from the fermented broth was 93.2%.

5.3.8. Characterization of purified 1,3-propanediol

The characterization of 1,3-propanediol was carried out to confirm the purity of the purified sample from the fermented broth. The HPLC analysis provided a single peak in the chromatogram at a retention time of 20.9, which is similar to the standard (Fig. 5.8). The structural similarity of the purified 1,3-propanediol was carried out using ^{13}C NMR and ^{1}H NMR. The ^{13}C NMR spectrum of purified 1,3-PDO (fig.5.9A) and Sigma commercial grade 1,3-PDO (fig.5.9B) and, ^{1}H NMR spectrum of purified 1,3-PDO (fig.5.10A) and Sigma commercial grade 1,3-PDO (fig.5.10B) were represented in the respective figures.

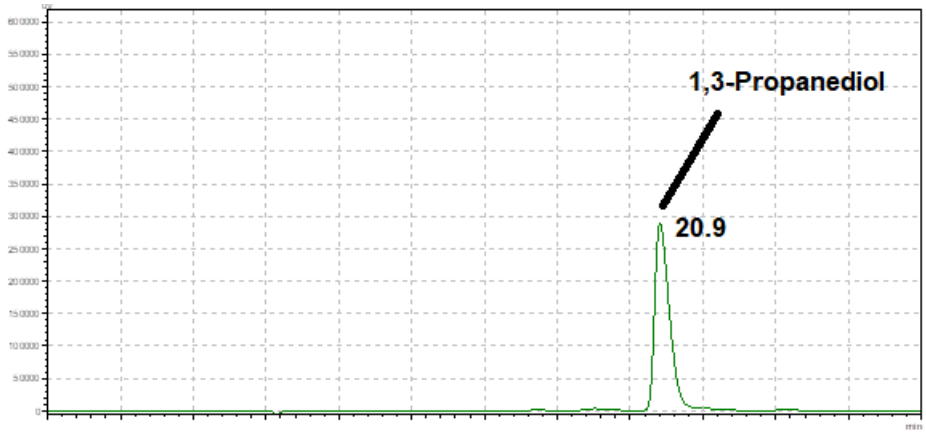
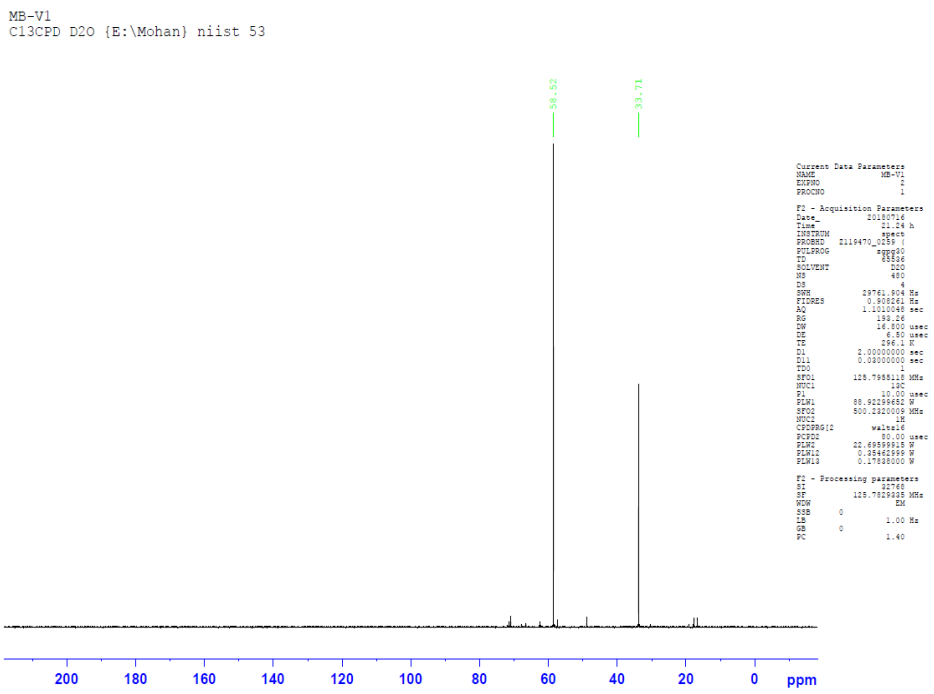


Figure.5.8. HPLC chromatogram of silica gel purified 1,3-PDO.

A



B

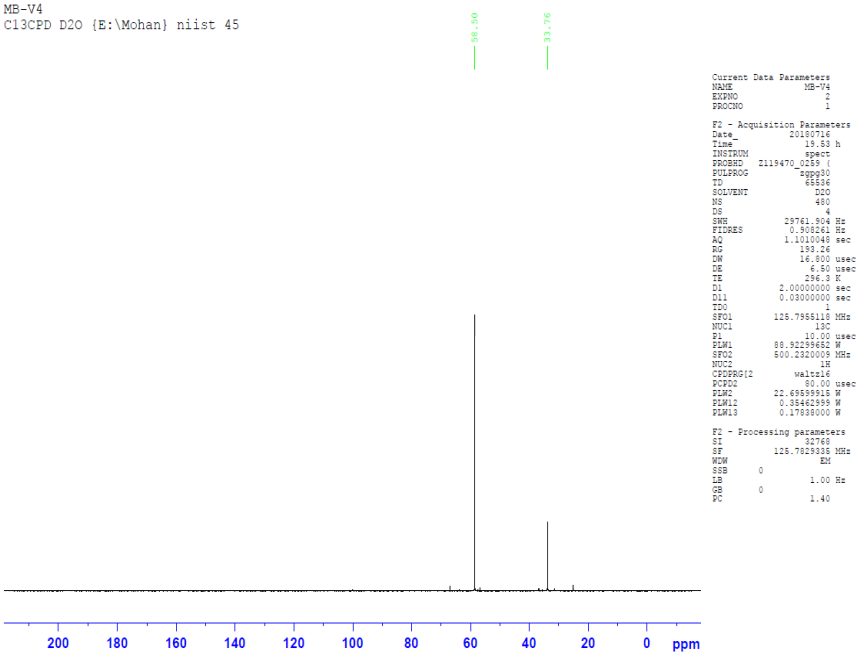
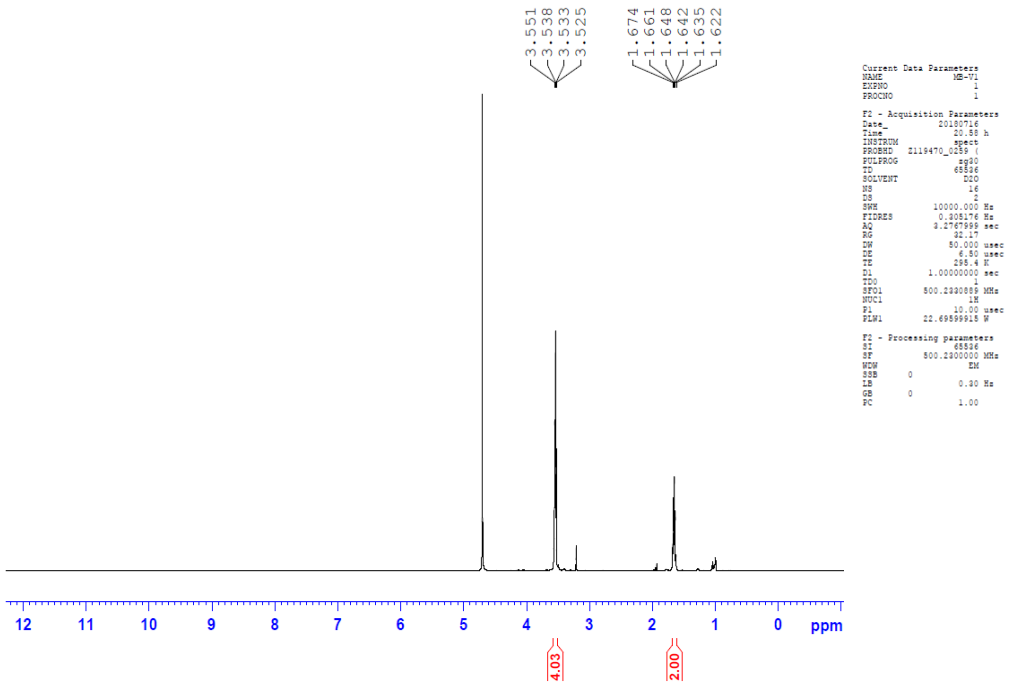


Figure.5.9. ^{13}C NMR spectrum of purified 1,3-PDO from fermented broth (A) and Sigma commercial grade 1,3-PDO (B)

A



B

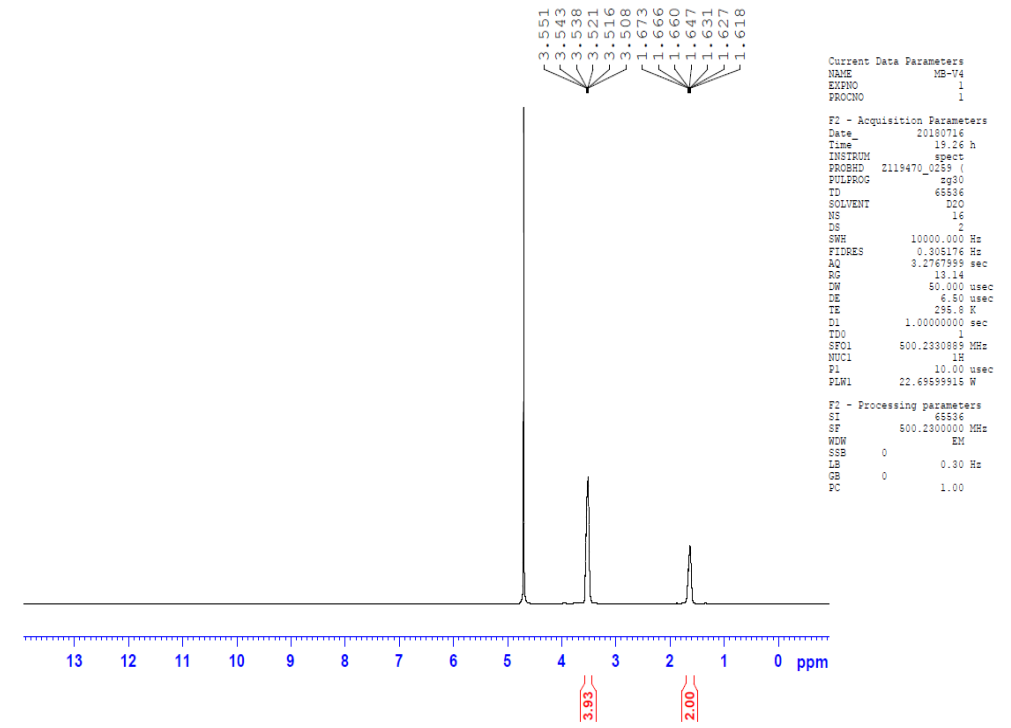


Figure.5.10. ^{1}H NMR spectrum of purified 1,3-PDO from fermented broth (A) and Sigma commercial grade 1,3-PDO (B)

5.4. Summary and Conclusion

An aqueous two phase extraction method was adopted for separation of 1,3-PDO from the fermented broth. The 1,3-PDO extraction using K_2CO_3/Isopropanol biphasic system is an optimal method. In this study a systematic experiments were carried out to understand the effect of inorganic electrolyte concentration on the partition co-efficient and recovery yield. The optimized inorganic salt concentration and process conditions, the partition co-efficient and recovery yield of 42.4 and 97.6 % was obtained respectively. The large amounts of lactic acid and acetic acid produced as byproducts during the fermentation was also observed to have partition into aqueous phase, which provides an add on advantage for simple and efficient recovery of 1,3-PDO. Later the 1,3-PDO extracted through organic phase was further purified using silica gel gradient column chromatography with chloroform and methanol as the eluent. The purified 1,3-PDO, was characterized by ^{13}C and ^{1}H NMR, where commercial grade 1,3-PDO purchased from Sigma Aldrich was used as the reference.

