PRODUCTION OF BIOETHANOL BY ELECTROCHEMICAL REDOX COUPLED WITH MICROBIAL CELLS USING LIGNOCELLULOSIC BIOMASS

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF DOCTOR OF PHILISOPHY

IN BIOTECHNOLOGY

BY

JARINA JOSHI

(January, 2020)

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DECLARATION

Thesis entitled "**Production of bioethanol by electrochemical redox coupled with microbial cells using lignocellulosic biomass**" which is being submitted to the Central Department of Biotechnology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of degree of Doctor of Philosophy (Ph.D.) is the research work carried out by me under the supervision of Prof. Dr. Tribikram Bhattarai, Central Department of Biotechnology, Tribhuvan University and co supervised by Prof. Dr. Lakshmaiah Sreerama , Department of Chemistry and Earth Sciences, Qatar University, Doha, Qatar and Prof. Dr. Amar Prasad Yadav, Central Department of Chemistry, Tribhuvan University, Nepal. This research is original and has not been submitted earlier in part or full in this and in any other form to any university or institute, here or elsewhere, for the award of any degree.

Jarina Joshi

RECOMMENDATION

This is to recommend that **Jarina Joshi** has carried out research entitled "**Production of bioethanol by electrochemical redox coupled with microbial cells using lignocellulosic biomass"** for the award of Doctor of Philosophy (Ph. D.) in **Biotechnology** under our supervision. To our knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph. D. degree.

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LETTER OF APPROVAL

Date: 20th January, 2020

On the recommendation of **Prof. Dr. Tribikram Bhattarai** (Supervisor), **Prof. Dr. Lakshmaiah Sreerama** (Co supervisor) and **Prof. Dr. Amar Prasad Yadav** (Co Supervisor), this Ph. D. thesis submitted by **Jarina Joshi** entitled "**Production of bioethanol by electrochemical redox coupled with microbial cells using lignocellulosic biomass"** is forwarded by Central Department Research Committee (CDRC) to the Dean IOST, TU.

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Jarina Joshi

January, 2020

Production of Bioethanol by Electrochemical Redox Coupled with Microbial Cells using Lignocellulosic Biomass

ABSTRACT

Bioethanol, blended with gasoline (petrol), is used as liquid transportation fuel worldwide. Local production and use of bioethanol supports local economies, decreases a country's carbon footprint and promotes self-sufficiency. The latter is especially important for bio-resource rich, land-locked countries such as Nepal that are seeking alternative transportation fuels and technologies to produce them. Bioethanol is a renewable resource that is dominantly produced from either sucrose or starchy biomass. As Nepal is rich in agricultural sector, use of residual lignocellulosic biomass from plants can be a better alternative for bioethanol production. The lignocellulosic biomass composition of plants differ depending on the locality and seasonal changes. We have evaluated the suitability of four different sources of lignocellulosic biomass, viz., *Ipomoea carnea*, *Phragmites karka, Saccharum spontaneum* and *Zea mays* cobs for obtaining reducing sugar which can be used for bioethanol production in Nepal. *S. spontaneum* was found to be the best among the four as an economic source of lignocellulosic biomass since it has better degradation capabilities, high caorific value and relatively high total reducing sugar (TRS) content (612.2±11.5 $mg \cdot g^{-1}$ biomass). Hot water pretreatment of *S. spontaneum* biomass at 100^{o}C for 2 h followed by hydrochloric acid hydrolysis (TRS = 330.4±20.5 $mg \cdot g^{-1}$ biomass) were found to be the best for release of fermentable sugars. The variations in characteristics of lignocellulosic biomass before and after pretreatment with hot water at 100^{o}C for 2 h were investigated by using differential thermo gravimetric curve (DTG), X-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy methods. The XRD and FTIR analysis showed that pretreatment reduced the amorphous nature of cellulose and increased crystalline characteristics. The content of glucose (untreated: 246.7±4.0 and pretreated: 235.1±5.0 $mg \cdot g^{-1}$ biomass) and xylose (untreated: 86.6±3.9 and pretreated: 62.5±3.0 $mg \cdot g^{-1}$ biomass) determined in untreated and pretreated (hot water at 100^{o}C for 2 h) biomass suggested that while the cellulose loss during pretreatment was minimal, hemicellulose content was lost significantly. The conventional method to produce ethanol is via microbial fermentation and it comes with limitations. We have

demonstrated the feasibility of using a bio-electrochemical system to improve ethanol production by yeast. In this system, externally supplied 4V was used to drive the chemical reactions to generate higher levels of ethanol in the yeast cultures. In the present study, we have identified two highly efficient ethanol producing yeast strains, viz., *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalous* (CDBT7) out of twelve isolates and used them in a bioelectrochemical cell to enhance ethanol production. CDBT2 and CDBT7 were cultured in anodic and cathodic compartments with fine platinum coated platinum anode and neutral red coated graphite-felt cathode, ethanol production was drastically enhanced by 52.8±0.44% in average. The above experiments were repeated using lignocellulosic biomass hydrolysates (*Saccharum spontaneum* by pretreating with hot water for 2 h at 100^{o}C followed by hydrolysis with 0.5M HCl) as substrate resulted better enhancement in ethanol production (61.8±0.12%). Use of cellulose acetate in place of nafion membrane as anodic and cathodic partition better enhanced ethanol production by 6.30±0.22%. The enhancement in expression of alcohol dehydrogenase and pyruvate decarboxylase was seen when voltage was supplied. The results concluded that CDBT2 and CDBT7 yeast strains produced ethanol efficiently from both glucose and lignocellulosic biomass hydrolysate. The ethanol production was enhanced in electrochemical cell in the presence of low level of external voltage. Ethanol production was further enhanced with the better involvement of electron transport systems, when neutral red was deposited on cathode and platinum nanoparticles were coated on the platinum anode and cellulose acetate as partition membrane. This can be an optimal method for commercial ethanol production from biomass hydrolysate after up scaling.

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LIST OF SYMBOLS

Ω	Ohm
Σ	Approximately
%	Percentage
Δ	Delta
$	Dollar

LIST OF ACRONYMS AND ABBREVIATIONS

µm	Micro meter
5-HMF	5-hydroxy methyl furfural
AAS	Atomic absorption spectroscopy
ADH	Alcohol dehydrogenase
AFEX	Ammonia fibre explosion
ANN	Artificial neural network
bp	Base pair
C_{ba}	Heat capacity of benzoic acid
Cal	Calorie
CBP	Consolidated bioprocessing
CCR	Carbon catabolite repression
CDBT	Central Department of Biotechnology
cDNA	Complementary deoxyribonucleic acid
CIFTIR	Crystalanity Index Fourier Transformed Infrared
cm	Centimetre
CoA	Coenzyme A
Ct	Critical point
CV	Cyclic Voltametry
DNA	Deoxyribonucleic acid
DNSA	Dinitriphenyl sacillic acid
DTG	Differential thermogravimetry
ECC	Electro chemical cell
EDTA	Ethylene diamine tetra acetic acid
FAD	Flavin adenine dinucleotide
FPU	Filter paper unit
g	Gram
GA	Genetic algorithm
Gxf1	Xylose transporter
h	Hour
H_{ba}	Calorific value of benzoic acid

H_{ct}	Calorific value of cotton thread
HDPE	High density polyethylene
H_{fs}	Calorific value of sample
HPLC	High performance liquid chromatography
ID	Identity
IITG	Indian Institute of Technology, Guwahati
ISP	Integrated storage pretreatment
kg	Kilo gram
kV	Kilo volt
L	Litre
LDPE	Low density polyethelene
M	Molar
mA	Milli ampere
m_{ba}	Mass of benzoic acid
m_{ct}	Mass of cotton thread
mg	Milligram
min	Minute
mm	Millimetre
mM	Millimolar
mV	Millivolt
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NAD^+	Nicotinamide adenine dinucleotide (positively charged)
NADH	Nicotinamide adenine dinucleotide
NAST	Nepal Academy of Science and Technology
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometre
NR	Neutral red
NREL	National Renewable Energy Laboratory
^oC	Degree centigrade
PDC	Pyruvate decarboxylate

PEG	Polyethylene glycol
pM	Pico molar
PMMA	Poly methyl methacrylate
ppb	Parts per billion
ppm	Parts per million
PQQ	Pyrollo quinoline quinone
Pt	Platinum
PYN	Peptone yeast extract nitrogen
qPCR	Quantitative Polymerase Chain Reaction
RFA	Radio Free Asia
RNA	Ribonucleic acid
rpm	Rotation per minute
RT PCR	Real Time Polymerase Chain Reaction
SHCF	Separate hydrolysis and co fermentation
SSCF	Simultaneous saccharification and co fermentation
TBP	Tri-n-butyl phosphate
TCA	Tricarboxylic acid
TFC	Transcription factor
TGA	Thermogravimetric analysis
TRS	Total reducing sugar
TU	Tribhuvan University
USA	United States of America
V	Volt
v/v	Volume per volume
w/v	Weight per volume
Wh	Watt hour
WO	Wet oxidation
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XR	Xylose reductase
YMA	Yeast maltose agar
ΔT_c	Observed change in temperature for Benzioc acid
ΔT_{fs}	Observed change in temperature for sample

CHAPTER 1

1 INTRODUCTION

1.1 Background

Globally, petroleum products fulfill the majority of liquid energy demand. Very few countries have these resources and again bringing petroleum products to land locked country is challenging. Further, continued use of petroleum products is associated with problems such as pollution and global warming. There has been a great deal of effort placed on research to find alternative renewable liquid energy source with cost effective technologies. Bioethanol is one such resource which can be used as an alternative to petroleum products and even for generation of electricity. Bioethanol holds great potential for electricity production in fuel cells (Cropenergies, 2008). According to the Cropenergies bulletin, ethanol fuel cells can produce about 6300 $Wh \cdot kg^{-1}$, whereas methanol, Li-ion, Ni metal hydride, Ni-Cd can only produce 5400, 500, 200 and 100 $Wh \cdot kg^{-1}$ respectively.

Dependence on petroleum-based transportation fuels continues to be a major challenge for developing countries like Nepal. The challenge is further amplified because Nepal happens to be land locked country and has no fossil fuel reserves (Joshi et al., 2011). Nepal has to spend a major share of its resources to import fossil fuels via other countries. This leads to severe threat of energy security of the country. Accordingly, serious attempts are being made to develop alternative energy sources including bioethanol in Nepal (Silveira & Khatiwada, 2010). Bio-derived ethanol has several other important uses. It is dehydrated to ethylene and used for making polyethylene. Polyethylene can be converted into different plastic products (Table 1). In early 20^{th} century, ethylene was mainly produced from ethanol. But because of high costs, the process was shifted to the petrochemical route in which ethylene is produced *via* steam cracking of hydrocarbons at 750–950°C. Lately, ethylene production is again been shifted back to bioethanol as it is a good renewable product and the technologies are improving as well as the process is becoming cost effective. Several companies, e.g., Dow, Braskem and Solvay, are currently engaged in bioethanol to polyethylene projects (Rose & Palkivits, 2011).

Table 1: Use of bio-ethanol derived ethylene as a platform chemical for synthesis of commodity monomers and their corresponding polymers (Kejian & Chuanbing, 2013)

Ethylene Products	Uses
LDPE (low density polyethelene)	Thin films, Bags, Coatings
HDPE (high density polyethelene)	Pipes, Toys, Utensils
PEG (polyethelene glycol)	Pharmaceutical drug delivery, Industrial flocculation and Binders
PGA (poly glycolic acid)	Sutures, Scaffolds
PMMA (poly methyl methacrylate)	Transparent glass substituents

1.1.1 Substrate for Ethanol Production

Ethanol is produced conventionally by microbial fermentation using renewable sources of feedstock, specially sucrose and starch. Sucrose and starch presents the drawback in competing with sources of human and animal nutrition. For that reason, nonfood biomass sources, mainly lignocellulosic materials, such as invasive plants like *Saccharum spontaneum,* agricultural residues like corn cob (Joshi et al., 2018) or municipal solid wastes are gaining interest (Naik et al., 2010). Lignocellulosic biomass has emerged as an attractive alternative sugar source for ethanol production. Lignocellulosic biomass such as dendromass and phytomass are nature based materials consisting of complex heterogenic macromolecules such as cellulose, hemicellulose and lignin, as well as numerous other low molecular mass organic and inorganic components that can be used for ethanol production (Rasmussen et al., 2017).

1.1.1.1 Lignocellulosic Biomass

Lignocellulose (second generation biomass) is richly found in plant sources. Lignocellulosic biomass generally includes hardwood and softwood, municipal solid waste, agricultural residue, dedicated energy crops and weedy plants (Premjet et al., 2018). Lignocellulose is the most abundant renewable biomass with a worldwide annual production of an estimated 1000 Gigatonne, which include wheat straw, sugarcane bagasse, corn stalks, rye straw, rice straw and barley straw as well as various types of organic waste (Hermosilla et al., 2017).

Lignocellulosic biomass is a complex combination of cellulose, hemicellulose, lignin as major component (Figure 1). Besides it also contains pectin, pigments, proteins

and small amount of metal ions which collectively forms ash. Cellulose fibrils are interwoven with non-cellulosic polysaccharides, hemicellulose and lignin which bind cellulose through covalent and non-covalent interactions, stabilizing further the plant cell walls and increasing recalcitrance to enzymatic attack (Kristensen et al., 2008; Henrik & Peter, 2010).

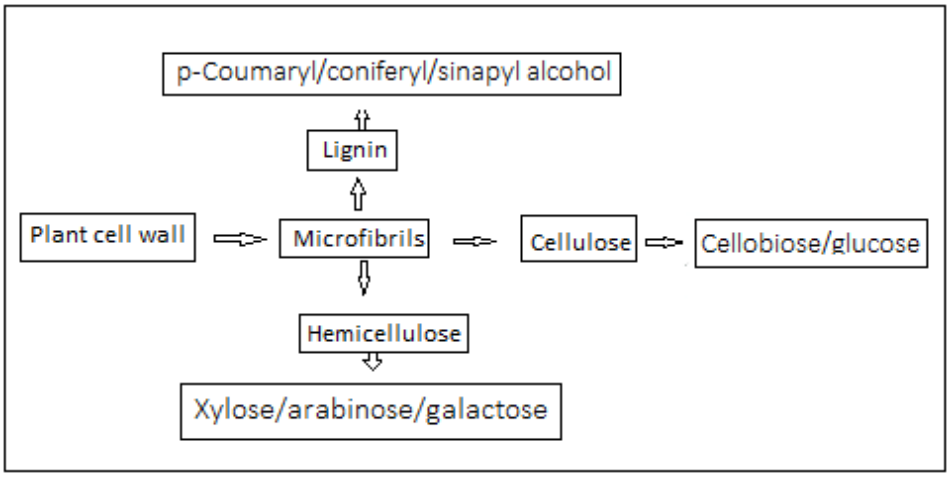

Figure 1: Plant cell wall with microfibrils of lignocellulosic biomass composed of cellulose, hemicellulose and lignin.

1.1.1.1.1 Cellulose

Cellulose (Figure 2) is a long chain of more than 500 glucose molecules linked through β-1,4-glycosidic bonding with the smallest repeating subunit of cellobiose (Zhang et al., 1997). Several of these polysaccharide chains are arranged in parallel arrays to form cellulose microfibrils. The individual polysaccharide chains are bound together in the microfibrils by hydrogen bonds. The microfibrils are bundled together to form macrofibrils with extremely tough texture called crystalline cellulose. They are inflexible due to the presence of strong hydrogen bonds unlike in starch molecule which is flexible polymer of glucose. These days cellulose is considered as most cost effective waste material to use to form value added renewable products (Kane & French, 2018).

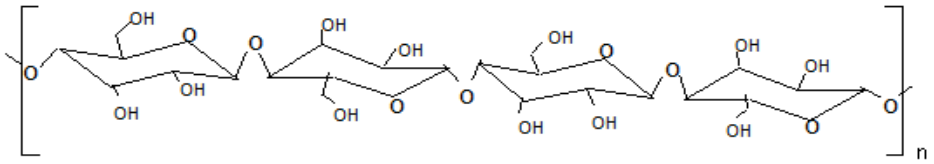

Figure 2: Cellulose structure [polymer of glucose/cellobiose with β(1-4) glycosidic linkage]

1.1.1.1.2 Hemicellulose

Hemicellulose derived from lignocelluloses of plants include the basic chain containing residues of D-xylose, D-mannose, D-glucose or D-galactose and other glycosyls as branched chains linked to this basic chain. Hemicellulose is more easily degraded in acidic medium than cellulose. The various ways of linkage between glycosyls are as shown in Figure 3, such as 1-2, 1-3, 1- 4 and 1-6 links (Yang, 2008).

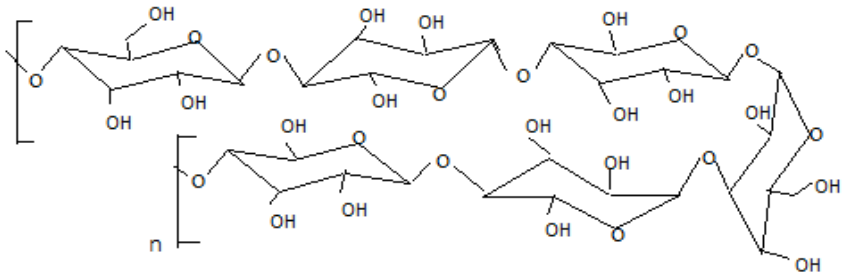

Figure 3: Hemicellulose with β(1-4) xylose, mannose, glucose and β(1-3) galactose linkage.

Softwoods contain the highest content of mannan hemicellulose; some hardwoods also have mannan hemicellulose, but grass has very less mannan contents. Hardwood hemicelluloses contain mostly xylans. Heteroglucans (xyloglucans) have a repeating backbone unit of β(1-4) linked glucopyranosyl molecules whereas heteroxylans (glucuronoxylans) consist of a linear backbone of β-xylopyranosyl units linked by β(1,4) glycosidic bonds (Kane, 2015).

1.1.1.1.3 Lignin

Lignin is a complex composed of complicated phenyl propane units nonlinearly and randomly linked. The three main monomers present in lignin are coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Because of the different monomers, lignin can be divided into three types: syringyl lignin polymerized by syringyl propane, guaiacyl lignin polymerized by guaiacyl propane, and hydroxy-phenyl lignin polymerized by hydroxyphenyl propane. Usually, gymnosperm mainly contains guaiacyl lignin, the dicotyledon mainly contains guaiacyl-syringyl lignin, the mono-cotyledon mainly contains guaiacyl-syringyl-hydroxy-phenyl lignin (Wei & Song, 2001).

1.1.2 Pretreatment of Lignocellulosic Biomass

The lignocellulosic biomass obtained from the plants is highly recalcitrant in effective release of sugars from them, which is a major challenge for its utilization.

Therefore, the saccharification of the biomass needs an effective pretreatment technique to expose cellulose and hemicellulose for efficient hydrolysis. Studies have shown that <20% glucose is released from lignocellulosic biomass without pretreatment, while that yield can be increased more with proper pretreatment procedures. Pretreatment is often expensive and it constitutes ~20% of the overall process cost for lignocellulosic bioethanol production (Yang & Wyman, 2008).

Adaptations of different pretreatment methods result in different effects on the biomass in terms of structure and composition. Pretreatment procedure varies for the same plant species depending on the growing condition and habitat. Additionally, biomass pretreatment needs to be optimized for obtaining highly digestible pretreated biomass with no significant sugar degradation, formation of a minimum level of toxic compounds, high yield of sugars after subsequent hydrolysis, fermentation compatibility, operation in reasonably sized and moderately priced reactors, lignin recovery with minimum heat and power requirements etc (Alvira et al., 2010).

1.1.2.1 Physical Method of Pretreatment

1.1.2.1.1 Chopping and Milling

Chopping and milling is considered as mechanical pretreatment method. The main objective behind mechanical pretreatment is to reduce the particle size and disruption of crystallinity of lignocellulose so that specific surface area for action of hydrolysis increases (Karunanithy & Muthukumarappan, 2011).

1.1.2.1.2 Extrusion

In extrusion method, the materials were modified by subjecting to heating, mixing and shearing by passing through extruder. Screw speed and barrel temperature are believed to disrupt the lignocellulose structure by shortening of the fibers. It increases accessibility of carbohydrates to hydrolytic action and making their conversion into fermentable sugar easier (Yang & Wyman, 2008).

1.1.2.1.3 Pyrolysis

In pyrolysis, biomass is subjected to very high temperature, above 300ºC in absence of oxygen. This leads to decomposition of cellulose into gaseous products and

residual char. At low temperature, the reaction speed is very slow, so that volatile products are formed which are environment hazardous. Acid hydrolysis when combined with pyrolysis, lignocellulosic residues results in reducing sugars, about 80– 85% cellulose can be converted into fermentable sugar (Fan et al., 1987).

1.1.2.2 Physicochemical Pretreatments

1.1.2.2.1 Microwave Pretreatment

Microwave based treatment is considered as physiochemical process because thermal and non-thermal (chemical) effects are involved. Pretreatments are carried out by immersing the biomass in dilute chemical reagents and exposing the slurry to microwave radiation for residence times ranging from 5 to 20 min. (Keshwani & Cheng, 2013).

1.1.2.2.2 Steam Pretreatment

Steam pretreatment method is frequently used in pretreatment of various lignocellulosic materials. Actually, it is a chemical method closely similar to dilute acid hydrolysis. In this method, dry lignocellulosic material is placed under high pressure environment. The desired material is placed under high-pressure saturated steam at typical temperatures between 180 and 260ºC for 1–15 min. The pressure is released that results highly exposed cellulose (Amores et al., 2013).

1.1.2.2.3 Ammonia Fiber Explosion (AFEX)

In the AFEX process, biomass is treated with liquid anhydrous ammonia at temperatures between 60 and 100°C at high pressure for a variable period of time. The pressure is then released, resulting in a rapid expansion of the ammonia gas that causes swelling and physical disruption of biomass fibers and partial decrystallization of cellulose (Alizadeh et al., 2005).

1.1.2.2.4 Wet Oxidation

Wet oxidation (WO) is the process of treating material with water and either air or oxygen at temperatures above 120°C. Two types of reactions occur: a low-temperature hydrolytic reaction and a high-temperature oxidative reaction. It has been

demonstrated that combination of alkali and WO reduces the formation of toxic furaldehydes and phenol aldehydes. It is known that WO mainly catalyzes the transfer of hemicelluloses from the solid phase to the liquid phase, but it does not catalyze the hydrolysis (Martin et al., 2007; Barkodia et al., 2016).

1.1.2.3 Chemical Pretreatment

Pretreatment using chemicals are most studied technique used for the recovery of sugar from the cellulosic material. Chemical reagents used for degradation of lignin from cellulosic waste are oxidizing agents, alkali, acids and salts. Some powerful oxidizing agents such as ozone and H_2O_2 have also been used for removal of lignin. It does not produce hazardous material during the process and the reactions are carried out at room temperature and pressure (Hah et al., 2017).

1.1.2.3.1 Alkaline Pretreatment

In alkali pretreatment method generally bases are used, such as hydroxides of calcium, sodium, ammonia and potassium. The use of bases in pretreatment causes the degradation of ester and glycosidic side chains which results into cellulose swelling, partial decrystallization of cellulose, removal of lignin and partial solvation of hemicellulose. Alkaline pretreatments enhance cellulose digestibility and are more effective for lignin solubilization (Girolamo et al., 2014).

1.1.2.3.2 Acid Pretreatment

In comparison to the other pretreatment procedures, particularly dilute acid pretreatment of lignocellulosic biomass is mostly used. Dilute acid pretreatment has some superiority over concentrated-acid hydrolysis to solve the issues like acid recovery, toxicity and special maintenance against corrosion materials. Acid pretreatment can be useful in case of several biomass feed-stocks like herbaceous material (grass), hardwoods and agricultural wastes. Most of the substrates give better results by solubilizing the hemicelluloses. Sulphuric acid pretreatment can be applied to various lignocellulosic materials like wheat straw, paddy straw, sugarcane bagasse. Chemical pretreatment methods such as using mild or concentrated acid, mild alkaline pretreatment, ozone or peroxide in pretreatment are faster but it may produces inhibitory end products such as furfurals, 5-hydroxymethylfurfural,

phenolic acids and aldehydes (Devi & Kumar, 2017). Hydrochloric acid can be used in delignification of several lignocellulosic materials such as sorghum straw, ryegrass and palm oil wastes (Barkodia et al., 2016).

1.1.2.3.3 Ozonolysis

Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw, bagasse, green hay, peanut, pine, cotton straw and poplar sawdust. The degradation was essentially limited to lignin and hemicellulose which was slightly attacked but cellulose was hardly affected. Ozonolysis pretreatment has several advantages like it effectively removes lignin, it does not produce toxic residues for the downstream processes and the reactions are carried out at room temperature and pressure. Despite it has important drawback, that is; a large amounts of ozone needed, which can make the process economically unviable (Hah et al., 2017)

1.1.2.3.4 Organic solvent

Organic solvents and inorganic solvents are used in combination to break the bonds between cellulose and hemicellulose. The organic solvents used in the process include methanol, ethylene, ethanol, acetone, glycol, triethylene glycol and tetrahydrofurfuryl alcohol. Organic acids such as acetylsalicylic, oxalic and salicylic acid can also be used as catalysts in the organic solvent pretreatment process. Benefit of using this method is that solvents used in the process can be drained from the reactor, evaporated, condensed and recycled to reduce the cost. Drawback of this process is that the removal of solvent becomes necessary from fermentation vessel because it is inhibitory to the growth of organisms, enzymatic hydrolysis, and fermentation (Zahid et al., 2012).

1.1.2.4 Biological Pretreatment

Biological pretreatments involve microorganisms mainly fungi, i.e. brown, white and soft-rot fungi that degrade lignin and hemicellulose and very little amount of cellulose. White-rot fungi, successfully removes lignin from lignocellulosic materials producing lignin degrading enzymes such as peroxidases and laccases. Several white-rot fungi such as *Ceriporia lacerata, Cyathus stercolerus, Phanerochaete*

chrysosporium, *Pycnoporus brumalis*, *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* have been examined on different lignocellulosic biomass showing high delignification efficiency (Lee et al., 2007; Zhou et al., 2017). Biological pretreatments are more effective, economical, eco-friendly and less health hazardous as compared to the physicochemical or chemical-based pretreatment methods. However, biological pretreatment is a very slow process that also requires careful control of growth conditions to perform efficient treatment. Another major drawback associated with microorganism mediated pretreatments is that some microorganisms degrade cellulose and hemicellulose with lignin degradation which is undesirable. Pretreatment using biological agent also requires a large amount of space for successful action. Because of these drawbacks, the biological pretreatment faces technical and economic barriers, therefore is commercially less attractive (Barkodia et al., 2016). The co-culture of bacteria and/or fungi as consolidated bioprocessing (CBP) is highly beneficial in the breakdown of complex biopolymers due to their high enzyme activity. Bacteria such as *Streptomyces sp.*, *Clostridium sp.*, *Cellulomonas sp.*, *Bacillus sp.*, etc. and several fungi such as *Trichoderma reesei*, *Phanerochaete chrysosporium*, *Aspergillus niger*, *Trichoderma viride*, etc.) are known to hydrolyze the natural biopolymers (Sharma et al., 2019).

1.1.3 Hydrolysis

Hydrolysis of pretreated lignocellulosic feedstock produces simple fermentable sugars. Hydrolysis of cellulose is significantly more difficult than that for starches, due to its crystalline form and presence of hydrogen bonding. The hydrolysis reaction can be catalyzed by acids or enzymes (Kim, 2018). Besides as raw materials for ethanol production, the hydrolysed products are also used for several other commercial compound productions. Xylose contained in hemicelluloses can be thermally transformed into furans (2-furfuraldeyde, hydroxymethyl furfural), short chain organic acids (formic, acetic, and propionic acids) and keto compounds (hydroxy-1-propanone, hydroxy-1-butanone). Furfural can be further processed to form some building blocks of innovative polymeric materials like 2, 5-furandicarboxylic acid. In addition, levulinic acid could be formed by the degradation of hydroxymethyl furfural). Xylose may be converted into xylitol by catalytic

hydrogenation which has several medical importance as sugar supplement for diabetic, prevent tooth decay etc (Ran et al., 2014).

1.1.3.1 Acid hydrolysis

The main advantage of the acid hydrolysis is that acids can penetrate lignin without any preliminary pretreatment of biomass, thus breaking down the cellulose and hemicellulose polymers to form individual sugar molecules. Acids like sulphurous, sulphuric, hydrochloric, hydrofluoric, phosphoric, nitric and formic acid are usually used for hydrolysis. Sulphuric and hydrochloric acids are the most commonly used acid for hydrolysis of lignocellulosic biomass (Lenihan et al., 2010). The concentrated acid hydrolysis occurs at low temperatures however, this process requires large amounts of acids causing corrosion problems to the equipments. For dilute acid hydrolysis, low amount of acid is needed to achieve acceptable rates of cellulose conversion. The high temperature also increases the rates of hemicellulose sugars decomposition thus causing the formation of toxic compounds such as furfural and 5-hydroxymethyl furfural (HMF). These compounds inhibit growth of yeast cells and the subsequent fermentation stage, causing a lower ethanol production (Maarten et al., 2009).

1.1.3.2 Enzymatic Hydrolysis

Cellulase is the major enzyme complex that catalyzes cellulose hydrolysis. Three major types of enzymatic reactions are reported for cellulase (1) endoglucanases or endo 1-4-β-D-glucan glucanohydrolases, (2) exoglucanases or exo 1-4-β-D-glucan glucanohydrolases (also known as celloextrinases) and (3) β-glucosidases or β-glucoside glucohydrolases. Endoglucanase react with internal amorphous cellulose sites to produce shorter chains of varying lengths and expose chain ends. Exoglucanases hydrolyze the ends of cellulose produced by endoglucanose to produce cellobiose as the major product. β-glucosidases convert cellodextrins and cellobiose to glucose. Genencor in USA and Novozymes in Europe produce celullases commercially. Hemicellulose offers an important amount of fermentable sugars, its enzymatic hydrolysis is more complicated than that one of cellulose. The main enzymes needed are glycoside hydrolase and carbohydrate esterase enzyme families (endoxylanase, beta-xylosidase, alpha-L-arabinofuranosidase, alpha-

glucuronidase, alpha-galactosidase, acetylxylan esterase and feruloyl-esterase) for degrading hemicellulose. These requirements make the enzymatic hydrolysis of hemicelluloses difficult and expensive (Gandla et al., 2018).

1.1.4 Fermentation

Fermentation is the final step of the conversion of lignocelulosic biomass to produce bioethanol. Bioethanol production by fermentation is of utmost importance to quantify the performance of the whole process (Sahu, 2016). A variety of microorganisms (yeast, fungi and bacteria) and technologies are developed for efficient ethanol fermentation. Some of the microbes have metabolic ability to ferment different types of sugars (Dodaris et al., 2013). A number of genetically engineered yeast and bacterial strains of *Saccharomyces spp, Pichia stipitis, Candida shehatae, Klebsiella oxytoca* and *Zymomonas mobilis* were reported for the production of bioethanol from different monosugars (Rajendran et al., 2015). Recently co-culturing and sequential use of yeast strains have been explored as promising for the high-level bioethanol production from complex sugars (Yadav et al., 2011). Genetically engineered *Escherichia coli* KO11 was developed to produce efficient ethanol from both pentose and hexose sugars. Yeast strains developed by protoplast fusion were also reported as glucose and xylose-fermenting yeast (Imamoglu & Sukran, 2014). Genetically engineered *S. cerevisiae* constructed either by introducing genes encoding xylose isomerase (XI) from bacteria and fungi or genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from fungi has also been reported as xylose and arabinose fermenting strains of recombinant yeast (Karhumaa et al., 2005).

1.1.4.1 Key Enzyme Involved in Ethanol Production

The production of enzyme pyruvate decarboxylase (PDC) followed by alcohol dehydrogenase (ADH) in yeasts like *S. cerevisiae* make it possible to produce ethanol by fermentation. The enzyme PDC catalyzes non-oxidative decarboxylation of α-keto acids to corresponding aldehyde and carbon dioxide. The most extensively examined enzymes of this group are from *S. cerevisiae* and *Z. mobilis*. In addition to decarboxylation of pyruvate, PDC also catalyzes the enantio-selective formation of 2-hydroxy ketons via. carboligase side reactions. On the other hand, alcohol

dehydrogenases are the members of the oxidoreductase family (Eram & Ma, 2013). They belong to the dehydrogenase/reductase superfamily of enzymes and catalyze the reversible inter-conversion of alcohols to corresponding aldehydes or ketons. ADHs can be classified based on their cofactor requirements as flavin adenine di-nucleotide (FAD)-dependent ADHs, pyrollo-quinoline quinone (PQQ), heme or cofactor F420 dependent ADHs and NAD(P)-dependent ADHs. However majority of them are NAD(P)-dependent (Savarimuthu et al., 2014).

1.1.4.2 Electrochemical Enhancement of Fermentation Technique

Electrochemical enhancement of fermentation technology merges traditional fermentation with electrochemistry. A supplied electrical field influences the microbial metabolism in either a reductive or oxidative manner. This approach is very much beneficial for selectivity, increase of carbon efficiency, limit the use of additives for redox balance or pH control, enhance microbial growth or in some cases enhance product recovery (Schievano et al., 2016). The supplied electric field is found to alter the mass transfer rate and fluid flow mechanism of fermentation system (Rosa et al., 2019). Electro-fermentation is a novel process that consists of electrochemically controlled microbial fermentative metabolism. The electrodes can act as either electron sinks or sources that allow unbalanced fermentation. They can also modify the medium by changing the redox balance. Such electrochemical modification exerts significant effects not only on microbial metabolism and cellular regulation but also on interspecies interactions and the selection of microbial populations (Moscoviz et al., 2016). The breakdown of substrate on the anode by microbes provides protons to the cathode. Salt bridge architecture as well as membrane is used for ion transfer (Haider et al., 2017). Jeon & Park (2010) observed 1.5 times enhancement in ethanol production using *Zymomonas mobilis* and *Saccharomyces cerevisiae* combination for ethanol production using electrofermentation.

1.1.4.3 Electron Transfer

Most microorganisms cannot transfer electrons directly to the electrodes. Electron transfer mediators are widely used to enhance the power output for such cases. Various endogenous and synthetic electron mediators are commonly used in cells.

Neutral red, methylene blue, thionine, iron (III) EDTA, Meldola's blue, Mn^{4+} are the synthetic mediators and humic acids, anthraquinone, the oxyanions of sulphur (sulphate and thiosulphate) are the endogenous mediators (Park & Zeikus, 2000). The endogenous mediators transfer electrons from inside the cell membrane to the electrode but there are only a limited number of organisms that can transfer electrons across the membrane by themselves to the electrode. As compared to the use of prokaryotes in the biofuel cell, much less researches have been carried out on the use of eukaryotes such as yeasts as biocatalysts in the electrolytic cell. Yeasts such as *Saccharomyces cerevisiae* and *Candida melibiosica* have the electrochemical capabilities to be used as attractive biocatalysts in a biofuel cells (Hubenova & Mitov, 2008).

1.2 Rational

Using an electrolytic cell for the production of bioethanol facilitates the improvement in ethanol production because of the enhancement in electrochemical redox reactions occurring inside the cell. The externally supplied voltage is used to drive the fermentation to generate ethanol. The substrate oxidized at the anode liberates protons and electrons that are transferred to the cathode where these protons and electrons are utilized by the microbes for the reduction of NAD^+ to NADH. The NADH generated can then be utilized for the metabolism of the microbes to produce ethanol. The generation of additional NADH increased NADH/NAD^+ ratio thus shifting the metabolic flux to decrease NADH/$NAD^{+,}$ resulting in more ethanol production. Immobilization of yeasts and electron transfer mediators to the electrodes, further facilitate the transfer of electrons from the electrodes thereby improving the ethanol production. Ethanol provides a green technology by reducing the CO_2 emission about 80%, resulting in the decrease in the globally increasing problems of greenhouse gases. Use of lignocellulosic biomass for ethanol production is a CO_2 balance technology as there is recycling of CO_2 between biomass and atmosphere. Further use of pentose fermenting yeast strains along with hexose fermenting yeast offers much more advantage as it helps for utilization of pentoses and hexoses both obtained from lignocellulosic biomass degradation which is prerequisite to meet economic challenge in the production of bioethanol utilizing lignocellulosic biomass. Bioethanol as a renewable source of energy and a green technology can be used to fuel direct ethanol fuel cells for the generation of

electricity and increasing applications to bioelectronics, biosensors and polyethylene production. Also use of yeasts as biocatalysts in biofuel cells helps to investigate the efficiency of such yeast powered biofuel cells which has gained much less attention as compared to cells utilizing prokaryotes. Ethanol offers advantages over methanol as an attractive alternative for alcohol fuel cell. Methanol has several major disadvantages such as relative toxicity, inflammability with lower boiling points and majorly being a non-renewable energy source. Rather ethanol has higher boiling points, lower toxicity and can be produced in large scale easily.

1.3 Objectives

1.3.1 General Objective

Enhancement in bioethanol production from efficiently pretreated lignocellulosic biomass in a redox coupled electrochemical cell using yeast.

1.3.2 Specific Objectives

- Characterization of common lignocellulosic biomass.
- Determination of efficient pretreatment technique for lignocellulosic biomass.
- Obtaining best yeast for ethanol production at optimized condition using glucose as carbon source.
- Construction of electrochemical cell and optimization of ethanol production.
- Study on best mediator or combination of mediators for electron transfer.
- Ethanol production from pretreated lignocellulosic biomass.