Chapter 6

Synthesis of transparent chitosan and chitosan exopolysaccharide biodegradable composite blend films and their characterization

Chapter 6: Synthesis of transparent chitosan and chitosan exopolysaccharide biodegradable composite blend films and their characterization

6.1. Introduction

Plastics are the part of day to day life since 1950's. Packaging industry is the sole largest consumer of plastics produced. Globally an estimate of 320 million tons of plastic is used for food packaging from which a significant amount will be converted as waste. These synthetic polymers (plastics) are durable, non-degradable and cannot be eliminated from usage. These plastic wastes from various sources was found to be accumulated and resulting in growing landfills, if burned the gases emitted has greenhouse effects and prone to air pollution. These non-degradable plastics also become an extreme threat to natural resources, mostly affecting the water bodies and living beings in it. However there are many measures taken to recycle the reusable plastics and incinerate non-reusable plastics, still the percentage of waste tackling through these measures is not sufficient compared to rate of production and consumption. Hence with increased population growth and market demand for plastics along with the limitation of non-renewable (petroleum) substrates, there is a great attention and interest towards renewable sources for production of biopolymers or bio-plastics. Various research groups worked on microbial synthesis of polyhydroxyalkanoates (PHA) (Volova et al., 2019), polyhydroxy butyrate (PHB) (Soto et al., 2019), and poly lactic acid (PLA) (Lunt, 1998) as substituent for synthetic polymers, but these polymers has limitations to be addressed in production yields, ecological compatibility before attaining commercial market (Kaplan 1998).

Edible and biodegradable carbohydrate based polymers composed of polysaccharides (starch, chitosan, kefiran, pullulan, and carboxymethyl cellulose), proteins (gelatin, casein, and soy protein) with different characteristic features like anti-microbial, and anti-oxidant activity, was considered as suitable and sustainable renewable alternatives for petroleum derived plastics (Tajik et al., 2013; González et al., 2017). In these polysaccharides, starch was widely used for bio-plastics production but the starch polymer has various limitations like retro gradation during storage i.e., reorganizing of the amylose and amylopectin structure resulting in new crystalline structures eventually the characteristic properties of the starch polymer is changed (González et al., 2017). But the heteropolymer chitosan is also well known for film forming property. Chitosan is a random heteropolysaccharide of NAG and its deacetylated part NG. This heteropolymer is considered as second most abundant naturally available polymer after cellulose with a global production of 10^{10}-10^{12} tons/year. Chitosan can be obtained from insects/crustacean shells and few fungal cell

walls (Crouvisier-Urion et al., 2016). The polycationic behavior of chitosan ($R\text{-}NH3^{+}$) after the deacetylation ($R\text{-}NH\text{-}Co\text{-}CH_3$) provides a good film forming property to the polymer and also increases water solubility (Yoshida et al., 2009). To stabilize the polycationic chitosan, counter ions either in the form of lactic, acetic or formic acid were required to stabilize the film. The film properties can be increased by addition of plasticizers like glycerol, iso-sorbide, and sorbitol, or anti-oxidants like tocopherol and ferulic acid. The importance of chitosan being used as film forming component is due to its biodegradable, biocompatible nature along with ease in solubility and non-toxicity. Chitosan has profound applications in biomedicine (Burn & Wound dressings), food & feed, cosmetics (hair & skin care), pharmaceuticals (encapsulating agent), agriculture (biopesticide) and waste water treatment (bio-flocculent) (Karthik et al., 2014).

1,3-propanediol (1,3-PDO), a monomer derived from glycerol or corn glucose has recently gained much importance in building of polymers especially a polyester, polytrimethylene terephthalate (PTT) formed by transesterification of 1,3-PDO and terephthalic acid. Being structurally similar to glycerol despite lack of one hydroxyl (-OH) group at 2^{nd} carbon, this monomer has also properties of plasticizing the composite films.

The chitosan films are rigid and require plasticizers to reduce the frictional forces between the adjacent polymer chains. Mostly chitosan films can be used in packaging. During synthesis of biodegradable plastics for packaging, the main characteristics to be considered are mechanical strength, transparency, flexibility, gases and water vapor permeability (Aider, 2010; González et al., 2017; Leceta et al., 2013a; Leceta et al., 2013b; Ma et al., 2018; ; Martinez-Camacho et al., 2010). The ideal green plasticizers used for improving the physiochemical properties of the biopolymers should be non-toxic, miscibility with the polymer, efficient in improving the strength, resistance to leaching and low cost. Various bio-plasticizers reported were iso-sorbide, glycerol, sugar alcohols, citrate, castor oil, fatty esters (Bocqué et al., 2016).

The aim of the research was to synthesize transparent and high performance CEPS and CEPS-PDO plasticized biodegradable composite films that can be used in packaging industry and also the influence of 1,3-propanediol as a green plasticizer compared to commercially available plasticizers. The characterization of these films reveals the unique properties and competitiveness with the commercial synthetic plastics.

6.2. Experimental section

6.2.1. Materials

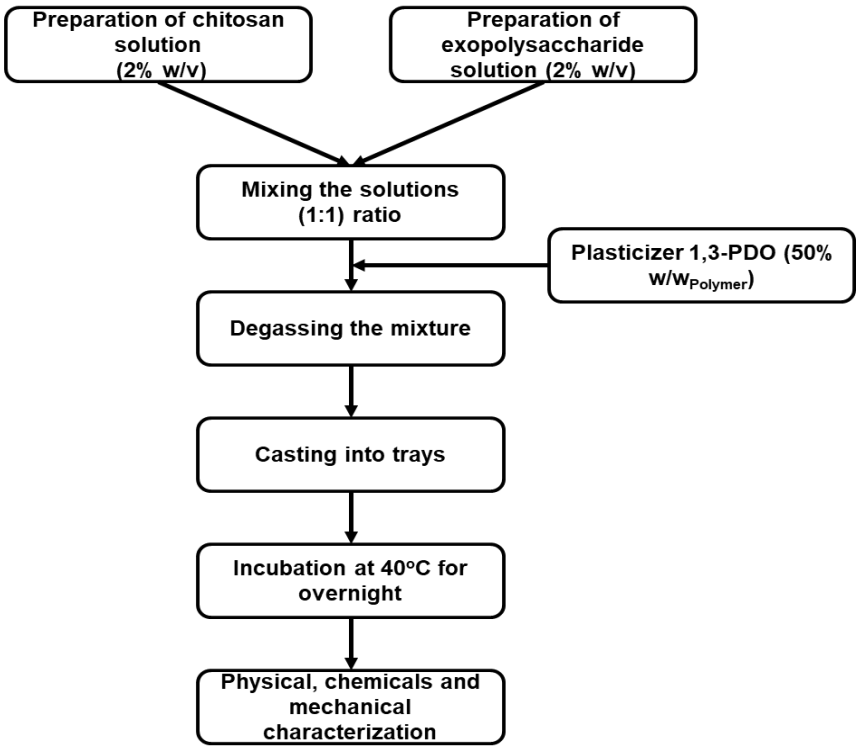
Chitosan was procured from Hi-media Labs, Mumbai, India. Dextran like EPS (Exopolysaccharides) used for the composite blends was purified from food grade lactic acid bacterium *Lactobacillus plantarum* BR2 at Microbial Processing and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (Sasikumar et al., 2017). 1,3-propanediol, used as the plasticizer was produced and purified as per the methodology explained in the previous chapters of this thesis.

6.2.2. Film formation

The films were made using solvent evaporation or solvent casting method (Rodríguez-Núñez et al., 2014; Priyadarshi et al., 2018a). At first (1% v/v) acetic acid solution was prepared using glacial acetic acid and distilled water, later known amount (2% w/v) of chitosan was added and mixed using magnetic stirrer until the solution is clear (fig.6.1 A), later the solution was degassed using Elma ultrasonic sonicator. The viscous chitosan solution then formed was poured into casting trays made of polyethylene terephthalate films. For the composite blend films, 1:1 w/w ratio of dextran like exopolysaccharide (2% w/v) solution (fig.6.1 B) was added to the chitosan solution, before degassing, similarly for plasticized CEPS composite films, 0.5:1 w/w of 1,3-propanediol to whole polymer (chitosan + EPS) was added before degassing and later the degassed solutions were poured into different casting trays and incubated at 40^{o}C overnight (Scheme.6.1). The films thus obtained were used for further characterization.

A **B**

Figure.6.1. (A) Chitosan solution (2% w/v) (B) Exopolysaccharide solution (2% w/v)



Scheme.6.1. Flow chart of film synthesis by solvent evaporation or solvent casting method.

6.2.3. Characterization of the films

6.2.3.1. Film thickness and Tensile strength

The thickness of the film was measured using a Screw gauge. The value for the thickness measurement used for tensile strength calculations was obtained by measuring the thickness of the film samples at three positions along the length of 20 x 110 mm films casted specifically for calculating the tensile strength of the film and using a mean value. The precision of the thickness measurements was ±5%. The tensile strength of the material was tested using Universal Testing Instrument Model H5KS (Tinius Olsen, Horsham, USA) fitted with a 100N static load cell, according to standard testing method (ASTM 1995) crosshead speed of 50mm/min and extension of 100mm and the initial grip separation was 50mm.. The films were cut into strips 10mm wide and 110mm long and mounted between cardboard grips (40mm x 30mm) using adhesive.

6.2.3.2. Scanning electron microscopy (SEM)

The study was employed using JSM-5600 LV scanning electron microscope of JEOL, Tokyo, Japan. In order to understand the surface morphology of the edges of the broken films obtained after tensile strength analysis. The dried samples were

mounted on a metal stub and sputtered with gold in order to make the sample conductive, and the images were taken at an accelerating voltage of 10KV.

6.2.3.3. Fourier transform infrared spectroscopy (FTIR)

The monomeric composition of these composite blended films were characterized using FT-IR spectra recorded using Shimadzu IR Tracer 100 Fourier Transform Infrared spectrophotometer (Shimadzu, Japan).

6.2.3.4. Thermo gravimetric analysis (TGA)

The thermal behaviors of the films were studied by thermo gravimetric analysis using TG-DTA 6200 (SII Nano-technology Inc., Japan). The substance (9mg) was subjected to a temperature range of 30 – 300 °C under normal atmosphere at a rate of 15°C/min and the corresponding weight loss was determined.