1.4 Hypothesis

Lignocellulosic biomass differs in sugar content depending on the plant source used. Screening studies may be used to select high fermentable sugar containing biomass. Best pretreatment and hydrolysis methods can be developed for the biomass by testing different pretreatment and hydrolysis methods. Local yeasts isolated from different substrate differ in fermentation capacity and efficient fermenting yeast can be selected for fermentation of the hydrolyzed biomass. The fermentation of lignocellulosic biomass can be enhanced using electrochemical cell with the supply of external voltage. The designed electrochemical cell utilizes the yeast metabolism for fermentation enhancement and hydrolyzed biomass as the source of sugar.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Economics of Lignocellulosic Conversion Processes

Lignocellulosic biomass can be considered as a potential feedstock for generation of energy. The biomass can be gasified to produce syngas which can be used to generate heat and electricity. The biomass can be used to produce fuel such as ethanol or hydrogen (Balat et al., 2009). Numerous studies have discussed the economics of biomass conversion processes over the last 20-30 years. Overall costs for production from biomass can be split into two major contributions: the feed and the processing costs. The feed cost covers the cost of purchasing the feedstock. Processing cost covers the cost of installing and running the manufacturing plants, ie; the cost of installation and running of plant, the labor as well as the energy and chemical consumed. Biomass costs represent almost about 35-50% of the ethanol production cost and the costs are dependent on biomass types, location, yield, weather, harvesting systems, collection methods, storage, and transportation (Sokhansanj & Fenton, 2006). According to Haque & Epplin, (2012), based on conversion rates, for 100 gallon ethanol production per ton of biomass, the breakeven price ranges $0.58 per liter of ethanol produced. Usually it is observed that, the cost of oil refining is dominated by the cost of feed, whereas the cost of lignocellulose conversion to different products (e.g., ethanol) is dominated by technology. The economics of biofuel derived from starch and sugar is intermediate to these two. There is obviously a trade-off between feed stock cost and plant cost. Feed stocks, such as vegetable oil, may be expensive ($ 500-700 per ton) but they are easy to convert. Others like lignocellulose may be cheap ($ 34-7 per ton dry) but are very difficult to convert. However, the cost of biomass conversion plant decreasing with increasing energy efficiency. A study had estimated that ethanol from cornstover was $0.90 L^{-1} in 2012 (Stephen et al., 2012) and the cost was reduced to almost $0.20 L^{-1} in 2018 (Hossain et al, 2019). Khatiwada et al. (2016) performed life cycle assessment to evaluate the climate change impact like greenhouse gas emission in production and use of ethanol from lignocellulosic biomass. It was observed that lignocellulosic biomass conversion is natural carbon balance process where ethanol is produced from plants.

The carbon dioxide produced by ethanol combustion is utilized by plant during its growth in presence of sunlight.

2.2 Lignocellulosic Biomass in context of Nepal

2.2.1 Topography and Physiography of Nepal

The climate in Nepal varies from sub-tropical to alpine within a short distance (about 193 km from plains to mountains) due to tremendous variation in topography and altitude (60 to 8,848 m). These factors along with mountain slope have created numerous micro-environments as alpine, cool temperate, warm temperate, subtropical and tropical climates. Accordingly, Nepal is divided into three geographical belts (Leipzig, 1996).

i. Mountains: About 28% of the total area is covered by mountains and has only a small proportion of the population. The agriculture is mainly livestock based with little or no cultivation. Conditions are extreme and food deficits are common. The snow line lies on around 2,500 m in winter and 4,000 m in summer. Snow fall is frequently observed above 1,500 m and very rarely observed below 1,500 m altitude .

ii. Hills: About 55% of the total land area is occupied by hills and has around 38% of the agricultural land. High ridges and steep slopes around numerous streams characterize the area, which is mainly responsible for giving rise to many microclimates with biotic variations. Hills account for about 50 % of the population.

iii. Terai (plains): Almost around 17% of the land area of the country and about 45% of the total population live in terai region. It represents over 55% of cultivated area and around 60% of crop production. It is known as the granary of Nepal. About 33% of the arable land in this region is irrigated.

2.2.2 Lignocellulosic Biomass

Except for mountainous region, all the other regions are rich in vegetation, especially weeds and agricultural growths. These vegetation leave a bulk of residue as lignocellulosic biomass like corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corn fiber, cotton stalk, corn cob and various weeds such as *Saccharum spontaneum, Lantana camara, Eichhornia crassipies* (water hyacinth), etc. *Ipomoea carnea* (locally known as Ajamari), *Phragmites karka* (locally known as Narkat),

*Saccharum spontaneum (*locally known as Kans) and *Zea mays* (corn) cobs are good source of lignocellulosic biomass. The first three are commonly found weeds, grown naturally in abundance in both the hilly and plain regions of Nepal that may be harvested periodically with relative ease. The last one can be considered as agriculture residue after use. They are rich in Cellulose and hemicellulose contents (Table 2).

Table 2: Cellulose, hemicellulose and lignin contents in common high energy possessing lignocellulosic biomass that grows in hilly areas.

Lignocellulosic feed stocks	**Cellulose %**	**Hemicellulose %**	**Lignin %**	**References**
Corn cob	45	35	15	Adepu et al., 2017
Rice straw	29-35	26-32	16-21	Adepu et al., 2017
News paper	12	40-55	25-40	Adepu et al., 2017
Saccharum spontaneum	45.3	24.9	7.4	Sandesh et al., 2017
Ipomea carnea	67.4	16.7		Srivastava & Shukla, 2015
Wheat straw	37.1	34.0	18.0	Sandesh et al., 2017
Sugar cane baggage	38.3	30.9	20.9	Sandesh et al., 2017
Corn stover	34.3	32.8	15.1	Sandesh et al., 2017
Artemisia vulgaris	40.92	29.1	28.3	Nei et al., 2019

I. carnea is a fast growing species with ability to absorb Cd and Hg. Accordingly, this species is used for bioremediation of heavy metals in polluted soils. Its seeds get widely dispersed by winds, water, birds and other means enabling them to colonize in new areas at distances far from their original. It is not found to attack by parasites, diseases, herbivores, etc. It is also considered as invasive alien species, contributing the large biodiversity in Nepal (Tiwari et al., 2005).

S. spontaneum is one of the widely distributed lignocellulosic biomass that grows very fast giving high forage yield and seed production. Hence, it is considered as a promising future biomass for bioethanol production. *S. spontaneum* is believed to have originated in India, Nepal and is distributed widely in other tropical and subtropical regions of Asia. It grows on riverbanks, road sides, waste lands and even in sandy soils (Holm et al., 1997). Medicinal and Aromatic Plants Network (MAP) of Nepal has given the taxonomic ID 1307 for the Nepali variety of *S. spontaneum*

(MAPs NET, 1997). *S. spontaneum* has attracted serious attention for its potential in ecological restoration (Pandey et al., 2015).

Z. mays is a field crop grown all around the world and its cob (the central core of corn shoot) is an agricultural waste that is thrown away as garbage in farm lands, waste bins and along road side (Vindis et al., 2009). Corn cobs are known to contain high energy content among 12 different types of biomass tested by Helsel & Wedin (1983).

P. karka is a herbaceous perennial grass producing somewhat woody culms that can be 4 - 10 m tall and 15 – 25 mm in diameter. In friendly condition, the plant can produce large areas of dense growth robustly. The species is planted in riverbeds for bioremediation of arsenic like heavy metals and to check erosion (Verma et al., 2014).

2.3 Obtaining Reducing Sugars from Lignocellulosic Biomass

Cellulose is the major component of lignocellulosic biomass and its concentration ranges from 40-50% of dry weight (Zhang et al., 2004), hemicellulose is present at a concentration of 25-35% (Saha & Cotta, 2007), lignin being the third major component is present at a concentration of 20-35% and pectin in trace amounts of 1-2% (Joshi et al., 2011). These components are variable for different biomasses as shown in Table 2. These components are exposed when lignocellulosic biomass is pretreated and hydrolyzed. The reducing sugar obtained is utilized for ethanol production. Table 3 showed the theoretical ethanol potentials of some lignocellulosic biomass.

Table 3: Composition of sugars in different biomass and their ethanol potential as described by Phyllis database for biomass and waste (*Energy research Centre of the Netherland)*

Biomass	**Ash %**	**Hemicellulose %**	**Cellulose %**	**Lignin %**	**Ethanol potential kg·kg^{-1}**
Poplar		17	49	18	0.37
Wheat straw	1.3	27.6	34	18	0.35
Rice straw	18.9	22.7	37	13.6	0.34
Barley straw	7.1	44	37	11	0.46
Potato rests	5	11.8	26		0.21

2.3.1 Pretreatment Strategies

Current technologies to produce ethanol largely depend upon sugarcane or starch based materials and tubers, however, the increasing stress on food prices and food security has evolved lignocellulosic materials as a source of ethanol. The lignin-hemicellulose-pectin complex forms a stringent seal around the cellulose, which makes the pretreatment step more important and a rate limiting step in overall fermentation process. The purpose of the pretreatment is to remove lignin and partly hemicellulose, changes cellulose crystallanity and increase the porosity of the biomass so that it will be easily accessible for hydrolysis (Yang & Wyman, 2008). Various techniques like steam explosion, acids, alkali, ammonia fiber explosion (AFEX), enzyme treatments are adopted for separating lignin, cellulose, hemicellulose and pectin from the biomass. All those techniques have different effect on biomass and have their own advantages and disadvantages (Table 4) (Hongyan et al., 2017).

Table 4: Advantages and disadvantages of different pretreatment techniques

Pretreatment	Advantages	Disadvantages
Steam explosion	Chemical free	Generation of degradation products
AFEX	Low degradation products	Low hydrolysis yields with woody crops
Ozonolysis	High reduction of lignin contents with no production of toxic residue	Expensive
Wet oxidation	Low degradation products	Oxygen use
Alkalies	Lignin removed, increase in surface area for hydrolysis	Chemicals use and high residence time
Acids	Alteration of lignin structure	Possibility of equipment corrosion, toxicity
CO_2 explosion	No contamination, more surface area exposed for hydrolysis	High amount of CO_2 requirement
Biological	Less energy is required	Low hydrolysis rate, time consuming, contamination

Enhanced pretreatment of lignocellulosic biomass is possible when pretreatment is done by using efficient and cheaper materials. Ionic liquids (Tony et al., 2012), high pressure steam (Brownell & Saddler, 1987) or storing them in moist form for certain time in presence of some antimicrobial agents (Passoth et al., 2013), i.e. integrated

storage and pretreatment (ISP). Storage of lignocellulosic biomass in moist condition increases hydrolyzing efficiency during pretreatment. Further inoculation of antimicrobial yeast like *Wickerhamomyces anomalus, Hansenula anomala, Pichia anomala* etc eventually protects biomass from enterobacteriaceae contamination (Olstorpe et al., 2012).

2.3.2 Hydrolysis Strategies

2.3.2.1 Dilute acid hydrolysis

Sulphuric and hydrochloric acids are the most commonly used catalysts for hydrolysis of lignocellulosic residues. In contrast to these acids, phosphoric acid can be more advantageous for hydrolysis. Phosphoric acid is less aggressive than other acids but give solutions with higher concentrations of growth inhibitors for microorganisms such as furfural or acetic acid. These hydrolysates obtained after the acid hydrolysis need to be processed if they are going to be used as fermentation media. In general the following operations are needed in sequence: concentration, detoxification, neutralization and supplementation with nutrients (Lenihan et al., 2011). Dilute acid (2% v/v H_2SO_4, 1:30 w/v) hydrolysis of sugarcane bagasse, although maximum xylose yield (0.81 $g \cdot g^{-1}$ hemicellulose) at 130°C, there was significant fraction of xylose that was converted to inhibitory products at high temperature (Belachew et al., 2018).

2.3.2.2 Enzymatic Hydrolysis

A cocktail of enzyme which can degrade cellulose, hemicellulose, pectin, etc., is necessary for efficient hydrolysis of biomass. Novozyme is a company which produces such cocktail enzyme (Myriam et al., 2017). The enzyme is very costly. Enzymatic degradation of cellulose to glucose is generally accomplished by synergistic action of at least three major classes of enzymes: endo-glucanases, exoglucanases and β-glucosidases. These enzymes are usually called together cellulase or cellulolytic enzymes (Wyman, 1996). Separate enzymatic hydrolysis and fermentation, simultaneous saccharification and fermentation, simultaneous saccharification and co-fermentation and consolidated bioprocessing are also adopted recently. The three most promising microbial species that have been developed by metabolic engineering in the last two decades are *S. cerevisiae, Z.*

mobilis and Escherichia coli. By genetic engineering it is possible to transfer cellulase genes from *Trichoderma spp*. to *Saccharomyces cerevisiae* and produce "superstrains" capable of hydrolyzing cellulose along with fermentation of glucose into ethanol (Ha et al., 2011).

2.4 Ethanol Fermentation

Fermentation technology is widely used for the production of various economically important compounds which have applications in the energy production, pharmaceutical, chemical and food industry. Although, fermentation processes are used from generations, the need for sustainable production of products, meet the market requirements in a cost effective manner has put forward a challenging demand. For any fermentation based product, the most important thing is the availability of fermented product equal to that of market demand (Rajeswari et al., 2014). Optimization of production medium is required to maximize the metabolite yield. This can be achieved by using a wide range of techniques from classical "one-factor-at-a-time" to modern statistical and mathematical techniques, viz. artificial neural network (ANN), genetic algorithm (GA) etc. Every technique comes with its own advantages and disadvantages and despite drawbacks, some techniques are applied to obtain best results. Use of various optimization techniques in combination also provides the desirable results (Singh et al., 2017).

Microbes especially yeasts are known for their increased capabilities of fermenting carbohydrate sources to produce ethanol with proper physical and chemical environment (Satheeskumar et al., 2015). *Saccharomyces cerevisiae* is the most commonly used yeast for ethanol production despite of its less ethanol tolerance and cannot metabolize xylose even though some researches has shown the presence of endogenous pathway in *Saccharomyces cerevisiae* (Hang et al., 2012). Complete utilization of substrate is a prerequisite to render lignocellulosic ethanol production economically competitive. Hence, all types of sugars in both cellulose and hemicellulose must be converted to ethanol and organisms capable of efficiently utilizing all the pentose and hexose sugars in the lignocellulosic biomass are a must. Other yeasts and bacteria are under investigation to ferment xylose and other pentoses into ethanol. Recombinant *S. cerevisiae* strain expressing xylose reductase (XR) and xylitol dehydrogenase (XDH) has been developed but no efficient metabolism of

xylose has been observed (Hahn-Hagerdal et al., 2007) till yet. Expression of xylose transporter (Gxf1) from *Candida intermedia* and expression of xylose isomerase (XI) from *Chlostridium phytofermentans* in yeast found to enhance ethanol production. Use of such xylose utilizing yeast strain of *Saccharomyces cerevisiae* with insertion of xylose transporter and Xylose isomerase gene will add the enhanced production of ethanol from xylose also (Van Maris et al., 2006; Joshi et al., 2019a). Investigation of yeasts which can utilize all types of carbohydrate with less carbon catabolite repression (CCR) is must for fast utilization of biomass and hence the products can be more economic (Gao et al., 2019).

Research has been done on more promising bacteria *Zymomonas mobilis* which can survive higher ethanol concentrations up to 16% v/v. The fermentation rate is also higher with *Zymomonas mobilis* in comparison to *Saccharomyces cerevisiae* (Kang et al., 2014). However, in spite of these attractive advantages, its substrate range is limited to glucose, fructose, and sucrose not for pentose sugar. *Z. mobilis* cannot tolerate toxic inhibitors present in lignocellulosic hydrolytes such as acetic acid. Several attempts have been made to engineer *Z. mobilis* to overcome its inherent deficiencies (Chen et al., 2009). However, when these engineered strains metabolize mixed sugars in the presence of inhibitors, the yield and productivity are much lower, thus preventing their industrial application (Joachimsthal & Rogers, 2000). Additional research tried to find genetically modified microorganisms which can effectively ferment both types of sugars into ethanol with *Escherichia coli* and *Klebsiella oxytoca* also (Hahn-Hagerdal et al., 2007). A novel technique, the consolidated bioprocessing (CBP) is on the way forward to proceed by producing all required enzymes and ethanol using a single type of microorganisms in a single reactor. CBP is considered as the ultimate evolution of biomass-to-bioethanol conversion technology, since it implies neither capital nor operating costs for dedicated enzyme production together with a reduced consumption of substrate for enzyme production. Unfortunately, industrial optimization of this technique may take several years for research to determine such microorganisms or compatible combinations of microorganisms only. Separate hydrolysis and co-fermentation (SHCF) and simultaneous saccharification and co-fermentation (SSCF) are possible alternatives to develop to minimize the cost. Co-fermenting both pentose and hexose sugars with a single strain or combination of strains in the same reactor significantly

improves the process economics and enhances the commercial production of lignocellulosic ethanol in the short term (Kazi et al., 2010). Various techniques are in progress for economic production of ethanol (RFA, 2017). Table 5 infers bioethanol production in world's scenario in 2017.

Table 5: Bioethanol production by country, 2017 (RFA, 2017)

Country	Bioethanol production (million gallon)
United States	15,250
Brazil	7295
European Union	1377
China	835
Canada	436
Thailand	322
Argentina	264
India	225
Rest of the World	490

2.5 Electrofermentation: A Way Forward Technique

Newer techniques for the improvement in production of fermented products by the use of electrochemical cells that decompose chemical compounds by means of electrical energy via electrochemical reactions involved in biochemical pathways. A microbial electrochemical cell is a mimic of a biological system in which microbes do not directly transfer their produced electrons to their characteristic electron acceptors; instead they are subsequently transferred to an anode, a resistance or power source and a cathode (Rabaey et al., 2003) as shown in Figure 4 below. A typical electrolytic cell is composed of three components: an anode, a cathode and a proton permeable system that separates the anode and cathode compartments. A small voltage is applied continuously to the electrode. The electrons and protons produced in anode are transferred continuously to the cathode. For the reaction to proceed continuously the electrons produced at the anode must pass through an external circuit and the protons must pass through the proton permeable system. Proton conducting membrane such as nafion membrane is commonly used as proton permeable membrane. Microbial electrochemical cells use whole living micro-organisms as catalysts. Microbes offer some major advantages over enzymes in that they can catalyze a more thorough oxidation of many substrates and can be less

susceptible to poisoning and loss of activity under normal operating conditions making them a popular choice for use in electrolytic cells. (Najafpour et al., 2011).

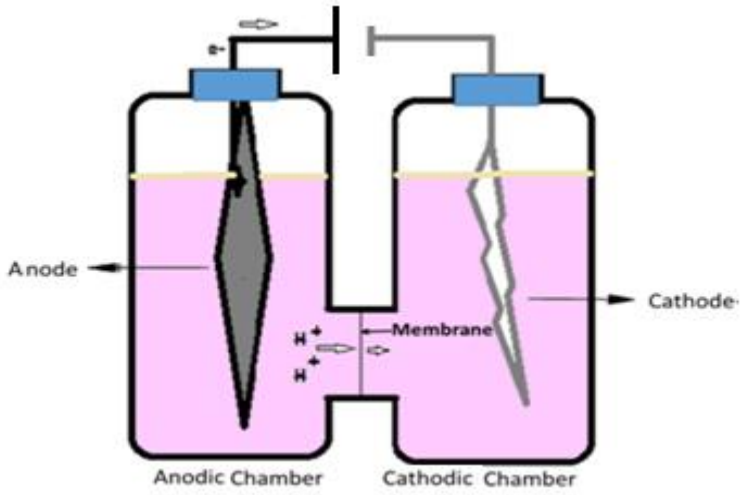

Figure 4: Fabricated electrochemical cell with e^- and H^+ transport from anodic to cathodic chamber

These electrolytic cells when provided with external voltage, substrates such as carbohydrates or water or else, at the anodic compartment are oxidized producing electrons and protons. The electrons are transferred through the external circuit, whereas, the protons are transferred through the electrolyte to the cathode where reduction reaction occurs. This requirement of electric potential (oxidation-reduction potential) can be exploited for the improvement of ethanol production (Jeon & & Park, 2010).

Different types of mediators such as neutral red, methylene blue, resazurine, 2-hydroxyl-1,4-naphthoquinone, pyocyanide, riboflavin, cytochrome, FeIII EDTA, thionine, methyl viologene, humic acid etc. (Espinoza et al., 2017) are known to increase the transfer of electrons to the electrode thus facilitating the regeneration of NADH and shifting the metabolic flux towards decreased NADH/NAD^+ balance and increased metabolite production, i.e.; ethanol in cathodic compartment.

Cathodic reaction:

$NAD^+ + H^+ + 2e^- \longrightarrow NADH$ (E ~ -330 mV)

NADH + Pyruvate $\longrightarrow$ NAD^+ + Ethanol

Anodic reactions:

NADH $\longrightarrow$ $NAD^+ + H^+ + 2e^-$

[or, $H_2O \longrightarrow 2H^+ + ½\ O_2 + 2e^-$ (E ~ -1.23mV)]

In *S. cerevisiae* like yeast NAD^+/NADH ratio is always high. So the applied potential favours for fast conversion of NAD^+ to NADH in cytosol and enhancement in production of ethanol in unbalanced condition (Veech et al., 1972; Choi & Sang, 2016; Atkins, 1998). There is the reverse condition possible in anodic compartment (Canelas et al., 2008).

2.6 Current Status and Future Perspective of Electrofermentation of Bioalcohol

Very less work has been done on electrofermentation of yeast for ethanol production. In a work by Jeon & Park (2010), when ethanol was allowed to produce by *Zymomonas mobilis* in cathodic compartment and *Saccharomyces cerevisiae* in anodic compartment, the ethanol production was enhanced by 1.5 times. However the ethanol production was enhanced in anodic compartment but ethanol production was suppressed in cathodic compartment. When the content of cathodic compartment was recycled to anodic compartment, the ethanol production was further enhanced. Ethanol production by *Clostridium thermocellum* and *Saccharomyces cerevisiae* was improved by 61% and 12% respectively in an electrochemical bioreactor system with 1.5 V of electric potential supply. The electric supply caused shift in the metabolite concentrations higher in *C. thermocellum* but less in *S. cerevisiae*. Acetate production was reduced with increased electric potential in both strains during fermentation. The high electric potential of 5 V adversely affected the *C. thermocellum* fermentation, but ethanol fermentation of *S. cerevisiae* enhanced even at a high electric potential of 10 V supply (Shin et al., 2002). Mathew et al. (2015) found the increase in cell number of *S. cerevisiae* when electrical potential was supplied to a single chambered electrochemical system upto 15V. Microbial electrolysis cell was found efficient also for the production of butanol by *Clostridium beijerinckii* IB4 (He et al., 2016). Electricity diminished the optimized fermentation time from 40 to 28 h. Reducing power such as NADH is essential for acetone/butanol/ethanol (ABE) fermentation. Externally carried electron increased the available NADH in microbial cell thus increasing fermented products.

2.7 Enzyme Expression and Ethanol Production

S. cerevisiae pyruvate decarboxylase, which converts pyruvate to aldehyde or ketone are of three isoforms ie PDC1, PDC5 and PDC6. PDC1 has high rate of decarboxylation, PDC5 has high decarboxylation efficiency and PDC6 has the least decarboxylation efficiency with low rate. Amongst the three, PDC1 is the most active decarboxylase in the yeast (Agarwal et al., 2013). Alcohol dehydrogenase (ADH1) is the mostly studied NAD(P)-dependent oxidoreductases occur in virtually all organisms and catalyze the reversible oxidation of primary and secondary alcohols into aldehydes and ketones (Jornvall et al., 2013). Amongst the several ADHs, ADH1 is normally constitutive under laboratory conditions, had a high Km value for ethanol (17 000–20 000 $\mu mol \cdot L^{-1}$) and, observed that is chiefly responsible for the production of ethanol during anaerobic growth (Thomson et al., 2005). RT PCR (qPCR) is fast and easy technique for gene expression analysis than other techniques such as northern blotting and *in situ* hybridization. Additionally, the detection method of qPCR is more sensitive and specific compared to the other assays (Bustin et al., 2005). SYBR Green is a popular DNA binding dye used in qPCR, which only fluoresces when bound to double-stranded DNA and not with single-stranded DNA. The cost of employing SYBR Green as a qPCR detection method is much lower than hydrolysis probes ie; Taqman probe (Navarro et al., 2015).

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Biomass Sample

Biomass samples of *Ipomoea carnea, Phragmites karka, Saccharum spontaneum* and *Zea mays* cobs were obtained from premises of Tribhuvan University campus, Kirtipur, Kathmandu, Nepal during the month of August. The sampling location was 27.6818^{o}N and 85.2865^{o}S. The sample was packed in plastic pouch and brought in laboratory for further work.

3.1.2 Sample for Yeast Isolation

Samples were collected from different yeast sources as shown in Table 6. All the samples were collected during September and October. They were kept in sterile zip locked bag and stored at 4^{o}C until further analysis.

Table 6: List of samples used for yeast isolation

S. No.	Sample	Sampling location	Substrate
1	Murcha	Lubhu, Lalitpur	Steamed rice
2	Manna	Lubhu, Lalitpur	Steamed wheat
3	Murcha	Bhaktapur	Steamed rice
4	Manna	Bhaktapur	Steamed wheat
5	Fresh black grape	Balkhu	Grape pulp
6	Oak tree	Tribhuvan University premises	Oak stem
7	Red wine yeast	USA	Red wine
8	Oak tree	Tribhuvan University premises	Oak stem bark

3.1.3 *Saccharomyces cerevisiae* Yeast strains

MKY09: A laboratory yeast strain kindly gifted by Prof. Eckhard Bole, University of Frankfurt, Germany.

Ethanol Red: An industrial yeast strain kindly gifted by Prof. Eckhard Bole, University of Frankfurt, Germany.

pGPD2/lac yeast strain: MKY09 transformed with pGPD2 plasmid inserted with laccase gene (pGPD2/lac) developed at Central Department of Biotechnology, Tribhuvan University, Nepal. Plasmid pGPD2/lac was constructed by cloning synthetic laccase gene of *Ganoderma lucidum* of size 1,576 bp in pGPD2 (expression vector purchased from Addgene Company) at HindIII and EcoRI restriction sites. The constructed plasmid was transformed into MKY09 strain (Bishwakarma, 2017).

3.1.4 Chemicals and Media

3.1.4.1 Materials and Chemicals

Woven graphite fibre of 10 mm thickness was purchased from Nippon Co., Japan. Platinum wire of 0.2 mm diameter was purchased from Sigma Co. Electrolytic vessels made up of pyrex glass were purchased from Adams and Chittenden Scientific Glass, California, USA. Nafion 117 membrane was purchased from DuPont, USA. High grade neutral red, D-glucose, gallic acid, furfural, 5-hydroxymethyl furfural, ethanol, aniline, thionyl chloride, cellulase (*Trichoderma resii*) were purchased from Sigma company. Hydrogen hexachloroplatinate (IV) hexahydrate was purchased from Kanto Chemicals Company. All the other common chemicals, dinitro salicylic acid, sodium potassium tartarate, Folin Ciocalteu reagent, yeast extract, D-glucose, peptone, ammonium sulphate were purchased from HiMedia company. Common chemicals hydrochloric acid, sulphuric acid, salicylic acid, sodium hydroxide, sodium bicarbonate were of laboratory grade.

3.1.4.2 Media

YMB (Yeast Maltose Broth) media composition: Yeast extract (3 $gm \cdot L^{-1}$), malt extract (3 $g \cdot L^{-1}$), peptone (5 $g \cdot L^{-1}$), glucose (10 $g \cdot L^{-1}$) and pH 4.5.

YMA (Yeast Maltose Agar) media composition: Yeast extract (3 $gm \cdot L^{-1}$), malt extract (3 $g \cdot L^{-1}$), peptone (5 $g \cdot L^{-1}$), agar (1.5 $g \cdot L^{-1}$), glucose (10 $g \cdot L^{-1}$) and pH 4.5.

PYN (Peptone Yeast extract and Nutrient) media: Peptone (3.5 $g \cdot L^{-1}$), yeast extract (3 $g \cdot L^{-1}$), KH_2PO_4 (2 $g \cdot L^{-1}$), $MgSO_4$ (1 $g \cdot L^{-1}$) and $(NH_4)_2SO_4$ (1 $g \cdot L^{-1}$).

3.2 Methodologies

3.2.1 Preparation of Lignocellulosic Biomass for Fermentation

The biomass sample after collection from field was brought to lab and further processing, biomass analysis, pretreatment and hydrolysis was done before being used for fermentation. The summary flow chart of the processing was as shown in Figure 5.

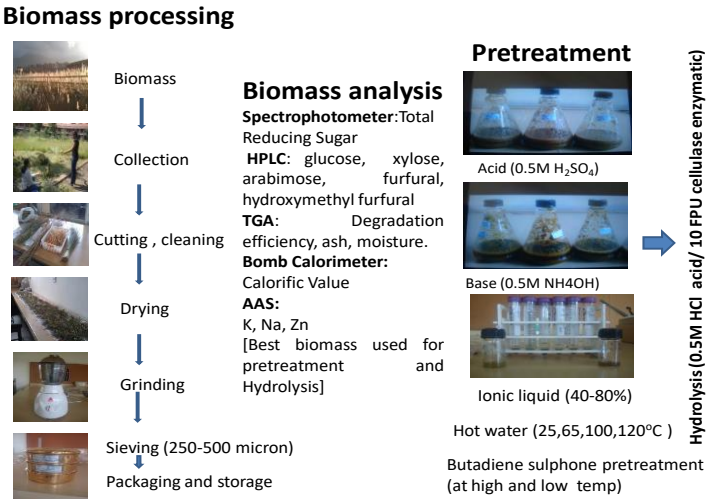

Figure 5: Flow chart of biomass processing, analysis, pretreatment and hydrolysis: Summary.

3.2.1.1 Biomass Sample Collection and Preparation for Storage

The aerial portion of the biomass of *I. carnea*, *P. karka* and *S. spontaneum* collected were first air dried for 24 h. The biomass were then cut into small pieces with average length of 2 cm. *Zea mays* (corn) cobs were also collected from the same location, however, the corn kernels were first deseeded to prepare the cobs. The air dried biomass was dried in a hot air oven at 60°C for 24 h. It was then ground and sieved to get powder with a particle size of 250-500 µm. The processed biomass was packaged in plastic bags and stored in a cool and dry cabinet at room temperature for further analysis.

3.2.1.2 Biomass Sample Analysis

3.2.1.2.1 Differential Thermo Gravimetric Analysis (TGA)

The degradation pattern of different components present in biomass were analyzed using Thermo Gravimetric Analyzer (STA 7200 HITACHI, Japan, done in

Department of Energy, IITG, Guwahati, India) (Kok & Emre, 2013). For approximately 10 mg grounded biomass sample, dynamic thermo gravimetric scans were conducted in the temperature range 30 to 900^{o}C at a heating rate of 10^{o}C min^{-1}. The experiments were carried out under nitrogen atmosphere at a flow rate of 100 mL·min^{-1}. A curve plotted with remaining biomass vs. temperature shows the biomass degradation pattern. Further sharp peaks obtained by plotting differential biomass vs. temperature reveals the temperature at which the specific component degraded. Moisture and ash contents were also determined using TGA curve by observing the change in biomass at 105^{o}C and remaining biomass after complete combustion respectively according to UNI EN 14774-2 (2010) and UNI EN 14775 (2010) methods.

3.2.1.2.2 Determination of Calorific Value

Bomb calorimeter (Parr Instrument Company, 2007, Central Department of Environmental Science, TU) was used for determination of calorific value (Ioannis et al., 2016). Briefly, ~1 g biomass was placed in a small container and allowed to touch with nichrome wire tied with cotton thread. The assembly was placed in the ignition port inside bomb calorimeter. The bomb calorimeter was filled with 20 bar oxygen at 25^{o}C. The bomb calorimeter was then submerged in a container filled with distilled water. The calorimeter jacket was maintained at temperature of about 25^{o}C. The cotton thread along with biomass was ignited. The heat released was measured in terms of temperature change. The calorific values of samples were calculated using the equation, $H_{fs} = [(C_{ba}.\Delta T_{fs} - m_{ct}.H_{ct})/m_{fs}]$, where, ΔT_{fs} was the observed change in temperature (^{o}C), m_{ct} was mass of cotton thread (g), H_{ct} was calorific value of cotton thread (J·g^{-1}), m_{fs} was mass of sample (g) and C_{ba} was heat capacity of benzoic acid (standard). $C_{ba} = [(m_{ba}. H_{ba} + m_{ct}.H_{ct})/ \Delta T_{c.}]$ where, m_{ba} was mass of benzoic acid (g), H_{ba} was calorific value of benzoic acid (J·g^{-1}) and ΔT_{c} was observed change in temperature (^{o}C)

3.2.1.2.3 Metal Ion Composition Analysis in Biomass

For the metal ion composition analysis, the protocol used was as described by Temminghoff & Houba, (2004). About 0.6 g of the biomass sample was weighed exactly in a 3-digit weighing balance and the sample was transferred to a 50-mL

volumetric flask. Exactly 3.3 mL of the digestion mixture (Appendix 1) was added and few carborundum beads were introduced. Then the mixture was swirled carefully until the sample got agitated. It was then allowed to stand overnight. The flask was heated on a hot plate at 180˚C for about 1 h. The flask was removed from the plate, cooled down and 5 drops of hydrogen peroxide was added. The flask was kept again on the hot plate and the temperature was increased to about 280^{o}C. The flask was heated for 10 min until the white fumes seen on the neck of flask. The flask was allowed to cool down. Again 5 drops of hydrogen peroxide was added and heated for another 10 min. The treatment was repeated until the digest had turned colorless. After cooling, added about 10 ml of distilled water, swirled until most of the precipitate got dissolved. The mark was made up with water. The digest was filtered to remove any SiO_2 that will otherwise dissolve gradually and then interfere in the determinations. Additionally two blank digestions were also prepared accordingly. The calibration solutions for analysis were prepared in the same final medium as the samples in order to get a matrix, which was the same as in the samples. The final medium was 0.8 M H_2SO_4. The digested mixtures along with blank were sent to Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur for analysis of Sodium, Potassium and Zinc using Atomic absorption spectroscopy (AAS). Sodium and Potassium were measured using Flame photometric AAS with standards runs of concentration 2, 4, 6, 8 and 10 ppm respectively and Zinc were measured using Graphite furnace AAS with standard of concentrations 0.3 ppb, 0.7 ppb, 1 ppb respectively.

3.2.1.2.4 Chemical Composition Analysis of Biomass

Structural carbohydrate contents of biomass were determined by two stage acid hydrolysis method as described in National Renewable Energy Laboratory (NREL) Standard Biomass Analytical Procedure (NREL, 2012). The stored biomass was further ground and sieved to about 60 µm size. Approximately 300.0 ± 10.0 mg of grounded biomass was mixed with 3.00 ± 0.01 ml of 72% sulfuric acid then was mixed properly with the help of a teflon rod. It was then capped tightly and incubated in a water bath set at 30 ± 3°C for 60 ± 5 min by continuously stirring the sample every 5 to 10 min. The sample was then removed from water bath and diluted by adding 84.00 ± 0.04 mL deionized water. Similar protocol was repeated for all

standard samples of glucose, xylose and arabinose (0.1-0.4 $mg \cdot mL^{-1}$ standard). The diluted samples were capped tightly and autoclaved at 120°C for 15 min and cooled. Exactly 20 mL of autoclaved liquid was poured into 50 mL flask. With solid calcium carbonate the sample was adjusted to pH 5-6. Decant the aliquot and filter supernatant with 0.2 µ filter. Store the sample in eppendorf tube for further analysis. Total structural carbohydrate in the aqueous portion of the hydrolyzed samples was analyzed by dinitro salicylic acid (DNSA) method as described by Miller (1959). Further, glucose, xylose, arabinose, 5-hydroxy methyl furfural and furfural were determined using HPLC (Agilent, Inc., USA) fitted with Metacarb 87H ion exchange column (300 x 6.6 mm) and Refractive Index detector. The column was eluted at a flow rate of 0.5 $mL \cdot min^{-1}$ using 0.67 mM H_2SO_4 at 60°C (Mood et al., 2015). The column was eluted continuously for 45 min. Respective peak and peak size were used for determining the composition by comparing with standard. The part of experiment was performed in Department of Chemistry, IITG, Guwahati, India.

3.2.1.3 Pretreatment of Biomass

Various procedures delineated below were used to determine the pretreatment efficiencies of biomass. An aliquot from pretreated biomass was collected by vacuum filtration and chemical analysis were performed to determine reducing sugars, phenols and furfural released from the biomass as described under chemical analysis procedures 3.2.1.3.1-3. All treatment procedures involved 10 g biomass mixed with 100 mL of pretreatment solvent in 250 mL stoppered flasks with constant stirring at 25°C, 65°C or 100°C for up to 24 h. All pretreatments were carried out in triplicates. Small aliquots were withdrawn at different time intervals and analyzed for the contents as described below. Pretreatment was also performed at 120°C as above, but the incubation was done for up to 2.5 h only and the samples were analyzed at 0.5 h intervals. The pretreatment solvents used were (i) 0.5M H_2SO_4 (sulfuric acid) (ii) 0.5M NH_4OH (ammonium hydroxide) (iii) water of 65°C, 100°C and 120°C (iv) 1-butyl-3-methyl imidazolium chloride with concentrations of 40%, 60% or 80% respectively or (v) butadiene sulfonate of 50% and 100% concentrations, respectively.

3.2.1.3.1 Determination of Total Reducing Sugars (TRS)

The supernatants obtained after pretreatment were analyzed for TRS using di-nitro salicylic acid (DNSA) method. Briefly, 200 µL of pretreated supernatant was added with 200 µL of DNSA reagent (Appendix 1). The aliquot incubated in a boiling water bath for 10 min, cooled on ice-bath for 5 min followed by addition of 2 mL distilled water. D-glucose (Sigma) 0 to 10 $mg \cdot mL^{-1}$ was used as standard. The absorbance due to reducing sugars was measured at 540 nm using UV-Visible spectrophotometer (Miller, 1959).

3.2.1.3.2 Determination of Phenol Content

Total phenol content in the supernatant was analyzed using Singleton et al. (1999) method. A 100 µL of sample was mixed with 1.0 mL of 10% Folin Ciocalteu reagent (freshly prepared) followed by 0.8 mL of 0.1 $g \cdot mL^{-1}$ sodium carbonate solution. The aliquot was incubated for 15 min at room temperature and absorbance was measured at 765 nm. Gallic acid (successively diluted solutions of 250 $\mu g \cdot mL^{-1}$ stock) was used as a standard.

3.2.1.3.3 Determination of Furfural Content

Furfural present in supernatants was determined by the method as described by Al Showiman (1998). A 0.1 mL aliquot of the sample was mixed with 0.4 ml of 50% ethanol, 20 µL aniline, 5 µL of 37% HCl and 0.8 mL distilled water. The absorbance of the sample was measured at 530 nm. Furfural (successively diluted solutions of 30 $\mu g \cdot mL^{-1}$ stock) solutions prepared in 50% ethanol were used to develop a standard curve.

3.2.1.4 Hydrolysis of Pretreated Biomass

The solid residue obtained after filtration of pretreated biomass was washed with distilled water several times until the washes reached neutral pH while testing with blue and red litmus paper. Thus obtained solids were first air dried for 24 h at room temperature followed by drying in hot air oven at 60^{o}C for 24 h. The dry pretreated biomass was then stored in sealed plastic pouches for further analysis.

3.2.1.4.1 Acid Hydrolysis

Approximately 1 g of dried sample was mixed with 10 mL of 0.5M HCl (Hydrochloric acid) and incubated at 90^{o}C for 24 h in water bath. After 24 h of hydrolysis, the samples were cooled on ice-water bath, vacuum filtered and the supernatants were tested for TRS, phenol and furfural as described in section 3.2.1.3.1-3.

3.2.1.4.2 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed by incubating 1 gm pretreated biomass with 10 mL of 10 filter paper unit (FPU) of cellulase enzyme (*Trichoderma resii*, Sigma Chemical Co., St Lous, MO, USA) at 45^{o}C for 24 h. The samples were then cooled on ice-water bath, vacuum filtered and the supernatants were tested for TRS, phenol and furfural as described in section 3.2.1.3.1-3.

3.2.1.5 Comparative Analysis of Untreated and Pretreated Biomass

3.2.1.5.1 X-Ray Diffraction (XRD) Analysis

Powder X-ray diffractometer (Bruker, Germany) was used to determine the crystallinity of untreated and pretreated biomass. Finely divided powdered samples were loaded in platform. CuKα (λ=0.154) was used as radiation source at 40 kV and 30 mA. Biomass samples were scanned at a speed of 1^{o} min^{-1} for 2θ ranging from 4^{o} - 40^{o} with an increment of 0.04^{o} and rotation speed of 200 rpm. XRD crystallinity index (CIXRD) was determined using the peak height method by taking 2θ ratio at 15.5 and 22.4^{o}. (Thyrel, 2014).

3.2.1.5.2 FTIR Analysis

The biomass (untreated and treated) sample-KBr mixture was prepared by thoroughly mixing approximately 2 mg biomass with 200 mg KBr. The mixture was kept in a specially designed plate and pressed with 20 bar pressure so as to make a uniform pellet. The pellet was scanned between 4000 to 400 cm^{-1} with 4 cm^{-1} resolution using FTIR spectrometer (Perkin Elmer, USA). The background spectrum of pure KBr was subtracted from the sample spectrum (Xu et al., 2013). It was done in Instrumentation laboratory, IITG, Guwahati, India.

3.2.1.5.3 Metal Ion Composition Analysis

The pretreated and crude biomass samples were digested and the digested solutions were used for metal ion composition analysis using AAS as described in section 3.2.1.2.3.

3.2.1.5.4 Differential Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was performed for pretreated and crude biomass as described in section 3.2.1.2.1. Differential thermogravitric curve was ploted by taking differential weight per ^{o}C.

3.2.2 Isolation, Characterization and Selection of Yeasts for Efficient Ethanol Production

3.2.2.1 Isolation of Yeasts

Yeast were isolated from the samples by taking finger printing in YMA media and or by overtaxing the sample in YMB, serially diluted and spread on YMA media. (Karki & Shrestha, 1999; Middlehoven, 2002). Distinctly isolated yeast colonies were sub cultured and stored in YMA slant and 15% glycerol.

3.2.2.2 Biochemical Characterization of Yeast Isolates

Isolated yeasts were studied for their efficiency of budding, D-glucose utilization, D-xylose utilization, ethanol production from glucose, ethanol production from xylose and salt tolerance respectively. Yeasts were allowed to grow in PYN media and observed microscopically to see budding. PYN media supplemented with glucose or xylose respectively were used to see ethanol production efficiency. PYN media supplemented with 1-20% salt (sodium chloride) were used for the salt tolerance test.

3.2.2.3 Molecular Characterization of Yeasts

3.2.2.3.1 Extraction of DNA from Yeasts

Total DNA were extracted from broth culture using DNA isolation kit (Promega). DNA pellet were dried for 15 min in air and finally re-suspended in 40 µL tris buffer. The genomic DNA was verified by running DNA in 0.8% agarose gel electrophoresis. Remaining DNA was stored for PCR analysis.

3.2.2.3.2 Amplification of D1D2 region

The partial DNA of 26S rRNA gene D1D2 region was amplified by PCR using the primer with forward sequence and reverse sequence D1D2 [NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′)] to PCR fragment of 680 bp (Cocolin et al., 2002). PCR was performed in 25 µL reaction volume containing: 1 µL (45 ng) genomic DNA, 1µL (25 mM) $MgCl_2$, 12.5 µL (2x) master mix, 1.5 µL (10 pM) of each primer pair and 7.5 µL nuclease free water. Thermocyclic conditions were 96ºC for 2 min for initial denaturation, followed by 35 cycles of 96ºC for 45 sec, 52ºC for 45 sec, 72ºC for 2 min. Final elongation at 72ºC for 10 min, finally stored at 4ºC. PCR were performed in Biorad thermo cycler.

Aliquots of 5.0 µL of PCR-amplified products and the 100-bp DNA Ladder (100 to 1500-bp) (Invitrogen Life Technologies, USA) were loaded onto 1.0% agarose electrophoresis gels (Sigma Chemical, USA). The run was performed with 1x Tris-acetate- EDTA (TAE) buffer, containing 40 mM Tris-HCl, pH 8.3, 20 mM acetic acid (Merck, Germany) and 1 mM EDTA (Sigma-Aldrich CO., USA), at 90 $V \cdot cm^{-1}$ for 45 min. The gels were stained with ethidium bromide. The stained gels were photographed using UV transilluminator (Eagle Eye II Video Imaging System, Stratagene, California, USA).

3.2.2.3.3 Phylogenetic Analysis

The PCR products were sent for sequencing (Paula Goncalves, Yeast Genomics Laboratory, Nova University, Lisbon, Portugal and Excelris lab Ahamdabad, India). The sequences obtained were edited, compiled, and aligned using BioEdit software.

Sequence similarity searches were performed using GenBank Blastn. A phylogenetic tree was generated using the neighbor-joining algorithm in MEGA6.

3.2.2.4 Study of Salt and Ethanol Tolerance of Yeast Isolates

All the isolated yeasts were cultured separately in PYN media supplemented with 0-22% sodium chloride or ethanol respectively and allowed to grow at pH 4.5 and temperature 28^{o}C for 96 h (Balakumar & Arasaratnam, 2012). Microbial growth pattern were observed spectrophotometrically (Genesis) at 600 nm against medium blank (Sherman, 2002).

3.2.2.5 Study on Glucose and Xylose Utilization and Ethanol Production

All the isolated yeasts were cultured separately in PYN media supplemented with glucose or xylose as a carbohydrate source. The growth of yeasts was observed by taking absorbance at 600 nm as described by Sherman (2002). Successively, ethanol production was also measured using the protocol of Seo et al. (2009). The broth was centrifuged at 4000 rpm for 15 min. Then 1 mL of the supernatant was added with 1 mL tri n-butyl phosphate (TBP). The mixture was vortexed for 15 min. Finally the vortexed mixture was centrifuged at 4000 rpm for 15 min to separate layers. About 750 µL of upper layer was transferred to another tube and added with equal amount of acidified 5% potassium dichromate reagent (Appendix 1). The process of vortexing and centrifuging was repeated. Then pooled out lower layer and observed the absorbance at 595 nm using spectrophotometer. The best yeast strains were submitted to NCBI for Gene Bank Accession No.

3.2.3 Optimization of Physiochemical Parameters for Ethanol Production

3.2.3.1 Effect of pH on Ethanol Production

The effect of pH on the fermentation process was determined by adjusting the pH of the PYN media. The pH of the media was adjusted in the range from 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 to 7.0 with 1M HCl and 1M NaOH. The media was then inoculated with 0.4% (18 h culture) of the inoculum and incubated at optimum temperature for 96 h. Ethanol concentration was measured after solvent extraction with tri-n-butyl phosphate and treating with acidified potassium dichromate as described in section 3.2.2.5.

3.2.3.2 Effect of Temperature on Ethanol Production

The effect of temperature on the fermentation process was determined by inoculating the inoculum to PYN medium and incubating at different temperature. The culture was incubated at temperatures 24ºC, 26ºC, 28ºC, 30ºC and 32ºC respectively for 96 h. The temperature was noted at which maximum ethanol produced.

3.2.3.3 Effect of Different Nitrogen Supplements on Ethanol Production

The effect of various nitrogen sources on fermentation was determined by incorporating single nitrogen sources at a time in the fermentation media composition. The nitrogen sources used were ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium oxalate, ammonium acetate, yeast extract, peptone and urea. The media were then inoculated and incubated at optimum temperature for 72 h. Ethanol production in different media were measured and the best source of nitrogen for ethanol production was then determined.

3.2.4 Ethanol Production in Electrochemical Cell

3.2.4.1 Construction of Electrochemical Cell (ECC)

Potent yeast strains were observed for improvement in ethanol production in electrochemical cell (ECC) with carbohydrate sources including lignocellulosic biomass hydrolysate. The carbohydrate source used was glucose and the source was supplemented with PYN media. Two compartment ECC was purchased from Adams and Chittedon, California USA as per design to induce the electrochemical oxidation and reduction reaction simultaneously. The anodic and cathodic compartments were separated by nafion membrane. Graphite felt (11 cm x 3cm x 1cm) was used as cathode and platinum wire (0.2 mm diameter and 1 m length) was used as anode (Appendix 3). Working volume of the cathodic and anodic compartments was adjusted to 300 mL each. Ethanol production was measured as described in section 3.2.2.5. The generalized cell representation of the system is represented as:
Pt/yeast, glucose (5%)//yeast, glucose (5%)/C.

3.2.4.2 Optimization of Ethanol Production in Electrochemical Cell

Ethanol was allowed to produce in ECC with supply of voltage. Catholyte and anolyte were PYN media supplemented with 5% glucose at optimized condition of pH and temperature. The optimized condition chosen were pH 5.5 and temperature 30^{o}C. CDBT2 and CDBT7 were chosen as best yeast strains for ethanol production. A 1.2 mL inoculum of respective yeast was inoculated in each 300 mL culture volume. Yeast inoculum was prepared by culturing yeast in PYN media supplemented with 2% glucose for 18 h (Balakumar & Arasaratnam, 2012). The yeasts were cultured in ECC at different conditions to observe the effect of cathodic and anodic potential on ethanol production. The ethanol production in each case was compared with ethanol production in respective normal fermentation where ethanol was produced without supply of voltage. ECC with media and without yeast culture and without voltage supply was observed as control system. Values reported are mean ± standard deviation of 3 different independent experiments.

3.2.4.2.1 Ethanol Production by CDBT2 and CDBT7 Combination

Ethanol was allowed to produce in ECC with CDBT2 and CDBT7 in cathodic and anodic chamber respectively (Pt/CDBT2, glucose (5%) // CDBT7, glucose (5%)/C) and vice versa (Pt/CDBT7, glucose (5%) // CDBT2, glucose (5%)/C) and the effect on ethanol production, cell growth and glucose reduction were observed with the supply of 4V. Small pieces of graphite electrode kept as cathode with cultures of CDBT2 and CDBT7 were vacuum evaporated and packed in sterile plastic pouch. The samples were sent to Jawaharlal Nehru University, New Delhi, India for capturing images by Scanning Electron Microscopy (SEM) to analyze the film formation by yeast CDBT2 and CDBT7 in graphite electrode.

3.2.4.2.2 Optimization of Voltage for Ethanol Production

To determine optimum voltage supply for ethanol production, different potential were supplied to ECC from 0 to 5V (± 0.1V) at the interval of 1V and ethanol production was measured. Because of the unavailability of system current flow was not observed.

3.2.4.3 Effect of Different Electron Transport Enhancing System in ECC

Ethanol was allowed to produce by culturing CDBT2 in both cathodic and anodic compartments, CDBT2 in anodic and CDBT7 in cathodic compartment and a mixed culture of CDBT2 and CDBT7 in both compartments. Further, the (cathode) graphite felt and (anode) platinum wire were coated with different electron transporters. The cathode electrode was immobilized with yeast, coated with neutral red (NR). Anode was coated with fine platinum nanoparticles. Ethanol production was measured and best combination of electron transport system was determined.

3.2.4.3.1 Immoblization of Yeast in Graphite Felt

About 2.4 mL of 18 h culture of yeast cells were centrifuged and pellet mixed with 10 mL of 25 mM phosphate buffer (pH 7.0) containing 4% sodium alginate and allowed to absorb into the graphite electrode for 30 min. The graphite electrode containing alginate and yeast cells was then soaked in a chilled 100 mM $CaCl_2$ solution for 30 min to induce calcium alginate coagulation. Lastly it was washed once with 25 mM phosphate buffer.

3.2.4.3.2 Coating of Neutral Red in Graphite Felt

The graphite felt soaked in methanol was dipped in 1% polyvinyl alcohol solution for 3 to 4 h and was dried in oven at about 80^{o}C for 24 h. The completely dried graphite felt was then soaked in pure chloroform containing 10% thionylchloride and 0.01% neutral red for 6 h. The graphite felt was then left for 12 h for air dry. It was then autoclaved and washed in running water till color persists. Finally it was dried at 60^{o}C for 24 h and used as cathode (Jeon & Park, 2010).

3.2.4.3.3 Coating of Platinum Nanoparticles in Platinum Electrode

A 1 m of platinum electrode was wiped with tissue paper. It was then coiled with the help of a 1 cm diameter rod. The coiled wire was dipped in distilled water and sonicated for 30 min. Further the coil was removed from water and dipped in acetone, sonicated again for 30 min. It was dried and used for coating platinum. The wire was dipped in a solution of hydrogen hexachloroplatinate (IV) hexahydrate. A constant

potential of 0.2V was supplied for 30 min for coating platinum to the wire using potentiostat (Hokuto Denko-115) (Appendix 3).

3.2.4.4 Comparison of Ethanol Production by CDBT7 vs. Other *S. cerevisiae*

To compare the ethanol production efficiency of CDBT2 with other *S. cerevisiae*, ethanol was allowed to produce in anodic compartment of ECC by culturing different sources of *S. cerevisiae* available in CDBT laboratory. CDBT2, MKY09 (laboratory strain from Germany, kindly provided by Prof. Eckhard Bole, University of Frankfurt), MKY09 with PGPD2/lac plasmid with laccase encoding gene (Laccase gene inserted in pGPD2 plasmid developed in Central Department of Biotechnology, Tribhuvan University by M.Sc. thesis student Sujan Bishwokarma Reg. No.5-1-48-301-2007; Bishwakarma, 2017) , Ethanol red, an industrial yeast from Prof. Eckhard Bole's laboratory. For the construction of pGPD2/lac plasmid, plasmid pGPD2 (expression vector) was purchased from Addgene Company. The plasmid was inserted with synthetic laccase gene of *Ganoderma lucidum* of size 1,576 bp cut from pUC57-laccase plasmid. pUC57-laccase plasmid was the laccase gene inserted in pUC57 plasmid from GeneScripts Company. The pUC57 plasmid was digested with EcoR1 and HindIII enzyme and the laccase gene cut was ligated to pGPD2 plasmid at EcoR1 and HindIII cut region. Later it was transformed into MKY09 strain. CDBT7 was cultured in cathodic compartment in all cases. The ethanol production was measured.

3.2.5 Ethanol Production by Lignocellulosic Biomass Hydrolysate as Carbohydrate Source

3.2.5.1 Ethanol Production from Hydrolysate

The hydrolysate obtained from best pretreatment and hydrolysis technique was used as lignocellulosic biomass hydrolysate (*S. spontaneum* pretreated with water at 100^{o}C for 2 h followed by 0.5M acid hydrolysis). It contained higher amount of reducing sugars with less interfering agents. The hydrolysate was supplied with PYN media and used for fermentation to get ethanol. The hydrolysates were kept in ECC chambers and operated in optimized condition for ECC (Appendix 3). Ethanol production was measured.

3.2.5.2 Comparison of Ethanol Production by Nafion and Cellulose Acetate Membrane

The above mentioned hydrolysate media was used for ethanol production with cellulose acetate membrane in a different systems in place of nafion membrane and compared the ethanol production efficiencies.

3.2.6 Quantitative Expression Analysis of Alcohol Dehydrogenase and Pyruvate Decarboxylase by RT PCR

RT PCR (qPCR) was done to observe the expression of alcohol dehydrogenase and pyruvate decarboxylase with or without supply of electric field.

3.2.6.1 Isolation of RNA from Yeasts

For isolation of RNA from yeast, Quick-RNA^{TM} MiniPrep kit from Zymo research was used (kindly provided by Sudeep Makaju, Applied Biotech Trading, Nepal). A 200 µl freshly cultured yeast sample was suspended and lysed using 600 µl RNA lysis buffer then centrifuged to remove particulate debris. The clear supernatant was transferred into spin-away filter with a collection tube by centrifugation. Equal volume of ethanol (95-100%) was added to the flow through and mixed well. Then the mixture was transferred to Zymo-spin IIICG column in a collection tube and centrifuged. The flow through was discarded. The column was first washed with 400 µl RNA prep buffer and centrifuged, flow through was discarded. Again washed two times with 700 µl and 400 µl of RNA wash buffer and centrifuged for 2 min to completely ensure removal of wash buffer. To elute RNA, 100 µl nuclease free water was added directly to the column matrix and centrifuged. The flow through consists of RNA which was immediately used to prepare cDNA for further study.

Synthesis of cDNA from RNA

BIO-RAD iScript^{TM} cDNA synthesis kit was used for preparation of cDNA. Reaction parameter for cDNA synthesis was done according to the information provided from the kit. All the components were stored at -20^{o}C in a constant temperature before use whereas nuclease free water was stored at room temperature. Table 7 showed the volume of components taken for cDNA synthesis. The components were well mixed by pipette. PCR cycle was set up in a thermal cycler with the condition shown in

Table 8. The synthesized cDNA was screened using 0.8% agarose gel and stored at -20ºC for further use.

Table 7: Reaction components in terms of volume for cDNA synthesis

Components	Volume per reaction (µL)
5x iScript reaction mix	4
iScript reverse transcriptase	1
Nuclease free water	7
RNA template	8
Total volume	**20**

Table 8: Thermocyclic condition for preparation of cDNA

S. N.	Steps	Temperature/Time
1	Step1 (priming)	25ºC for 5 min
2	Step2 (reverse transcription)	46ºC for 20 min
3	Step3 (RT inactivation)	95ºC for 1 min
4	Step4 (optional step)	4ºC (Hold)

Relative quantification of ADH1, PDC1 and TFC1 genes using RT PCR

Gene expression level was quantified by using RT PCR (Smidt et al., 2011). The relative quantification technique was used for the comparison of gene expression relative to the reference gene. TFC1 (transcription initiation factor 1) was used as reference gene or housekeeping gene for relative quantification of ADH1 and PDC1 gene. Advanced Universal SYBER green supermix dye was used. All the components stored were thawed to room temperature before use. These reagents and components were centrifuged to collect solutions at bottom of the tube and then stored on ice protected from light.

Reaction mixture was prepared according to the samples to be processed. All the components were mixed in a single reaction tube except the template. Volume for 15 µL for each tube was calculated in which 1 µL sample (template) was used and 14 µL master mix was prepared using remaining components. Table 9 showed the volume components of the samples used for RT PCR. Each well of the PCR plate was initially loaded carefully pippetting with 14 µL master mix and 1 µL cDNA templet

was added at the end. All experiments were done in triplicate to optimize the result. Then a clear film was used to cover the plate and sealed the content after loading all the samples. Primers used for RT PCR were shown in Table 10. Table 11 was the PCR cycle run for RT PCR to conduct.

Table 9: Reaction volume of RT PCR mixture per well.

Components	Volume for each tube (µL)
SYBER green supermix	7.5
Forward primer	0.35
Reverse primer	0.35
Nuclease free H_2O	5.8
cDNA template	1.0
Total volume	15.0

Table 10: List of primers used in RT PCR

S.N.	Primer Name	Primer Sequence(5'-3')
1.	ALD1F	CGTTTCCGAAGCCGCTATTG
	ALD1R	GCATACCGACCAAAACGGTG
2.	PDC1F	GCCAAACGATGCTGAATCCG
	PDC1R	CCTTGACGTCGTGTCTGGAA
3.	TFC1F	GCTGGCACTCATATCTTATCGTTTCACAATGG
	TFC1R	GAACCTGCTGTCAATACCGCCTGGAG

Table 11: Cycling conditions for RT PCR

S. N.	For reference gene(TFC1)	For test gene (ADH1 and PDC1)
Stage 1	95°C for 2 min	95°C for 2 min
Stage 2	95°C for 30 sec	95°C for 30 sec
Stage 3	64°C for 30 sec	57°C for 30 sec
Stage 4	72°C for 20 sec	72°C for 20 sec
Plate Read		
Stage 5	GO TO 2 , 34 cycles	
Stage 6	Melt curve	

When the PCR run was complete the data obtained was saved in a folder and analyzed (Appendix 4). Further calculation was done manually using the following formula (Yuan et al., 2006).

ΔC_t (test sample) = C_t (test sample) – C_t (reference)

ΔC_t (control) = C_t (control) – C_t (reference)

$\Delta\Delta C_t = \Delta C_t$ (test sample) – ΔC_t (control)

3.2.7 Statistical Analysis

All graphs and statistical calculations were performed with Graph Pad Prism 8.0.1. Standard errors were represented in terms of ± standard deviation (± SD). Values reported are mean ± standard deviation of three different independent experiments.

CHAPTER 4

4 RESULTS AND DISCUSSION

4.1 Characterization of Biomass

4.1.1 Biomass

The four different biomass samples *Ipomoea carnea, Phragmites karka, Saccharum spontaneum* and *Zea mays* (corn) cobs (Figure 6) were chosen. All these biomass except corn cob are agricultural derivatives of fast growing invasive plants. They are grown for the sake of different purposes like protecting from pests, landslides, water purification etc (Leipzig,1996; Tiwari et al., 2005; MAPs-Net, 1997; Pandey et al., 2015, Verma et al., 2014). The plants generally grow without any external effort and they are having more cellulose contents amongst the potent energy possessing biomass found around the premises (Table 2). Additionally, *Phragmites karka* is planted in riverbeds for bioremediation of arsenic like heavy metals besides to check erosion. *Phragmites karka* is halotolerant plant also (Verma et al., 2014). On the other hand corn cob is the waste material produced in high amount. According to Salman (2018), 0.96 million tons of corn cobs produced yearly in the Philippines only, which can be used for making value added products. Chemical analysis of the biomass was done in dried and powdered sample.

4.1.2 Chemical Analysis

The four biomasses were characterized for their sugar contents, thermal degradation pattern, calorific value, moisture content, ash content and metal ion contents before pretreatement (Table 12). The data showed the presence of maximum sugar content in *S. spontaneum.* Glucose and total reducing sugar (TRS) content were 246.7 ± 4.0 and 612.2 ± 11.5 $mg \cdot g^{-1}$ biomass respectively. The TRS reported herein was higher than that of the values reported by Chandel et al. (2009) ie; (539.1 ± 0.55 $mg \cdot g^{-1}$ in *S. spontaneum*) and lower than that of Singh et al. (2011) ie; (68% on oven dry weight basis in *S. spontaneum*). A number of varieties of *S. spontaneum* are known to grow around the world and *S. spontaneum* (switch grass) is one such variety.

According to Vogel et al. (2002) without exerting pressure on the land, it is estimated that in average 10.5 to 12.6 megagram per hacter per year switch grass can be produced and 334 L of ethanol can be produced from one megagram biomass where

as theoretical yield is approximately 450 L per megagram biomass (Mitchell et al., 2012). It grows in open land without any special effort, so it is a good source of fermentable sugar to produce bioethanol and other value added products. Compared to other species of biomass analyzed, *S. spontaneum* showed high ash content showing that it is rich in macro and micro nutrients. The high amount of total ash contents with high potassium and sodium ion contents of *S. spontaneum* favors the growth of yeast (Camacho et al., 1981).

Figure 6: Plants chosen as source of lignocellulosic biomass for study

4.1.3 Differential Thermogravimetric Analysis

The differential thermogravimetric curves was plotted to observe the degradation pattern of biomass (Figure 7). The peaks at around 300^{o}C revealed that *S. spontaneum* has fast degradation capability followed by *P. Karka, I. carnea* and *Zea mays* cobs. The main peak in the differential thermogravimetric curve corresponds to degradation of cellulose in lignocellulosic biomass and shoulder peak at lower temperature corresponds to the degradation of hemicelluloses. The sharp peak for *S.*

spontaneum nearly at 300^{o}C corresponds to high cellulose content, as well as fast degradation of cellulose in biomass. Lignin decomposes in the temperature range of 300–500^{o}C and shows heterogeneous peaks due to its heterogeneity and lack of a defined primary structure. The remaining constant mass beyond this range represents ash content (Singh et al., 2013).

Table 12: Analysis of untreated (crude) biomass for various sugars, fermentation inhibitory substances, calorific value, moisture and ash content.*

Parameter analyzed	Biomass analyzed			
	I. carnea	***P. karka***	***S. spontaneum***	***Zea mays* (cobs)**
Glucose (mg·g^{-1} biomass)	200.3 ± 10.6	223.0 ± 5.1	246.7 ± 4.0	232.6 ± 16.3
Xylose (mg·g^{-1} biomass)	27.0 ± 1.0	78.3 ± 3.5	86.6 ± 3.9	85.2 ± 5.5
Arabinose (mg·g^{-1} biomass)	0.9 ± 0.1	6.9 ± 0.8	6.5 ± 1.0	10.9 ± 1.0
5-HMF (mg·g^{-1} biomass)	82. 7 ± 3.6	78.2 ± 5.2	72.9 ± 3.9	90.8 ± 3.0
Furfural (mg·g^{-1} biomass)	2.19 ± 0.01	10.6 ± 1.0	5.7 ± 0.8	12.1 ± 1.0
TRS (mg·g^{-1} biomass)	560 ± 18.6	492 ± 6.5	612.2 ± 11.5	580.8 ± 8.0
Calorific value (cal·g^{-1})	4126 ± 228	4235 ± 42	4419 ± 224	4784 ± 273
Moisture (%)	6.1	6.7	6.3	8.3
Ash (%)	4.4	4.1	7.8	1.7
Potassium (mg·g^{-1} biomass)	26.6 ± 2.8	18.8 ± 2.5	12.7 ± 0.9	4.5 ± 0.44
Sodium (mg·g^{-1} biomass)	0.31 ± 0.02	0.33 ± 0.05	0.34 ± 0.01	<0.1
Zinc (mg·g^{-1} biomass)	<0.1	<0.1	<0.1	<0.1

*Biomass samples hydrolyzed (acid hydrolysis) were analyzed for glucose, xylose, arabinose, 5-hydroxymethyl furfural and furfural concentrations by HPLC method using a refractive index detector. TRS using protocol as described in NREL, 2012 and Miller, 1959. Calorific value was determined using a bomb calorimeter, moisture and ash contents were determined by TGA as described in 3.2.1.2.

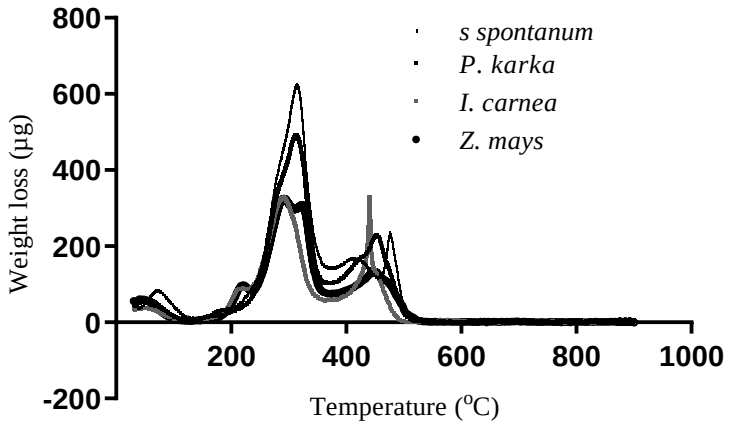

Figure 7: Differential thermogravimetric analysis of biomass. Preparation of biomass samples and their differential thermogravimetric analysis with continuous change of temperature at a rate of 10°C min^{-1} performed as described in 3.2.1.2.1.

4.2 Pretreatment and Hydrolysis of Biomass

From the results of chemical analysis given above in Table 12 and differential thermogravimetric analysis curve shown in Figure 7, *S. spontaneum* was found as good biomass source to convert into other value added products as *S. spontaneum* found to have higher carbohydrate content with high calorific value and fast degradation rate. So, it was selected as a biomass source for further processing. The dried and powdered biomass was pretreated and hydrolysed.

4.2.1 Pretreatment

The biomass was subjected to various pretreatments with 0.5M sulphuric acid, 0.5M ammonium hydroxide, hot water, various concentrations of 1-butyl-3-methyl imidazolium chloride (40, 60 and 100%) or butadiene sulphone (50 and 100%) at 25°C, 65°C, 100°C and 120°C. The liquid portions of the pretreated samples were analyzed for TRS, phenols and furfural. The results of these analysis are shown in Figures 8a, 9a, 10a (TRS); 8b, 9b, 10b (phenols) and 8c, 9c, 10c (furfural). Lower concentration of acid and base were used in this study as higher concentrations, although increase delignification process, decrease in the recovery of total reducing sugars (Soto et al., 1994). Study showed that, the most commonly used acid for pretreatment is sulfuric acid. However, the concentrated acid despite being powerful degrading agent is highly corrosive and not used any more (Kavitha et al., 2014). Alkaline pretreatment utilizes lower temperature and pressure as compared to other

pretreatment technologies with less sugar degradation. Additional advantages of alkali pretreatment is degradation of ester and glycolytic side chains causing structural alteration of lignin, swelling of cellulose, partial decrystallization of cellulose and partial solvation of hemicelluloses (Holm et al., 1997). It has been shown that ionic liquids display interesting properties such as chemical inertness, good thermal stability, very low to no toxicity and unique salvation ability; accordingly they are considered a good choice of matrices for pretreatment of lignocellulosic biomass. Further, butadiene sulphone in presence of water is believed to efficiently catalyze the breakage of xylan-lignin bonds (Atilio et al., 2013). Pretreated samples were hydrolyzed successively by 0.5M hydrochloric acid or 10 FPU cellulase enzyme to observe the effectiveness of pretreatment.

4.2.2 TRS, Phenol and Furfural Released During Hydrolysis

Post pretreatment biomass samples were hydrolyzed by 0.5M hydrochloric acid or 10 FPU (filter paper unit) cellulase to release enough TRS which can be used for fermentation. In this study, acid hydrolysis for 24 h at 90^{o}C released highest TRS from the sample of hot water pretreatment at 100^{o}C for 2 h that released 330.4 ± 20.5 mg TRS, 1.54 mg phenol and 88.88 ± 0.07 µg furfural respectively per gram biomass. Pretreatment with ammonium hydroxide (0.5M) for 12 h followed by cellulase enzyme hydrolysis at 65^{o}C released 347.2 ± 55.6 mg TRS, 2.03 mg phenol and 19.7 ± 4.2 µg furfural respectively per gram biomass, however this method is costlier than HCl hydrolysis and the increment in TRS is very low. Similar results with the release of 350 mg TRS per gram from *S. spontaneum* biomass when pretreated with 0.5% NaOH at 120^{o}C for 2 h and the biomass was hydrolyzed by 10 FPU cellulase (Kataria & Ghosh, 2014). In the same paper, Kataria & Ghosh showed the release of 69.08 mg TRS·g^{-1} biomass of *S. spontaneum* when pretreated with 2% H_2SO_4 for 90 min at 120^{o}C and the biomass was hydrolyzed with 10 FPU cellulase. Further the study of Singha et al. (2011) showed that the dilute acid pretreatment of biomass followed by enzymatic hydrolysis released TRS of only 231 mg·g^{-1} biomass suggesting that the process is inefficient. Conata et al. (2015) used several methods of pretreatment and found that pretreatment with 2.5% NaOH at 100^{o}C for 30 min produced the most efficient TRS recovery (202 mg TRS g^{-1} biomass). Comparing our results with other findings the hot water pretreatment at 100^{o}C for 2 h followed by hydrochloric acid hydrolysis seems to be best for TRS release from lignocellulosic

biomass. The process is not only simple but also economical since no costly enzymes were used and TRS loss due to pretreatment was also minimal (25.4 ± 2.9 $mg \cdot g^{-1}$ biomass, Figure 8a). Alkali pretreatment at 65^{o}C followed by enzymatic hydrolysis was also equally effective method, if the enzymes are cheap. The latter is still a challenge until protein engineering techniques could be exploited to develop more efficient cellulases. *Saccharomyces cerevisiae* is the most commonly used organism for ethanol production and has phenol tolerance of 1 $g \cdot L^{-1}$ as observed by Colombi et al. (2017) which is very higher than the phenol released in our condition. Further *S. cerevisiae* is capable of reducing furfural to furfuryl alcohol at low concentrations which helps for detoxification due to furfural at small amount (Villa et al., 1992). The concentrations of furfural, *S. cerevisiae* can tolerate is 3.75 $mg \cdot mL^{-1}$ in normal fermentation and 17 $mg \cdot mL^{-1}$ in membrane bioreactor (Ylitervo et al., 2013) and generally furfural concentration above 3 $mg \cdot mL^{-1}$ is antagonistic to cell growth (Palmqvist et al., 2000). Additionally, in our study, pretreatment of *S. spontaneum* biomass with hot water at 100^{o}C for 2 h produced only 7.2 ± 0.15 mg phenol and 103.2 µg furfural per gram biomass (Figure 8b and 8c). This corresponds to 0.72 ± 0.01 $mg \cdot mL^{-1}$ phenol and 10.3 $\mu g \cdot mL^{-1}$ furfural which was very low to show inhibitory effects though the pretreated biomass along with extract might be directly used for acid hydrolysis to avoid the loss of TRS due to pretreatment. TRS released after pretreatment of biomass at 120^{o}C followed by acid hydrolysis was lower than at 100^{o}C pretreatment (Figure 9a) and acid hydrolysis indicating that temperatures above 100^{o}C pretreatment, the TRS levels will be less and this is likely due to degradation of sugars during high temperature pretreatment.

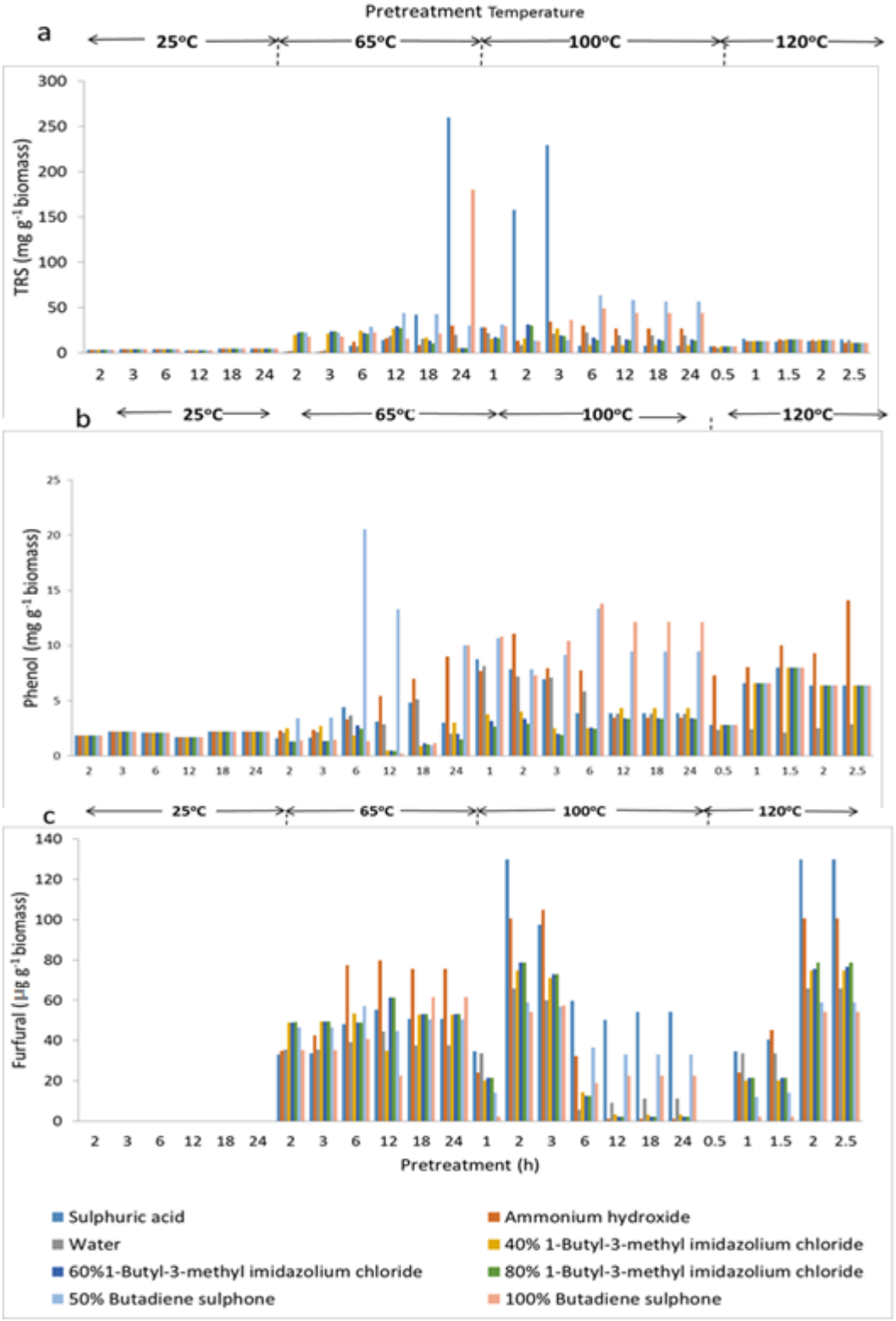

Figure 8: Product released after pretreatment of *S. spontaneum* biomass with various chemicals. Panel a: TRS. Panel b: Phenol. Panel c: Furfural.