6.2.3.5. UV-Vis Spectroscopy

The composite blend films were cut into 20mm x 20mm square pieces and were subjected to solid state UV-Visible spectroscopy using a Shimadzu 2100 UV-Visible spectrophotometer. The absorbance and transmittance of these composite films were recorded from 300 nm to 800 nm. The transparency of the film plays a crucial role in the acceptability of the film for food packaging uses, hence making this test important.

6.2.3.6. Wide angle X-ray diffraction (WAXD)

To understand the X-ray diffraction pattern of these composite films, which elucidate the information regarding the structural properties and intermolecular bonding nature, WAXD measurements were carried out using XEUSS SAXS/WAXS system using a Genix micro source from Xenocs. The methodology followed for the measurement and sample preparation was according to the report by Nagendra and associates (Nagendra et al., 2015).

6.2.3.7. Water transfer rate of blend films

Water transfer rate of the chitosan dextran blend films (control and with addition of ferulic acid) were tested according to ASTM E96 method, specifically the wet cup method. The test involved addition of 10mL de–ionized water to a 150mL screw cap bottle and which is sealed using a hollow cap and paraffin wax (fig.6.2). The setup was then transferred to a desiccator chamber with dry silica gel. The weights of each of the screw cap bottles were recorded every two hours and the loss in weight of

the water from the vessels is ultimately used to calculate the water transfer rate for each film.

Figure. 6.2. Experimental setup for testing the water transfer rate of CS (Control) and CEPSPDO composite films.

6.2.3.8. Antioxidant activity

Biopolymers or composite films with antioxidant or hydrogen peroxide radical scavenging activity would be an added benefit for the food and packaging applications. The antioxidant activity of chitosan exopolysaccharide blend composite films (CEPS) and plasticized composite films (CEPSPDO) were carried out using hydrogen peroxide radical scavenging assay (Hafsa et al., 2016) with slight modifications. The methanol extract of composite films was carried out by adding 500 mg film pieces in 15 ml methanol and sonicating the mixture for 2 hours, then the mixture was centrifuges to collect the supernatant. To perform the assay, the solution of 40 mM of 30 % hydrogen peroxide was prepared using 50 mM phosphate buffer (pH 7.4), was prepared. After the incubation of methanolic extract, 500 µl of the supernatant was added to 3 ml of assay mixture and incubated at 37^{o}C for 10 mins. Later absorbance was noted at 230 nm using Shimadzu UV spectrophotometer. 50mM phosphate buffer without added hydrogen peroxide was used as the blank. The antioxidant activity was measured using the equation 6.1.

H_2O_2 radical antioxidant activity (%) = $[1-(A_{Sample}/A_{Control})] \times 100$ (6.1)

A_{Sample}: Absorbance of sample

$A_{Control}$: Absorbance of control

6.2.3.9. Moisture content

As the known characteristic of a biopolymer, the composite films may absorb the moisture from the surrounding environment. The amount of moisture absorbed by the film from the surrounding environment, till equilibrium is attained attributes to

moisture content of the films. In this study, CEPS and CEPSPDO composite film moisture content was measured by drying the 20 mm x 20 mm square pieces of films in an oven at 105^{o}C till a constant weight is attained. Initial weight of the film was denoted as A_i and final dry weight as A_f. The moisture content was calculated using the equation 6.2 (Priyadarshi et al., 2018b).

Moisture content % = $\frac{A_i - A_f}{A_i}$ x 100 (6.2)

6.2.3.10. Water absorption

The water absorption capacity of the composite films was determined by immersing the dried films with weight A_f obtained from the above experiment, in 50 mL distilled water and increase in weight was monitored over a period of time until a constant weight was observed. After attaining the level of saturation, the final weight of the sample was recorded and denoted as Aw. The water absorption capacity of the composite film was measured using the equation 6.3 (Priyadarshi et al., 2018b).

Water absorption % = $\frac{A_w - A_f}{A_f}$ x 100 (6.3)

6.2.3.11. Film solubility

As the monomers used in the preparation of these blend films are of biological origin and can be easily miscible in water, the percentage of weight loss of films can be measured by observing the solubility. To determine the water solubility of the films, the films were cut into 20 mm x 20 mm square pieces and kept in a vacuum desiccator to remove the moisture content and then the initial weight of the sample was noted as F_i, later the film pieces were immersed in 50 mL distilled water and kept overnight at room temperature. Later the samples were dried at 105^{o}C, until a constant weight is attained and the final weight was recorded as F_f. The percentage solubility of composite films was measured using the equation 6.4 (Priyadarshi et al., 2018b).

Film solubility (%) = $\frac{F_i - F_f}{F_i}$ x 100 (6.4)

6.2.3.12. Biodegradability of composite films

Though the composite films synthesized in this study was using the polymers and monomers derived from biological origin, like chitosan from shrimp shell waste, EPS from food grade lactic acid bacterium, and 1,3-propanediol produced by *Lactobacillus brevis* N1E9.3.3 strain using biodiesel derived crude glycerol, it would be appropriate to prove that these bio-composite films can be degraded in natural environment, without causing any damage to natural ecosystem. Here, we made a

compost soil using vermicompost and biogas reactor digestate, later CS films and CEPSPDO films of 1 gram weight were placed inside the soil and incubated at room temperature, to observe the decrease in weight of the films.

6.3. Results and Discussion

6.3.1. Film casting and physical and mechanical characteristics

Intact and transparent chitosan (CS), chitosan exopolysaccharide (CEPS) and plasticized chitosan exopolysaccharide (CEPSPDO) films were obtained by solvent casting method. CS and CEPS films were fragile compared to CEPSPDO films, as the plasticizer 1,3-propanediol imparts flexible characteristics to the film.

A B

Figure.6.3. (A) Chitosan (CS) film (B) plasticized chitosan exopolysaccharide (CEPSPDO) film.

The functionality of a plasticizer can be explained as, the molecule with lesser molecular weight, weakens the intermolecular interactions between the polymeric chains, resulting in a less dense structure with increased elongation percentage. The physical properties of the above mentioned films were summarized in table 6.1. It was observed that, addition of exopolysaccharides to the chitosan solution decreased the tensile strength, force of elongation and elongation percentage, but addition of plasticizer to the exopolysaccharide blended chitosan films, all the properties were found to be increased, approximately 39.19 % increase in tensile strength, 67.38 % decrease in max force of elongation and 94 % increase in elongation percentage was observed due to plasticizing chitosan exopolysaccharide blends in comparison to chitosan films. Even the phenomenon was attributed by physical imaging of broken corners of films obtained after the mechanical testing (figure 6.4).

Film	Tensile MPa	Max Force N	Elongation %	Thickness Mm	Width mm
CS	31.13	39.85	10.64	0.05	20
CEPS	20.08	12.85	10.00	0.05	20
CEPSPDO	43.33	13.00	20.73	0.05	20

Table.6.1. Mechanical characteristics of the composite films obtained using Universal Testing Instrument Model H5KS (Tinius Olsen, Horsham, USA) (ASTM 1995).

The CS films (fig.6.4.A) has homogenous appearance, CEPS films (fig.6.4.B) looks little fragile and less compact, later the plasticization with 50% 1,3-propanediol (fig.6.4.C), the cross-section of the films look so compact, elastic and flexible. As CSEPS films are fragile without addition of plasticizers, further physiochemical characterization experiments were carried out using CS and CEPSPDO films.

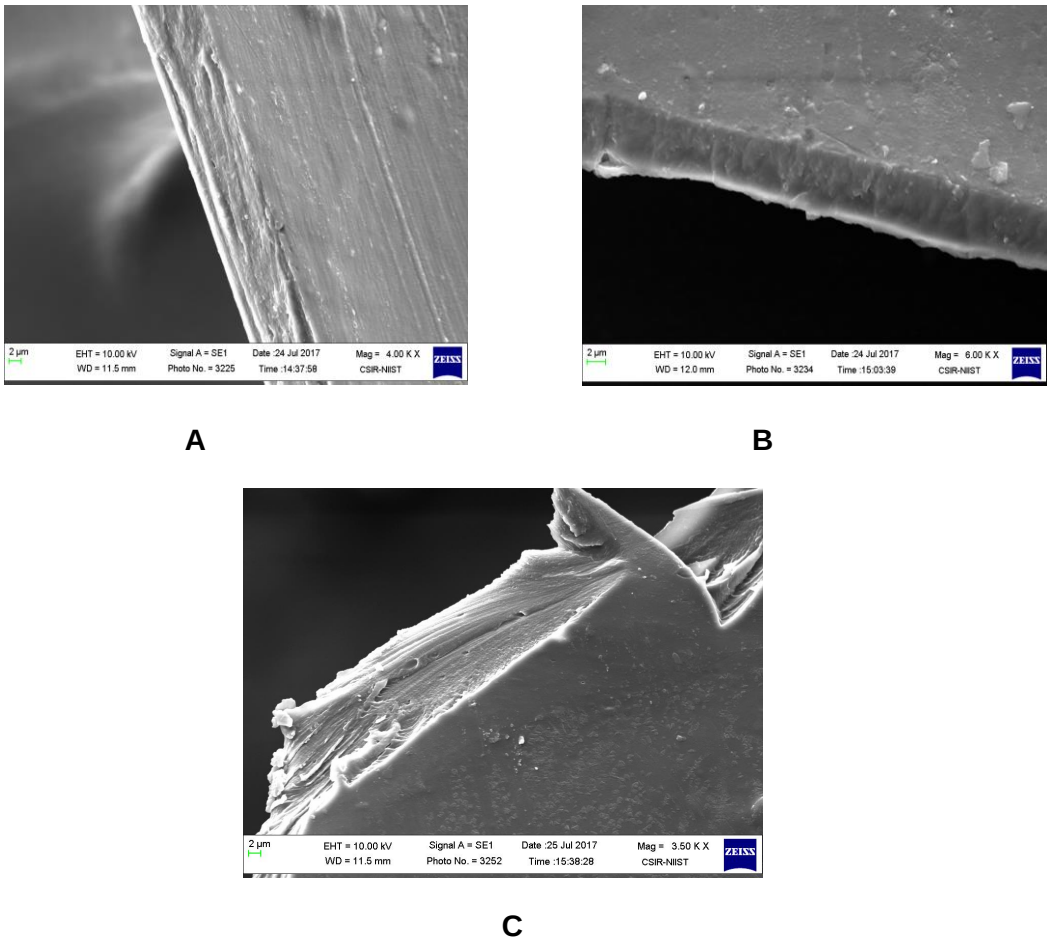


Figure.6.4. SEM images of composite blend films (A) chitosan (CS) (B) chitosan exopolysaccharide (CEPS) and (C) chitosan EPS and 1,3-PDO (CEPSPDO).

6.3.2. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum analyzes the free functional groups present in the molecule and to understand the change in the structure after synthesizing the composite films. In figure 6.5, we can observe the IR spectrum of CS, CEPS and CEPSPDO films, a strong band in the region 3000 – 3500 cm^{-1} corresponds to N-H and O-H bonds, and intramolecular hydrogen bonding. The characteristic band close to 3000 cm^{-1} indicates, C-H symmetric and asymmetric. A distinguishing peak after 1500 cm^{-1}, corresponds to free N-acetyl groups, specifically (1645 cm^{-1} and 1325 cm^{-1}), was observed in CS films, but there are no peaks at that particular region in CEPS and CEPSPDO composite films. The band close to 1000 cm^{-1} corresponds to C-O stretching. The dextran like exopolysaccharide has repeating units of glucose like units, which has majorly –OH groups, corresponds to band in the region 3000 – 3500 cm^{-1} and CH-groups symmetry and asymmetry close to 3000 cm^{-1}, similarly the plasticizer 1,3-propanediol structurally consists of –OH groups and –CH symmetric regions (Alonso et al., 2009; Fernandes Queiroz, Moacir, et al., 2014; Skornyakov et al., 1996).