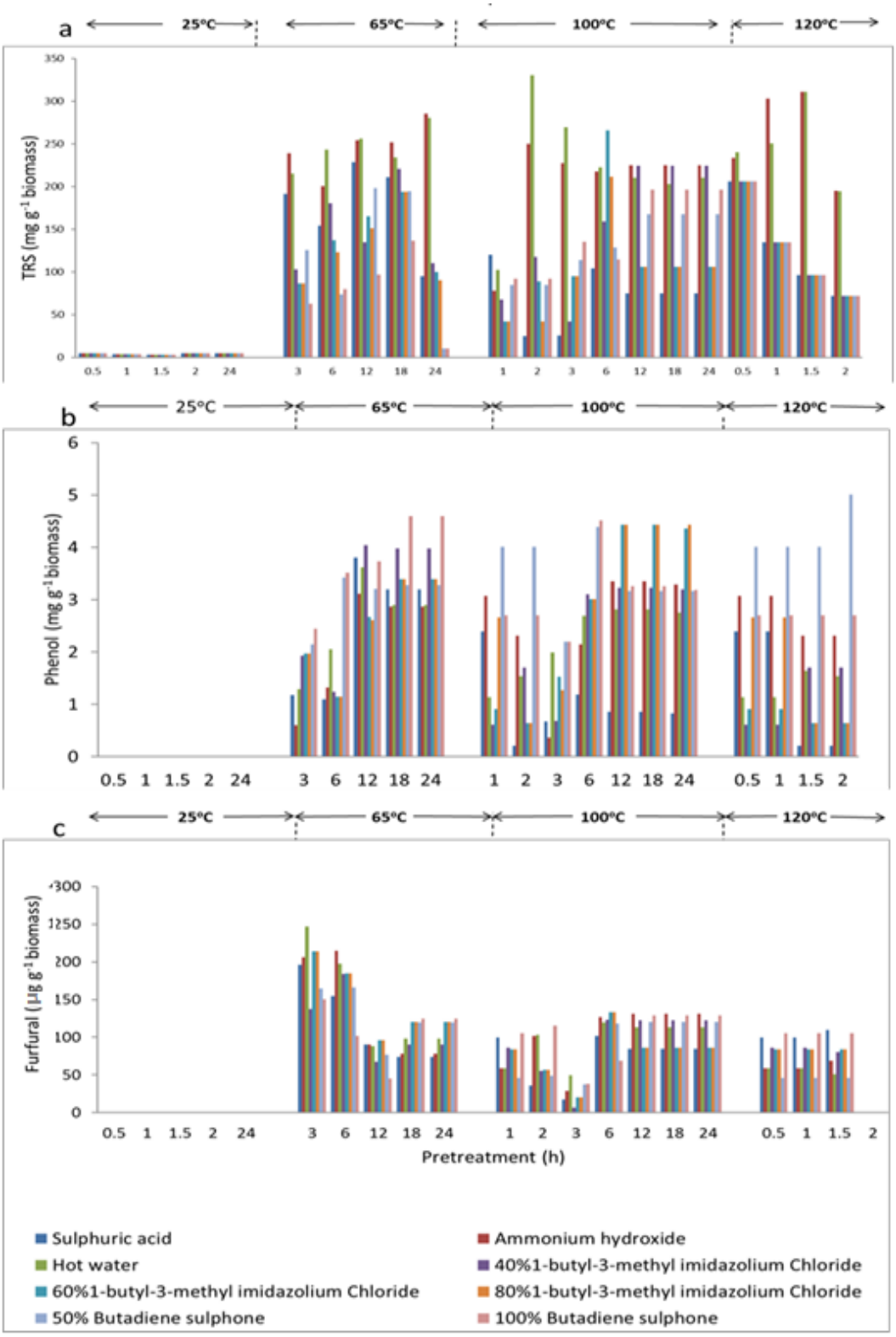

Figure 9: Product released after hydrochloric acid hydrolysis of *S. spontaneum* biomass pretreated with various chemicals. Panel a: TRS. Panel b: Phenol. Panel c: Furfural. Hydrolysis was performed at 90ºC as described in 3.2.1.4.1.

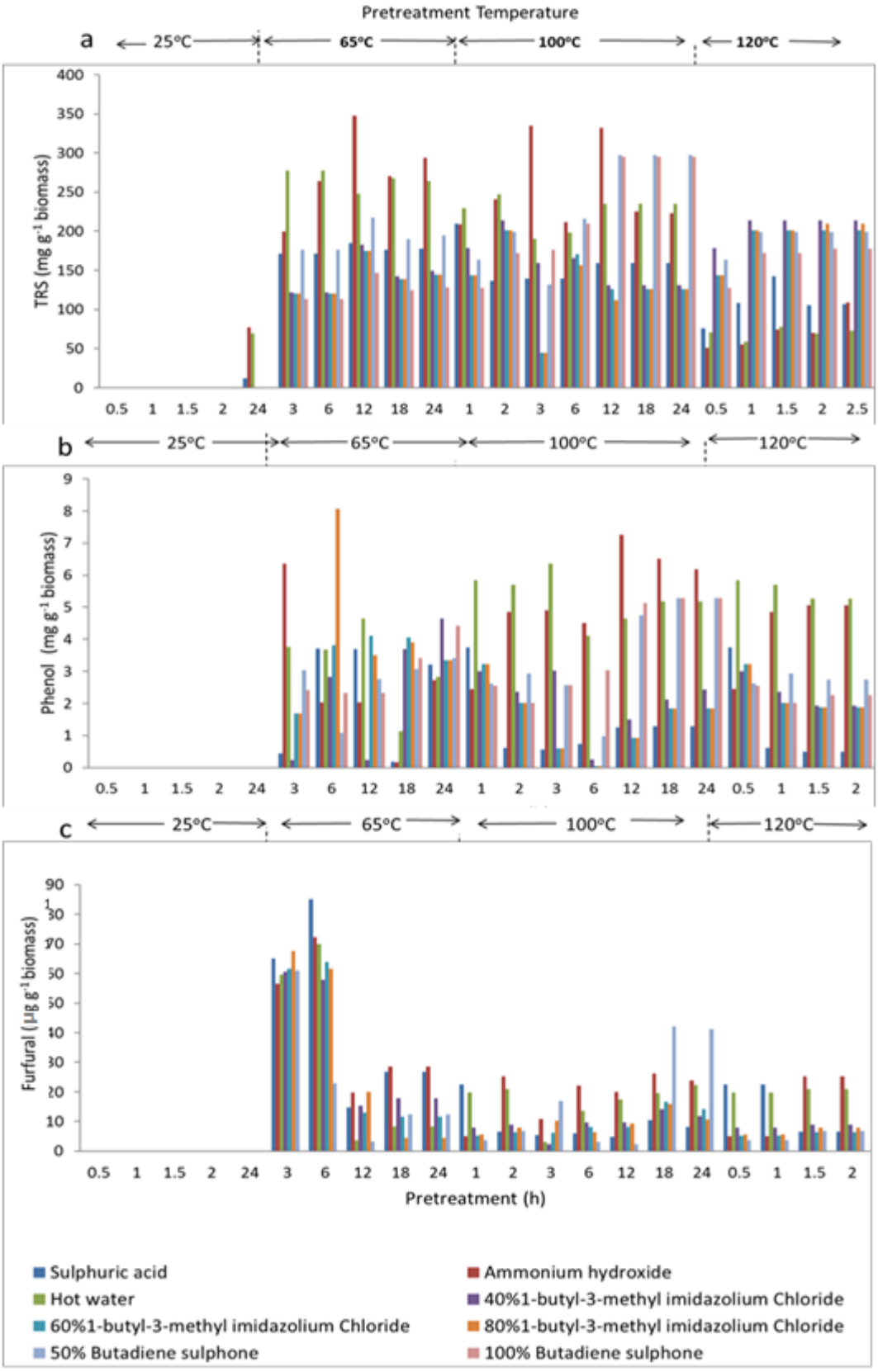

Figure 10: Product released from 10 FPU cellulase hydrolysis of *S. spontaneum* biomass pretreated with various chemicals. Panel a: TRS. Panel b: Phenol. Panel c: Furfural. Hydrolysis was performed at 45ºC as described in 3.2.1.4.2.

4.3 Characterization of Untreated (crude) and Pretreated *S. spontaneum* Biomass

S. spontaneum biomass pretreated at 100^{o}C for 2 h was found to be the best condition for obtaining higher TRS yield. The untreated and pretreated biomass (100^{o}C for 2 h) were analyzed by various techniques described below to further characterize the biomass.

4.3.1 Differential Thermogravimetric Analysis (TGA)

TGA was done to observe the degradation pattern of biomass. The differential thermogravimetric plot showed that the remaining % weight (ash content) of hot water pretreated *S. spontaneum* biomass was less than that of crude biomass (Figure 11). The lower remaining weight (ash) might be due to the removal of metal ions, silica and other components during pretreatment process. Moisture content in pretreated biomass was somewhat higher (Table 13) and this may be due to increased porosity in pretreated biomass. The shifting of peak at around 225 to 325^{o}C, higher temperature indicates the removal of hemicellulose and fast degrading lignin during pretreatment. Tapering of hemicelluloses / lignin peak in both biomass indicates the ease of combustion of biomass due to pretreatment. At temperatures below 380^{o}C, the pretreated biomass was found to be more stable as compared to untreated (crude) *S. spontaneum* biomass. This is because of removal of hemicellulose and lignin. Similarly, pretreated biomass showed decrease in weight at higher temperature comparing to untreated biomass even at beyond 540^{o}C. This was because of the presence of more crystalline cellulose which was difficult to degrade (Xiao et al., 2011).

4.3.2 Calorific Value Determination

Calorific values of biomass before and after pretreatment were 4380 ± 112 and 4130 ± 60 cal·g^{-1} biomass respectively (Table 13). The decrease in calorific value of pretreated biomass was due to partial removal of carbohydrate and lipid content during pretreatment. The increase in moisture content was due to increase in porosity of cellulose in pretreated biomass (Sotelo et al., 2010).

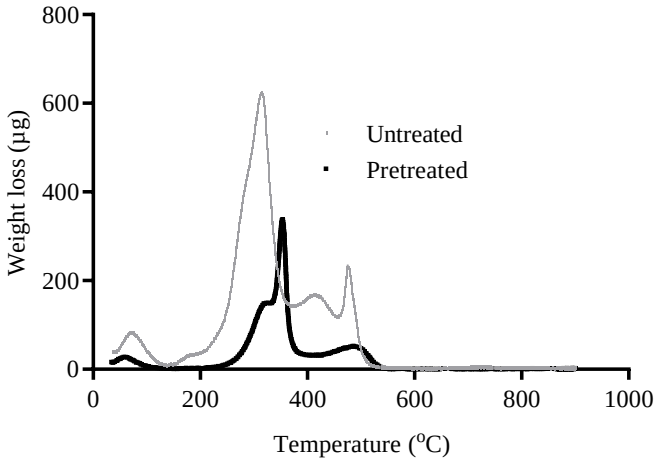

Figure 11: Differential thermogravimetric curves of untreated (Crude) and pretreated (hot water at 100°C for 2 h) *S. spontaneum* biomass showing the variation in biomass weight with increasing temperature.

Table 13: Calorific value, moisture contents and ash contents of untreated (crude) and pretreated (hot water at 100°C for 2 h) *S. spontaneum* biomass.

S. No.	Contents	Untreated biomass	Pretreated biomass
1	Calorific value (g^{-1} biomass)	4380 ± 112 cal	4130 ± 60 cal
2	Moisture	6.3%	6.7%
3	Ash	7.8%	4.1%

4.3.3 XRD Analysis

The amorphous nature of biomass can be analysed by XRD analysis. The biomass before pretreatment was more amorphous and after pretreatment the amorphous character decreased (Figure 12). In the X-ray defractograms, the peak at $2\theta = 22.4°$ became sharper, while the height of the other peak at 15.5° disappeared in the pretreated biomass. This was in turn due to decrease in amorphous nature of biomass (Fackler et al., 2011). Further, the calculated crystallinity index for the pretreated biomass increased from 41.05 to 45.9. This too was consistent with the previous reports published by Xiao et al. (2011). The increased crystallininy index was caused by the solubilization of hemicelluloses and lignin together with less ordered crystalline cellulose in pretreated biomass.

4.3.4 FTIR Analysis

FTIR was done to observe the change in biomass before and after pretreatment. In FTIR spectra, FTIR Crystallinity index (CIFTIR) is represented by intensity ratio of 1375/1512. In our samples (Figure 13), the CIFTIR were found to be 0.79 and 0.92 for untreated (crude) and pretreated biomass respectively showing the crystalline nature of pretreated biomass (Lionetto et al., 2012). FTIR spectra at 3500 cm^{-1} was due to OH stretching vibration of alcohol and phenol. FTIR spectra at 2900 cm^{-1} was due to C-H stretching vibration of aldehyde in polysaccharides. The band at 1735 cm^{-1} was characteristic of an unconjugated carbonyl group typical of xylan and hemicelluloses. Peak at 1010 cm^{-1} was due to Si-O stretching vibration. Peak at 750 ± 20 cm^{-1} might be due to C-H bending (Hospodarova et al., 2018).

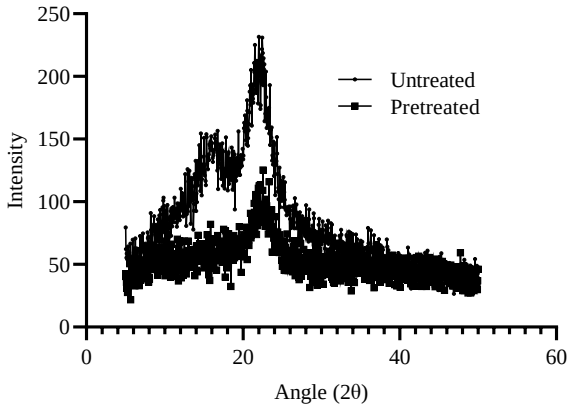

Figure 12: XRD analysis of untreated (crude) and pretreated *S. spontaneum* biomass. The biomass was pretreated with hot water at 100ºC for 2 h.

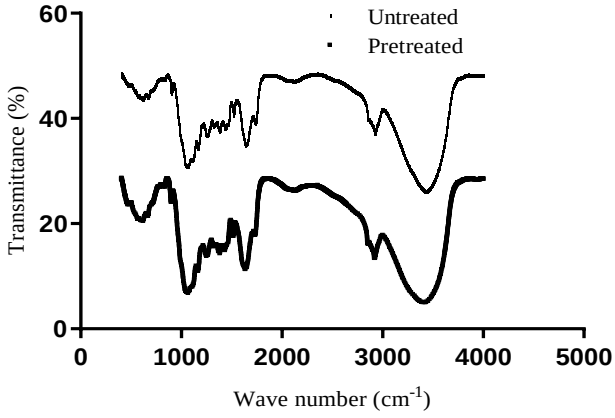

Figure 13: FTIR analysis of untreated (crude) and pretreated *S. spontaneum* biomass. The biomass was pretreated with hot water at 100ºC for 2 h.

4.3.5 Chemical Analysis of Untreated (Crude) and Pretreated Biomass

The untreated and pretreated biomass were analyzed for carbohydrate content using NREL (2012) as described above which involves the determination of glucose, xylose, arabinose and their derivatives (5-HMF and furfural) by HPLC (Table 14). The glucose concentration is an indicator of total cellulose concentration whereas the pentose sugar (xylose and arabinose) are an indicator of total hemicellulose concentration in the biomass (Sun & Cheng, 2005). Pretreatment with hot water at 100^{o}C leads to minimal decrease in cellulose content whereas xylose content is significantly decreased, suggesting loss of hemicellulose during pretreatment. This can be further confirmed by significant increases in furfural formation during pretreatment. On the other hand, 5-HMF formation was in smaller amounts and this was in turn due to minimal release of glucose during pretreatment.

Table 14: Sugar and fermentation inhibitory substance compositions in untreated and pretreated *S. spontaneum* biomass.

Biomass types	Glucose	Xylose	Arabinose	5-HMF	Furfural
Untreated (mg·g^{-1})	246.7 ± 4.0	86.6 ± 3.9	6.5 ± 1.0	72.9 ± 3.9	5.7 ± 0.8
Pretreated (mg·g^{-1})	235.1 ± 5.0	62.5 ± 3.0	6.6 ± 0.8	58.6 ± 3.9	9.9 ± 1.0

4.4 Isolation of Yeasts from Different Substrates and Molecular Characterization

4.4.1 Morphological Study

From the eight different substrates, 12 different yeast colonies were isolated. The isolated yeast were given the names CDBT1 to 12 respectively. The isolated yeast were white or creamy colonies with variability in consistency and texture as described by Cletus et al. (2011). All isolates have cottony or rubbery like appearance (Figure 14, Table 15).

4.4.2 Biochemical Characterization of Yeast

All the yeast were multiplied by budding and were good ethanol producer (Table 16). CDBT7 and CDBT8 were found to utilize xylose. CDBT7 could produce ethanol from xylose. CDBT2, CDBT3, CDBT7 and CDBT11 were found to tolerate high salt concentration. All the yeast strains showed normal growth till 4% ethanol except

CDBT8 (Figure 15). However CDBT2 was found to grow normally till 6% ethanol. Selection of ethanol tolerance strain is must when the yeast is planned to use for the industrial production of ethanol (Ekunsanmi & Odunfa, 1990). Among the various stresses that yeast cells encounter during ethanol fermentation, the important one are ethanol toxicity, adverse environmental factors, osmotic and salt pressure etc (Logothetis et al., 2007). The inability to adapt in these stresses makes slow or incomplete fermentation (Zhao & Bai, 2009). According to Sutticha et al. (2013) ethanol tolerance of 5% is considered as good isolate for ethanol production and most of isolates of *S. cerevisiae* could retain viability of 46% with 5% ethanol concentration within 48 h (Balakumar & Arasaratnam, 2012). In this aspect, CDBT2 can be a good strain for industrial ethanol production. Almost all yeast strains found to grow till 14% ethanol. There was a significant decrease in growth after 4-6% which was similar to the results by Chiranjeevi et al. (2013). CDBT2 was found to grow even in 16% ethanol concentration. However, according to Gonzalez et al. (2002), ethanol tolerance is found to vary slightly with media composition and culture condition.

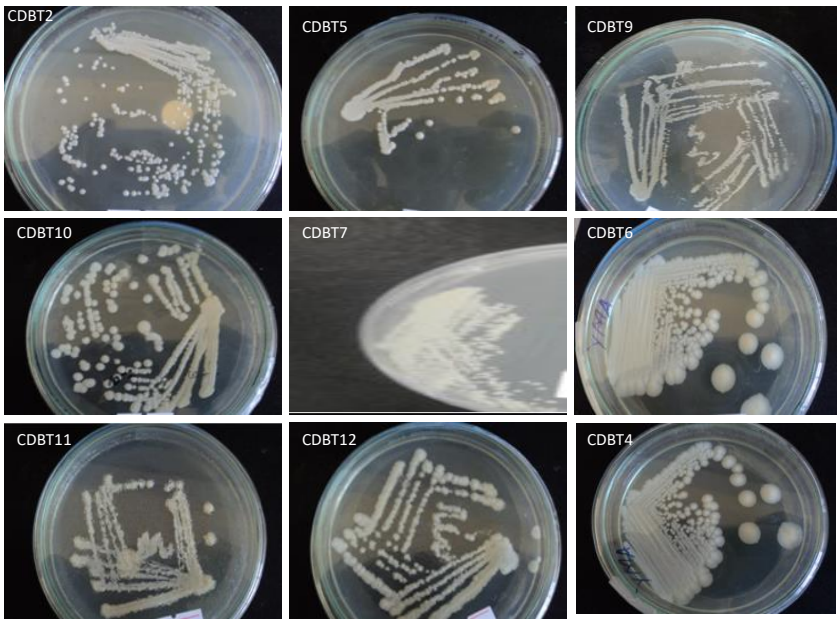

Figure 14: Yeast isolates grown in YMA media - colony morphologies of some isolated yeasts.

Table 15: Morphological characterization of yeast colonies

S. No.	Isolate Name	Colony morphology
1	CDBT1	Ovoid, smooth
2	CDBT2	Ovoid, smooth
3	CDBT3	Ovoid smooth
4	CDBT4	Ovoid, smooth
5	CDBT5	Ovoid, smooth
6	CDBT6	Diffuse
7	CDBT7	Diffuse
8	CDBT8	Ovoid, smooth
9	CDBT9	Diffused
10	CDBT10	Ovoid, smooth
11	CDBT11	Ovoid, smooth
12	CDBT12	Diffuse

Table 16: Study of different features and characters of yeasts

Yeast (CDBT)/ Characters	1	2	3	4	5	6	7	8	9	10	11	12
Budding	+	+	+	+	+	+	+	+	+	+	+	+
Growth/D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Growth/D-Xylose	-	-	-	-	-	-	+	+	-	-	-	-
Ethanol production from glucose	+	+	+	+	+	+	+	+	+	+	+	+
Ethanol production from xylose	-	-	-	-	-	-	+	+	-	-	-	-
Salt tolerance (growth seen/ % salt)	9	15	15	6	6	6	15	9	9	10	15	8

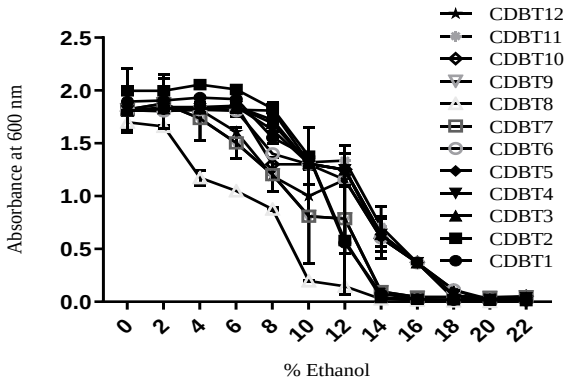

Figure 15: Effect of ethanol concentration on yeast growth. Ethanol with concentrations of 0 - 22% was taken as described in 3.2.2.4.

4.4.3 Molecular Characterization of Yeast

DNA of yeast were isolated using DNA isolation kit of Promega. The D1D2 region were amplified by D1D2 primer NL1 and NL4. The 680 bp amplified DNA were confirmed by electrophoresis in 1.0% agarose gel (Figure.16). The amplified products were sent for sequencing. CDBT1 to 8 were sequenced by Poula Gunclave's laboratory, Nova University, Poutugal. CDBT9 to 12 were sequenced in Excelris laboratory, Ahamdabad, India. The sequences were edited by BioEdit software (Hall, 1999) and analyzed by NCBI blast. Out of twelve yeasts, ten of them were *Saccharomyces cerevisiae* and CDBT7 and CDBT8 were *Wikerhamomyces anomalus* and *Cyberlindnera fabianii* respectively (Table 17). A phylogenetic tree was developed to see the relatedness between the yeasts (Fig.17) using MEGA6 software.

Figure 16: D1D2 Primer PCR Products of Yeast Isolates

Table 17: Molecular Characterization of isolates

S. No.	Isolate Naming	Isolate Identified as
1	CDBT1	*Sacharomyces cerevisiae*
2	CDBT2	*S. cerevisiae*
3	CDBT3	*S. cerevisiae*
4	CDBT4	*S. cerevisiae*
5	CDBT5	*S. cerevisiae*
6	CDBT6	*S. cerevisiae*
7	CDBT7	*Wickerhamomyces anomalus*
8	CDBT8	*Cyberlindnera fabianii*
9	CDBT9	*S. cerevisiae*
10	CDBT10	*S. cerevisiae*
11	CDBT11	*S. cerevisiae*
12	CDBT12	*S. cerevisiae*

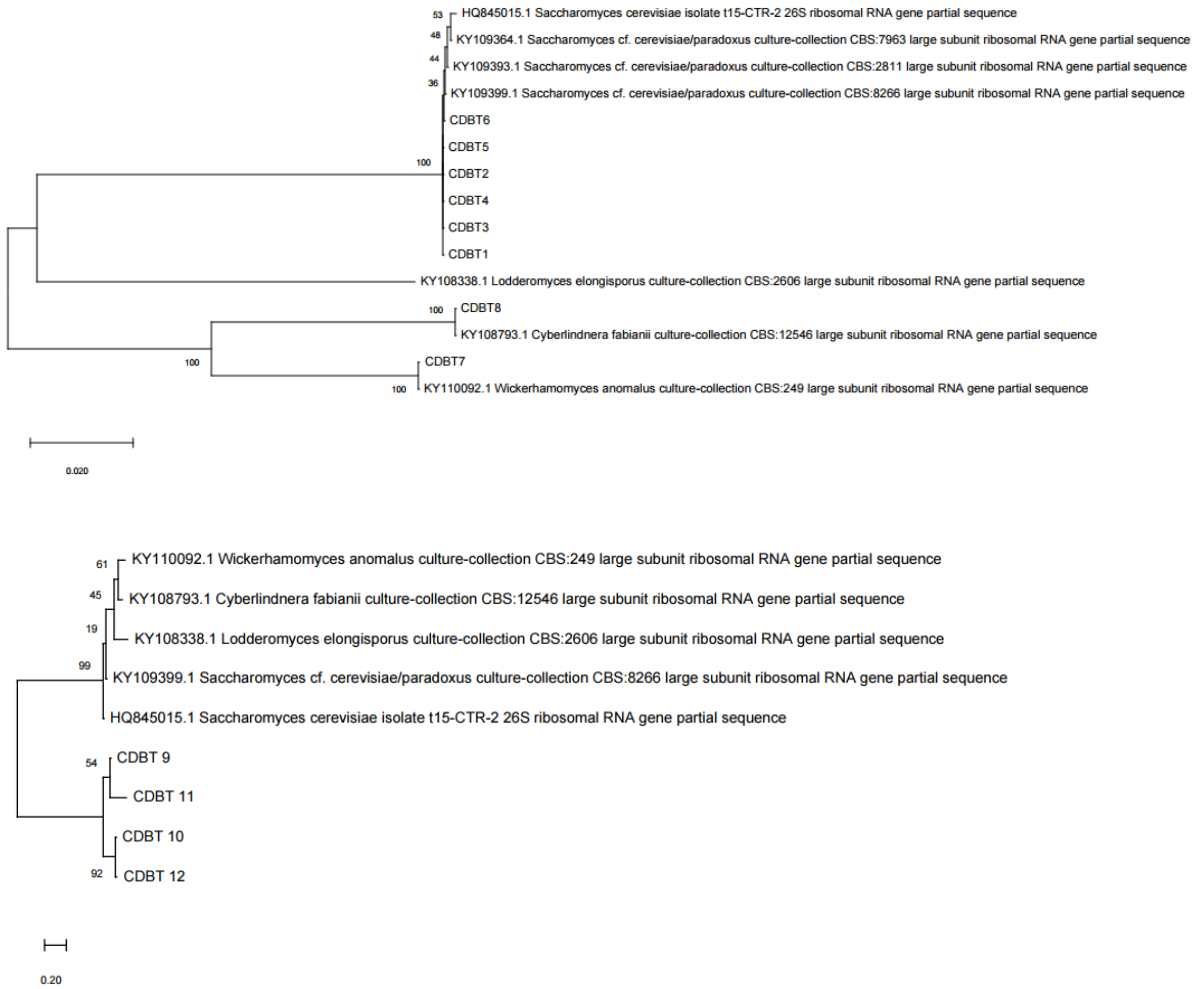

Figure 17: Phylogenetic tree based on sequences of the D1/D2 region of the rDNA 26S gene. The tree shows the position of CDBT isolates with respect to closely related yeast species. The tree was constructed based on the genetic distances obtained according to MEGA6 using the neighbor-joining method.

4.5 Optimization of Fermentation Condition for Ethanol Production

Yeast strains *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalus* (CDBT7) were selected amongst the twelve isolates. CDBT2 was best ethanol producer and ethanol tolerant strain of *S. cerevisiae* (Joshi et al., 2014a) and CDBT7 was xylose utilizer, ethanol producer and ethanol tolerant strain (Table 16, Figure 15). The combination of these two yeast were used for best utilization of glucose and xylose produced from lignocellulosic biomass hydrolysate to convert into value added product ie; ethanol (Joshi et al., 2019b; Mamman et al., 2008). Use of yeast *Wickerhamomyces anomalus* helps to utilize xylose and produce ethanol (Zha et al., 2013). *Wickerhamomyces anomalus* has ethanol production ability comparable to *S.*

cerevisiae despite of long fermentation time needed (Domizio et al., 2011; Ruyters et al., 2015). Besides, it has antifungal activities (Oro et al., 2018; Coda et al., 2011), glucosidase, arabinofuranosidase and xylosidase activities (Sabel et al., 2014). It is found to survive in extreme environmental condition, show high ethanol tolerance (12.5%) and high sugar tolerance (Walker, 2011; Lanciotti et al., 1998). Accession no. of CDBT2 and CDBT7 assigned by NCBI were MK910215 and MK910216 respectively .

4.5.1 Optimization of Ethanol Production from CDBT2

For optimization, PYN media supplemented with 5% glucose was used as fermentation media (Tony et al., 1989). Only 5% of glucose used here during optimization, as the concentration of reducing sugars obtained from lignocellulosic biomass was less than 5% ie 3.3% (Joshi et al., 2018). When the ethanol production efficiencies were studied by adjusting pH 3.5 to 7 (at the interval of 0.5 pH, Figure 18a), maximum amount of ethanol was produced at pH 5.5 in 3.5 days (12.4 ± 0.0 $mg \cdot mL^{-1}$). Optimized temperature for ethanol production was determined by adjusting 24 to 32^oC temperature at the interval of 2^oC (Figure 18b) and 30^oC was found to be the most favorable temperature for ethanol production (12.5 ± 0.02 $mg \cdot mL^{-1}$ produced in 3 days). Among the different possible nitrogen sources in medium, the supply of ammonium sulphate was found to be best for ethanol production followed by peptone and yeast extract with 12.4 ± 0.03 $mg \cdot mL^{-1}$, 11.50 ± 0.14 $mg \cdot mL^{-1}$ and 9.52 ± 0.04 $mg \cdot mL^{-1}$ ethanol production respectively (Figure 18c). Thus the optimized condition of CDBT2 growth for maximum ethanol production was found pH 5.5, temperature 30^oC, ammonium sulphate as main source of nitrogen supplement and growth period of 3 days.

4.5.2 Optimization of Ethanol Production from CDBT7

For optimization of ethanol production from CDBT7, pH of the media were adjusted to 3.5 to 7 (at the interval of 0.5 pH), it was found that maximum amount of ethanol was produced at pH 5.0, after 4 days (10.5 ± 0.09 $mg \cdot mL^{-1}$). Within 3 days, only 9.67 ± 0.52 and 9.40 ± 0.5 $mg \cdot mL^{-1}$ ethanol were produced at 5.0 and 5.5 pH respectively (Figure 19a). Similarly, when media temperature were adjusted at 24 to 32^oC (at the interval of 2^oC), 30^oC was found to be most favorable for ethanol production with

production efficiency of 10.5 ± 0.09 mg·mL^{-1} in 4 days (Figure 19b). Among the different possible nitrogen sources (Figure 19c), the supply of ammonium sulphate (10.1 ± 0.17 mg·mL^{-1} in 3rd day) was found to be best in terms of ethanol production followed by peptone and yeast extract. Thus the optimized condition of CDBT7 growth for ethanol production was found pH 5.0, temperature 30^{o}C and ammonium sulphate as main nitrogen source. Maximum ethanol production reached on 4th day.

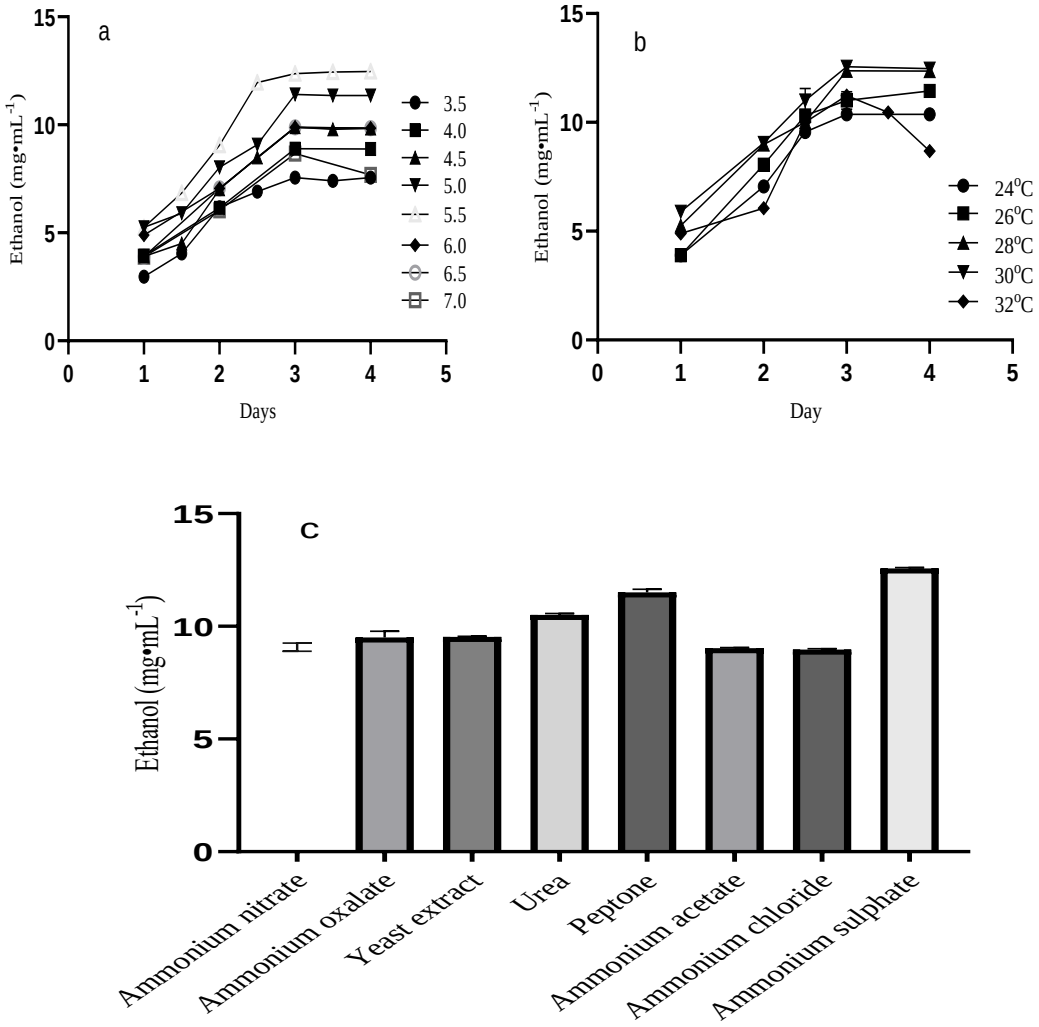

Figure 18: Ethanol production by strain CDBT2 grown in PYN media. Panel a: Effect of pH at 28^{o}C. Panel b: Effect of temperature at 5.5 pH. Panel c: Effect of nitrogen supplements at pH 5.5 and 30^{o}C. The data reported is average of three independent experiments.

4.6 Optimization of Ethanol Production in Electrochemical Cell

An electrochemical cell was optimized for ethanol production by varying different factors. Ethanol was allowed to produce at optimized condition of pH 5.5, 30^{o}C

temperature and ammonium sulphate, peptone and yeast extract as nitrogen source with electric supply of 4V (Jeon & Park, 2010).