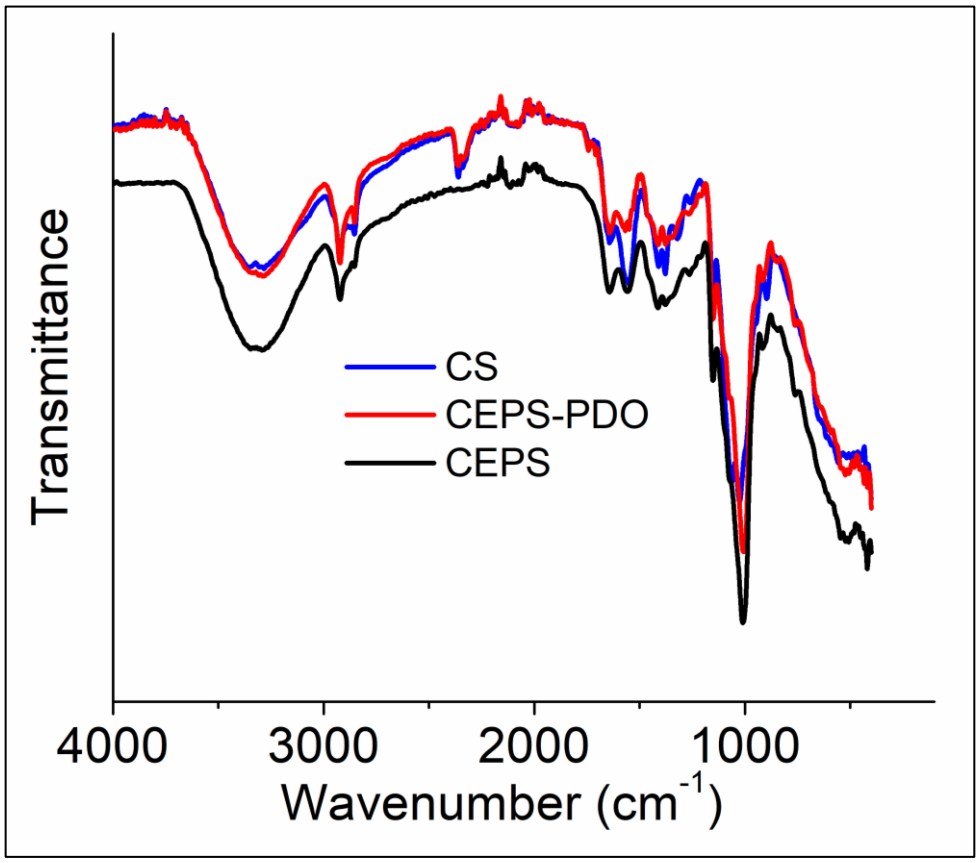


Figure.6.5. FT-IR Spectra of chitosan (CS), chitosan exopolysaccharide (CEPS) and plasticized chitosan exopolysaccharide (CEPSPDO) films.

6.3.3. UV-Vis Spectroscopy

In FTIR, we understood the structural characterization of CS and other blend composite films, whereas in UV-Vis spectrum, we can determine the presence of inter and intra covalent and non-covalent interactions in monomers of composite films. The covalent interactions may be detected due to presence of few organic molecules that can absorb the light at particular wavelength. In Fig.6.6, we can observe that, there are no deviations in the spectra observed from the wavelength 300 – 800 nm, except a deviation between 300 – 350 nm observed in the plasticized CEPS film, may be due to an interactions between 1,3-propanediol and free chemical groups of chitosan and exopolysaccharide (Kumirska et al., 2010; Bhat et al., 2008).

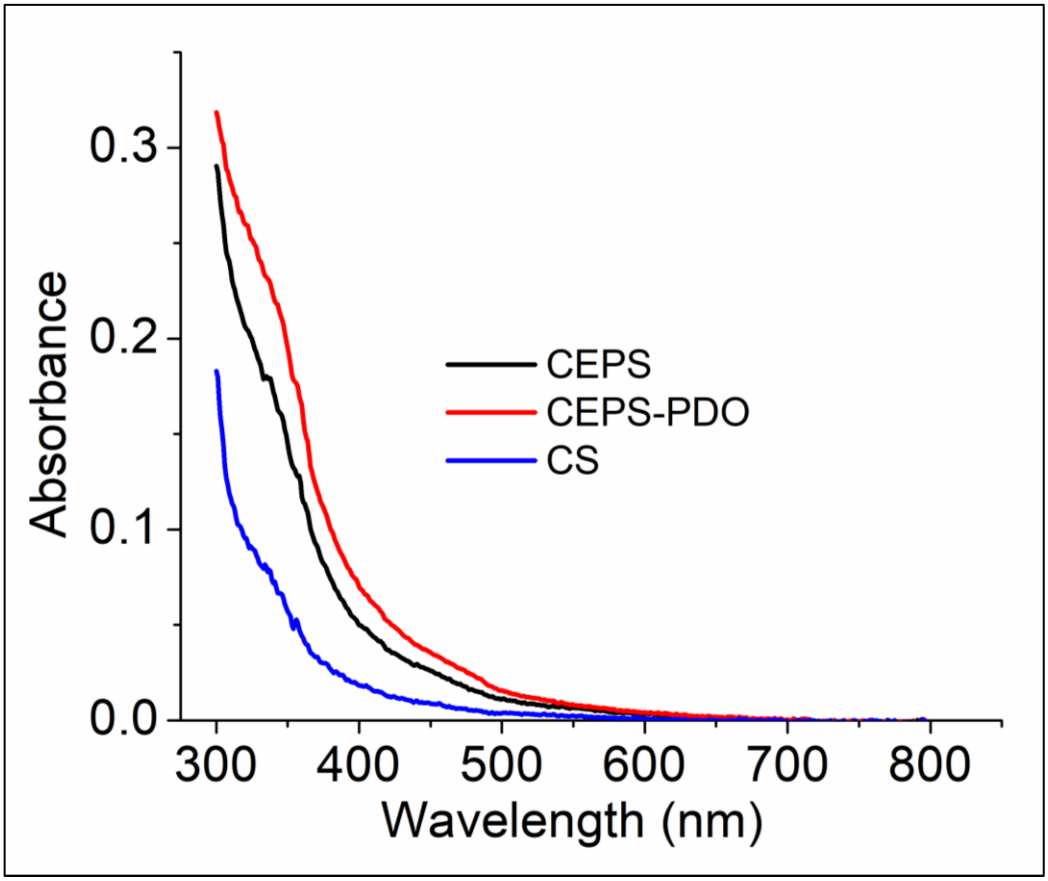


Figure.6.6. UV-absorbance Spectra of chitosan (CS), chitosan exopolysaccharide (CEPS) and plasticized chitosan exopolysaccharide (CEPSPDO) films.

6.3.4. Thermo gravimetric analysis (TGA)

The TGA analysis of CS, CEPS and CEPSPDO films, were carried out to understand the thermal stability and the degradation pattern in response to increase in temperature from 30 – 800^{o}C at a rate of 10^{o}C/min. the TGA pattern of films CS (A), CEPS (B) and CEPSPDO (C), represented in the figure.6.7, the pattern of thermal degradation in all the films were observed to similar, the reason would be the similar nature of bonding in chitosan and exopolysaccharides and even the presence of CH2OH groups in chitosan, exopolysaccharide and 1,3-propaendiol.

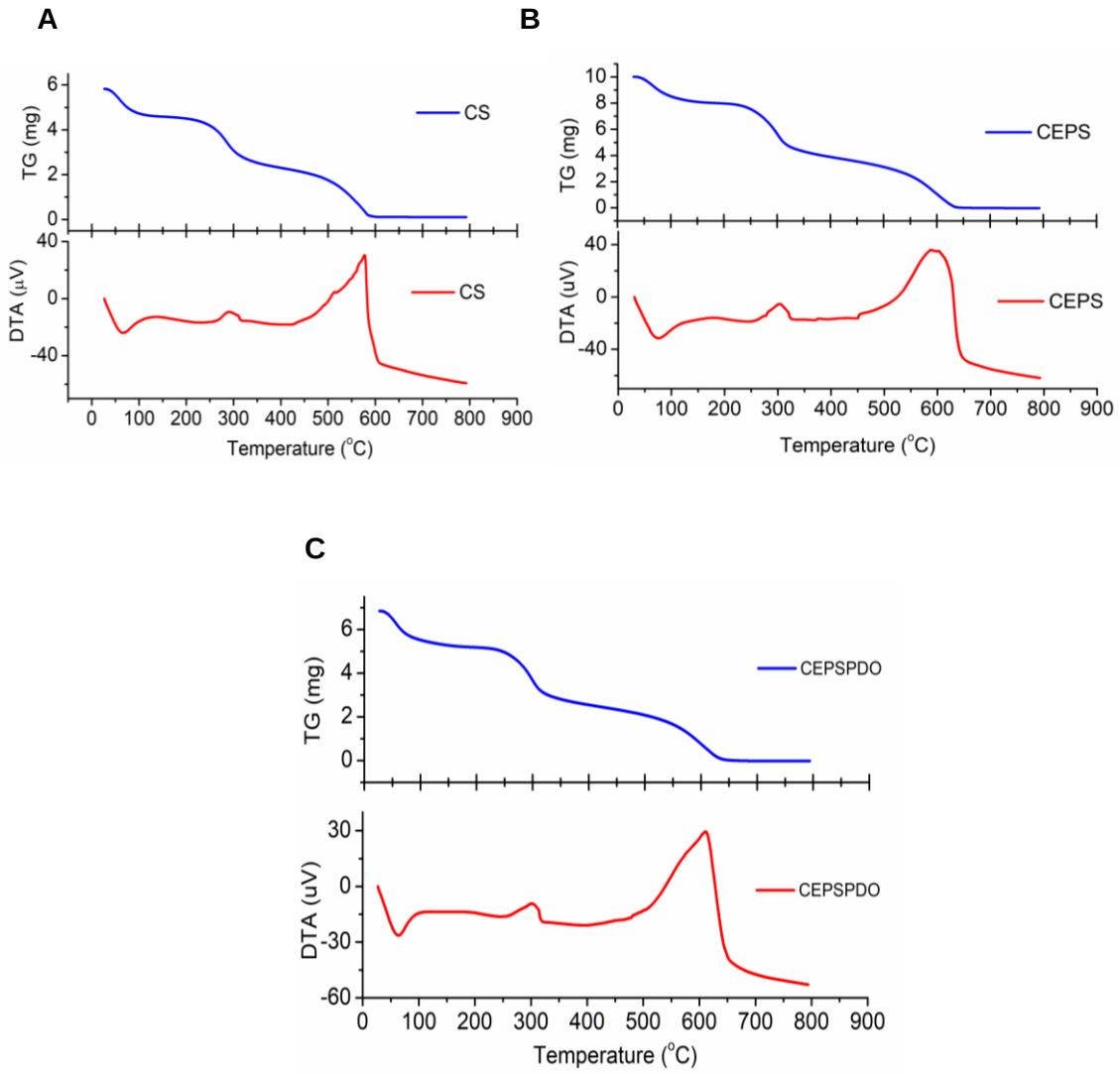


Figure.6.7. TGA and DTG pattern of chitosan (A), chitosan exopolysaccharides (B) and Plasticized chitosan exopolysaccharide (C) composite blend films.

If we observe the degradation pattern, it is in three stages, initial loss up to 100-105^{o}C, represents the removal of water molecules and other volatiles, until the second mass loss from 250 – 400^{o}C, the films were observed to be stable, the second degradation step corresponds to amine group degradation in chitosan polymer, and later the film was not observed to be stable, then there is a gradual decrease in the mass of the film, when the temperature reaches 600^{o}C, 100% degradation of the film was observed (fig.6.7.).

6.3.5. Wide and small angle X-ray scattering (WAXS/SAXS)

X-ray diffraction or scattering spectroscopy studies is widely used to characterize the materials of all forms. The characteristic peaks of chitosan films (CS) are in acceptance with the literature reports (Kumirska et al., 2010), where there is a strong reflection at 2θ of 20-21^{o} and a slight reflection at 2θ of 9-10^{o} (fig.6.8).

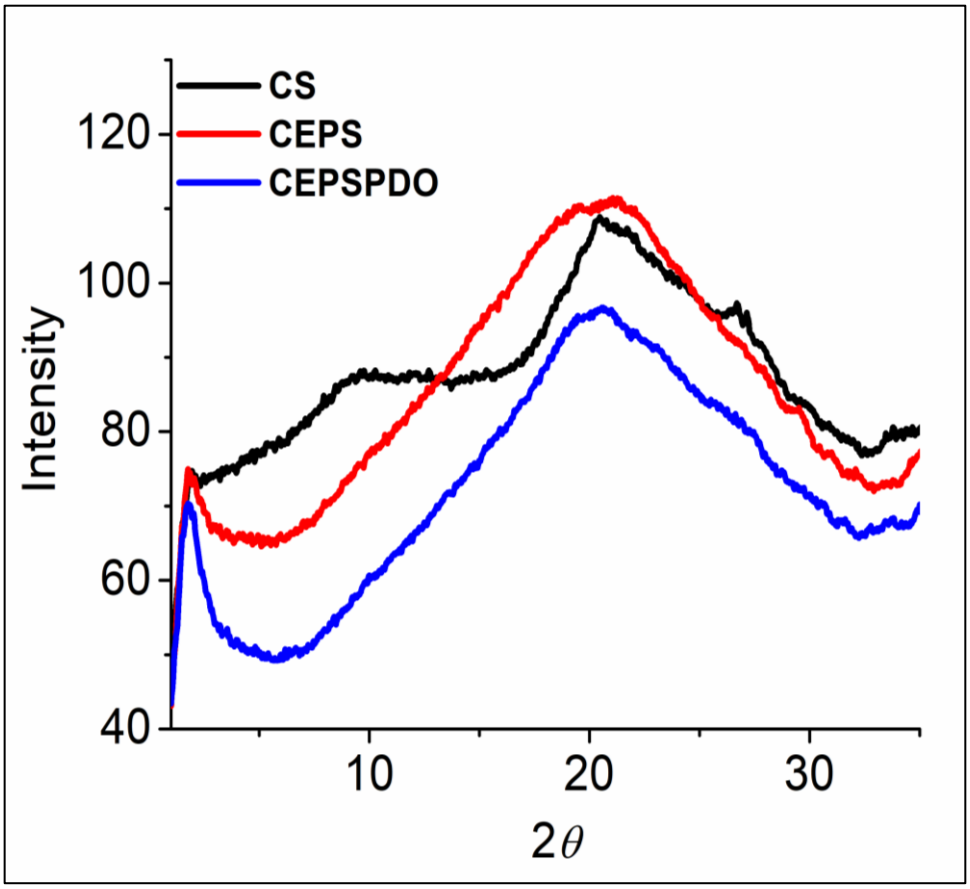


Figure.6.8. X-ray diffraction patterns of chitosan (CS), chitosan exopolysaccharide (CEPS) and Plasticized chitosan exopolysaccharide (CEPSPDO) composite films.

6.3.6. Water vapor transfer rate

In the food (dry and fresh) packaging, the packing material acts as the barrier between the surrounding environment and packed food. The phenomenon which plays a major role here is moisture content, if the barrier is easily accessible for the moisture, the dry food packed may observe the moisture from the environment or fresh produce may lose the moisture into the environment, which may cause deterioration of the food material and its quality. However in our studies (Table.6.2), the water vapor transfer rate between the chitosan and plasticized chitosan exopolysaccharide films has showed very less values in comparison to the reports given by Priyadarshi et al., 2018b, where they have plasticized and cross-linked the chitosan films using glycerol and citric acid respectively, even the results were promising with 1,3-propanediol as the plasticizer than glycerol, with the similar work of Reddy et al., and associates (Reddy & Yang., 2010).

6.3.7. Anti-oxidant activity

The Fenton reaction, in which the hydrogen peroxide can be oxidized to release hydroxyl group radicals, may happen when hydrogen peroxide enters the biological cells by crossing the barrier membrane and causing cell cytotoxicity. Anti-oxidants are group of synthetic or bio-derived chemical components, which prevent the oxidizing property of hydrogen peroxide. Hence in the food packaging industry, the packaging material with anti-oxidant property will be an added advantage, by preventing the oxidation of foods by oxidative agents. From the observed results between the chitosan films and plasticized chitosan and exopolysaccharide films, 28.6% (Table 6.2) reduced oxidative activity was observed, the reason of reduced activity is due to lower concentrations of chitosan in CEPSPDO films, almost half in comparison to chitosan (CS) films. But there are earlier literature reports that the anti-oxidant activity of the plasticized films can be improved by incorporating cross linkers like citric acid (Priyadarshi et al., 2018b), ferulic acid and palmitic acid. Similarly addition of *Eucalyptus globulus* essential oils in chitosan films, resulted in 60 % antioxidant activity (Hafas et al., 2015), but the cost of film production will be uneconomical to use these essential oils, hence utilization of bio-derived monomers and polymers like 1,3-PDO, exopolysaccharide and chitosan will be a preferred alternatives to petrochemical derived plastics.

Film type	Physiochemical properties			Anti-oxidant activity (%)	Water vapor transfer rate (g/m2/d)
	Moisture content (%)	Moisture absorption (%)	Film solubility (%)		
CS	8.42	137.17	19.15	40.1	429.6
CEPSPDO	10.30	193.91	51.48	28.6	424.8

Table.6.2. Physiochemical, water vapor transfer rate and anti-oxidant activity of chitosan and plasticized chitosan and exopolysaccharide composite blend films.

6.3.8. Moisture content, moisture absorption and Film solubility

The polymers used in CS and CEPSPDO films, chitosan and exopolysaccharides can interact with water molecules due to presence of hydroxyl groups and chitosan has even amine groups. As there is no chemical reaction between these polymers, the free hydroxyl groups and amine groups are available for hydrogen bonding between the polymer and water molecules, which was confirmed by increase in 22.32% moisture content, 41.36% moisture absorption rate. Even the explanation was in acceptance with the film solubility, 51.48 % of CEPSPDO films were soluble in water, compared to 19.15% of chitosan films (Table 6.2), which adds a huge advantage of these composite blend films. When the composite films were immersed in the water, the polymer components absorbs the water molecules, resulting in loosening of the compact polymer matrix, then other components like EPS and 1,3-PDO are easily miscible in water, hence these components were released from the composite film resulting in higher solubility. These days, it is very much discussed about the threat of petrochemical derived plastics to aquatic ecosystem, if those plastics are replaced with these bio-composite blend materials, the huge environmental disaster can be avoided.

6.3.9. Biodegradability of composite films

With an environmental concern and knowing the impact of petroleum derived plastics on the natural ecosystem, the composite films of chitosan, chitosan exopolysaccharide and plasticized chitosan exopolysaccharide films biodegradation pattern was observed in the natural environment, where the soil compost was prepared using vermicompost soil, and dried biogas digestate. Then the soil compost

was transferred into disposable water cups and 1 gram weighed films were kept in the soil and incubated for a week at room temperature. As the biogas digestate is rich source of aerobic and anaerobic microorganisms, and vermicompost serves as rich nitrogen source, all the films were degraded 100 %, where there is no sign of any particles of composite blend films were observed after incubation.

6.4. Summary & Conclusion

The composite blend films of chitosan and exopolysaccharides were prepared and later the chitosan exopolysaccharide film was plasticized using 1,3-propanediol with ease. The physical and structural characterization revealed the incorporation of 1,3-propaendiol as the plasticizer and increased the flexibility of the CEPS films. The physiochemical properties of CEPSPDO films were observed to be superior to the neat chitosan films. The CEPSPDO film has lesser water transfer rate, higher water solubility, than CS films. With the observed thermal, mechanical, physical and biodegradable properties, these composite blend films have recognizable characteristics unlike commercial petrochemical packaging material used in food industries.

Chapter 7

Summary and Conclusions

Summary

Bio-based 1,3-propanediol has huge potential in the world market than the chemically synthesized 1,3-PDO. The chemical has various applications as monomer in polymer industries, textiles, adhesives, coatings, pharmaceuticals and cosmetics. Although 1,3-PDO is known to synthesize from chemical and biological methods, the most suitable process is through fermentation (bioprocess), as environmental friendly microorganisms mediate the process as biocatalysts. The sustainable bioprocess for 1,3-PDO production provided a platform by supplying the monomers to synthesize bio-plastics like polytrimethylene terephthalate (PTT). PTT with inherited characteristics of PET and PBT changed the market potential by replacing the other two petrochemical polyesters in various applications.

The objectives and experimental aspects of the work carried out in this study reflects the dedication towards the development of a green process for 1,3-PDO production using native strain *Lactobacillus brevis* N1E9.3.3. The strain was isolated from a Corporation dumping yard in Thiruvananthapuram, Kerala, India. The following could be summarized as the major results of the research work carried out.

Through onsite enrichment technique 17 strains, which can withstand high glycerol concentrations were isolated and of which a strain named N1E9.3.3 was observed to be higher 1,3-PDO producer. The strain was identified as Lactobacillus brevis, with a highest homology to a type strain Lactobacillus brevis ATCC 14869 using 16s rDNA identification method.

Optimization of process parameters like initial pH of the production media was carried out using single parameter approach in shake flask experiments. Further optimization of media components was conducted using multiple parameter statistical optimization designs like Placket – Burman, Steepest ascent followed by Central Composite (Response surface methodology) design. Later the experimental result obtained by response surface methodology was validated using artificial neural networks. The optimal conditions were identified to be a production medium consisting (grams per litre) of 22.9g Meat extract B; 12.5g Yeast extract; 3g Peptone; 5g Sodium acetate; 3g Sodium citrate; 0.5g Ammonium dihydrogen ortho phosphate; 1g dipotassium hydrogen phosphate; 0.37g Magnesium sulphate; 0.058g Manganese sulphate; 6mg Vitamin B_{12}; 4mg Cobalt chloride; and 1: 1 ratio (w/w) of glucose and glycerol at pH 8.5 and incubated at 37^{o}C, 200 rpm.