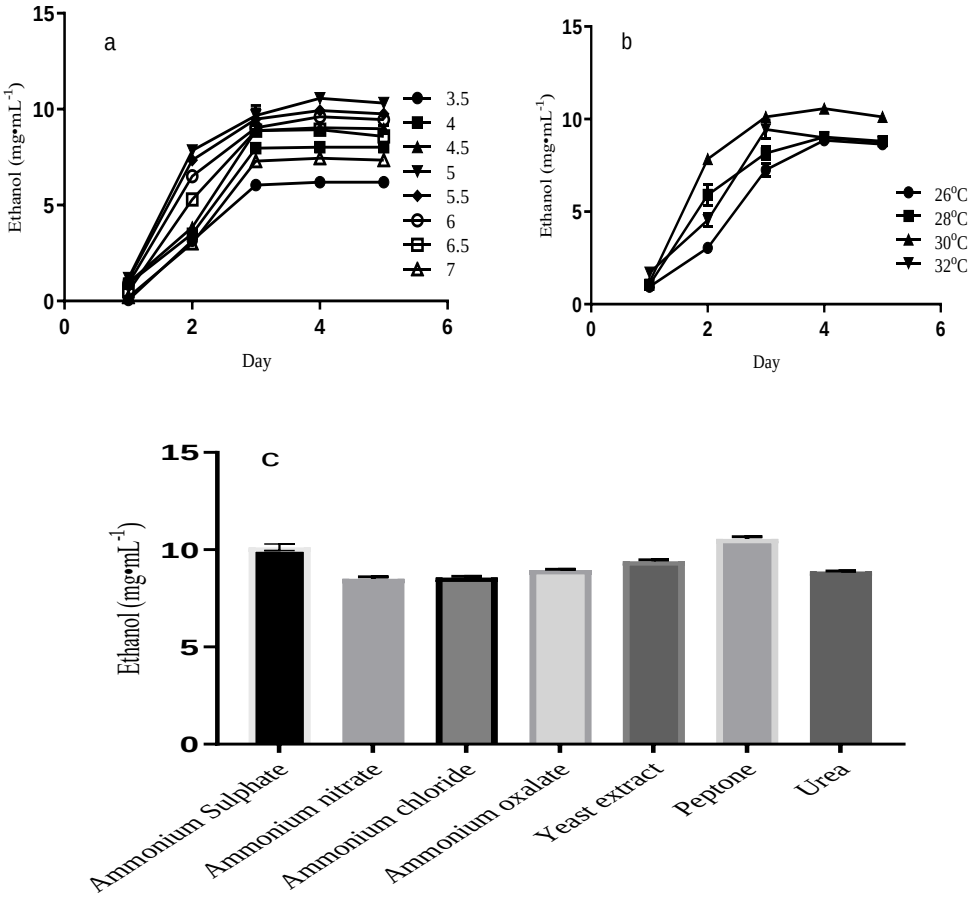

Figure 19: Ethanol production by strain CDBT 7 grown in PYN media. Panel a: Effect of pH at 28°C. Panel b: Effect of temperature at 5.5 pH. Panel c: Different nitrogen supplements at pH 5 and 30°C. The data is average of three independent experiments.

4.6.1 Different combination of CDBT2 and CDBT7 in electrochemical cell

CDBT2 and CDBT7 strains were grown in cathodic and anodic chambers in various combinations. In one experiment CDBT2 and CDBT7 were cultured in anodic and cathodic compartments respectively, then in another experiment they were cultured in reversed compartments. Though pH 5.0 was optimum for CDBT7, pH 5.5 was used in this study, as the effect on ethanol production by CDBT7 was not significantly different in those pH ranges. Out of two combinations, ethanol production was better when CDBT2 cultured in anodic compartment and CDBT7 cultured in cathodic compartment (in average 13.8 ± 0.77 mg·mL^{-1} on 3rd day) than the reversed

combination (in average 11.8 ± 0.71 mg·mL^{-1} on 3rd day). It was observed that time required for optimum production of ethanol for CDBT7 was reduced from 4 days to 3.5 days. However optimized condition for the combination of CDBT2 and CDBT7 culture was taken at 3 days as the amount of ethanol increased in between 3 to 3.5 days for CDBT7 was very less and for CDBT2 ethanol production was optimum at 3 days. (Figure 20a, 20b; Table 18).

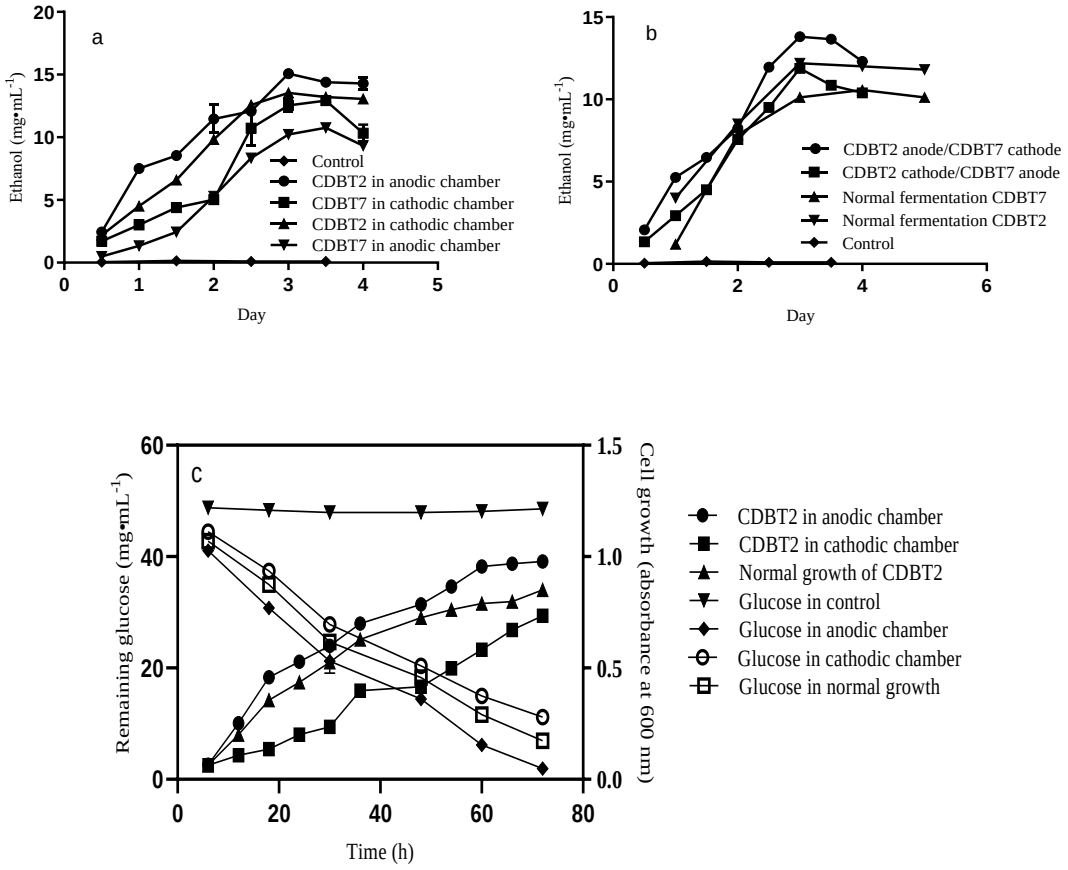

Figure 20: Ethanol production in Electrochemical cell (ECC) by CDBT2/CDBT7 vice versa. Panel a: Individual ethanol production in cathodic and anodic chamber. Panel b: Average of ethanol production in both chambers. Panel c: Cell growth pattern and remaining glucose concentration when CDBT2 cultured in anodic and cathodic chambers.

4.6.2 Ethanol Production in Cathodic Compartment

Both CDBT2 and CDBT7 cultured in cathodic compartment with 4V supply yielded more ethanol than normal fermentation without voltage supply with the enhancement of 7.14 ± 0.64% and 23.7 ± 0.51% respectively (Table 18, Figure 20). CDBT7 showed more % enhancement than CDBT2. This may be because CDBT7 (*W. anomalus)*, had formed film in graphite cathode so that electron was transported

through cathode easily resulting in more ethanol production. The scanning electron micrograph of graphite electrode showed the formation of film by CDBT7, where as CDBT2 when cultured in graphite cathode did not found to form film (Figure 21).

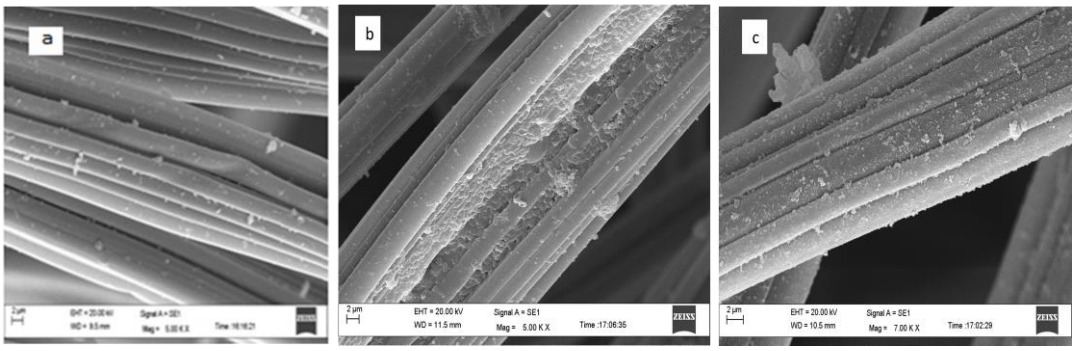

Figure 21: Scanning electron microscopic images of graphite felt electrodes. Panel a: Plane graphite felt. Panel b: Graphite felt cultured with CDBT7 in cathode. Panel c: Graphite felt cultured with CDBT2 in cathodic chamber.

Du & Pretorius, (2000) also reported the film formation by *W. anomalus* in broth culture. The voltage supply enhanced redox potential of film enhancing more electron transport (Mohamoud, 2014). Mohamoud, 2014 reported 40% enhancement in film redox potential when electricity was passed to electrode with polyaniline/ polyvinyl composite. Canelas et al. (2008) observed that ethanol formation requires the maintenance of NAD^+/NADH ratios. In healthy eukaryotes, usually NAD^+/NADH ratios in cytosol are relatively high and the range varies widely (60-700). Canelas et al. (2008) also observed that the cytosolic free NAD^+/NADH ratio in *S. cerevisiae*, under steady and highly dynamic state ranges between 101 ± 14 and 320 ± 45 respectivly, where as whole cell NAD^+/NADH ratio was 7.5 ± 2.5. Further it was observed that, in *S. cerevisiae* NAD^+/NADH ratio was reduced when there is the presence of electron donor and the ratio was increased in presence of electron acceptor. In our case when yeast strain was cultured in cathodic compartment, there was increment in ethanol production because the cathode was the electron donor, thus resulting more conversion of NAD^+ to NADH which had directed the conversion of pyruvate to ethanol. In spite of the ratios reported above, eukaryotes could survive even when the NAD^+/NADH reached up to 7-10 (Veech et al., 1972). According to Gunawardena et al. (2008), NAD^+ is mostly available in cytosol. Voltage polarizes ions present in cytosol and positively charged ions (NAD^+) migrate toward cell membrane making the transfer of electron from cathode to attached cell easy and fast

(Ann & Neil, 1986) thus converting more NAD^+ to NADH. The pyruvate formed by glycolysis has fate of converting into different products. During fermentation pyruvate is first decarboxylated to acetaldehyde. The increasing level of NADH favors more production of ethanol in yeast by activating alcohol dehydrogenase to convert acetaldehyde to ethanol by consumption of NADH. It is also reported that increasing NADH inhibit pyruvate dehydrogenase complex (Harris et al., 2002) present in mitochondrial matrix, hence conversion of pyruvate to acetyl coenzyme A, the reaction of TCA cycle, will be inhibited (Figure 22). It favors the ethanol production in cathodic compartment. The reduction in growth of CDBT2 seen in cathodic compartment (Figure 20c) should be also due to the increase in anaerobic fermentation and reduction in aerobic metabolism.

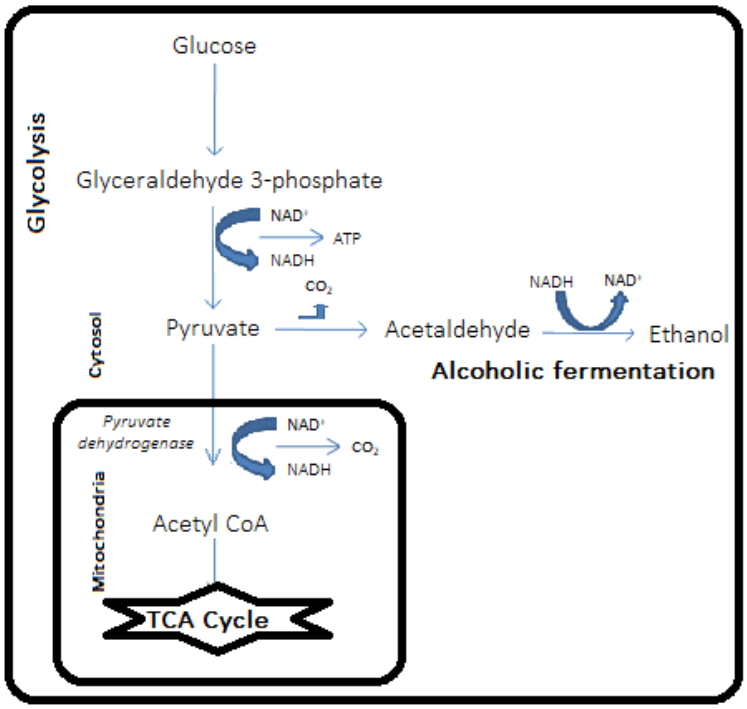

Figure 22: Roll of NADH and NAD^+ in ethanol fermentation and aerobic metabolism.

4.6.3 Ethanol Production in Anodic Compartment

The fermentation efficiency of both CDBT2 and CDBT7 when cultured in anodic compartment were enhanced than in normal fermentation by 19.8 ± 0.50% and 1.00 ± 0.14% respectively (Table 18). The enhancement in ethanol production in anodic compartment may be due to the fast and enhanced growth of yeast cells as observed in Figure 20c. The enhanced growth in cell number may be due to the supplied voltage in ECC. Canelas et al. (2008) observed that, in *S. cerevisiae* NAD^+/NADH

ratio was decreased in presence of electron acceptor. When yeast strain were cultured in anodic compartment which is electron acceptor, converted NADH to NAD^+ which had directed fast conversion of glucose to pyruvate so that we could see enhancement in growth and ethanol formation as well as pyruvate could be utilized in both cases (Figure 22). Further, Mathew et al. (2015) found the increase in cell number as voltage supply increased up to 15V. According to Song et al. (2014) voltage changes glucose metabolism in *S. cerevisiae* under both aerobic and anaerobic condition. The supplied voltage polarizes ionic charges in yeast cells, which lowers the tunnel barrier for transferring electrons and increase glucose oxidation (Yau et al., 2013). In our case, about 63.84% of the total consumed glucose was utilized for ethanol production when CDBT2 cultured in anodic compartment, whereas only 57.16% was utilized during normal fermentation (Figure 20). The increment in glucose utilization may be for enhanced cell growth and ethanol fermentation. CDBT7 cultured in anodic compartment showed very less enhancement in ethanol production (Table 18) compared to CDBT2. This may be because of the slow growth and slow fermentation of CDBT7 (Domizio et al., 2011; Ruyters et al., 2015).

Table 18: Ethanol produced by CDBT2 and CDBT7 cultured in different compartments in two sets[#]

S. No.	CDBT2 in AC	CDBT7 in CC	CDBT2 CC	CDBT7 in AC	CDBT2*	CDBT7*
Ethanol ($mg \cdot mL^{-1}$)	15.1 ± 0.28	12.5 ± 0.49	13.5 ± 0.0	10.2 ± 0.0	12.6 ± 0.42	10.1 ± 0.14
% increment	19.8 ± 0.50	23.7 ± 0.51	7.14 ± 0.64	1.00 ± 0.14		

AC= Anodic chamber; CC= Cathodic chamber

[#] Set 1: 2nd and 3rd column (CDBT2 in AC and CDBT7 in CC); Set 2: 4th and 5th column (CDBT2 in CC and CDBT7 in AC)

*Fermentation under normal conditions (no external source of electricity).

4.6.4 Voltage Optimization of ECC

When 0 to 5V (± 0.1V) of energy was supplied externally to the CDBT2 in anodic and CDBT7 in cathodic compartment, 4V (± 0.1V) supply gave the highest amount of ethanol (Figure 23a). Ethanol produced in anodic and cathodic compartments were 15.1 ± 0.28 and 12.5 ± 0.49 $mg \cdot mL^{-1}$ at 4V supply in 3 days. For reduction of NAD^+/NADH, voltage requirement is -330 mV (Veech et al, 1972). Our experiment consumed 4V to show optimal ethanol production (Joshi et al., 2014b). The requirement of such huge amount of voltage may be because of ohmic drop exerted

by the designed ECC. Mathew et al. (2015) reported the gradual increment in growth of *S. cerevisiae* till 15V supply. In our case increasing voltage beyond 4V reduced ethanol production. Beyond 4V, there may be high chance of water oxidation (beyond 1.23V; Atkins, 1998) and possibility of conversion of Platinum to Platinum dioxide (PtO_2), an insulating oxide which hindered the electrode activity reducing transfer of electrons (Wang et al., 2006)) at the anode. In other end, in cathode at such high voltage (>4V) reduction of H^+ to H_2 gas at the surface of cathode which had inhibited cathodic activity (Hsu et al., 2008) Ethanol production was not seen in ECC without microbial inoculation (Figure 23b).

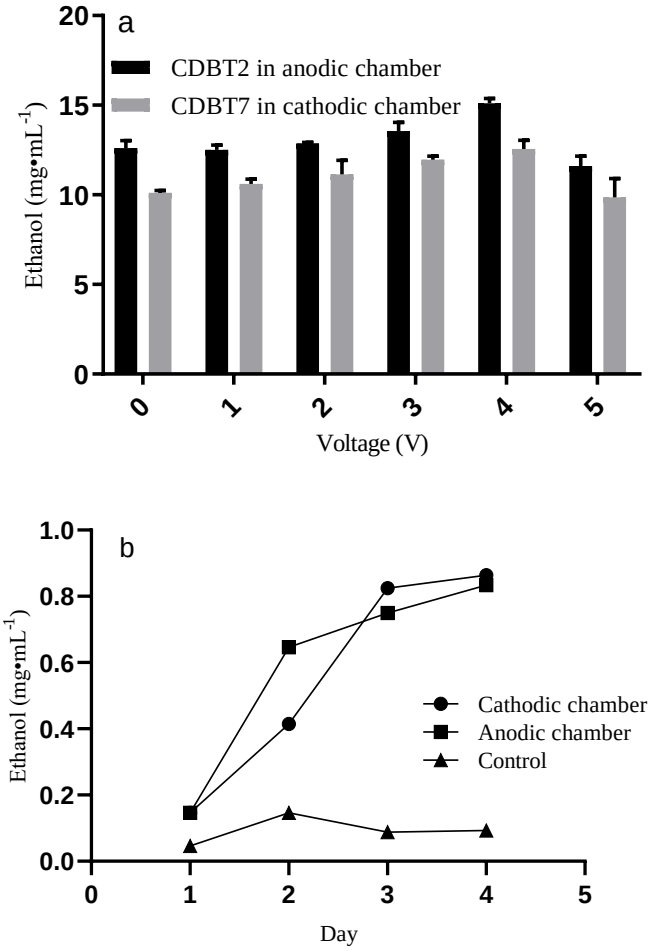

Figure 23: Ethanol production in Electrochemical cell (ECC) by CDBT2/CDBT7. Panel a: Optimization of ethanol production with the supply of external voltage. Panel b: Ethanol production in ECC without yeast culture [Control: ECC w/o culture and w/o voltage supply]

4.7 Comparative Ethanol Production by Different *S. cerevisiae* Strains in Anodic Compartment against CDBT7 in Cathodic Compartment

The efficiency of ethanol production of different available *S. cerevisiae* strains in anodic chamber and CDBT7 in cathodic compartment were studied (Figure 24a).

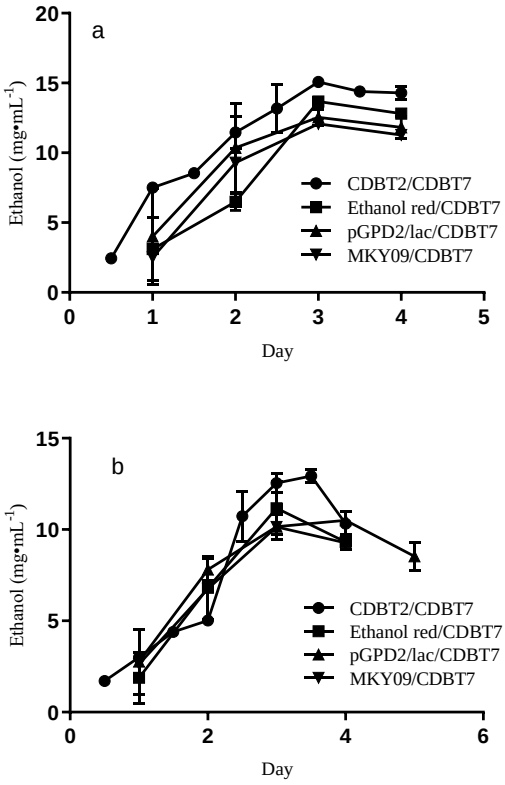

Figure 24: Comparative ethanol production by different S. cerevisiae strains cultured in anodic compartment of Electrochemical cell against CDBT7 cultured in cathodic compartment. Panel a: Ethanol production in anodic compartment. Panel b: Ethanol production in cathodic compartment.

Ethanol was produce by culturing *S. cerevisiae* strains, CDBT2, Ethanol red, MKY09, pGPD2/lac in anodic and CDBT7 in cathodic compartment. The data revealed that CDBT2 produced highest amount of ethanol (15.1 ± 0.31 $mg \cdot mL^{-1}$, Figure 24a) than other *S. cerevisiae* including Ethanol red (13.6 ± 0.20 $mg \cdot mL^{-1}$). Similarly, it also showed that lacase gene expression in yeast cell by genetic modification didn't affect ethanol production in electrochemical cell. The ethanol productions by MKY09 and pGPD2/lac were almost same, 12.0 ± 0.0; 12.5 ± 0.55 $mg \cdot mL^{-1}$ respectively. CDBT2 produced 11.0 ± 0.36% more ethanol than the industrial strain, Ethanol Red. Thus the strain CDBT2 can be a potent industrial strain for genetic modification and ethanol production. However, the ethanol production of

CDBT7 in cathodic compartment did not changed significantly (Figure 24b) when different yeast strains were used in anodic chamber.

4.8 Effect of different Electron Transporters

4.8.1 Effect of Yeast Cultures

To determine the effect of yeast cultures on electron transport system, yeast strains CDBT2 and CDBT7 were cultured in ECC in the following combinations; (i) CDBT2/CDBT2 in anodic/cathodic compartments; (ii) CDBT2/CDBT7 in anodic and cathodic compartments and (iii) co-culture of CDBT2 and CDBT7 in both compartments (Figure 25a). In combination of CDBT2/CDBT2, CDBT2 produced more ethanol in the anodic compartment (15.5 ± 0.14 $mg \cdot mL^{-1}$) as compared to cathodic compartment (13.4 ± 0.07 $mg \cdot mL^{-1}$). The average ethanol production in this combination was 14.4 ± 0.15 $mg \cdot mL^{-1}$ and the increment in ethanol production was 27.5 ± 0.44%. In combination of CDBT2/CDBT7, CDBT2 strain produced more ethanol in anodic chamber (15.1 ± 0.28 $mg \cdot mL^{-1}$) as compared to CDBT7 strain in cathodic compartment, (12.5 ± 0.49 $mg \cdot mL^{-1}$). The average ethanol production in this combination was 13.8 ± 0.56 $mg \cdot mL^{-1}$ and the increase in ethanol production was 21.5 ± 0.71% (Table 19A). When CDBT2 and CDBT7 strains were co-cultured in both compartments, ethanol production was significantly lower as compared to individual cultures of CDBT2 and CDBT7 in anodic and cathodic compartments (14.4 ± 0.90%). This may be because, the growth of *W. anomalus* may suppressed the growth of *S. cerevisiae* (Ruyters et al., 2015), when cultured together, thus decreased the overall ethanol production. A slight enhancement in ethanol production was observed than normal fermentation (3.96 ± 0.83%).

4.8.2 Effect of Immoblization of Yeast

In ECC without voltage supply, CDBT7 immobilized in presence of calcium alginate on graphite cathode was used in cathodic compartment with CDBT2 on anodic compartment produced 4.95 ± 0.07 $mg \cdot mL^{-1}$ and 12.6 ± 0.42 $mg \cdot mL^{-1}$ ethanol in respective compartments. There was a significant decrease in ethanol production (Figure 25b) compared to normal fermentation without voltage supply (22.7 ± 0.60%). This may be because immobilization reduces substrate diffusion thus reducing ethanol production. The same system when used in an ECC with the

application external voltage input (4V), the ethanol production was significantly enhanced (35.6 ± 0.81%) than normal immobilized culture without voltage supply.

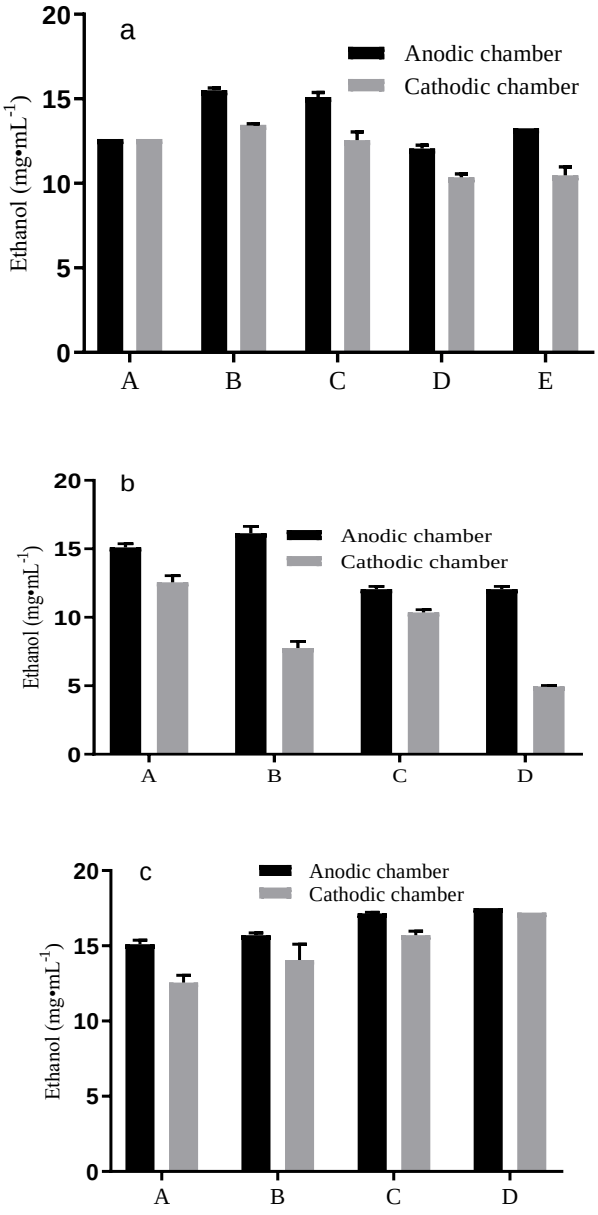

Figure 25: Ethanol production with different electron transport enhancement materials. Panel a: Use of yeasts in various combinations in ECC [A:. CDBT2 normal fermentation; B: CDBT2 / CDBT2 in Electrochemical cell; C: CDBT2/CDBT7 in Electrochemical cell, D: CDBT2 /CDBT7 normal fermentation, E: CDBT2/CDBT7 co culture]. Panel b: Use of graphite electrode immobilized with CDBT7 yeast strain [A: Graphite cathode/Pt anode; B: Graphite cathode (immoblized with CDBT7)/Pt anode; C: Normal fermentation CDBT2/CDBT7; D: Normal fermentation CDBT2/CDBT7 immoblized in graphite]. Panel c: Use of electrodes coated with different electron transporters [A: Normal Platinum anode/graphite cathode; B: Platinum coated Platinum anode/graphite anode; C: Neutral red coated graphite cathode/ Platinum anode; D: Neutral red coated cathode/ Pt coated anode].

The net increment in ethanol production was only 5.3 ± 0.82% than that of normal fermentation of individual culture without immoblization. The average ethanol production was 11.9 ± 0.70 $mg \cdot mL^{-1}$ (Table 19B). The increase in ethanol production in ECC with voltage input may be because immobilization of cells in the form of film enhanced electron transport in electrode (Mohamoud, 2014). However the net production of ethanol was less than that of free cell culture with CDBT7 in cathodic compartment and CDBT2 in anodic compartment (13.7 ± 0.89% less).

4.8.3 Effect of Electron Transport Enhancing Materials

Coating of platinum electrode anode with fine particles (nanoparticles) of platinum using hexachloroplatinate (IV) and cathode with plane graphite electrode resulted 7.24 ± 1.20% increment in ethanol production. When neutral red was coated in graphite cathode and culturing CDBT2 strain in anodic compartment resulted further 10.8 ± 1.11% increament in ethanol production. When both the coated electrode were used together, there was further enhancement in ethanol production by 5.41 ± 0.30%. The net increment in ethanol production was 15.8 ± 0.28% and 37.6 ± 0.49% in anodic and cathodic compartments respectively than uncoated electrodes. (Figure 25c). The ethanol productions were 17.5 ± 0.01 $mg \cdot mL^{-1}$ in anodic compartment and 17.2 ± 0.01 $mg \cdot mL^{-1}$ cathodic compartment with net increment of 52.8 ± 0.44% than normal fermentation (Table 19C). He et al. (2016) obtained 60.3% enhancement in butanol production by *Clostridium spp*. when neutral red was used as electron transporter. The paper demonstrated that neutral red increased the butanol production better than other electron transporters like viologen dyes. Further neutral red can strongly bind to cell membranes (Park et al., 1999). Neutral red has a redox potential of about -325 mV which is similar to that of NADH redox potential (-320 mV). Neutral red can reduce NAD^+ thus increasing the label of $NADH/NAD^+$ giving rise to more ethanol production (Park et al., 1999).

Table 20 showed the percentage conversion of glucose to ethanol. Conversion of glucose to ethanol was more when voltage was supplied to ECC. Further enhancement was seen with the use of better electron transport system. Conversion percentage was more in cathodic compartment than in anodic compartment.

Table 19: Changes in ethanol production with implementation of different system for electron transport enhancement: Summary

S. N.	Culture types*	Ethanol in Anodic Chamber ($mg \cdot mL^{-1}$)	Ethanol Cathodic Chamber ($mg \cdot mL^{-1}$)	Average Ethanol ($mg \cdot mL^{-1}$)	Average Increase (%)
A	*Yeast Combinations*				
1	CDBT2 / CDBT7 Normal Fermentation with no Applied Voltage	12.6 ± 0.42	10.1 ± 0.14	11.3 ± 0.44	
2	CDBT2 / CDBT7 in ECC with 4V Applied Voltage (Pt/CDBT2(5% glucose)//(5% glucose)CDBT7/C)	15.1 ± 0.28	12.5 ± 0.49	13.8 ± 0.56	21.5 ± 0.71
3	CDBT2 / CDBT2 in ECC with 4V Applied Voltage (Pt/CDBT2(5% glucose)//(5% glucose)CDBT2/C)	15.5 ± 0.14	13.4 ± 0.07	14.4 ± 0.15	27.5 ± 0.44
4	CDBT2+CDBT7 Mixed/Co-Culture (Pt/CDBT2,7(5% glucose)//(5% glucose)CDBT2,7/C)	13.2 ± 0.0	10.4 ± 0.71	11.8 ± 0.71	3.96 ± 0.83
B	*Immoblized Yeast*				
1	CDBT2 / CDBT7 with CDBT7 Immobilized on Graphite Cathode without Applied Voltage	12.6 ± 0.42	4.95 ± 0.07	8.77 ± 0.42	
2	CDBT2 / CDBT7 with CDBT7 Immobilized on Graphite Cathode and 4V Applied Voltage (Pt/CDBT2(5% glucose)//(5% glucose)CDBT7immoblized in cathod/C)	16.1 ± 0.49	7.75 ± 0.50	11.9 ± 0.70	5.3 ± 0.82
C	*Electron Transport Systems*				
1	CDBT2 / CDBT7 Pt Coated Pt Anode/Graphite Cathode (Pt, nano coated/CDBT2(5% glucose)//(5% glucose)CDBT7/C)	15.7 ± 0.16	14.0 ± 1.06	14.8 ± 1.07	30.9 ± 1.15
2	CDBT2 / CDBT7 Pt Anode and Neutral Red Coated Cathode (Pt/CDBT2(5% glucose)//(5% glucose)CDBT7/C, neutral red coated)	17.1 ± 0.07	15.7 ± 0.30	16.4 ± 0.30	44.6 ± 0.53
3	CDBT2 / CDBT7 Pt Coated Pt Anode/Neutral Red Coated Cathode (Pt, nano coated/CDBT2(5% glucose)//(5% glucose)CDBT7/C, neutral red coated)	17.5 ± 0.01	17.2 ± 0.01	17.3 ± 0.01	52.8 ± 0.44

* Normal fermentation: Yeast cultured in ECC without external voltage supply. The yeast strain listed first was used in the anodic compartment and the organism listed second was cultured in the cathodic compartment. Average increase was based on normal fermentation without voltage supply.

Table 20: Percentage conversion of glucose to ethanol by CDBT2 and CDBT7

S. N.	Conditions	CDBT2 in AC	CDBT7 in CC
1	Normal fermentation*	59.2 ± 0.42	47.4 ± 0.14
2	Fermentation with external supply of 4V	63.3 ± 0.32	65.4 ± 0.70
3	Fermentation with 4V along with Platinum and neutral red coated anode and cathode	71.0 ± 0.50	77.2 ± 0.01

AC= Anodic chamber; CC= Cathodic chamber

*Fermentation under normal conditions (no external source of electricity).

4.9 Electrochemical Ethanol Production from *S. Spontaneum* Hydrolysate

Ethanol was allowed to produce in ECC in optimized condition. Platinum coated platinum anode and neutral red coated graphite cathode were used as electrodes. CDBT2 and CDBT7 were cultured in anodic and cathodic compartments. *S. spontaneum* hydrolysate supplemented with PYN media was used as fermentation media (Figure 26) [the cell representation abbreviated as: Pt(Pt coated)/CDBT2, *S. spontanum* hydrolysate // CDBT7, *S. spontanum* hydrolysate/C (Neutral red coated)]. There was drastic enhancement in ethanol production by 60.8 ± 0.10% in anodic and 63.0 ± 0.07% in cathodic compartments with total enhancement of 61.9 ± 0.12% (Table 21). The enhancement was higher than that in PYN media supplemented with glucose. This may be because; the NaCl produced during neutralization after preteatment might be responsible for enhancement in ethanol as NaCl decrease resistance for electron transfer. According to Yang et al., (2014), when the concentrations of NaCl were increased from 5 to 30 $g \cdot L^{-1}$ in media, the internal resistances of the system found to decrease from 2432.0 to 2328.4Ω (Yang et al., 2014). Similarly, Kamcev et al. (2018) also showed the increase in electrical conductivity as salt concentration increased, hence increasing electron flow. External voltage supply found to decrease the time required for ethanol fermentation. There is regular increase in ethanol production in both cathodic and anodic compartment. The more increment in ethanol production in cathodic compartment than anodic compartment revealed that glucose and xylose are used continuously by CDBT7 strain.

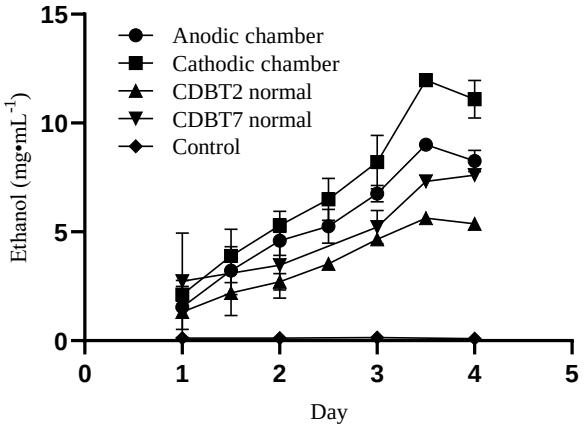

Figure 26: Ethanol production from *S. spontaneum* hydrolysate (hot water pretreatment for 2 h followed by 0.5M acid hydrolysis) in ECC after 3.5 days culture [Control: ECC without yeast culture and without external voltage supply. Normal: ECC with yeast culture and without external voltage supply].

4.10 Effect of cellulose acetate membrane in place of proton exchange membrane

Figure 27 showed the ethanol production when nafion membrane was replaced by cellulose acetate membrane. There was slight increament in ethanol production (6.30 ± 0.22%) when cellulose acetate was used in place of nafion membrane. The reason might be because cellulose acetate is nonspecific membrane and nafion membrane is proton specific membrane. Cellulose acetate membrane allowed unutilized xylose to transport from anodic chamber to cathodic chamber. CDBT7 utilized xylose and glucose both in cathodic chamber producing more ethanol than CDBT2 when hydrolysate was used as substrate where as reverse case was observed when sole glucose was used as substrate. CDBT7 strain might be with less carbon catabolite repression (CCR) so that it could utilize both glucose and xylose simultaneously with enhanced production of ethanol. According to Gao et al. (2019), future effort for efficient utilization of lignocellulosic biomass hydrolysate for ethanol production should focus on integrating the capacities of transporting and converting major sugars available in biomass hydrolysate in CCR free manner. One of the strategy for the successful utilization of this is the isolation of more and more xylose utilizing yeast which has xylose metabolism with less CCR effect. Recently *Spathaspora passalidarum* strain was isolated which could convert xylose to ethanol with minimal glucose repression (Rodrussamee et al., 2018). It would be better if CDBT7 is with

less CCR effect. Additionally the enhancement in ethanol production might be partially because of the slight decrease in resistance of cellulose acetate membrane to transport ions than nafion membrane ie; 263 and 267 Ω respectively (Tang et al., 2010).