In the batch bioreactor studies carried out in Parallel bioreactor (Infors, Multifors HT) using pure and crude glycerol, maximum titers of 1,3-PDO of 55.12 and 51.51 g/L with a volumetric yield of 0.64 $g_{1,3\text{-PDO}}/g_{Glycerol}$ was observed using optimized media composition at 8% glucose and glycerol concentration respectively. The titers of 1,3-PDO obtained using *L. brevis* N1E9.3.3 strain, are maximum than any lactic acid bacteria reported in batch fermentation using either pure or crude glycerol. Although this native strain has potent nature to be a commercial producer, as lactic acid bacteria grow in a complex nutrient media, cost of the production media is very high due to complex nitrogen sources like meat extract, yeast extract and peptone, to tackle this limitation, a new media composition was developed using fish protein hydrolysate as the sole nitrogen source.

Various raw materials like CSL, cotton seed cake, ground nut cake, soybean were reported as cost effective nitrogen sources for growth and development of lactic acid bacteria. In this study, we observed that 4% (w/v) fish protein hydrolysate was optimal for 1,3-PDO production resulting in 57.9 g/L and 57.84 g/L with a volumetric yield of 0.72 0.64 $g_{1,3\text{-PDO}}/g_{Glycerol}$ in glycerol – glucose co-fermentation strategy carried out in 3 L stirred tank bioreactor (Infors Minifors HT). With the experimental data on 1,3-PDO production and growth experiments, we can conclude that fish protein hydrolysate can be a cost effective and efficient alternative nitrogen source for meat extract, yeast extract and peptone.

Separation of 1,3-propanediol from the fermented broth was carried out using aqueous two phase extraction. Under the optimal concentrations of 45% K_2CO_3 + 15% K_2HPO_4, and 1: 1 (v/v) of isopropanol to the volume of fermented broth, 95 % recovery of 1,3-PDO, 73.83 % and 49.47 % removal of lactic and acetic acid was observed respectively. Later the final purification of the organic phase separated crude 1,3-PDO, was carried out using silica gel gradient column chromatography with chloroform and methanol as the mobile phase with increasing polarity. Further characterization of purified 1,3-PDO was carried out using ^{13}C and ^{1}H NMR. The results clearly indicated the purity of 1,3-PDO obtained from the fermented broth.

1,3-propanediol has a major role in synthesis of polyesters and bio-plastics, but glycerol is well known plasticizer for these polyesters, and bio-plastics, as 1,3-PDO is derived from glycerol and also have a structural similarity, in this study we provided a new application for 1,3-PDO as a plasticizer in chitosan films. As major polymers for these composite films, chitosan and dextran like exopolysaccharides purified from food grade lactic acid bacteria (*Lactobacillus plantarum*) was used. The casted films were

characterized to understand the properties, the 1,3-PDO plasticized films displayed significant physical and mechanical properties like 39% increase in tensile strength, and 94% increase in elongation percentage. The composite films were observed to be biodegradable and with 50 % water solubility. Hence we can conclude that as a derivative of glycerol, 1,3-PDO also has properties of a plasticizer.

Conclusion

Though a commercial bioprocess for 1,3-PDO production was established, several studies were carried out in respect to isolation of novel microorganisms, engineering potent producers to improve the titers and etc. With reference to decades of research on 1,3-PDO, only two microorganisms Clostridium butyricum and Klebsiella pneumoniae are considered as best producers, but two strains has its own limitations to be commercialized. To claim the microorganism to be a potent producer and to apply in an industrial scale, detailed studies on development of upstream process and downstream process at feasible prices is necessary.

Results of the present study pertaining to development of a bioprocess for 1,3-PDO production, showcased the potent nature of *Lactobacillus brevis* N1E9.3.3 strain to be a 1,3-PDO producer. The microorganism having a GRAS (genetically regarded as safe) status, can be grown in a cost effective growth and production media developed using fish protein hydrolysate. The results of downstream process also shows the ease of purification of 1,3-PDO from the fermented broth using aqueous two phase extraction.

So with these experimental results we can conclude that a bioprocess with optimized upstream and downstream processes for 1,3-propanediol production using biodiesel derived crude glycerol through *Lactobacillus brevis* N1E9.3.3 strain was developed.

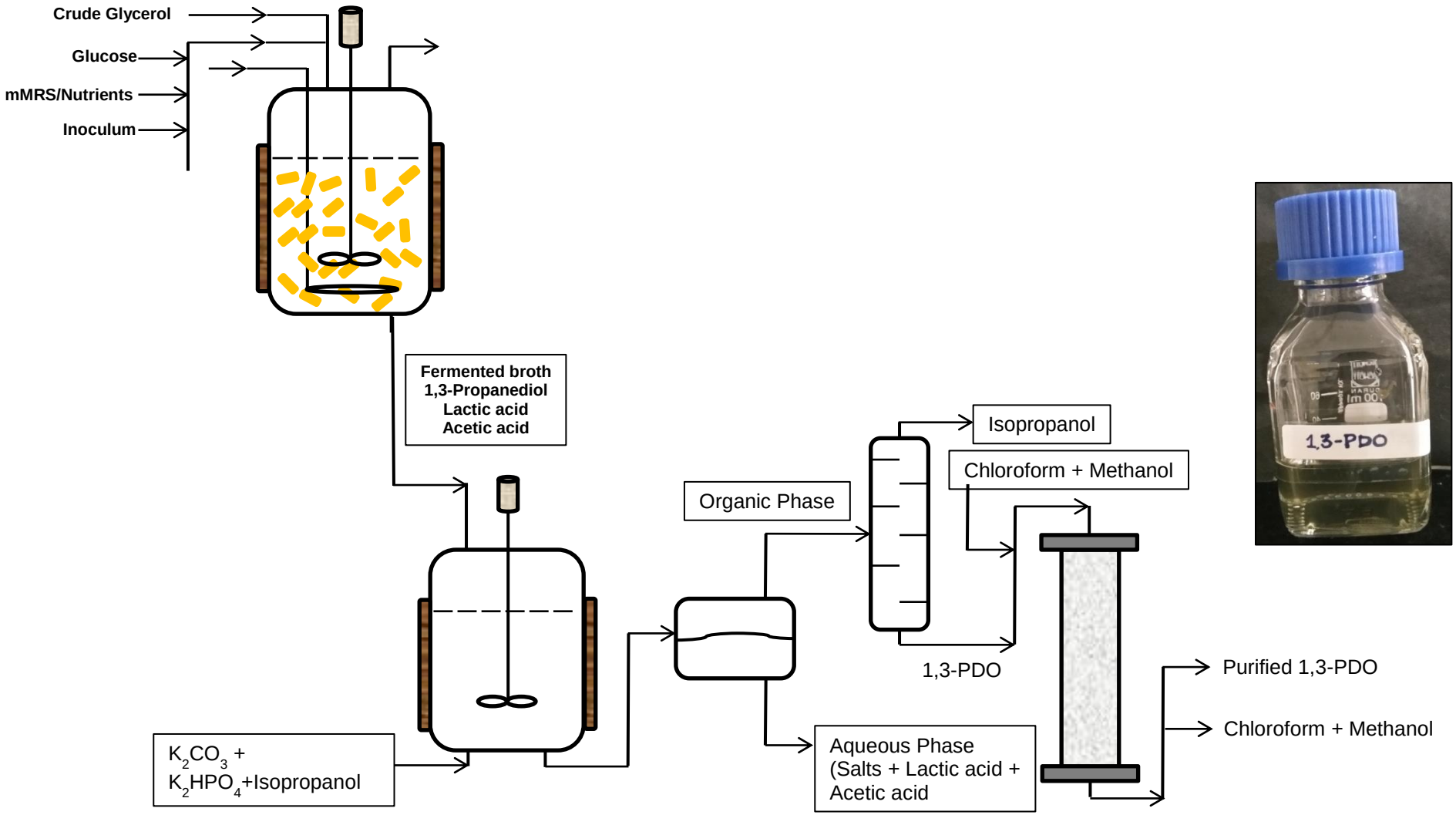
Significance of the study

1,3-Propanediol as a monomer, is well known for synthesis of polytrimethylene terephthalate (PTT) by polycondensation reaction with terephthalic acid. But glycerol glut, resulted in expanding the applications of 1,3-PDO, in other fields along with synthesis of polyesters. Hence 1,3-PDO is a potential bulk chemical with an industrial significance. In this study, the focus is on developing an efficient bioprocess for 1,3-PDO production using biodiesel derived crude glycerol, and significant findings of this study as follows

i. Selection of a potent 1,3-PDO producer *Lactobacillus brevis* N1E9.3.3, with substrate (> 100 g/L) and product (> 75 g/L) tolerance.
ii. Development of a cost effective growth and production media for 1,3-PDO production and other products like lactic acid using lactic acid bacteria.
iii. Developing an aqueous two phase extraction using dual inorganic electrolytes (K_2CO_3 and K_2HPO_4) resulting in an 95% recovery of 1,3-PDO from the fermented broth.
iv. Increase in 94% elongation percentage (Physical property for plastics and packaging industry) by addition of 1,3-PDO as a plasticizer, this study provided a new application for the monomer.

Future Perspective

At present the LAB strains are confined to food and dairy industry, the availability of vast genomic database, next generation techniques, metabolic profiling can discover metabolic pathways for novel chemicals and intermediates that have applications in food, pharma and white biotechnology. These strains have ability to rule the market by producing various value added chemicals worth billions like 1,3-propanediol. To specify the interesting facts about LAB strains are ability to utilize various carbon sources, specifically *Lactobacillus brevis*, can utilize both hexoses and pentoses simultaneously without carbon catabolite repression. Hence with this idea, whether sorghum juice or other lignocellulosic hydrolysates as carbon sources for growth in both batch and fed-batch mode of fermentation can provide similar or improved titers of 1,3-PDO will be the problem of interest. To date various genetic engineering tools are available for metabolic engineering of LAB strains, hence a genetic engineering approach for modifying the lactic acid metabolic pathway, either via gene deletion or gene disruption could improve the flux of reducing equivalents towards reductive pathway and resulting in increased titers of 1,3-propanediol.



Scheme. 7.1. Illustration of bioprocess developed using fish protein hydrolysate as the Nitrogen source and crude glycerol as the substrate in upstream process and aqueous phase two phase extraction in downstream process for production and purification of 1,3-propanediol.

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StdOrder	RunOrder	PtType	Blocks	Meat Extract (g/L)	Yeast Extract (g/L)	MgSO4 (g/L)	MnSO4 (g/L)	Vit B12 (mg/L)	Glycerol (g/L)	Glucose (g/L)	1,3-PDO (g/L)
75	1	1	3	20.5	10.37	0.42	0.08	7.34	40	30	20.18
100	2	1	3	18.5	11.35	0.42	0.08	7.34	60	50	21.35
97	3	1	3	18.5	10.37	0.46	0.07	7.34	60	50	19.73
90	4	1	3	20.5	11.35	0.42	0.07	7.34	40	50	21.05
72	5	1	3	20.5	11.35	0.46	0.08	6.73	40	30	20.24
78	6	1	3	18.5	11.35	0.46	0.07	6.73	60	30	20.63
74	7	1	3	20.5	11.35	0.46	0.07	7.34	40	30	20.62
85	8	1	3	20.5	10.37	0.46	0.07	6.73	40	50	21.31
82	9	1	3	20.5	11.35	0.42	0.07	7.34	60	30	20.14
93	10	1	3	20.5	10.37	0.42	0.07	6.73	60	50	21.5
86	11	1	3	18.5	11.35	0.46	0.07	6.73	40	50	20.83
101	12	0	3	19.5	10.86	0.44	0.075	7.035	50	40	22.47
94	13	1	3	18.5	11.35	0.42	0.07	6.73	60	50	21.44
91	14	1	3	20.5	10.37	0.46	0.08	7.34	40	50	21.25
98	15	1	3	20.5	11.35	0.46	0.07	7.34	60	50	22.10
92	16	1	3	18.5	11.35	0.46	0.08	7.34	40	50	21.03
70	17	1	3	18.5	11.35	0.42	0.07	6.73	40	30	21.51
69	18	1	3	20.5	10.37	0.42	0.07	6.73	40	30	20.36
77	19	1	3	20.5	10.37	0.46	0.07	6.73	60	30	20.37
79	20	1	3	18.5	10.37	0.42	0.08	6.73	60	30	21.14
83	21	1	3	20.5	10.37	0.46	0.08	7.34	60	30	20.74
84	22	1	3	18.5	11.35	0.46	0.08	7.34	60	30	22.62
102	23	0	3	19.5	10.86	0.44	0.075	7.035	50	40	22.06
89	24	1	3	18.5	10.37	0.42	0.07	7.34	40	50	20.26
99	25	1	3	20.5	10.37	0.42	0.08	7.34	60	50	21.51
96	26	1	3	20.5	11.35	0.46	0.08	6.73	60	50	21.62
71	27	1	3	18.5	10.37	0.46	0.08	6.73	40	30	20.35

StdOrder	RunOrder	PtType	Blocks	Meat Extract (g/L)	Yeast Extract (g/L)	MgSO4 (g/L)	MnSO4 (g/L)	Vit B12 (mg/L)	Glycerol (g/L)	Glucose (g/L)	1,3-PDO (g/L)
95	28	1	3	18.5	10.37	0.46	0.08	6.73	60	50	21.00
88	29	1	3	20.5	11.35	0.42	0.08	6.73	40	50	20.84
81	30	1	3	18.5	10.37	0.42	0.07	7.34	60	30	21.52
73	31	1	3	18.5	10.37	0.46	0.07	7.34	40	30	20.76
76	32	1	3	18.5	11.35	0.42	0.08	7.34	40	30	19.66
80	33	1	3	20.5	11.35	0.42	0.08	6.73	60	30	21.29
87	34	1	3	18.5	10.37	0.42	0.08	6.73	40	50	21.00
111	35	1	4	18.5	10.37	0.42	0.07	6.73	60	30	19.88
119	36	1	4	18.5	10.37	0.42	0.07	6.73	40	50	20.38
112	37	1	4	20.5	11.35	0.42	0.07	6.73	60	30	20.38
120	38	1	4	18.5	10.37	0.42	0.07	6.73	40	50	22.21
130	39	1	4	18.5	11.35	0.42	0.08	6.73	60	50	22.10
125	40	1	4	18.5	10.37	0.42	0.07	6.73	40	50	20.38
135	41	0	4	18.5	10.37	0.42	0.07	6.73	40	50	22.45
136	42	0	4	19.5	10.86	0.44	0.075	7.035	50	40	21.93
134	43	1	4	20.5	11.35	0.46	0.08	7.34	60	50	22.33
117	44	1	4	18.5	10.37	0.42	0.08	7.34	60	30	21.77
105	45	1	4	20.5	10.37	0.42	0.08	6.73	40	30	17.25
106	46	1	4	18.5	11.35	0.42	0.08	6.73	40	30	19.58
103	47	1	4	18.5	10.37	0.46	0.07	6.73	40	30	20.40
133	48	1	4	18.5	10.37	0.46	0.08	7.34	60	50	22.68
126	49	1	4	20.5	11.35	0.42	0.08	7.34	40	50	21.31
129	50	1	4	20.5	10.37	0.42	0.08	6.73	60	50	21.72
113	51	1	4	20.5	10.37	0.46	0.08	6.73	60	30	20.94
107	52	1	4	20.5	10.37	0.42	0.07	7.34	40	30	19.49
127	53	1	4	18.5	10.37	0.46	0.07	6.73	60	50	20.92
121	54	1	4	20.5	10.37	0.46	0.08	6.73	40	50	20.57
131	55	1	4	20.5	10.37	0.42	0.07	7.34	60	50	22.04
132	56	1	4	18.5	11.35	0.42	0.07	7.34	60	50	22.31

StdOrder	RunOrder	PtType	Blocks	Meat Extract (g/L)	Yeast Extract (g/L)	MgSO4 (g/L)	MnSO4 (g/L)	Vit B12 (mg/L)	Glycerol (g/L)	Glucose (g/L)	1,3-PDO (g/L)
108	57	1	4	18.5	11.35	0.42	0.07	7.34	40	30	19.22
128	58	1	4	20.5	11.35	0.46	0.07	6.73	60	50	22.02
104	59	1	4	20.5	11.35	0.46	0.07	6.73	40	30	20.78
115	60	1	4	20.5	10.37	0.46	0.07	7.34	60	30	21.56
118	61	1	4	20.5	11.35	0.42	0.08	7.34	60	30	19.86
110	62	1	4	20.5	11.35	0.46	0.08	7.34	40	30	19.90
116	63	1	4	18.5	11.35	0.46	0.07	7.34	60	30	21.25
114	64	1	4	18.5	11.35	0.46	0.08	6.73	60	30	21.71
123	65	1	4	20.5	10.37	0.46	0.07	7.34	40	50	21.01
124	66	1	4	18.5	11.35	0.46	0.07	7.34	40	50	20.58
109	67	1	4	18.5	10.37	0.46	0.08	7.34	40	30	19.63
122	68	1	4	18.5	11.35	0.46	0.08	6.73	40	50	20.30
144	69	-1	5	19.5	10.86	0.44	0.091	7.035	50	40	22.76
148	70	-1	5	19.5	10.86	0.44	0.075	7.035	83.63	40	18.49
154	71	0	5	19.5	10.86	0.44	0.075	7.035	50	40	22.99
146	72	-1	5	19.5	10.86	0.44	0.075	8.060	50	40	24.37
140	73	-1	5	19.5	12.50	0.44	0.075	7.035	50	40	22.91
143	74	-1	5	19.5	10.86	0.44	0.058	7.035	50	40	23.73
150	75	-1	5	19.5	10.86	0.44	0.075	7.035	50	73.63	16.86
137	76	-1	5	16.11	10.86	0.44	0.075	7.035	50	40	23.55
138	77	-1	5	22.86	10.86	0.44	0.075	7.035	50	40	22.91
147	78	-1	5	19.5	10.86	0.44	0.075	7.035	16.36	40	14.21
158	79	0	5	19.5	10.86	0.44	0.075	7.035	50	40	24.06
152	80	0	5	19.5	10.86	0.44	0.075	7.035	50	40	23.77
151	81	0	5	19.5	10.86	0.44	0.075	7.035	50	40	24.05
156	82	0	5	19.5	10.86	0.44	0.075	7.035	50	40	24.06
142	83	-1	5	19.5	10.86	0.50	0.075	7.035	50	40	23.63
159	84	0	5	19.5	10.86	0.44	0.075	7.035	50	40	24.06
141	85	-1	5	19.5	10.86	0.37	0.075	7.035	50	40	23.53

StdOrder	RunOrder	PtType	Blocks	Meat Extract (g/L)	Yeast Extract (g/L)	MgSO4 (g/L)	MnSO4 (g/L)	Vit B12 (mg/L)	Glycerol (g/L)	Glucose (g/L)	1,3-PDO (g/L)
139	86	-1	5	19.5	9.21	0.44	0.075	7.035	50	40	21.21
157	87	0	5	19.5	10.86	0.44	0.075	7.035	50	40	23.90
149	88	-1	5	19.5	10.86	0.44	0.075	7.035	50	6.3	13.25
160	89	0	5	19.5	10.86	0.44	0.075	7.035	50	40	24.06
155	90	0	5	19.5	10.86	0.44	0.075	7.035	50	40	23.64
153	91	0	5	19.5	10.86	0.44	0.075	7.035	50	40	23.90
145	92	-1	5	19.5	10.86	0.44	0.075	6.009	50	40	22.84
60	93	1	2	20.5	11.35	0.42	0.07	6.73	60	50	22.64
67	94	0	2	19.5	10.86	0.44	0.075	7.035	50	40	24.06
40	95	1	2	18.5	11.35	0.46	0.07	7.34	40	30	19.59
41	96	1	2	18.5	10.37	0.42	0.08	7.34	40	30	19.63
37	97	1	2	20.5	10.37	0.46	0.08	6.73	40	30	19.41
59	98	1	2	18.5	10.37	0.42	0.07	6.73	60	50	19.84
46	99	1	2	18.5	11.35	0.42	0.08	6.73	60	30	21.14
48	100	1	2	18.5	11.35	0.42	0.07	7.34	60	30	19.91
51	101	1	2	18.5	10.37	0.46	0.07	6.73	40	50	20.79
56	102	1	2	18.5	11.35	0.42	0.07	7.34	40	50	22.67
53	103	1	2	20.5	10.37	0.42	0.08	6.73	40	50	20.82
66	104	1	2	20.5	11.35	0.42	0.08	7.34	60	50	22.33
36	105	1	2	20.5	11.35	0.42	0.07	6.73	40	30	22.52
49	106	1	2	18.5	10.37	0.46	0.08	7.34	60	30	22.54
42	107	1	2	20.5	11.35	0.42	0.08	7.34	40	30	20.50
47	108	1	2	20.5	10.37	0.42	0.07	7.34	60	30	22.53
63	109	1	2	20.5	10.37	0.46	0.07	7.34	60	50	21.33
38	110	1	2	18.5	11.35	0.46	0.08	6.73	40	30	21.25
55	111	1	2	20.5	10.37	0.42	0.07	7.34	40	50	22.07
52	112	1	2	20.5	11.35	0.46	0.07	6.73	40	50	23.01
61	113	1	2	20.5	10.37	0.46	0.08	6.73	60	50	22.80
44	114	1	2	20.5	11.35	0.46	0.07	6.73	60	30	20.22