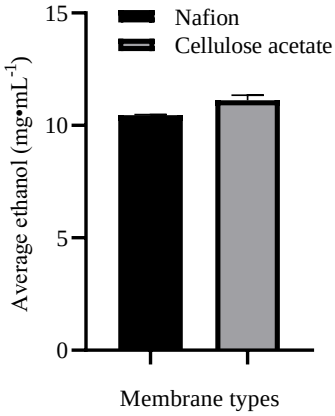

Figure 27: Comparison of ethanol production by nafion and cellulose acetate membrane with *S. spontaneum* hydrolysate (hot water pretreatment for 2 h followed by 0.5M acid hydrolysis) in ECC after 3.5 days culture.

Table 13: Ethanol produced by CDBT2 and CDBT7 cultured with *S. spontaneum* biomass hydrolysate[#]

S. No.	CDBT2 in AC	CDBT7 in CC	Average Production	CDBT2*	CDBT7*
Ethanol ($mg \cdot mL^{-1}$)	9.01 ± 0.1	11.9 ± 0.05	10.4 ± 0.11	5.60 ± 0.03	7.30 ± 0.06
% Increase	60.8 ± 0.10	63.0 ± 0.07	61.9 ± 0.12		

AC= Anodic chamber; CC= Cathodic chamber
[#]Pt, nano coated/CDBT2(biomass hydrolysate)//(biomass hydrolysate)CDBT7/C, neutral red coated
*Fermentation under normal conditions (no external source of electricity).

4.11 Alcohol Dehydrogenase and Pyruvate Decarboxylase Expression Analysis in Normal and Electrochemically Enhanced Fermentation by RT-PCR

RNA were isolated from the normally grown CDBT2 and CDBT2 grown in electrochemical cell using Zymo kit. Zymo kit produces highly-concentrated, DNA-free RNA that is suitable for RT-PCR, hybridization, sequencing etc as written in kit. Isolated RNA was confirmed by running RNA in 1% agarose gel (Figure 28a, 28b). Immediately after RNA isolation, cDNA was prepared using SuperScript III Reverse Transcriptase Kit (Invitrogen, Waltham, USA). Confirmative analysis of the

transcribed RNA was done in 1% agarose gel which showed smearing of cDNA (Figure 28c). Real time qPCR was done to quantify the relative expression of genes Pyruvate Decarboxylase (PDC1) and Alcohol Dehydrogenase (ADH1) in normal and

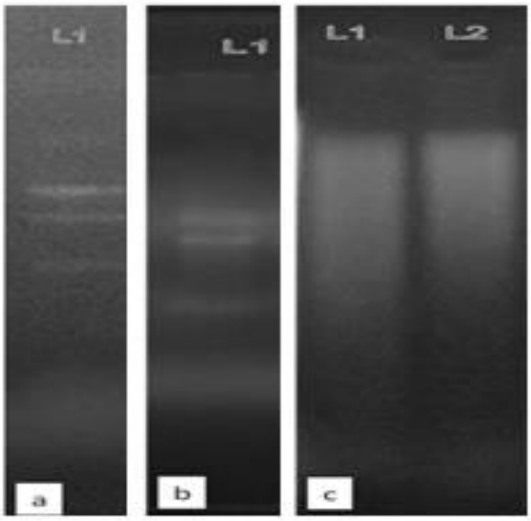

Figure 28: RNA analysis in yeast strains and cDNA preparation. Panel a: RNA isolated from yeast culture (normal growth). Panel b: RNA isolated from yeast culture (electrochemically enhanced). Panel c: cDNA prepared immediately after RNA isolation and confirmative analysis of the transcribed RNA on 1% agarose gels.

electrochemically enhanced yeasts. Expression levels of PDC1 and ADH1 were analyzed taking same amount of template for both reference or housekeeping gene (TFC1) and test genes. TFC1 gene was taken as reference. TFC1 is the subunit of RNA polymerase III transcription initiation factor complex; one of six subunits of the RNA polymerase III transcription initiation factor complex which is very essential in cell. Its location is chromosome II (484742--- 486691). PDC1 is the major of three pyruvate decarboxylase isozymes ie the key enzyme in alcoholic fermentation which decarboxylates pyruvate to acetaldehyde; which is also found to involve in amino acid catabolism. It has highest rate of decarboxylation amongst. It is located in Chromosome XII (232390 --- 234081) in *S. cerevisiae* (Kellermann et al., 1986). ADH1 is one of the main fermentative isozyme required for the reduction of acetaldehyde to ethanol in yeast out of four isozymes (ADH 1, 2, 3 and 5), the last step in the glycolytic pathway. It is located in Chromozome XV (159548 --- 160594). ADH1 has a paralog, ADH5, which arose from the whole genome duplication. Relative expression of PDC1 & ADH1 was then calculated comparing the expression of TFC1 gene as reference using the formula expressed by Lival et al. (2001).

$\Delta C_t = C_t$ (sample) – C_t (Reference)

$\Delta\Delta C_t = \Delta C_t$ (Test sample) – ΔC_t (Control)

Where, C_t is the cycle time calculated as per graph obtained from RT PCR [Appendix 3]. Gene expressed in normal growth of CDBT2 was taken as control and electrochemically grown CDBT2 was taken as test sample. The smooth sharp melting curve [Appendix 4] for CDBT2 indicated that the amplified products were pure in all cases of ADH1, PDC1 and TFC1 genes. The decrease in C_t data obtained in Table 22 clearly revealed high expression of both the genes than in normal condition. The fold of expression of ADH1 and PDC1 in case of CDBT2 were 2.78 ± 0.80 and 1.12 ± 0.37 respectively (Table 23).

Table 22: Average C_t values of ADH1, PDC1 and TFC1 genes obtained from RT PCR.

S. N.	Culture Types	ADH1	PDC1	TFC1
1	CDBT2 (normal growth)	3.35 ± 0.51	3.45 ± 0.10	2.49 ± 0.21
2	CDBT2 (electrochemically enhanced)	1.95 ± 0.26	3.36 ± 0.01	2.57 ± 0.29

Table 23: Increment in expression of ADH1 and PDC1 gene due to voltage supply

S. No.	Yeast strains	Fold expression in ECC	
		ADH1	PDC1
1	CDBT2	2.78 ± 0.80	1.12 ± 0.37

Overall Discussion

Out of four different biomass samples *Ipomoea carnea, Phragmites karka, Saccharum spontaneum* and *Zea mays* (corn) cobs, maximum sugar content was found in *S. spontaneum* with *g*lucose and total reducing sugar (TRS) content 296.8 ± 4.1 and 612.2 ± 11.5 $mg \cdot g^{-1}$ biomass respectively. The TRS reported herein was higher than that of the values reported by Chandel et al. (2009) ie; 539.1 ± 0.55 $mg \cdot g^{-1}$ in *S. spontaneum*.

The study observed two best conditions for TRS release. Firstly, *S. spontaneum* biomass when pretreated at 100^{o}C for 2 h followed by acid hydrolysis for 24 h at 90^{o}C released 330.4 ± 20.5 mg TRS per gram biomass with very less amount of phenol and furfural. Secondly, pretreatment of *S. spontaneum* with ammonium hydroxide (0.5M) for 12 h followed by 10 FPU cellulase hydrolysis at 65^{o}C released 347.2 ± 55.6 mg TRS per gram biomass. Similar results with the release of 350 mg TRS per gram from *S. spontaneum* biomass when pretreated with 0.5% NaOH at 120^{o}C for 2 h and the biomass was hydrolyzed by 10 FPU cellulase (Kataria & Ghosh, 2014). Out of the two processes, first process was not only simple but also economical since no costly enzymes were used and TRS loss due to pretreatment was also minimal (25.4 ± 2.9 $mg \cdot g^{-1}$ biomass).

Further, pretreatment of *S. spontaneum* biomass with hot water at 100^{o}C for 2 h produced only 7.2 ± 0.15 mg phenol and 103.2 µg furfural per gram biomass. This corresponds to 0.72 ± 0.01 $mg \cdot mL^{-1}$ phenol and 10.3 $\mu g \cdot mL^{-1}$ furfural which is very low to show inhibitory effects in fermentation. Hence, the separation of pretreated biomass may not be necessary and the acid hydrolysis can be done directly after pretreatment, to avoid the loss of TRS due to pretreatment.

Out of twelve yeasts isolated, *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalus* (CDBT7) were selected for further study. CDBT2 was the best ethanol producer and ethanol tolerant strain of *S. cerevisiae* isolates and CDBT7 was xylose utilizer, ethanol producer and ethanol tolerant strain. PYN media supplemented with 5% glucose was used for ethanol production. The optimized condition for ethanol production by the strains were pH 5.5, 30^{o}C temperature with the supplementation of ammonium sulphate, peptone, yeast extract as nitrogen source

in the media. At this condition maximum ethanol production were 12.5 ± 0.02 $mg \cdot mL^{-1}$ and 10.5 ± 0.09 $mg \cdot mL^{-1}$ by CDBT2 and CDBT7 strains respectively in 3rd and 4th day. This medium and culture conditions were used for ECC experiments.

In ECC ethanol fermentation was analyzed with the external electric supply of 4V by culturing CDBT2 in anodic chamber and CDBT7 in cathodic chamber. Platinum wire and graphite felt were used as anode and cathode respectively. Anodic and cathodic chambers were separated by nafion membrane. There was enhancement in ethanol production in both anodic and cathodic chambers (7.14 ± 0.64% in anodic and 23.7 ± 0.51% in cathodic chambers) in 3rd day. The enhancement in cathodic chamber may be due to decreasing NAD^+/NADH ratio as cathode are electron donors. Similarly the enhancement in anodic chamber may be because of electron accepting nature of anode (Canelas et al., 2008) which favored more pyruvate production and increased cell growth and ethanol production. Higher expression of alcohol dehydrogenase and pyruvate decarboxylate also favored the ethanol production by CDBT2.

Coating of platinum anode with fine particles (nanoparticles) of platinum and graphite cathode with neutral red resulted net increment in ethanol production by 15.8 ± 0.28% in anodic and 37.6 ± 0.49% in cathodic compartments respectively than by uncoated electrodes. There was 52.8 ± 3.84% enhancement in total of both chamber than normal fermentation without voltage supply. Jeon and Park (2010) also observed about 50% increment in ethanol production when external voltage was supplied. Out of total glucose consumed by yeasts, more percentage conversion of glucose to ethanol was found in cathodic compartment ie; 77.2 ± 0.01% where as respective conversion in anodic compartment was 71.0 ± 0.50%. Further experiments with *S. spontaneum* biomass hydrolysate instead of glucose were done using coated electrodes.

The hydrolysate fermentation in ECC showed drastic enhancement in ethanol production in both compartments (enhancement of 60.8 ± 0.10% in anodic and 63.0 ± 0.07% in cathodic compartments with total enhancement of 61.9 ± 0.12%). The enhancement may be because of increasing conductivity by NaCl generated during neutralization after hydrolysis. When cellulose acetate was used in place of nafion membrane, there was slight increment in ethanol production (6.30 ± 0.22%). The increment may be due to decrease in resistance of membrane and also the chances of

xylose transfer from anodic compartment to cathodic compartment, where *W. anomalus* can ferment the xylose. RT PCR revealed that there was overexpression of both ADH1 and PDC1 gene when external voltage was supplied during fermentation. The overall experimental results concluded that CDBT2 and CDBT7 yeast strains produced ethanol efficiently from both glucose and lignocellulosic biomas hydrolysate. Applying external voltage enhanced the production of ethanol. Ethanol production was further enhanced with the better application of electron transport system, when neutral red was deposited on cathode and fine platinum nanoparticles were coated on the platinum anode. This method when applied for ethanol production from biomass hydrolysates obtained significant enhancement. Scale up of the technique can be applied for commercial ethanol production.

CHAPTER 5

5 CONCLUSION AND RECOMENDATION

5.1 Conclusion

Electrochemical cell has been successfully used for enhancement in ethanol production from lignocellulosic biomass by using two highly efficient ethanol fermentating yeasts. From the results and analysis of the data obtained in laboratory, following conclusions have been derived:

1. *Saccharum spontaneum* biomass showed great potential for ethanol production among the four biomass ie; *Ipomoea carnea, Phragmites karka, Saccharum spontaneum* and *Zea mays* cobs. The biomass were characterized on the basis of total reducing sugar contents, monomeric carbohydrate contents, calorific values, degradation capabilities.

2. Pretreatment of biomass with water at 100^{o}C and acid hydrolysis was found efficient and economical method to release reducing sugars from the biomass. The hydrolysate obtained from the technique has very less phenol and furfural contents.

3. Pretreatment of biomass with water at 100^{o}C for 2 h showed the reduction in hemicelluloses content with more crystalline nature compared with crude biomass without any pretreatment.

4. Among the 12 yeast isolates, *Saccharomyces cerevisiae,* CDBT2 and *Wickerhamomyces anomalus,* CDBT7 were found good for ethanol production from lignocellulosic biomass hydrolysate as the yeasts are good ethanol producer, ethanol tolerant, and the later one can produce ethanol from xylose also.

5. Ethanol production from the yeast has been greatly enhanced by supplying external voltage. *Saccharomyces cerevisiae* in anodic compartment and *Wickerhamomyces anomalus* in cathodic compartment were found best

combination for ethanol production. Further enhancement was seen when neutral red and fine platinum were coated in cathode and anode electrodes respectively.

6. Cathodic compartment has been able to convert more percentage of total glucose utilized into ethanol than anodic compartment.

7. Genetic modification of yeast has not shown any effect on ethanol production in electrochemical cell.

8. More enhancements in ethanol production has been observed when ethanol was allowed to produce by CDBT2 and CDBT7 yeasts in electrochemical cell using lignocellulosic biomass hydrolysate pretreated with hot water at 100oC and hydrolyzed with dilute acid.

9. A slight enhancement in ethanol production was observed when nafion membrane was replaced with cellulose acetate membrane.

10. Enhancement of alcohol dehydrogenase and pyruvate decarboxylate expression was observed when yeast was cultured supplying external voltage.

5.2 Recommendation

Cheaper electrode like steel can be tested for improvement in ethanol production. For industrial use of the technique in future, scale up of the electrofermentation process must be done as further work. During scale up, vessel should be improved to reduce resistance.

CHAPTER 6

6 SUMMARY

This thesis described about the possibility of production of ethanol from lignocellulosic biomass in an electrochemical with better yield than normal fermentation. This thesis comprised of five different chapters and a reference section.

The first chapter describes about general introduction of the thesis. According to the chapter, crisis of transportation fuel is a huge problem in the country like Nepal. To alleviate this problem, bioethanol can be a good alternative as it is renewable and ecofriendly. Besides as fuel, bioethanol can be used for other different purposes such as making biopolymers (bioplastics) and in ethanol biofuel cell for electricity generation. Bioethanol can be produced in rural or urban areas by using easily available lignocellulosic biomass. Lignocellulosic biomass is rich in cellulose, hemicellulose, lignin and other contents like pectin. Cellulose and hemicellulose upon degradation produces reducing sugars which can be used for converting into value added products like ethanol.

Second chapter described about the pertinent literatures reviewed. The review illustrated that, the extent of ethanol that can be produced from lignocellulosic biomass depends on composition of sugars, which differ between different plant species and also on their habitat. Selection of glucose and xylose utilizing yeast is must for producing ethanol efficiently from lignocellulosic biomass. The production of ethanol can be enhanced in an electrochemical cell with supply of external voltage.

The third chapter described about materials and methods. *Ipomoea carnea, Phragmites karka, Saccharum spontaneum* and *Zea mays* cobs, are the widely and sufficiently available waste biomasse were studied for their utilization in ethanol production. The biomasse were characterized in terms of sugar contents, calorific values and degradation capabilities. Sugar contents were analyzed by HPLC. Energy contents were determined by bomb calorimeter and biomass degradation efficiencies were determined using thermogravimetric analysis. The pretreatment of *S. spontaneum* biomass was evaluated using sulphuric acid, ammonium hydroxide, 1-butyl-3-methyl imidazolium chloride, butadiene sulphone and hot water. After

pretreatment, the biomass was subjected to acid or enzymatic hydrolysis. The total reducing sugar, phenol and furfural present in the hydrolysates were determined. The best technique was adopted for ethanol production in electrochemical cell at optimized condition.

The fourth chapter described about results and discussion. According to our result, among the four biomasse, *S. spontaneum* was found to be the best as it has high reducing sugar contents, better degradation capabilities. The two methods of pretreatment (i) ammonium hydroxide at 65^{o}C for 12 h followed by cellulase hydrolysis and (ii) hot water pretreatment at 100^{o}C for 2 h followed by hydrochloric acid hydrolysis were found to be good for *S. spontaneum*, however the hot water pretreatment followed by acid hydrolysis was more economical as costly enzymes are not used. Pretreatment made the biomass more crystalline. In the study, two highly efficient ethanol producing yeasts strains *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalus* (CDBT7) were used for ethanol production in electrochemical cell. The percentage enhancement in ethanol production in ECC was significantly higher in lignocellulosic biomass hydrolysate than in media with glucose suggesting that the type of substrate used in the electrochemical cell has an effect on ethanol production. The NaCl present in hydrolysate may have enhanced the conductivity in the cell. The enhancement in two enzymes responsible for ethanol production were observed when voltage was supplied during culture. It indicated that the enhancement in fermentation in ECC may be due to the enhancement in the enzyme production. When cellulose acetate membrane was used in place of nafion membrane slightly more ethanol production was observed.

Fifth chapter showed the conclusion of the finding of the study. The main finding is that the ethanol production from *S. spontanum* hydrolysate obtained by pretreatment with hot water at 100^{o}C for 2 h followed by acid hydrolysis in ECC using CDBT2 and CDBT7 strains was enhanced significantly. So this can be an optimum method for commercial ethanol production from biomass hydrolysate after up scaling.

Chapter six summarized the thesis writeup.

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APPENDIX

Appendix 1

1. Preparation of reagents

Digestion mixture:

- Pour 18 ml water in a 250-ml erlenmeyer flask.
- Add few Carborundum Beads.
- Add 100 ml of Sulphuric Acid (96 % (w/w) of 18 mol/l, U =1.84 g cm^{-3} concentrated acid) with caution in small portions at a time.
- Dissolve 6 g of salicylic acid with the aid of a magnetic stirrer.

Dinitro salicillic acid (DNSA) reagent:

- Prepare 20 ml of 2M NaOH solution.
- Add about 1g of DNSA by stirring.
- To this solution add about 30g of sodium potassium tartarate tetrahydrate in small lots.
- Made the final volume 100 ml with the distilled water.
- Stored the solution in an amber colored bottle.

Acidified 5% potassium dichromate solution:

- Prepare 5M Sulphuric acid from concentrated acid.
- Dissolve 5 gm of potassium dichromate in 100 ml of 5M Sulphuric acid.
- Use freshly prepared.

Appendix 2

Figure A1: Photographs of some biomass in its native states Left: *S. Spontanum*, Right: *Phragmites kark*

Figure A2: Photographs Left: Collected biomass and Right: Air drying of biomass

Figure A3: Photographs Left: Sieve for obtaining required particle size of biomass, Right: Biomass with different particle size stored in plastic pouch.

Figure A4: Photographs Left: Biomass keeping in pretreatment vessel for pretreatment. Right: Biomass stored in plastic pouch after pretreatment and drying.

Appendix 3

Figure A5: Left: Platinum wire.. Right:. Electroplating of platinum wire

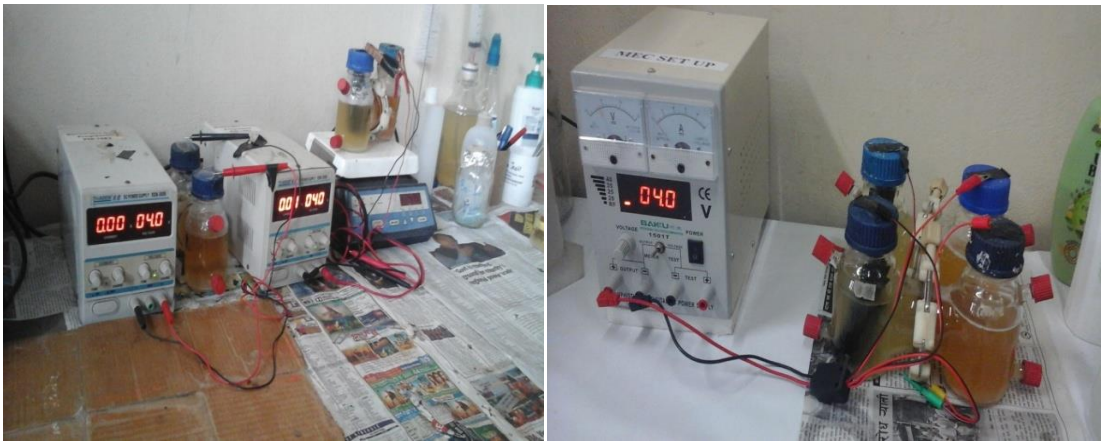

Figure A6: Electrochemical cell (PYN media supplied with glucose and with voltage supply).

Figure A7: Electrochemical cell with *S. spontaneum* hydrolysate as media along with normal fermentation of cultures.

Figure A8: Left: Membrane coated with cellulose acetate. Right: Electrochemical Cell with cellulose acetate coated membrane.

Appendix 4

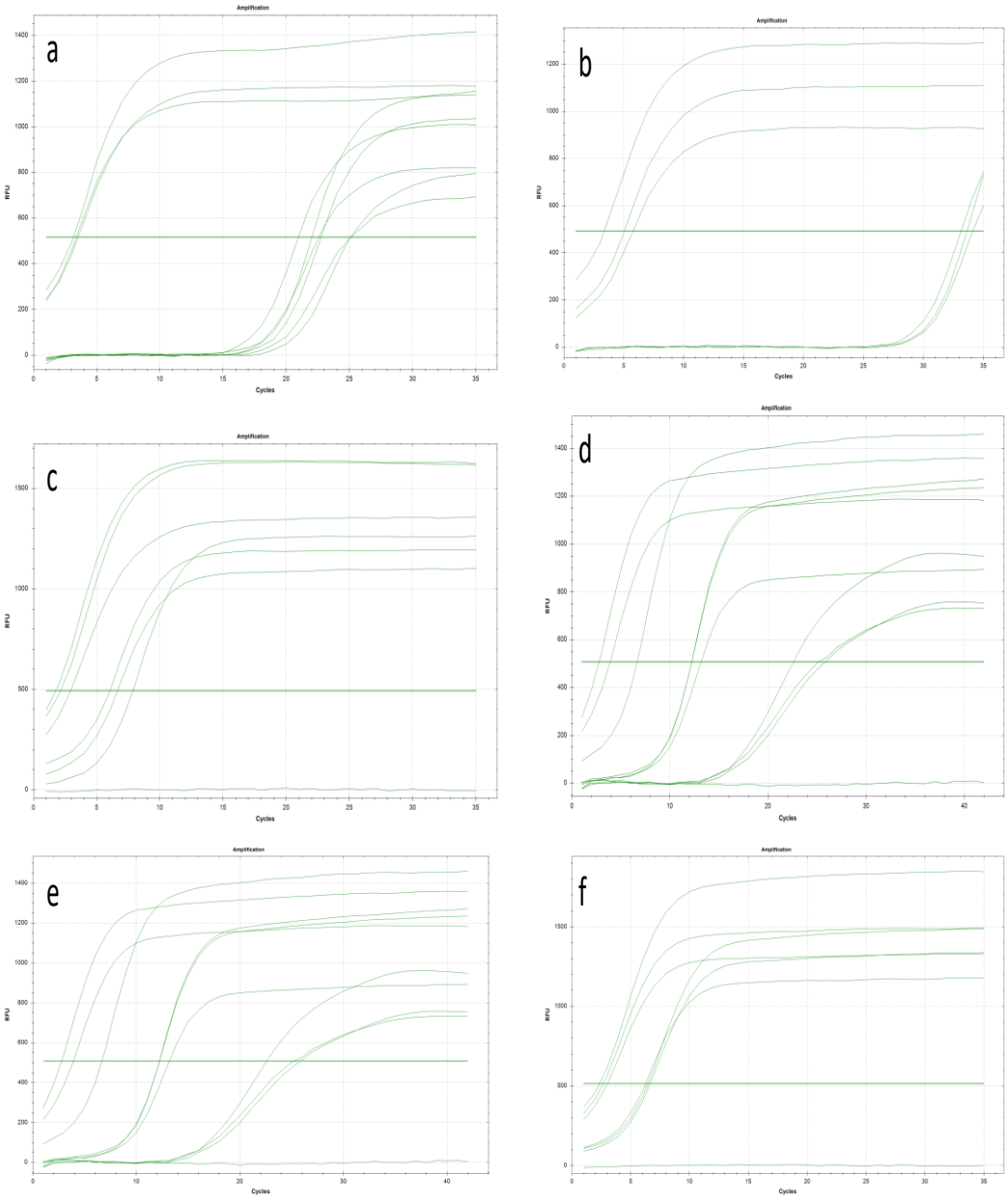

Figure A9: RT PCR gene expression curves showing C_t values of different genes a. PDC1 CDBT7 with voltage supply, CDBT7 without voltage supply, CDBT2 without voltage supply. b. PDC1 CDBT2 with voltage supply c. ADH1 CDBT7 with voltage supply, CDBT2 with voltage supply d.ADH1 CDBT7 without voltage supply, CDBT2 without voltage supply e. TFC1 CDBT7 with voltage supply, CDBT7 without voltage supply, CDBT2 with voltage supply f. TFC1 CDBT2 without voltage supply.

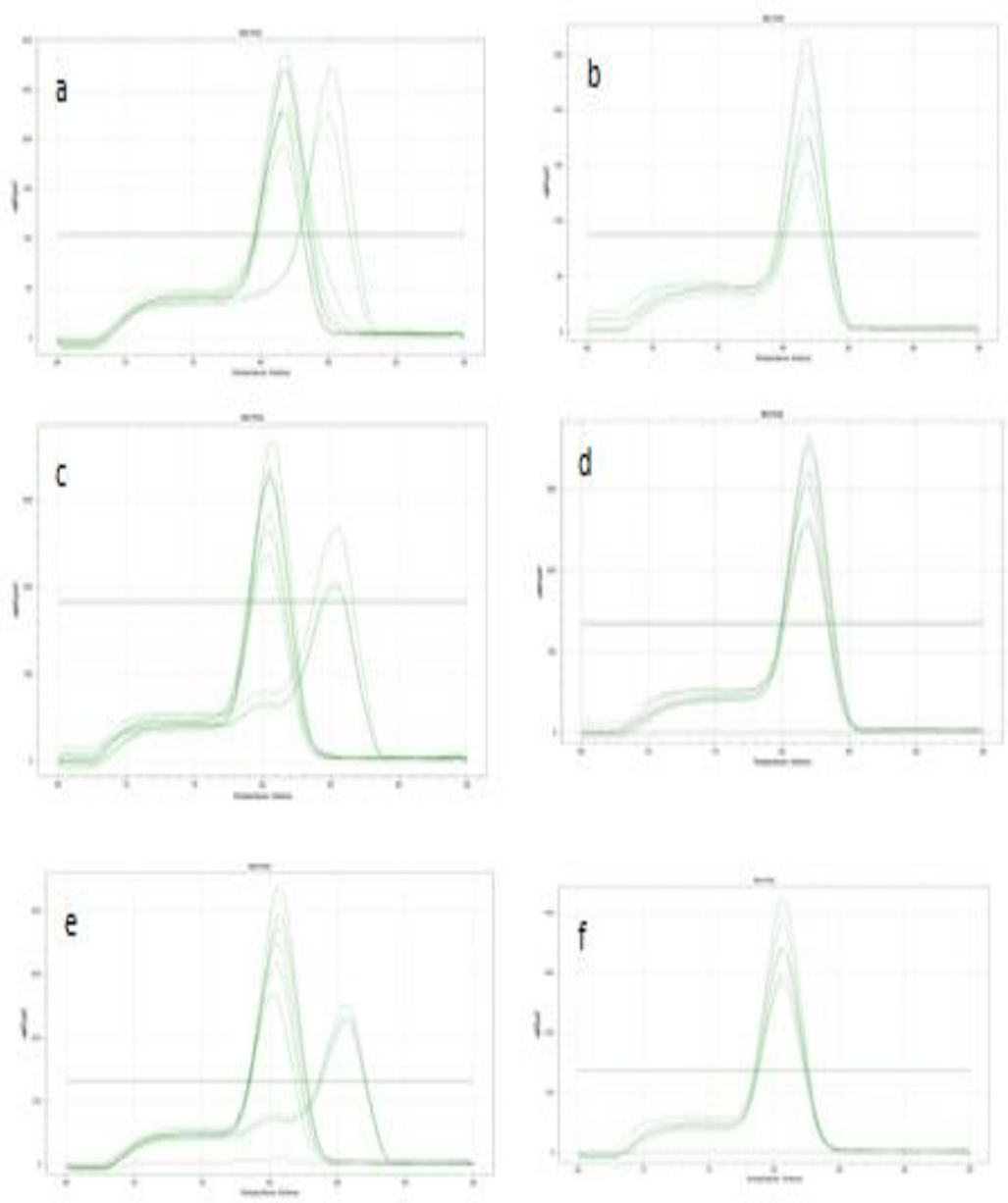

Figure A10: Differential melting curve of amplified DNA product obtained from RT PCR of different genes a. PDC1 CDBT7 with voltage supply, CDBT7 without voltage supply, CDBT2 without voltage supply. b. PDC1 CDBT2 with voltage supply c. ADH1 CDBT7 with voltage supply, CDBT2 with voltage supply d.ADH1 CDBT7 without voltage supply, CDBT2 without voltage supply e. TFC1 CDBT7 with voltage supply, CDBT7 without voltage supply, CDBT2 with voltage supply f. TFC1 CDBT2 without voltage supply.

Appendix 5

Figure A11: Thermo gravimetric analyzer (TGA) (STA 7200 HITACHI) attached with PC available in Biotechnology Lab, IITG, Guwahati.

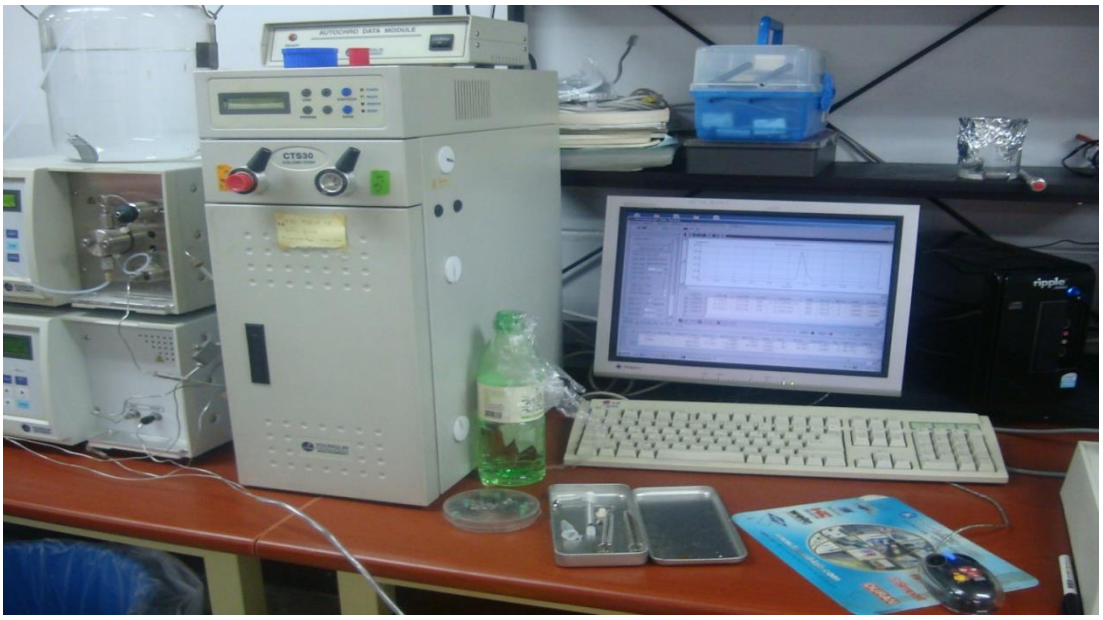

Figure A12: HPLC analyzer (Agilent with RI detector) attached with PC available in Department of Chemistry IITG, India.

Appendix 6

Figure A13: Photographs Left: Cleaning graphite electrode. Right: Installing ECC.

Figure A14: Photographs Left: With Professor Pranab Goswami and lab mentor, IITG, Guwahati, India. Right: With facultiess, IITG, Guwahati, India.

Figure A15: Photographs Left: In Super Critical Fluid Lab, Seoul National University, Korea. Top Right: With Professor Doo Hun Park, Syokyong University, Korea.

Figure A16: Photographs: With Professor Doo Hun Park and lab colleagues, Syokyong University, Korea..

Figure A16: Photograph of paper presentation in AP ISMET-2018, BITs Pilani, Goa, India.

Appendix 7

List of Journal Papers

Jarina Joshi, Pradip Dhungana,Bikram Prajapati, Rocky Maharjan, Pranita Poudyal, Mukesh Yadav, Milan Mainali, Amar Prasad Yadav, Tribikram Bhattarai and Lakshmaiah Sreerama (2019). Enhancement of ethanol production in electrochemical cell by *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalus* (CDBT7). *Front. Energy Res. - Bioenergy and Biofuels.* **7**:70.

Jarina Joshi, Tribikram Bhattarai, and Lakshmaiah Sreerama. (2018). Efficient methods of pretreatment for the release of reducing sugars from lignocellulosic biomass native to Nepal and characterization of pretreated lignocellulosic biomass. (2018). *International Journal of Advanced Biotechnology and Research* (IJBR). **9**(3):9-23.

Jarina Joshi, Rejeena Shrestha, Rojlina Manandhar, Krishna Manandhar, Lakshmaiya Sreerama and Tribikram Bhattarai. (2014). "Improvement of ethanol production by electrochemical redox combination of yeast cells" *International Journal of Biological Sciences and Applications, American Association of Science and Technology,* **1**(3): 44-51.

Jarina Joshi, Rejeena Shrestha, Rojlina Manandhar, Dinita Sharma, Krishna Manandhar, Lakshmaiah. Sreerama, Doo Hyun Park and Tribikram Bhattarai (2014). Optimization of external potential for ethanol production by yeasts in electrochemical cell. *Rentech Symposium Compendium.* **4**:44.

Book Chapter

Bishnu Joshi, **Jarina Joshi**, Tribikram Bhattarai and Lakshmaiah Sreerama. (2019). Currently used microbes and advantages of using genetically modified microbes for ethanol production (Chapter 15). *Bioethanol Production from Food Crops. Sustainable Sources, Interventions, and Challenges. Academic Press*. 293-316.

List of Related Paper Published During the Period

Jarina Joshi. (2014). Enhanced production of ethanol from red potatoes grown in hilly regions of Nepal using various nitrogen sources. International Journal of Applied Sciences and Biotechnology (IJASBT) 2(1): 41-44.

Bimala Dhakal and **Jarina Joshi** (2015). Neutral red immoblized graphite felt anodic microbial fuel cell for wastewater treatment and generation of electricity. *J. Bioprocess Bioprocessing Biotech.* **5**(11):261.

Prabin Shrestha, Bishnu Joshi, **Jarina Joshi**, Rajani Malla and Lakshmaiah Sreerama (2016). Isolation and physicochemical characterization of laccase from *Ganoderma lucidum*-CDBT1 isolated from its native habitat in Nepal. *BioMed Research International.* **2016**: 10 pages.

Mukesh Yadav, Garima Bista, Rocky Maharjan, Pranita Poudyal, Milan Mainali, Lakshmaiah Sreerama, **Jarina Joshi** (2019). Secretory laccase from *Pestalotiopsis* species CDBT-F-G1 fungal strain isolated from high altitude: Optimization of its production and characterization. *Appl. Sci.* **9**: 340.

List of Paper Presented

Jarina Joshi . Enhancement in ethanol production in bioelectrochemical cell using *Saccharomyces cereviseae* and *Wickerhamomyces*. *(Asia Pacific-International Society of Microbial Electrochemistry and Technology) AP-ISMET-2018* organized by ISMET and BITs Pilani, KK Birla Goa Campus. 13th -16th Nov, 2018.

Jarina Joshi. Fate of *Saccharum spontaneum* for fermentative production of Bioethanol. *South Asian Biotechnology Conference (SABC-2017),* 16-18th Mar, 2017.

Jarina Joshi. Fermentable sugar and phenol production by pre-treatments of *Saccharum spontanum* followed by hydrolysis. *Biotechnology Workshop 2015*, NARC, Khumaltar 6th July, 2015.

Jarina Joshi "Comparison of Fermentable Sugar and Phenol Production during Pre-treatments of *Saccharum spontaneum* followed by Hydrolysis." *Nepal Biotechnology Society Annual Conference*. 22nd November, 2014.

Jarina Joshi. Optimization of external potential for ethanol production by yeasts in electrochemical cell. *RENTECH 2014*. 12 September 2014.

List of Training / Workshop Participated

Advanced training on "Microbial Fuel cell and Instrumentation". Professor Pranab Goswami's Lab, Department of Biotechnology, Indian Institute of Technology, IITG, Guwahati, India from 20th September to 19th November, 2015.

Workshop on "Scientific writing and publishing" National Institute of Environmental Health Science (USA) and Progressive Sustainable Development, Nepal. 3rd-4th November, 2014.

Trainning on "Alcohol Fermentation and Applying Electrochemical Bioreactor for Cultivation of *Zymomonas mobilis*." Doo Hyun Park's Lab, Seokyeong University, Seoul, Korea from July 29, 2013 to August 14, 2013.