Std Order	Run Order	Pt Type	Blocks	Meat Extract (g/L)	Yeast Extract (g/L)	MgSO4 (g/L)	MnSO4 (g/L)	Vit B12 (mg/L)	Glycerol (g/L)	Glucose (g/L)	1,3-PDO (g/L)
65	115	1	2	18.5	10.37	0.42	0.08	7.34	60	50	21.98
57	116	1	2	18.5	10.37	0.46	0.08	7.34	40	50	22.08
62	117	1	2	18.5	11.35	0.46	0.08	6.73	60	50	21.79
58	118	1	2	20.5	11.35	0.46	0.08	7.34	40	50	21.35
39	119	1	2	20.5	10.37	0.46	0.07	7.34	40	30	20.84
64	120	1	2	18.5	11.35	0.46	0.07	7.34	60	50	19.82
50	121	1	2	20.5	11.35	0.46	0.08	7.34	60	30	22.60
43	122	1	2	18.5	10.37	0.46	0.07	6.73	60	30	21.45
54	123	1	2	18.5	11.35	0.42	0.08	6.73	40	50	20.66
68	124	0	2	19.5	10.86	0.44	0.07	7.03	50	40	22.74
45	125	1	2	20.5	10.37	0.42	0.08	6.73	60	30	19.91
35	126	1	2	18.5	10.37	0.42	0.07	6.73	40	30	20.24
7	127	1	1	20.5	10.37	0.46	0.08	7.34	40	30	17.87
25	128	1	1	20.5	10.37	0.46	0.07	6.73	60	50	19.21
5	129	1	1	18.5	10.37	0.42	0.07	7.34	40	30	18.11
34	130	0	1	19.5	10.86	0.44	0.07	7.03	50	40	20.60
32	131	1	1	18.5	11.35	0.46	0.08	7.34	60	50	20.45
31	132	1	1	20.5	10.37	0.46	0.08	7.34	60	50	19.99
24	133	1	1	18.5	11.35	0.42	0.08	7.34	40	50	18.99
16	134	1	1	18.5	11.35	0.42	0.08	7.34	60	30	20.08
3	135	1	1	18.5	10.37	0.42	0.08	6.73	40	30	17.27
33	136	0	1	19.5	10.86	0.44	0.07	7.03	50	40	19.33
30	137	1	1	20.5	11.35	0.42	0.07	7.34	60	50	21.07
12	138	1	1	20.5	11.35	0.46	0.08	6.73	60	30	19.53
11	139	1	1	18.5	10.37	0.46	0.08	6.73	60	30	19.61
21	140	1	1	18.5	10.37	0.46	0.07	7.34	40	50	18.89
27	141	1	1	18.5	10.37	0.42	0.08	6.73	60	50	19.71
17	142	1	1	20.5	10.37	0.42	0.07	6.73	40	50	19.95
29	143	1	1	18.5	10.37	0.42	0.07	7.34	60	50	18.53

Std Order	Run Order	Pt Type	Blocks	Meat Extract (g/L)	Yeast Extract (g/L)	MgSO4 (g/L)	MnSO4 (g/L)	Vit B12 (mg/L)	Glycerol (g/L)	Glucose (g/L)	1,3-PDO (g/L)
9	144	1	1	20.5	10.37	0.42	0.07	6.73	60	30	19.81
22	145	1	1	20.5	11.35	0.46	0.07	7.34	40	50	19.59
1	146	1	1	20.5	10.37	0.46	0.07	6.73	40	30	18.86
19	147	1	1	18.5	10.37	0.46	0.08	6.73	40	50	18.39
26	148	1	1	18.5	11.35	0.46	0.07	6.73	60	50	18.58
20	149	1	1	20.5	11.35	0.46	0.08	6.73	40	50	19.03
23	150	1	1	20.5	10.37	0.42	0.08	7.34	40	50	18.85
8	151	1	1	18.5	11.35	0.46	0.08	7.34	40	30	18.41
28	152	1	1	20.5	11.35	0.42	0.08	6.73	60	50	19.80
4	153	1	1	20.5	11.35	0.42	0.08	6.73	40	30	17.99
14	154	1	1	20.5	11.35	0.46	0.07	7.34	60	30	18.33
2	155	1	1	18.5	11.35	0.46	0.07	6.73	40	30	17.66
10	156	1	1	18.5	11.35	0.42	0.07	6.73	60	30	19.35
13	157	1	1	18.5	10.37	0.46	0.07	7.34	60	30	19.52
18	158	1	1	18.5	11.35	0.42	0.07	6.73	40	50	19.50
6	159	1	1	20.5	11.35	0.42	0.07	7.34	40	30	18.42
15	160	1	1	20.5	10.37	0.42	0.08	7.34	60	30	19.66

Annexure I

Central composite (RSM) experimental design for 1,3-propaendiol production.

Annexure II

Research Publications

1. **Narisetty Vivek,** Pandey A, Binod P. Biological valorization of pure and crude glycerol into 1, 3-propanediol using a novel isolate Lactobacillus brevis N1E9. 3.3. **Bioresource technology**. 2016 Aug 31;213:222-30.
2. **Narisetty Vivek**, Christopher M, Kumar MK, Castro E, Binod P, Pandey A. Pentose rich acid pretreated liquor as co-substrate for 1, 3-propanediol production. **Renewable Energy**. 2017 Jan 30.
3. **Narisetty Vivek**, Astray G, Gullón B, Castro E, Parameswaran B, Pandey A. Improved 1, 3-propanediol production with maintained physical conditions and optimized media composition: Validation with statistical and neural approach. **Biochemical Engineering Journal**. 2017 Oct 15;126:109-17.
4. **Narisetty Vivek**, T VAswathi, Petit Riff Sven, Ashok Pandey and Parameswaran Binod. Self-cycling fermentation for 1,3-Propanediol production: Comparative evaluation of metabolite flux in cell recycling, simple batch and continuous processes using *Lactobacillus brevis* N1E9.3.3 strain. **Journal of biotechnology**. 2017 Oct 10;259:110-9.
5. **Narisetty Vivek**, Pandey A, Binod P. An efficient dual inorganic electrolytes for aqueous two phase extraction of 1,3-propanediol from the fermented broth. **Bioresource technology**. 2018 Feb 20; 254:239-246.

Annexure III

Patent

1. Parameswaran Binod & **Narisetty Vivek**, Microbial process for the production of 1,3- propanediol in high titers using fish protein hydrolysate as nitrogen source by native microorganism using waste by-products (Submitted for evaluation).

Annexure IV

Review Articles

1. Sindhu R, Binod P, Pandey A, Madhavan A, Alphonsa JA, **Narisetty Vivek**, Gnansounou E, Castro E, Faraco V. Water hyacinth a potential source for value addition: An overview. **Bioresource technology**. 2017 Apr 1;230:152-62.
2. **Narisetty Vivek**, Sindhu R, Madhavan A, Anju AJ, Castro E, Faraco V, Pandey A, Binod P. Recent advances in the production of value added chemicals and lipids utilizing biodiesel industry generated crude glycerol as a substrate–Metabolic aspects, challenges and possibilities: An overview. **Bioresource technology**. 2017 Sep 1;239:507-17.
3. **Narisetty Vivek**, Nair LM, Mohan B, Nair SC, Sindhu R, Pandey A, Shurpali N, Binod P. Bio-butanol production from rice straw–Recent trends, possibilities, and challenges. Bioresource Technology Reports. 2019 May 7:100224.
4. **Narisetty Vivek**, Hazeena SH, Rajesh RO, Godan TK, Anjali KB, Nair LM, Mohan B, Nair SC, Sindhu R, Pandey A, Binod P. Genomics of Lactic Acid Bacteria for Glycerol Dissimilation. Molecular biotechnology. 2019 Jun 4:1-7.

Annexure V

Book Chapters

1. **Narisetty Vivek**, P Binod and A Pandey, Bio-catalytic production of 1,3-propanediol, In: Currents Development in Biotechnology and Bioengineering, Elsevier (In press).
2. **Narisetty Vivek**, and P Binod, Biosynthesis of 1,3-Propanediol; Genetics and applications: Springer.
3. **Narisetty Vivek**, P Binod and A Pandey, Bio-1,3-propanediol as monomer in synthesis of polyurethane and polyesters; Green Chemistry, RSC publication.

Annexure VI

Conference Publications

1. **Narisetty Vivek**, Pandey, A., Binod, P. *"*Isolation and screening of 1,3-propnanediol producers and optimization of fermentative parameters for increase in productivity of *Klebsiella pneumoniae"* presented at ICETB 2014 conference, New Delhi, India.
2. **Narisetty Vivek**, Pandey, A., Binod, P. "A novel onsite enrichment for crude glycerol utilizing and diol producing facultative anaerobic bacteria" presented at NHBT 2015 conference, Thiruvanathapuram, Kerala, India.
3. **Narisetty Vivek**, Christopher M, Kumar MK, Castro E, Binod P, Pandey A. "Pentose rich acid pretreated liquor as co-substrate for 1, 3-propanediol production" at 1st International conference on Bioenergy, Bioproducts and Environmental sustainability, Spain, 2-26 October 2016.
4. **Narisetty Vivek**, Petit Riff Sven, T VAswathi, Ashok Pandey and Parameswaran Binod. "Performance of suspended and immobilized *Lactobacillus brevis* N1E9.3.3 strain in batch, fed-batch and continuous processes for 1, 3-PDO production". Presented at ICCB 2016 conference, Vellore, Tamilnadu India.
5. **Narisetty Vivek**, Pandey A, Binod P. "An efficient dual inorganic electrolytes for aqueous two phase extraction of 1,3-propanediol from the fermented broth". Presented at ETBWC 2017 conference, Nagpur, India.
6. **Narisetty Vivek,** Pandey A, Binod P. "Adsorption of lactic acid and acetic acid on synthesized and commercial anion exchange resins from simulated solutions and fermented broth" at International conference BioSD 2018, held at CSIR-IICT, Hyderbad, 2018.

Annexure VII

AcSIR Course work

Level 100			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-1-001	Biostatistics	1	Completed
BIO-NIIST-1-002	Bioinformatics	1	Completed
BIO-NIIST-1-003	Basic Chemistry	1	Completed
BIO-NIIST-1-004	Research Methodology, Communication, Ethics & Safety	1	Completed

Level 200			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-2-001	Biotechniques and Instrumentation	1	Completed
BIO-NIIST-2-206	Protein sciences and Proteomics	2	Completed
BIO-NIIST-2-239	Basic Molecular biology	2	Completed

Level 300			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-3-001	Seminar Course	1	Completed
BIO-NIIST-3-381	Bioprocess Technology	2	Completed
BIO-NIIST-3-382	Biopolymer	2	Completed

Level 400			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-4-001	Review Writing	1	Completed
BIO-NIIST-4-002	Project Proposal	1	Completed

AcSIR 800	**Completed**

Annexure VIII

Instruments used during the research period	Instrument Brand Name
Agarose gel electrophoresis unit	Biorad
Gel documentation system	Chemidoc (Biorad)
Laminar flow hood cabinet	CleanAir
Centrifugal vacuum concentrator	Eppendorf
PCR thermal cycler	Biorad
Temperature controlled heating block	Eppendorf
Fume-hood	Esco global
DNA sequencer	Hitachi
Fermentor (3L total volume)	Infors
Rotary incubation shaker	Infors
Autoclave	Labline
Microscope (clinical)	Leica
Weighing balance	Mettler Toledo
Centrifuge	Remi, Kubota, Eppendorf
4°C walk-in cold storage room	Rinac
Heating oven	RRL-T NC hot air drier
Nitrogen generator	Schmidlin
HPLC	Shimadzu
UV-Vis spectrophotometer	Shimadzu
Magnetic stirrer	Tarsons
Vortex mixture	Tarsons
Bath sonicator	iUltrasonic
-20°C deep freezer	Voltas
-80°C deep freezer	Haier
pH meter	EuTech