Appendix 8

Copies of consent taken to send the samples for analysis

Government of Nepal
Ministry of Agriculture Development
Department of Agriculture
Plant Protection Directorate
National Plant Quarantine Program

Phyto-sanitary Certificate

Certificate number:8/1/1/14/072/73
From the Plant Protection Organization of **Nepal** to Plant Protection Organization of India

1. Description of consignment:

Exporter's name and address:	Ms. Jarina Joshi, Centeral Dept.Of Biotechnology, TU, Kathmandu, Nepal.
Name and address of consignee of plants or plant products:	Ms. Jarina Joshi, Hand Carry India.
Number and description of packages:	30 Small Pkt In One Bag.
Objective/end use:	Research.
Identification mark:	X
Place of origin:	**Nepal**
Type of conveyance:	By Air.
Declared entry point:	Delhi, India.
Name of product and declared quantity:	Narkot dust, Kans dust, Rice stem dust, Corn cob dust, besharam dust (each 6pkt= 30 Pkt)
Scientific name of plants:	*Pragmites spp, Saccharum spontaneuss, Oryza sativa stem, Zea mays cob, Ipomoea spp.*

This is to certify, upon the inspection, examination and test of the above mentioned plants and plant products or other regulated articles in accordance with the formal procedures, that they are free from quarantine pests as mentioned by the importing country and they conform to the importing country's Phyto-sanitary requirements and controlled non-quarantine pests.

2. Additional declaration: This certificate is issued only for research consigment .

3. Disinfection/disinfection treatment:

Date of treatment: x	Treatment:
Chemical (active ingredients):	Duration and temperature: x
Concentration: x	Additional information:x
Date of inspection: 15 Sept 2015	

The consignment has to be exported no later than five days of 15 Sept 2015, TIA, KTM Customs.
Date of issue: 15 Sept 2015
Place of issue: Hariharbhawan
Certificate issuing authority's:

Signature:

Name: Mahesh Chandra Acharya
Post: Senior Plant Quarantine Officer

Seal

Government of Nepal Ministry of Agriculture Development Department of Agriculture National Plant Quarantine Program Hariharbhawan, Lalitpur

Appendix 9

Photocopies of Published journals and certificates of paper presentations attached in proceeding papers.

frontiers in Energy Research

ORIGINAL RESEARCH
published: 31 July 2019
doi: 10.3389/fenrg.2019.00070

Enhancement of Ethanol Production in Electrochemical Cell by *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalus* (CDBT7)

Jarina Joshi[1]*, *Pradip Dhungana*[1], *Bikram Prajapati*[1], *Rocky Maharjan*[1], *Pranita Poudyal*[1], *Mukesh Yadav*[1], *Milan Mainali*[1], *Amar Prasad Yadav*[2], *Tribikram Bhattarai*[1] and *Lakshmaiah Sreerama*[3]

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Bioethanol (a renewable resource), blended with gasoline, is used as liquid transportation fuel worldwide and produced from either starch or lignocellulose. Local production and use of bioethanol supports local economies, decreases country's carbon footprint and promotes self-sufficiency. The latter is especially important for bio-resource-rich land-locked countries like Nepal that are seeking alternative transportation fuels and technologies to produce them. In that regard, in the present study, we have used two highly efficient ethanol producing yeast strains, *viz*., *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalous* (CDBT7), in an electrochemical cell to enhance ethanol production. Ethanol production by CDBT2 (anodic chamber) and CDBT7 (cathodic chamber) control cultures, using 5% glucose as substrate, were 12.6 ± 0.42 and 10.1 ± 0.17 mg·mL^{-1} respectively. These cultures in the electrochemical cell, when externally supplied with 4V, the ethanol production was enhanced by 19.8 ± 0.50% and 23.7 ± 0.51%, respectively, as compared to the control cultures. On the other hand, co-culturing of those two yeast strains in both electrode compartments resulted only 3.96 ± 0.83% enhancement in ethanol production. Immobilization of CDBT7 in the graphite cathode resulted in lower enhancement of ethanol production (5.30 ± 0.82%), less than free cell culture of CDBT7. CDBT2 and CDBT7 when cultured in platinum nano particle coated platinum anode and neutral red-coated graphite cathode, respectively, ethanol production was substantially enhanced (52.8 ± 0.44%). The above experiments when repeated using lignocellulosic biomass hydrolysate (reducing sugar content was 3.3%) as substrate, resulted in even better enhancement in ethanol production (61.5 ± 0.12%) as compared to glucose. The results concluded that CDBT2 and CDBT7 yeast strains produced ethanol efficiently from both glucose and lignocellulosic biomass

hydrolysate. Ethanol production was enhanced in the presence of low levels of externally applied voltage. Ethanol production was further enhanced with the better electron transport provision i.e., when neutral red was deposited on cathode and fine platinum nanoparticles were coated on the platinum anode.

Keywords: electrochemical cell, lignocellulosic biomass, *Saccharomyces cerevisiae*, *Wickerhamomyces anomalous*, *Saccharum spontaneum*, bioethanol

INTRODUCTION

Dependence on petroleum-based transportation fuels is a major challenge for developing countries which don't have fossil fuel reserves. The challenge is severe in the landlocked countries such as Nepal (Joshi et al., 2011). In fact, landlocked countries spend a major share of their GDP to import fossil fuels via other countries. Given the above, serious attempts are being made to develop alternative energy sources that are expected to alleviate the above challenge. Bioethanol is one of those renewable and eco-friendly fuels (Khatiwada and Silveira, 2011). Besides being used as a fuel, bioethanol can also be used for other purposes such as making bioplastics (Rose and Palkovits, 2011) and development of ethanol fuel cells for electricity generation (Saisirirat and Joommanee, 2017).

Second generation biofuels such as bioethanol (obtained from lignocellulose) can be produced in rural as well as urban areas using easily available lignocellulosic biomass. Lignocellulose is the most abundant biomass worldwide with annual production of about 1,000 giga-metric tons. The biomass is a mixture of cellulose, hemicellulose, lignin and other contents such as pectin. Cellulose and hemicelluloses upon degradation produces fermentable sugars, e.g., glucose and xylose. These sugars can be further converted into important products, including ethanol, by fermentation. The extent of ethanol that can be produced by lignocellulosic biomass depends on composition of sugars, which varies in plants and the habitat in which they grow (Hermosilla et al., 2017; Joshi et al., 2018). To date the cost of ethanol production from lignocellulosic biomass depends on technique used. The better the technique, the lower is the cost of production (Haque and Epplin, 2012).

Yeasts such as *S. cerevisiae* are widely used for ethanol fermentation. Yeasts with high salt and ethanol tolerance are most valuable in this process. Further to produce ethanol efficiently from lignocellulosic hydrolysates, yeasts should be able to utilize both glucose as well as pentoses such as xylose and arabinose. This is because lignocellulosic biomass is rich in both glucose and xylose (a pentose) (Joshi et al., 2018). This is the main drawback of using *S. cerevisiae* alone as it cannot ferment both glucose and xylose. Accordingly, identifying yeast strains that can ferment both glucose and xylose is critical, if not, at the least use two different strains of yeast that are capable of utilizing these sugars independently. In this study, we have identified and used highly efficient, salt and ethanol tolerant, yeast strains, *viz., S. cerevisiae* (CDBT2) (Joshi et al., 2014) and xylose utilizing *W. anomalous* (CDBT7) for ethanol production using lignocellulosic biomass.

For enhancing ethanol production, electro-fermentation technology that merges traditional fermentation with electrochemistry can be adopted. Electro-fermentation is a novel process in which the microbial fermentative metabolism may be controlled electrochemically. The benefits of this process are that for the process (i) is selective, (ii) increases sugar (carbon) utilization efficiency, (iii) minimizes the use of additives for redox balance or pH control, (iv) enhance cell growth and (v) in some cases enhance product recovery (Schievano et al., 2016). The electrodes used in the electrochemical cell can act as electron sinks, as source of electrons or polarize ions present in microbes that allow an unbalanced growth. Such electrochemical modifications also exert significant effects on not only metabolism and cellular regulation, but also on interspecies interactions as well as the selection of microbial populations (Moscoviz et al., 2016). The novel yeast strains of *S. cerevisiae* (CDBT2) and *W. anomalous* (CDBT7), identified in our laboratory, are used in this study to determine better utilization of glucose and xylose from lignocellulosic biomass and enhancement in ethanol production in an electrochemical cell. The lignocellulosic biomass used in the study was obtained from *Saccharum spontaneum* pretreated with hot water at 100°C for 2 h followed by 0.5 M hydrochloric acid hydrolysis (Joshi et al., 2018).

MATERIALS AND METHODS

Materials

Woven graphite felt (10 mm thickness) was purchased from Nippon Co., Nippon, Japan. Platinum wire (0.2 mm diameter), high grade neutral red, ethanol, and thionyl chloride were purchased from Sigma Chemical Co., St. Louis, MO, USA. Electrochemical cell (ECC) vessels made up of Pyrex glass were purchased from Adams and Chittenden Scientific Glass Co., California, USA. Nafion 117 membrane was purchased from DuPont Co., Wilmington, DE, USA. Hydrogen hexachloroplatinate (IV) hexahydrate (Kanto Chemicals Company, Japan) was kindly provided by Prof. Dr. Amar Prasad Yadav, Central Department of Chemistry, Tribhuvan University, Nepal. D-Glucose, dinitro salicylic acid (DNSA), sodium potassium tartrate, yeast extract, peptone, ammonium sulfate and sodium alginate were purchased from Hi-Media Company, Bangalore, India. All other chemical were of analytical grade and were available locally.

Biomass samples of *S. spontaneum* was harvested from the premises of Tribhuvan University Campus, Kirtipur, Kathmandu, Nepal during the month of August. The sampling

location was 27.6818°N and 85.2865°S. The aerial portion of the collected sample was air dried for 24 h and cut into about 2 cm pieces. It was further dried in oven at 60°C for 24 h. The dry samples were ground using a blender. The blended biomass was sieved using 250–500 μm sieves and packed into plastic pouches for further use in fermentation studies.

Methods

Ethanol Production in Electrochemical Cell

Development of CDBT2 and CDBT7 inoculums

Preserved yeast strains CDBT2 (Gene Bank accession # MK910215) and CDBT7 (Gene Bank accession # MK910216) were used to develop inoculums. Inoculums were prepared by inoculating a loop-full of agar cultures of CDBT2 and CDBT7 strains into PYN (Peptone, Yeast extract and Nutrient) media (Peptone: 3.5 gm·L^{-1}, yeast extract: 3 gm·L^{-1}, KH_2PO_4: 2 gm·L^{-1}, MgSO4:1 gm·L^{-1}, and $(NH_4)_2SO_4$: 1 gm·L^{-1}) (Balakumar and Arasaratnam, 2012) supplemented with 5% glucose. They were cultured in orbital shaker for 18 h at 30°C and pH 5.0.

Construction of an electrochemical cell (ECC)

The anodic and cathodic compartment of ECC was assembled tightly using a rubber gasket inserted with nafion membrane that separates the two chambers (**Figure 1**). Working volume of the cathodic and anodic compartments were ∼300 mL each. Normal graphite felt (11 cm × 3 cm × 1 cm) was used as cathode and platinum wire (0.2 mm diameter, 1 m length) was used as anode. Each time 1.2 mL of respective inoculum, prepared above, was added with 300 mL PYN media in each case.

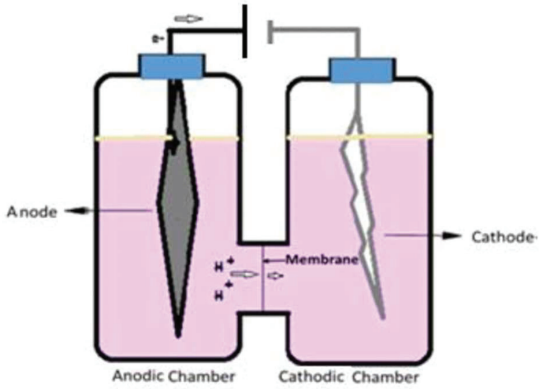

FIGURE 1 | The electrochemical cell (ECC): The ECC was assembled using the Pyrex glass fuel cell assembly as shown in the figure, separated by a nafion membrane using a leak-proof rubber gasket. The electrodes were inserted through small openings in each of the chamber caps and then connected to a power pack to supply external voltage.

Optimization of ethanol production in an ECC

The yeast strains CDBT2 and CDBT7 were cultured in ECC (**Figure 1**) and evaluated for ethanol production in the presence of carbohydrate sources, i.e., glucose (5%) and lignocellulosic biomass hydrolysate (with 3.3% reducing sugar). Lignocellulosic biomass hydrolysate was prepared by collecting the hydrolysate that was formed after pretreating *Saccharum spontaneum* biomass (250–500 μm size) with hot water at 100°C for 2 h followed by hydrolyzing with 0.5M HCl for 24 h. The 5% glucose or biomass hydrolysate acted as carbohydrate sources (Joshi et al., 2018). PYN media composition was added as supplements. The fermentation media in ECC without culture and the external source of voltage served as control. The two compartments of the ECC were filled with PYN media supplemented with glucose or lignocellulosic biomass hydrolysate, inoculated with yeast strains and allowed to produce ethanol in the presence and absence of applied voltage at an optimized pH 5.5 and 30°C (Joshi et al., 2014).

Ethanol production by yeast strains CDBT2 and CDBT7 under applied voltage

In the first fermentation reaction, the yeast strains CDBT2 and CDBT7 were cultured in cathodic and anodic chambers, respectively. In a second fermentation reaction, the yeast strains CDBT2 and CDBT7 were cultured in anodic and cathodic chambers, respectively. In each of the above reactions, the ECC was supplied with 4V constantly. Ethanol production by yeast strains CDBT2 and CDBT7 in ECC was monitored at intervals of 12 h as described by Seo et al. (2009). The sample broth (1 mL) collected from ECC was initially centrifuged at 4000 × g for 15 min to remove cell debris. The supernatant was then mixed with 1 mL tri n-butyl phosphate (TBP), vortexed for 15 min and centrifuged at 4000 × g for 15 min to separate layers. About 750 μL of upper TBP layer was transferred to another tube and mixed with equal amount of acidified 5% potassium dichromate reagent. The mixture was vortexed, centrifuged as above and lower green layer (potassium dichromate reagent layer) was separated and its absorbance was measured at 595 nm using spectrophotometer against blank. Cell growth was monitored by measuring turbidity of culture broth at 600 nm. The reduction in reducing sugar concentrations in the broth samples were measured by DNSA method (Miller, 1959).

Formation of film in graphite electrode by yeast

Biofilm formation was analyzed by Scanning Electron Microscopy (SEM). After completion of experiment in ECC, graphite electrodes kept as cathode with cultures of CDBT2 and CDBT7 were vacuum evaporated and packed in sterile plastic pouch. The samples were sent to Advanced Instrumental Lab, Jawaharlal Nehru University, New Delhi, India for SEM. The biofilm formation was confirmed from the micrograph.

Ethanol production by CDBT2 and CDBT7 at different voltage

To determine optimum supply of external voltage for ethanol production, the ECC voltage was varied between 0 and 5V (± 0.1 V), at an interval of 1V and ethanol production was measured as above.

Comparison of ethanol production by CDBT7 vs. several strains of S. cerevisiae

To compare the ethanol production efficiency of CDBT2 with other *S. cerevisiae*, ethanol production in the anodic compartment of ECC was monitored by culturing *S. cerevisiae* strains obtained from different sources. Yeast strains MKY09 (a laboratory yeast strain) and Ethanol Red (an industrial yeast strain) were kindly provided by Prof. Eckhard Bole, University of Frankfurt, Germany. Yeast strain MKY09 transformed with pGPD2 plasmid inserted with laccase gene (pGPD2/lac) was developed at Central Department of Biotechnology, Tribhuvan University, Nepal (Bishwakarma, 2017). The plasmid pGPD2/lac was constructed by cloning synthetic laccase gene of *Ganoderma lucidum* of size 1,576 bp in pGPD2 expression vector purchased from Addgene Company. The constructed plasmid was transformed into MKY09 strain. Yeast strain CDBT7 was cultured in cathodic compartment in all cases. The two chambers were separated by nafion membrane. The ethanol production was measured as described above.

Effect of different electron transport enhancing system in ECC

Production of ethanol was monitored in cathodic and anodic compartments by culturing yeast strains CDBT2 and CDBT7 in various combinations as follows. In reaction 1, yeast strain CDBT2 was cultured in both the compartments. In reaction 2, yeast strains CDBT2 and CDBT7 were cultured in anodic and cathodic compartments, respectively. In reaction 3, yeast strains CDBT2 and CDBT7 were co-cultured in both cathodic and anodic compartments. In all reactions, ethanol production was monitored as above. In the next set of reactions, the graphite felt (cathode) and platinum wire (anode) were coated with different electron transport enhancers and used as cathode and anode in ECC. In first case, the graphite electrode was immobilized with yeast cells using calcium alginate gel and used as cathode. Briefly, about 2.4 mL of 18 h culture of yeast strains were first centrifuged, the pellet mixed with 10 mL of 25 mM phosphate buffer (pH 7.0) containing 4% sodium alginate, and allowed to absorb into the graphite electrode for 30 min. The graphite electrode containing alginate and yeast cells was then soaked in a chilled 100 mM $CaCl_2$ solution for 30 min to induce calcium alginate coagulation and finally washed with 25 mM phosphate buffer. In the second case, the platinum electrode was coated with platinum nanoparticle electrochemically in anode. For this platinum wire was dipped in 10% hydrogen hexachloroplatinate (IV) hexahydrate solution in distilled water (Kanto Chemical Co.) with constant supply of 0.2 V using Hokuto Denko-151 potentiostat (Hokuto Denko Corporation, Japan) for 30 min. In the third case, the cathode was coated with neutral red as described by Jeon and Park (2010). Briefly, the graphite felt was first soaked in methanol, then dipped in 1% polyvinyl alcohol solution for 3 to 4 h and was dried in an oven at about 80°C for 24 h. The completely dried graphite felt was then soaked in pure chloroform containing 10% thionylchloride and 0.01% neutral red for 6 h. The graphite felt was then left for 12 h to air dry. It was then autoclaved and washed in running distilled water till color persists. Finally, it was dried at 60°C for 24 h and used as cathode. Best combination of electron transport system amongst was determined on the basis of higher ethanol production.

Ethanol Production by Lignocellulosic Biomass Hydrolysate as Carbohydrate Source

Ethanol production from hydrolysate

Lignocellulose biomass hydrolysate supplemented with PYN media was used for further fermentation to produce ethanol. The hydrolysates were kept in ECC chambers. After autoclaving, the chambers were inoculated with CDBT2 and CDBT7 yeast strains in anodic and cathodic chambers, respectively. The two chambers were separated by a nafion membrane. Ethanol production was measured as described above.

Comparison of ethanol production by nafion and cellulose acetate membrane

The experiments above were repeated in an ECC fitted with a cellulose acetate membrane to separate the two chambers. Ethanol production was measured as above and compared to those determined when the separating membrane was nafion membrane.

Statistical Analysis

All the data presented were the average of three redundant data. All graphs and statistical calculations were performed with Graph Pad Prism 8.0.1. Standard errors were represented in terms of ± standard deviation (±SD).

RESULTS

Optimization of Ethanol Production in an Electrochemical Cell (ECC)

The standard conditions under which fermentation was performed in the ECC (schematic of ECC assembly shown in **Figure 1**) using various yeast strains were pH 5.5, 30°C, PYN broth supplemented with 5% glucose and an external supply of 4V. The fermentation system in which CDBT2 strain was cultured in anodic compartment and CDBT7 strain in cathodic compartment was found to be most efficient for ethanol production than culturing CDBT2 strain in cathodic compartment and CDBT7 strain in anodic compartment (**Figures 2A,B**, **Table 1**). Scanning electron microscopic imaging showed the formation of a film of CDBT7 in graphite felt electrode (**Figure 3**). The fermentation efficiency was enhanced in both cathodic and anodic chamber cultures as compared to fermentation reactions carried out in the absence of applied voltage. There was observed faster and enhanced growth of CDBT2 in anodic compartment (**Figure 2C**). On the other hand, growth of CDBT2 strain when cultured in cathodic compartment was relatively limited. The CDBT7 strain when cultured in anodic compartment, showed relatively lower enhancement in ethanol production (**Table 1**). On application of external voltage in the range of 0 to 5V (± 0.1V), while

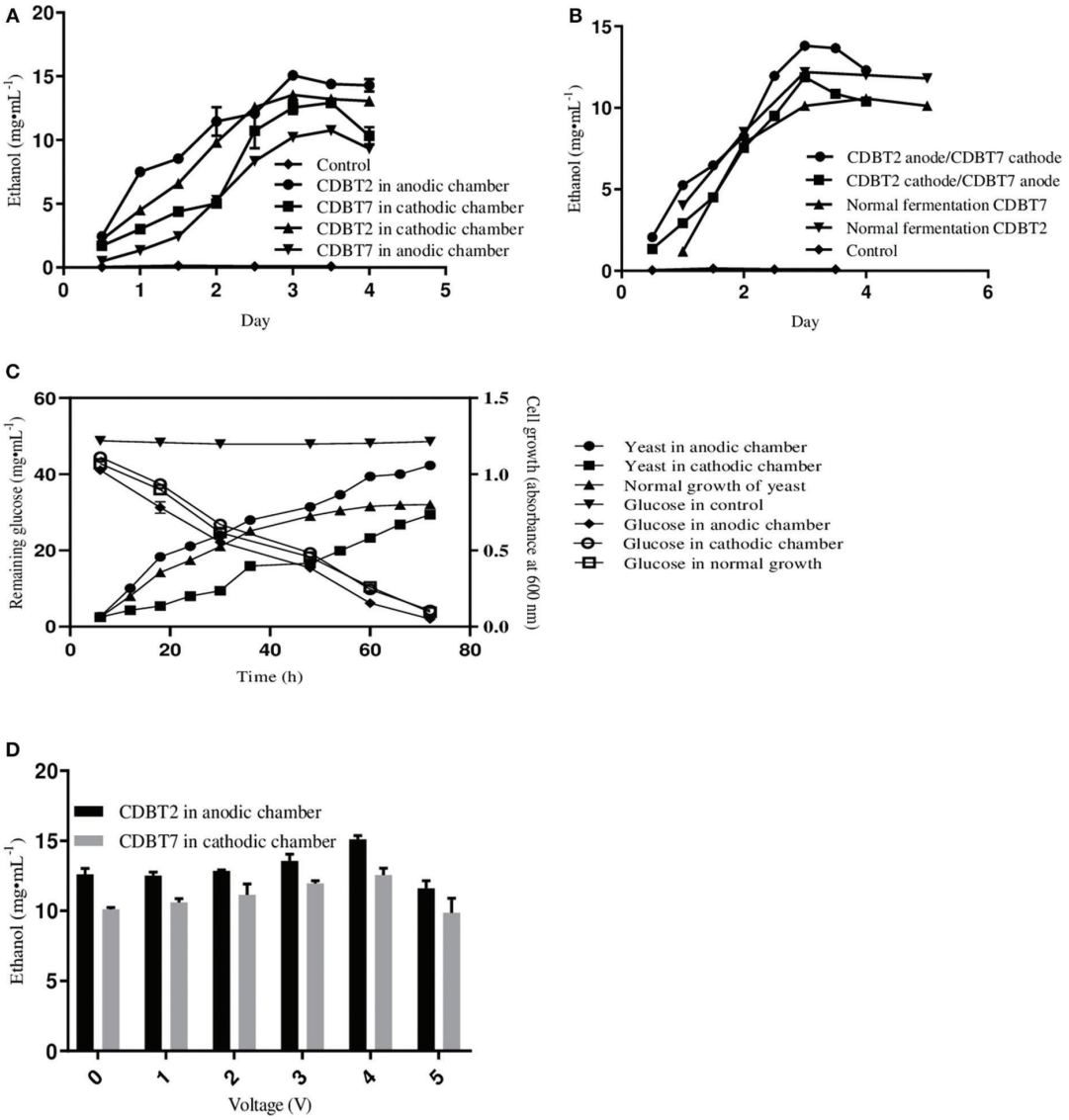

FIGURE 2 | Ethanol production in ECC by yeast strains CDBT2/CDBT7 with the supply of 4V externally. **(A)** Ethanol production in cathodic and anodic chambers by CDBT2 and CDBT7 individually. **(B)** Average of ethanol production in both chambers. **(C)** Cell growth and glucose utilization when CDBT2 cultured in anodic and cathodic chambers with and without applied external voltage (normal growth—without the supply of external voltage). **(D)** Effect of applied electrical current on ethanol production by CDBT2 and CDBT7. (Control: ECC w/o/yeast culture and w/o voltage supply; normal fermentation: ECC w/o voltage supply).

CDBT2 was cultured in the anodic compartment and CDBT7 cultured in cathodic compartment, application of 4V produced the highest amount of ethanol (**Figure 2D**). The enhancement of ethanol production observed was 19.4 ± 0.18% when CDBT2 was cultured in anodic compartment and 23.7 ± 0.51% when CDBT7 was cultured in cathodic compartment. Supply of external voltage through ECC in the absence of yeast inoculums (negative control) did not produce detectable levels of ethanol.

Comparison of Ethanol Production by Various *S. cerevisiae* Strains Cultured in Anodic Compartment Coupled With CDBT 7 Strain Cultured in Cathodic Compartment

To determine the efficiency of ethanol production using different *S. cerevisiae* strains in ECC, ethanol production was monitored by culturing CDBT2, ethanol red, MKY09, and MKY09-pGPD2/lac in anodic compartment against CDBT7 in cathodic

TABLE 1 | Ethanol produced by CDBT2 and CDBT7 cultured in different compartments.

S. No.	CDBT2 in AC	CDBT7 in CC	CDBT2 CC	CDBT7 in AC	CDBT2*	CDBT7*
Ethanol ($mg{\cdot}mL^{-1}$)	15.1 ± 0.28	12.5 ± 0.49	13.5 ± 0.0	10.2 ± 0.0	12.6 ± 0.42	10.1 ± 0.14
% increment	19.8 ± 0.50	23.7 ± 0.51	7.14 ± 0.64	1.00 ± 0.14		

AC, Anodic chamber; CC, Cathodic chamber.
**Fermentation under normal conditions (no external source of electricity).*

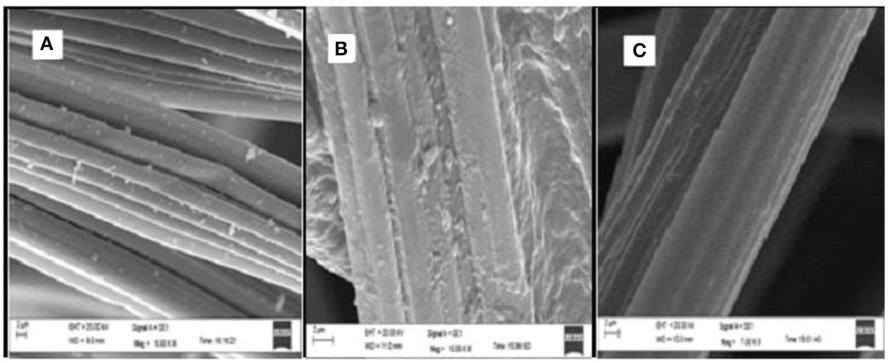

FIGURE 3 | Scanning electron microscopic images of graphite felt electrodes. **(A)** Plane graphite felt. **(B)** Graphite felt cathode post CDBT7 growth and ethanol production in the cathodic chamber. **(C)** Graphite felt cathode post CDBT2 growth and ethanol production in the cathodic chamber.

compartment. Once again, CDBT2 produced the highest amount of ethanol (**Figure 4A**) as compared to other *S. cerevisiae* strains. Further, genetic modification of MKY09 strain by transforming it with a laccase gene (MKY09-pGPD2/lac) did not affect ethanol production in ECC, rather both MKY09 and MKY09-pGPD2/lac strains produced similar amounts of ethanol. In addition, yeast strain CDBT2 cultured in anodic chamber, coupled to CDBT7 strain cultured in cathodic chamber showed enhancement of the ethanol production by CDBT7 (**Figure 4B**).

Effect of Different Electron Transport Systems

Effect of Yeast Cultures

To determine the effect of yeast cultures on electron transport systems, yeast strains CDBT2 and CDBT7 were cultured in ECC in the following combinations; (i) CDBT2/CDBT2 in anodic/cathodic compartments; (ii) CDBT2/CDBT7 in anodic and cathodic compartments and (iii) co-culture of CDBT2 and CDBT7 in both compartments. In combination of CDBT2/CDBT2, it was found that CDBT2 yeast strain produced more ethanol in the anodic compartment (15.5 ± 0.14 $mg{\cdot}mL^{-1}$) as compared to cathodic compartment (13.4 ± 0.07 $mg{\cdot}mL^{-1}$). The average ethanol production in this combination was 14.4 ± 0.15 $mg{\cdot}mL^{-1}$ (**Table 2**) and the increase in ethanol production was 27.5 ± 0.44%. In combination of CDBT2/CDBT7, CDBT2 strain again produced more ethanol in anodic chamber (15.10 ± 0.28 $mg{\cdot}mL^{-1}$) as compared to CDBT7 strain in cathodic compartment (12.5 ± 0.50 $mg{\cdot}mL^{-1}$). The average ethanol production in this combination was 13.8 ± 0.56 $mg{\cdot}mL^{-1}$ (**Figure 5A**, **Table 2**) and the increase in ethanol production was 21.5 ± 0.71%. When CDBT2 and CDBT7 strains were co-cultured in both compartments, ethanol production was significantly lower as compared to individual cultures.

Effect of Immobilization of Yeast

Yeast strain CDBT7 immobilized in presence of calcium alginate on cathode, when used for ethanol production in an ECC with CDBT2 strain in anodic compartment, produced 12.6 ± 0.42 $mg{\cdot}mL^{-1}$ and 4.95 ± 0.07 $mg{\cdot}mL^{-1}$ ethanol by CDBT2 and CDBT7 strain respectively without the applied voltage i.e., there was a significant decrease in ethanol production (**Figure 5B**). The same system when used in ECC with the application external voltage input (4V), the ethanol production was significantly enhanced. The ethanol production in the latter case were 16.1 ± 0.49 $mg{\cdot}mL^{-1}$ (CDBT2 in anodic compartment) and 7.75 ± 0.5 $mg{\cdot}mL^{-1}$ (CDBT7 strain in the cathodic compartment), respectively. The average ethanol production was 11.5 ± 0.70 $mg{\cdot}mL^{-1}$ and the increase in ethanol production was 36.2 ± 0.54% than without voltage supply. However, the average ethanol production in ECC was less than without immobilization of CDBT7 in cathode. In summary, immobilization did not show the better enhancement in ethanol production.

Effect of Electron Transport Enhancing Materials

Coating of platinum electrode with fine particles (nanoparticles) of platinum using hexachloroplatinate (IV) and graphite cathode with neutral red, and culturing CDBT2 strain in anodic

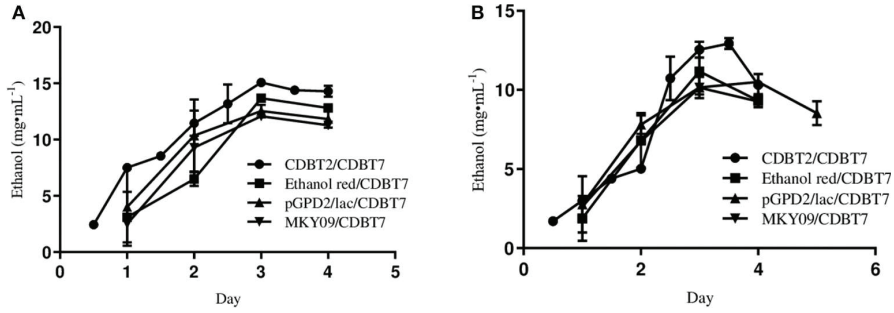

FIGURE 4 | Ethanol production by various *S. cerevisiae* strains cultured in anodic compartment of electrochemical cell with CDBT7 cultured in cathodic compartment: a comparative study. **(A)** Ethanol production in anodic compartment. **(B)** Ethanol production in cathodic compartment.

TABLE 2 | Ethanol production in an ECC with various combinations of anodic and cathodic systems for electron transport enhancement: summary.

S. no.	Culture types*	Ethanol in anodic chamber ($mg \cdot mL^{-1}$)	Ethanol cathodic chamber ($mg \cdot mL^{-1}$)	Average ethanol ($mg \cdot mL^{-1}$)	Average increase (%)
(A) YEAST COMBINATIONS					
1	CDBT2/CDBT7 Normal Fermentation with no Applied voltage	12.60 ± 0.42	10.10 ± 0.14	11.3 ± 0.44	
2	CDBT2/CDBT7 in ECC with 4V	15.1 ± 0.28	12.5 ± 0.49	13.8 ± 0.56	21.5 ± 0.71
3	CDBT2/CDBT2 in ECC with 4V	15.5 ± 0.14	13.4 ± 0.07	14.4 ± 0.15	27.5 ± 0.44
4	CDBT2+CDBT7 mixed/co-culture with 4V	13.2 ± 0.0	10.4 ± 0.71	11.8 ± 0.71	3.96 ±0.83
(B) IMMOBILIZED YEAST					
1	CDBT2/CDBT7 with CDBT7 Immobilized on Graphite Cathode without Applied Voltage	12.6 ± 0.42	4.95 ± 0.07	8.77 ± 0.42	
2	CDBT2/CDBT7 with CDBT7 immobilized on graphite cathode and 4V supply	16.1 ± 0.49	7.75 ± 0.50	11.9 ± 0.70	5.3 ± 0.82
(C) ELECTRON TRANSPORT SYSTEMS					
1	CDBT2/CDBT7 Pt coated Pt anode/graphite cathode	15.7 ± 0.16	14.0 ± 1.06	14.8 ± 1.07	30.9 ± 1.15
2	CDBT2/CDBT7 Pt anode and neutral red coated cathode	17.1 ± 0.07	15.7 ± 0.30	16.4 ± 0.30	44.6 ± 0.53
3	CDBT2/CDBT7 Pt coated Pt anode/neutral red coated cathode	17.5 ± 0.01	17.2 ± 0.01	17.3 ± 0.01	52.8 ± 0.44

**Normal fermentation: Yeast cultured in ECC without external voltage supply. The yeast strain listed first was used in the anodic compartment and the organism listed second was cultured in the cathodic compartment. Values reported are mean ± SD of 3 different independent experiments.*

compartment and CDBT7 strain in cathodic compartment produced 17.5 ± 0.01 $mg \cdot mL^{-1}$ and 17.2 ± 0.01 $mg \cdot mL^{-1}$ ethanol, respectively (**Figure 5C**). A significant increase in ethanol production (52.8 ± 0.44%) was observed than normal fermentation without voltage supply (**Table 2**).

Electrochemical Ethanol Production From *Saccharum spontaneum* Hydrolysate

When lignocellulosic (*Saccharum spontaneum)* hydrolysate with 3.3% reducing sugar was used as substrate for fermentation in ECC, there was significant enhancement in ethanol production in anodic (9.0 ± 0.1 $mg \cdot mL^{-1}$; increase = 60.8 ± 0.10%) as well as in cathodic (11.9 ± 0.05 $mg \cdot mL^{-1}$; increase = 63.0 ± 0.07%) compartments, with an average enhancement of 61.9 ± 0.12% than normal fermentation without voltage supply. Ethanol production by CDBT2 and CDBT7 without external voltage supply were 5.6 ± 0.03 and 7.3 ± 0.06 $mg \cdot mL^{-1}$, respectively (**Figure 6**, **Table 3**). The increase in amount of ethanol produced, when the fermentation substrate was lignocellulosic hydrolysate, was much higher when compared to fermentation with glucose as substrate under identical conditions. In addition, when the membrane barrier was changed to cellulose acetate in place of nafion membrane, there was a further increase (6.30 ± 0.22%) in ethanol production (**Figure 7**, **Table 3**).

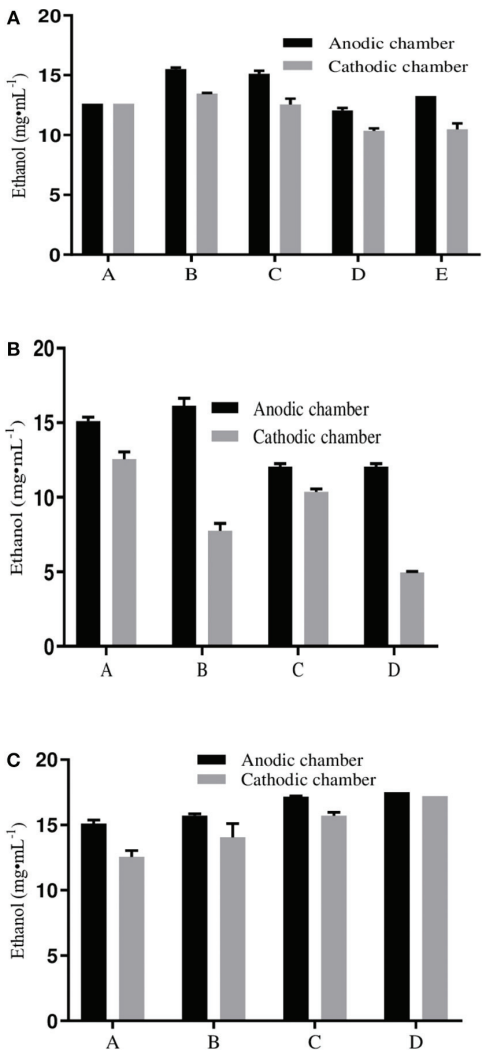

FIGURE 5 | Ethanol production with different electron transport enhancement systems. **(A)** Use of yeasts in various combinations in ECC (A: CDBT2 normal fermentation; B: CDBT2/CDBT2 in Electrochemical cell; C: CDBT2/CDBT7 in Electrochemical cell, D: CDBT2/CDBT7 normal fermentation, E: CDBT2/CDBT7 mixed culture). **(B)** Use of graphite electrode immobilized with CDBT7 yeast strain [A: Graphite cathode/Pt anode; B: Graphite cathode (immobilized with CDBT7)/Pt anode; C: Normal fermentation CDBT2/CDBT7; D: Normal fermentation CDBT2/CDBT7 immobilized in graphite]. **(C)** Use of electrodes coated with different electron transporters (A: Normal Platinum anode/graphite cathode; B: Platinum coated Platinum anode/graphite cathode; C: Neutral red coated graphite cathode/Platinum anode; D: Neutral red coated cathode/Pt coated anode).

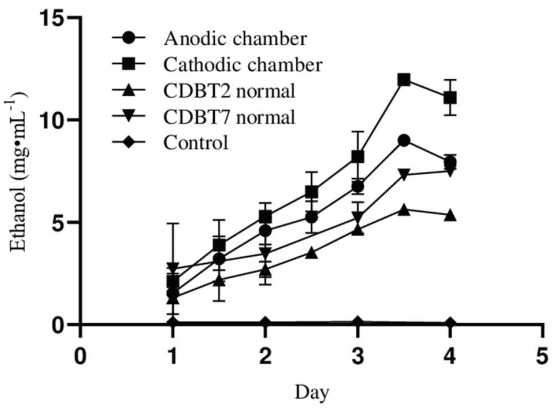

FIGURE 6 | Ethanol production from lignocellulosic hydrolysate (*S. spontaneum* pretreated with hot water for 2 h followed by 0.5M acid hydrolysis) in ECC with CDBT2 and CDBT7 strains cultured in anodic and cathodic compartments, respectively (Control: ECC without yeast culture and without external voltage supply. Normal: ECC with yeast culture and without external voltage supply).

DISCUSSION

S. cerevisiae CDBT2 and *W. anomalus* CDBT7 yeast strains were selected for the study because both are good ethanol producers and the later has been shown to be capable of converting xylose into ethanol. The pH optima for ethanol production using yeast strains CDBT2 and CDBT7 were pH 5.5 and temperature 30°C, respectively. The conditions were consistent with our previous experiments with optimization of yeast strains CDBT2 (Joshi et al., 2014). The ECC system with CDBT2 in anodic compartment and CDBT7 in cathodic compartment, produced the highest amount of ethanol as compared to reverse system tested. This may be because CDBT7 strain forms film on graphite cathode resulting in fast electron transport through cathode. This was further supported by the fact that (i) SEM images of cathode surface clearly show CDBT7 biofilm formation (**Figure 3**), and (ii) use of CDBT7 immobilized cathode resulted in significant fold increase in ethanol production when cultured in ECC (**Table 2**) (Toit and Pretorius, 2000). Further, the study by Mohamoud (2014) suggests that supply of external voltage enhances film redox potential and thus results in enhancing more electron transport and more ethanol production. According to that study there was 40% enhancement in film redox potential when electricity was passed to electrode with polyaniline/polyvinyl composite. Canelas et al. (2008) observed that ethanol formation requires the maintenance of NAD^+/NADH ratios. In healthy eukaryotes, NAD^+/NADH ratios usually are relatively high and the range varies widely (60–700). Canelas et al. (2008) also observed that the cytosolic free NAD^+/NADH ratio in *S. cerevisiae* under steady and highly dynamic state ranges between 101 ± 14 and 320 ± 45 where as whole cell NAD^+/NADH ratio was 7.5 ± 2.5. Further it was observed that in *S. cerevisiae* NAD^+/NADH ratio was reduced when there is presence of electron donor and the ratio was increased in the presence of electron acceptor. In our case, when yeast strain was cultured in cathodic compartment, there was increment in ethanol production because the cathode was the electron donor, thus resulting more conversion of NAD^+ to NADH

which has directed the conversion of pyruvate to ethanol. Reversibly when yeast strain cultured in anodic compartment which is electron acceptor, converted NADH to NAD^+ which had directed fast conversion of glucose to pyruvate so that we could see enhancement in growth and ethanol formation as well. In spite of the ratios reported above, eukaryotes could survive even when the NAD^+/NADH ratio was as low as 7–10 (Veech et al., 1972). The supplied external voltage input polarizes ions present in cytosol and as a result positively charged NAD^+ ion bound to cell membrane make the transfer of electrons from cathode easier and faster (Gunawardena et al., 2008; McGillivray and Gow, 2009) thus makes it easy to access for reduction of NAD^+ to NADH in cathode resulting more ethanol production. According to Yau et al. (2013), the applied voltage was believed to polarize ionic charges in yeast cells, which may lower the tunnel barrier for transferring electrons during glucose oxidation resulting more pyruvate and hence more cell growth and ethanol production.

The pyruvate formed during glycolysis is converted to (i) acetyl CoA under aerobic conditions in eukaryotes, (ii) lactate during homolactic fermentation in mammals, or (iii) ethanol during anaerobic fermentation in yeast and bacteria. When an external voltage was supplied, NAD^+ was directly converted to NADH, increasing the level of NADH. This is known to cause an imbalance in the growth of yeasts that favors production of ethanol by forcing the yeast to convert pyruvate to acetaldehyde and then to ethanol by consuming NADH. It has also been shown that increased NADH allosterically inhibits pyruvate dehydrogenase (Harris et al., 2002) and prevents conversion of pyruvate to acetyl coenzyme A. Accordingly, pyruvate can be diverted to ethanol formation. Song et al. (2014) have shown that external input of voltage can be used to control the kinetics of glucose metabolism in *S. cerevisiae* under both aerobic and anaerobic conditions. Here, intracellular electron carriers such as NAD^+, NADH and the transplasma membrane electron transfer (tPMET) system located in the plasma membrane plays important role for direct transport of electrons through cell membrane. tPMET system consists of cytochromes and various redox enzymes such as NADH oxidase which provides redox activity to the membrane at specific sites.

In reality, for reduction of NAD^+/NADH ratio, applied voltage requirement is around −0.33V (Veech et al., 1972). In our experiments, we can see that optimal ethanol production was obtained at 4V ± 0.1V. The higher voltage, we believe, was because of ohmic drop exerted by the designed ECC. Mathew et al. (2015) have reported the gradual increase in growth by 1.1 fold and ethanol production by 2 fold when *S. cerevisiae* was cultured with external voltage supply till 15V. In our study, increasing voltage beyond 4V reduces ethanol production. This may be because, increasing voltage might oxidize Platinum to Platinum dioxide which acted as insulator and decreased the activity of electrode (Wang et al., 2006). Similarly in cathode, there might be overproduction of hydrogen gas which might decrease the activity of graphite electrode (Hsu et al., 2008). Further, when mixed culture of yeast strains CDBT2 and CDBT7 were used, ethanol production was relatively low. This may be because when *S. cerevisiae* and *W. anomalous* were cultured together, growth of *W. anomalous* may have suppressed the growth of *S. cerevisiae* thus decreased the overall ethanol production (Ruyters et al., 2015).

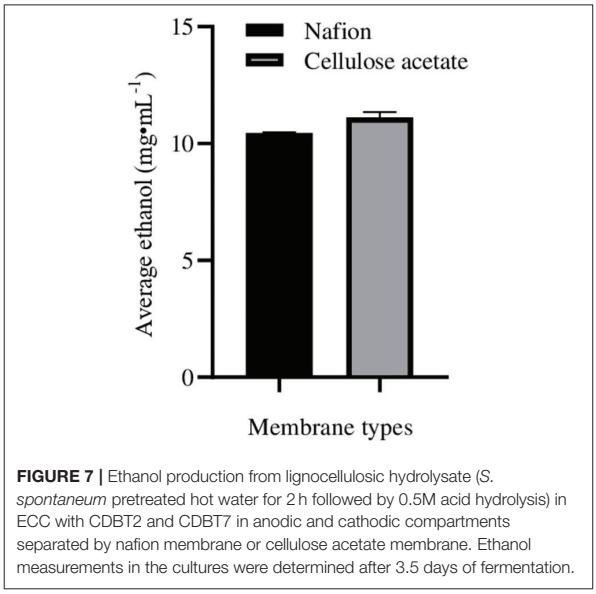

FIGURE 7 | Ethanol production from lignocellulosic hydrolysate (*S. spontaneum* pretreated hot water for 2 h followed by 0.5M acid hydrolysis) in ECC with CDBT2 and CDBT7 in anodic and cathodic compartments separated by nafion membrane or cellulose acetate membrane. Ethanol measurements in the cultures were determined after 3.5 days of fermentation.

Steinbusch et al. (2010) have reported an increase in more than 2 fold enhancement of ethanol production in the presence of an external source of electrical current when acetate was used as substrate for fermentation. He et al. (2016), have reported 60.3% enhancement in butanol production by *Clostridium spp*. when neutral red was used as electron transporter. He et al. have also demonstrated that neutral red increased the butanol production better than other electron transporters such as viologen dyes (dyes containing pyridine groups). Further neutral red can strongly bind to cell membranes (Park et al., 1999). It has a redox potential of about −0.325V which is similar to that of the redox potential of NADH (−0.32 V). Accordingly neutral red could interact with NADH and, thus, increase the level of NAD^+/NADH giving rise to more ethanol. The decrease in ethanol production in immobilized culture was because immobilization reduces substrate diffusion and hence ethanol production was less than in normal growth whereas it enhances fast electron transport at the intersection of the electrode.

The increase in ethanol production due to applied external source of voltage was further more when acid hydrolyzed *Saccharum spontaneum* hydrolysate was used as substrate. This may be because; the NaCl present in the neutralized hydrolysate may help in enhancement of ethanol production by decreasing resistance. In fact, Yang et al. (2015) have shown that when the concentrations of NaCl was increased from 5 to 30 $g{\cdot}L^{-1}$ in the fermentation media, the internal resistances of the system decreases from 2432.0 to 2328.4 Ω. Similarly, Kamcev et al. (2018) also observed that increasing salt concentration increases electrical conductivity hence increases electron flow and reduces resistance. Accordingly, the increased conductivity is likely

TABLE 3 | Ethanol produced by CDBT2 and CDBT7 cultured with lignocellulosic biomass hydrolysate.

S. no.	CDBT2 in AC	CDBT7 in CC	Average production	CDBT2*	CDBT7*
Ethanol ($mg{\cdot}mL^{-1}$)	9.01 ± 0.1	11.9 ± 0.05	10.4 ± 0.11	5.60 ± 0.03	7.30 ± 0.06
% Increase	60.8 ± 0.10	63.0 ± 0.07	61.9 ± 0.12		

AC, Anodic chamber; CC, Cathodic chamber.
**Fermentation under normal conditions (no external source of electricity).*

to promote ethanol production. The increased production of ethanol from lignocellulosic biomass could also be partly due to the presence of various natural products/substance present in the mixture that could promote growth of yeast strains. Alteration of cellulose acetate membrane in place of nafion membrane enhanced ethanol production. The latter could be due to transport of xylose across cellulose acetate membrane from anodic chamber to cathodic chamber, as well as decrease in internal resistance due to ion accumulation on the membrane surface (Tang et al., 2010).

CONCLUSION

In summary, a combination of yeast strains *S. cerevisiae* CDBT2 and *W. anomalous* CDBT7 effectively and efficiently produce ethanol from both glucose and lignocellulosic biomass hydrolysate. Use of CDBT2/CDBT7 strains in an ECC efficiently utilize reducing sugars as indicated by near complete utilization of reducing sugars. Ethanol production by CDBT2 and CDBT7 yeast strains can be enhanced by supplying low levels of external voltage. *S. cerevisiae* CDBT2 was most efficient at ethanol production in the anodic compartment, whereas *W. anomalous* CDBT7 yeast strain was most efficient in ethanol production in the cathodic compartment. Further enhancement of ethanol production was observed when ECC was operated with fine platinum nanoparticles coated on the platinum anode, and neutral red was deposited on graphite cathode. Hot water pretreated and mineral acid hydrolyzed lignocellulosic biomass (an economic method) can be used as substrate for fermentation in ECC with CDBT2 and CDBT7 strains. An additional advantage of using hydrolyzed lignocellulosic biomass was that it further enhances ethanol production in ECC. Given the data reported herein, yeast strain CDBT2 could serve as a potent industrial strain for genetic modification and ethanol production. One of the limitation of this study is to go for further scaled up. It is strongly believed that the scale up of this study is entirely feasible with the availability of large electrochemical fermentation cell and is next phase of our study.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

AUTHOR CONTRIBUTIONS

JJ performed laboratory work and prepared manuscript draft. TB, LS, and AY edited manuscript. PD, BP, PP, MY, MM, and RM helped in laboratory works. All the authors revised the manuscript and approved the submitted version.

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Research Article

Efficient Methods of Pretreatment for the Release of Reducing Sugars from Lignocellulosic Biomass Native to Nepal and Characterization of Pretreated Lignocellulosic Biomass

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ABSTRACT
The lignocellulosic biomass composition of plants differs depending on the locality and seasonal changes. We have evaluated the suitability of four different sources of lignocellulosic biomass, viz.,*Ipomoea cornea, Phragmites karka, Saccharum spontanum*and *Zea mays* cobs for obtaining reducing sugar commercially which can be used for bioethanol production in Nepal. *S. spontaneum* was found to be the best among the four as a cheap source of lignocellulosic biomass since it has better degradation capabilities, high calorific value and relatively high total reducing sugar (TRS) content (612.2±11.5 mg g^{-1} biomass). *S. spontaneum* biomass was pretreated with 0.5M sulphuric acid, 0.5M ammonium hydroxide, 1-butyl-3-methyl imidazolium chloride, butadiene sulphone and hot water and further subjected to acid or enzymatic hydrolysis to determine total sugar content. Among the pretreatment methods employed, 0.5M ammonium hydroxide at 65^{o}C for 12 h followed by cellulase enzyme hydrolysis (TRS = 347.3±55.6 mg g^{-1}) and hot water pretreatment at 100^{o}C for 2 h followed by hydrochloric acid hydrolysis (TRS = 330.4±20.5 mg g^{-1}) were found to be the best, although the latter method is more economical. The variations in characteristics of lignocellulosic biomass before and after pretreatment with hot water at100^{o}C for 2h were investigated by thermo gravimetric analysis (TGA), X-ray diffraction (XRD) and FTIR methods. The XRD and FTIR analysis showed that pretreatment reduced the amorphous nature of cellulose and increased crystalline characteristics. The content of glucose (untreated:246.7±4.0 and pretreated:235.1±5.0 mg g^{-1} biomass) and xylose (untreated:86.6±3.9 and pretreated: 62.5±3.0 mgg^{-1} biomass) determined in untreated and pretreated (hot water at 100^{o}C for 2 h) suggests that while the cellulose loss during pretreatment is minimal, hemicellulose content is lost significantly.

Key Words: lignocelluloses;*S. spontanum*; pretreatment; hydrolysis; cellulose; hemicellulose.

I. INTRODUCTION
Dependence on petroleum-based transportation fuels continues to be a major challenge for developing countries. The challenge is further enhanced if the country happens to be landlocked and has no fossil fuel reserves. Accordingly, landlocked countries such as Nepal have to spend a major share of their resources to import fossil fuels via other counties. This leads to severe threat of energy security of the country. Accordingly, serious attempts are being made to develop alternative energy sources including bioethanol in Nepal [24].

Currently, majority of global ethanol production is based on starch and sugarcane, which compete

with their use as food and/or feed. Lignocellulosic biomass has emerged as an attractive alternative sugar source for ethanol production. Lignocellulosic biomass such as dendromass and phytomass are nature based materials consisting of complex heterogenic macromolecules such as celluloses, hemicelluloses and lignin, as well as numerous other low molecular mass organic and inorganic components that can be used for ethanol production [23].

Ipomoea cornea (locally known as Ajamari), *Phragmites karka* (locally known as Narkat), *Saccharum spontanem (*locally known as Kans) and *Zea mays* (corn) cobs are good source of lignocellulosic biomass. The first three are commonly found weeds, grown naturally in abundance in both the hilly and plain regions of Nepal that may be harvested periodically with relative ease. *I.cornea* is a fast growing species with ability to absorb Cd and Hg. Accordingly, this species is used for bioremediation of heavy metals in polluted soils [14]. *S. spontaneum* is one of the widely distributed lignocellulosic biomass that grows very fast giving high forage yield and seed production. *S. spontaneum* is believed to have originated in India and is distributed widely in tropical and subtropical regions of Asia. It grows on river banks, road sides, waste lands and even in sandy soils [9]. Medicinal and Aromatic Plants Network (MAP) of Nepal has given the taxonomic ID 1307 for the Nepali variety of *S. spontaneum* [16].*Z. mays* is a field crop grown all around the world and its cob (the central core of corn shoot) is an agricultural waste that is thrown away as garbage in farm lands, waste bins and along road side. Corn cobs are known to contain high energy content among 12 different types of biomass tested by Helsel and Wedin [6].

The lignocellulosic biomass obtained from the plants is highly recalcitrant, accordingly effective release of sugars from them is a major challenge. Therefore, the saccharification of the biomass needs an effective pretreatment technique so as to expose cellulose and hemicellulose for efficient hydrolysis [37]. Pretreatment methods result in different effects on the biomass in terms of structure and composition. Further pretreatment procedures vary for the same plant species depending onthe growing conditions and the region from which they originate. In addition, parameters such as (i) obtaining highly digestible pretreated solids (ii) high biomass concentration (iii) no significant sugar degradation (iv) formation of a minimum level of toxic compounds (v) high yield of sugars after subsequent hydrolysis(vi) fermentation compatibility (vii) operation in reasonably sized and moderately priced reactors (viii) lignin recovery with minimum heat and power requirements etc., need to be considered and optimized for the biomass used [2]. Accordingly, with an ultimate goal of developing proper procedures for commercial production of ethanol from local biomass sources (agricultural wastes) in Nepal, in this paper, we describe the best biomass source for fermentable sugars and proper pretreatment procedures to be used to get maximum total reducing sugar yields. Total reducing sugar content in the biomass tested after acid and/or enzymatic hydrolysis of differently pretreated biomass. The changes in biomass characteristics before and after pretreatment were examined by thermal degradation analysis (TGA), X-ray diffraction analysis (XRD), Infra-red spectral analysis (FTIR) and the calorific values together with the analysis of difference in reducing sugar, phenol and furfural content before and after pretreatment.

II. MATERIALS AND METHODS

2.1. Sample collections and preparation

Biomass samples of *Ipomoea cornea, Phragmites karka, Saccharum spontanem* and *Zea mays* (corn) cobs were obtained from Tribhuvan University campus, Kirtipur, Kathmandu, Nepal during the month of August. The sampling location was 27.6818^{o}N and 85.2865^{o}S. In the case of first three species, the aerial portion of the biomass were harvested, air dried for 24 h. The biomass was then cut into small pieces with average length of 2 cm. *Zea mays* (corn) cobs were also collected from the same location as above, however, the corn kernels were first deseeded to prepare the

cobs. The biomass was dried in a hot air oven at 60°C for 24 h. It was then ground and sieved to get powder with a particle size of 250-500 µm. The processed biomass was packaged and sealed in plastic bags and stored in a cool and dry cabinet at room temperature for further analysis.

2.2. Thermo gravimetric analysis (TGA) of biomass

Component degradation pattern were analyzed using Thermo Gravimetric Analyzer (STA 7200 HITACHI, Japan) [16]. Dynamic thermo gravimetric scans were conducted in the temperature range 30 to 900°C at a heating rate of 10°C min^{-1}. The experiments were carried out under nitrogen atmosphere at a flow rate of 100 ml min^{-1} with biomass load of approximately 10 mg. Moisture and ash contents were also determined using TGA curve according to UNI EN 14774-2 [33] and UNI EN 14775 [34] methods respectively.

2.3. Determination of calorific value

Bomb calorimeter (Parr Instrument Company, 2007) was used for determination of calorific value [10]. Briefly, ~1 g biomass was placed in contact with nichrome wire tied with cotton thread. The assembly was placed in the ignition port. The bomb calorimeter was filled with 20 bar oxygen at 25°C. The bomb calorimeter was then submerged in a container filled with distilled water. The calorimeter jacket was maintained at temperature 25°C. The heat released was measured in terms of temperature change. The calorific values of samples were calculated using the equation, $H_{fs} = [(C.\Delta T_{fs} - m_{ct}.H_{ct})/m_{fs}]$, where, ΔT_{fs} was the observed change in temperature (°C), m_{ct} was mass of cotton thread (g), H_{ct} was calorific value of cotton thread (J g^{-1}), m_{fs} was mass of sample (g) and C was heat capacity of benzoic acid (standard). $C= [(m_{ba}. H_{ba} + m_{ct}.H_{ct})/ \Delta T_{c.}]$ where, m_{ba} was mass of benzoic acid (g), H_{ba} was calorific value of benzoic acid (J g^{-1}) and ΔT_c was observed change in temperature (°C)

2.4 Chemical composition analysis of biomass

Structural carbohydrate contents of biomass were determined by two stage acid hydrolysis method as described in NREL Standard Biomass Analytical Procedure [19]. The stored biomass was further ground and sieved to 60 µm size and subjected to acid hydrolysis. Total structural carbohydrate in the aqueous portion of the hydrolyzed samples was analyzed by di-nitro salicylic acid (DNSA) method as described by Miller, 1959 [18]. Further, glucose, xylose, arabinose, 5-hydroxy methyl furfural and furfural were determined using HPLC (Agilent, Inc., USA) fitted with Metacarb 87H ion exchange column (300 x 6.6 mm) and Refractive Index detector. The column was eluted at a flow rate of 0.5 ml min^{-1} using 0.00065M H_2SO_4 at 60°C [19].

2.5. Pretreatment of Biomass

Various procedures delineated below were used to determine the pretreatment efficiencies of biomass. An aliquot from pretreated biomass was collected by vacuum filtration and chemical analysis were performed to determine reducing sugars, phenols and furfural released from the biomass as described under chemical analysis procedures above. All treatment procedures involved 10 g biomass mixed with 100 ml of pretreatment solvent in 250 ml stoppered flasks with constant stirring at 25°C, 65°C and 100°C for up to 24 h. All pretreatments were carried out in triplicates. Small aliquots were withdrawn at different time intervals and analyzed for sugar content as described below. Pretreatment was also performed at 120°C as above, but the incubation was done for up to 2.5 h only and the samples were analyzed at 0.5 h intervals. The pretreatment solvents used were (i) 0.5M H_2SO_4 (Sulfuric acid) (ii) 0.5M NH_4OH (ammonium hydroxide) (iii) water (iv) 1-butyl-3-methyl imidazolium chloride with concentrations of 40%, 60%, 80% respectively and (v)

butadiene sulfonate of 50% and 100% concentrations, respectively.

2.6. Chemical analysis of pretreated biomass

2.6.1: Determination of total reducing sugars (TRS)

The supernatants obtainedafter pretreatment were analyzed for TRS using di-nitro salicylic acid (DNSA) method. Briefly, 200 mm^{-3} of pretreated supernatant was added with 200 $mm^{-3}$of DNSA reagent. The aliquot incubated in a boiling water bath for 10 min, cooled on ice-bath for 5 min followed by addition of 2 cm^{-3} distilled water. D-glucose (Sigma) was used as standard. The absorbance due to reducing sugars was measured at 540 nm.

2.6.2: Determination of phenol content

Total phenol content in the supernatants were analyzed using Singleton method [28]. A 100 $mm^{-3}$of sample was mixed with 1.0 cm^{-3} of 10% Folin Ciocalteu reagent followed by 0.8 $cm^{-3}$of 0.1g cm^{-3}sodium carbonate solution. The aliquot was incubated for 15 min at room temperature and absorbance was measured at 765 nm. Gallic acid (successively diluted solutions of 250 µg cm^{-3} stock) was used as a standard.

2.6.3: Determination of furfural content

Furfural present in supernatants were determined by the method described by Al Showiman [1]. A 0.1 cm^{-3} aliquot of the sample was mixed with 0.4 cm^{-3} of 50% ethanol, 20 mm^{-3} aniline, 5 mm^{-3} of 37% HCl and 0.8 cm^{-3} distilled water. The absorbance of the sample was measured at 530 nm. Furfural (successively diluted solutions of 30 µgcm^{-3} stock) solutions prepared in 50% ethanol were used to develop a standard curve.

2.7. Hydrolysis of pretreated biomass

The solid residue obtained after filtration of pretreated biomass was washed with distilled water several times until the washes reached neutral pH (tested using blue and red litmus paper). Thus obtained solids were first air dried for 24 h at room temperature followed by drying in hot air oven at 60°C for 24 h. The dry pretreated biomass was then stored in sealed plastic pouches for further analysis.

2.7.1. Acid hydrolysis

Aliquots of dried samples (1 g each) were mixed with 10 cm^{-3} of 20 mg cm^{-3} HCl(Hydrochloric acid) and incubated at 90°C for 24 h in water bath. After 24 h of hydrolysis, the samples were cooled on ice-water bath, vacuum filtered and the supernatants were tested for TRS, phenol and furfural as described above.

2.7.2 Enzymatic hydrolysis

Enzymatic hydrolysis was performed by incubating 1 g pretreated biomass with10 cm^{-3} of diluted (10FPU $g^{-1}$of biomass) cellulase enzyme (*Trichoderma resii,*Sigma Chemical Co., St Lous, MO, USA) at 45°C for 24 h. The samples were then cooled on ice-water bath, vacuum filtered and the supernatants were tested for TRS, phenol and furfural.

2.8.Comparative analysis of untreated and pretreated biomass

2.8.1. X-Ray diffraction (XRD) analysis of biomass

Powder X-ray diffractometer (Bruker, Germany) was used to determine the crystallinity of untreated and pretreated biomass. CuKα (λ=0.154) was used as radiation source at 40 kV and 30mA. Biomass samples were scanned at a speed of 1°min^{-1} for 2θ ranging from 4° -40° with an increment of 0.04° and rotation speed of 200 rpm. XRD crystallinity index (CIXRD) was determined using the peak height method as described [32].

2.8.2FTIR analysis of biomass

The biomass (untreated and treated) sample-KBr pellets were prepared by thoroughly mixing 2 mg biomass with 200 mg KBr at 20 bar pressure. The pellets were scanned between 4000 to 400 cm^{-1} with 4cm^{-1} resolution using FTIR spectrometer (Perkin Elmer, USA). The background spectrum of pure KBr was subtracted from the sample spectrum [36].

3. RESULTS AND DISCUSSION

3.1. Characterization of Biomass

The four different biomass samples were characterized for their sugar contents, thermal

degradation pattern, calorific value, moisture and ash content before pretreatement (Table 1).The data shows the presence of maximum sugar content in *S. spontaneum.* Glucose and total reducing sugar (TRS) content were 296.8±4.1and 612.2±11.5 mg g^{-1} biomass respectively. The TRS reported herein are in between the values reported (539.1±0.55 mg g^{-1} of *S. spontaneum*) by Chandel and associates [4] and (68% on oven dry weight basis of *S. spontaneum*) by Singh *et al* (2011) [25]. A number of varieties of *S. spontaneum* are known to grow around the world and Switch grass is one such variety. According to Kataria et al., [11] without excreting pressure on the land, it is estimated that 1318 million barrels of ethanol can be produced using Switch grass alone. It grows in open land without any special effort, accordingly a good source of fermentable sugars to produce bioethanol and other value added products.

Table 1: Analysis of untreated (crude) biomass for various sugars, fermentation inhibitory substances, calorific value, moisture and ash content.*

Parameter analyzed	Biomass analyzed			
	I. cornea	***P. karka***	***S. spontaneum***	***Zea mays* (cobs)**
TRS (mg g^{-1} biomass)	560.7±18.6	492.4±6.5	612.2±11.5	580.8±8.0
Glucose (mg g^{-1} biomass)	200.3±10.6	223.0± 5.1	246.7 ± 4.0	232.6±16.3
Xylose (mg g^{-1} biomass)	27.0±1.0	78.3±3.5	86.6±3.9	85.2±5.5
Arabinose (mg g^{-1} biomass)	0.9±0.1	6.9 ± 0.8	6.5 ± 1.0	10.9 ±1.0
5-HMF (mg g^{-1} biomass)	82. 7±3.6	78.2 ± 5.2	72.9 ± 3.9	90.8 ± 3.0
Furfural (mg g^{-1} biomass)	2.19±0.01	10.6 ± 1.0	5.7 ±0.8	12.1 ±1.0
Calorific value (cal g^{-1})	4126± 228	4235± 42	4419± 224	4784± 273
Moisture (%)	6.1	6.7	6.3	8.3
Ash (%)	4.4	4.1	7.8	1.7

*Biomass samples hydrolyzed (acid hydrolysis) were analyzed for glucose, xylose, arabinose, 5-hydroxymethyl furfural and furfural concentrations by HPLC method using a refractive index detector. Calorific valuewasdetermined using a bomb calorimeter, moisture and ash content were determined by TGA as described in materials and methods.

The differential thermogravimetric curve presented (Figure 1) revealed that *S. spontaneum* has fast degradation capability followed by *P. Karka, I. cornea and Zea mays (cobs)* indicating that less energy is needed for degradation of *S. spontaneum* biomass. The main peak in the thermogravimetric curve corresponds to degradation of cellulose in lignocellulosic biomass and shoulder peak at lower temperature corresponds to the degradation of hemicelluloses. The sharp peak for *S. spontaneum* nearly at 300°C corresponds to high cellulose content, as well as fast degradation of cellulose in biomass. Lignin decomposes in the temperature range of 300–500°C and shows heterogeneous peaks due to its heterogeneity and lack of a defined primary structure. The remaining constant mass beyond this range represents ash content [26].

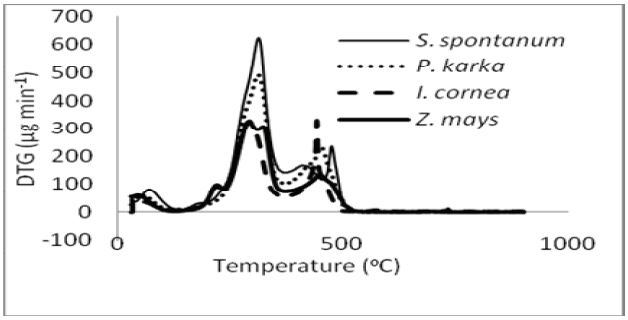


Figure 1: Differential thermogravimetric analysis of *S. spontaneum* biomass. Preparation of biomass samples and their differential thermogravimetric analysis with continuous change of temperature at a rate of 10°C min^{-1} as described in materials and methods.

3.2. Pretreatment and hydrolysis of *S. spontaneum*

Given the results described above in table 1 and figure 1, *S. spontaneum* is good source for fermentable sugars and it also exhibits high calorific value. Accordingly, it was used as a biomass source for further pretreatment processing in the study. The biomass was subjected to pretreatment with sulphuric acid, ammonium hydroxide, hot water, various concentrations of 1-butyl-3-methyl imidazolium chloride (40, 60 and 100%) and butadiene sulphone (50 and 100%) at 25°C, 65°C, 100°C and 120°C. Post pretreatment, samples were withdrawn at different intervals of time, the solids were hydrolyzed by Hydrochloric acid or 10 FPU (filter paper unit) cellulase enzyme. The liquid portion of the pretreated samples were analyzed for TRS, phenols and furfural. The results of these analysis are shown in Figures 2a, 3a, 4a (TRS); 2b, 3b, 4b (Phenols) and 2c, 3c, 4c (Furfural). Lower concentration of acid and base were used in this study as higher concentrations, although increase delignification process, but decrease in the recovery of total reducing sugars [30].

In this study, during treatment, highest TRS release was measured in hot water pretreatment at 100°C for 2 h followed by acid hydrolysis Hydrochloric acid for 24 h at 90°C that released 330.4±20.5 mg TRS, 1.54 mg phenol and 88.88±0.07 µg furfural respectively per gram biomass. Pretreatment with ammonium hydroxide for 12 h followed by cellulase enzyme hydrolysis at 65°C released 347.26±55.6 mg TRS, 2.03 mg phenol and 19.75±4.27 µg furfural respectively per gram biomass, however this method is found to be costly than earlier as the increment in TRS is very less. In a similar study, Kataria and associates [12] showed the release of 350 mg TRS per gram *S. spontaneum* biomass when pretreated with 0.5% NaOH at 120°C for 2 h and the biomass was hydrolyzed by 10 FPU cellulase. In another study, Kataria and Ghosh [11] showed the release of 69.08 mg TRS per gram biomass of *S. spontaneum* when pretreated with 2% H_2SO_4 for 90 min at 120°C and the biomass was hydrolyzed with 10 FPU cellulose showing that dilute acid pretreatment of *S. spontaneum* biomass is not a good method for the efficient recovery of fermentable sugars. In a relatively recent study, Singh and associates [27] showed the dilute acid pretreatment of biomass followed by enzymatic hydrolysis released 231 mg g^{-1} biomass suggesting that the process is inefficient. This further supports our observations. Cotana and associates [5] used several methods of pretreatment and found that pretreatment with 2.5% NaOH at 100°C for 30 min produced the most efficient TRS recovery (202g TRS per kg biomass). While we have not used NaOH to pretreat *S. spontaneum* biomass, it appears, the alkali method we have described herein is more efficient in recovery of TRS.

The most commonly used acid for pretreatment is sulfuric acid [14]. The concentrated acid, despite being powerful degrading agent is highly corrosive and not used any more. Alkaline pretreatment utilizes lower temperature and pressure as compared to other pretreatment technologies. The advantage is that alkali causes less sugar degradation. Additional advantages include, degradation of ester and glycolytic side chains causing structural alteration of lignin, swelling of cellulose, partial decrystallization of cellulose and partial solvation of hemicelluloses [9]. Comparing our results with other findings the hot water pretreatment at 100°C for 2 h followed by hydrochloric acid hydrolysis seems to be best for TRS release from lignocellulosic biomass. The process is not only simple but also economical since no costly enzymes are used. Alkali pretreatment at 65°C followed by enzymatic hydrolysis is also equally effective method, if the enzymes are cheap. The latter is still a challenge until protein engineering techniques could be exploited to develop more efficient cellulases.

Saccharomyces cerevisiae is the most commonly used organism for ethanol production and is capable of reducing furfural to furan alcohol at low concentrations which helps for detoxification

due to furfural at small amount. The concentrations of furfural, *S. cerevisiae* can tolerate is 3.75 mg cm^{-3} in normal fermentation and 17 g dm^{-3} in membrane bioreactor [20]. According to Palmqvist *et al*. [22] furfural concentration above 3 g dm^{-3} is antagonistic to cell growth. In our study, pretreatment of *S. spontaneum* biomass with hot water at 100°C for 2 h produced only 103.2 µg furfural per gram biomass. This corresponds to 10.3 mg dm^{-3}, which is very low to show any inhibitory effects during fermentation. Similarly, the amount of phenol released was minimal to negligible under all pretreatment reagents and all temperatures in our study.

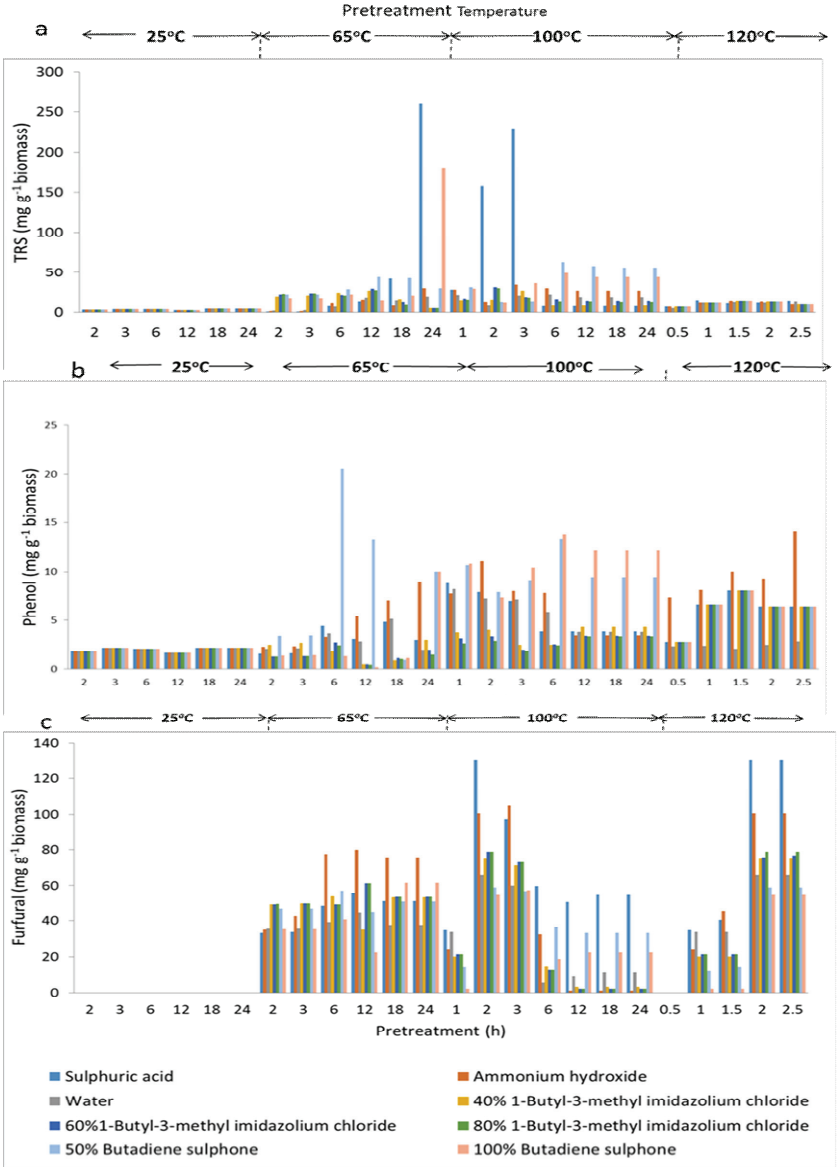


Figure. 2: Product released from *S. spontaneum* biomass pretreated with sulphuric acid, ammonium hydroxide, water, 1-butyl-3-methyl imidazolium chloride and butadiene sulphone. a. TRS b. Phenol c. Furfural. Pretreatments were performed at 25, 65,100 and 120° C as described in materials and methods.

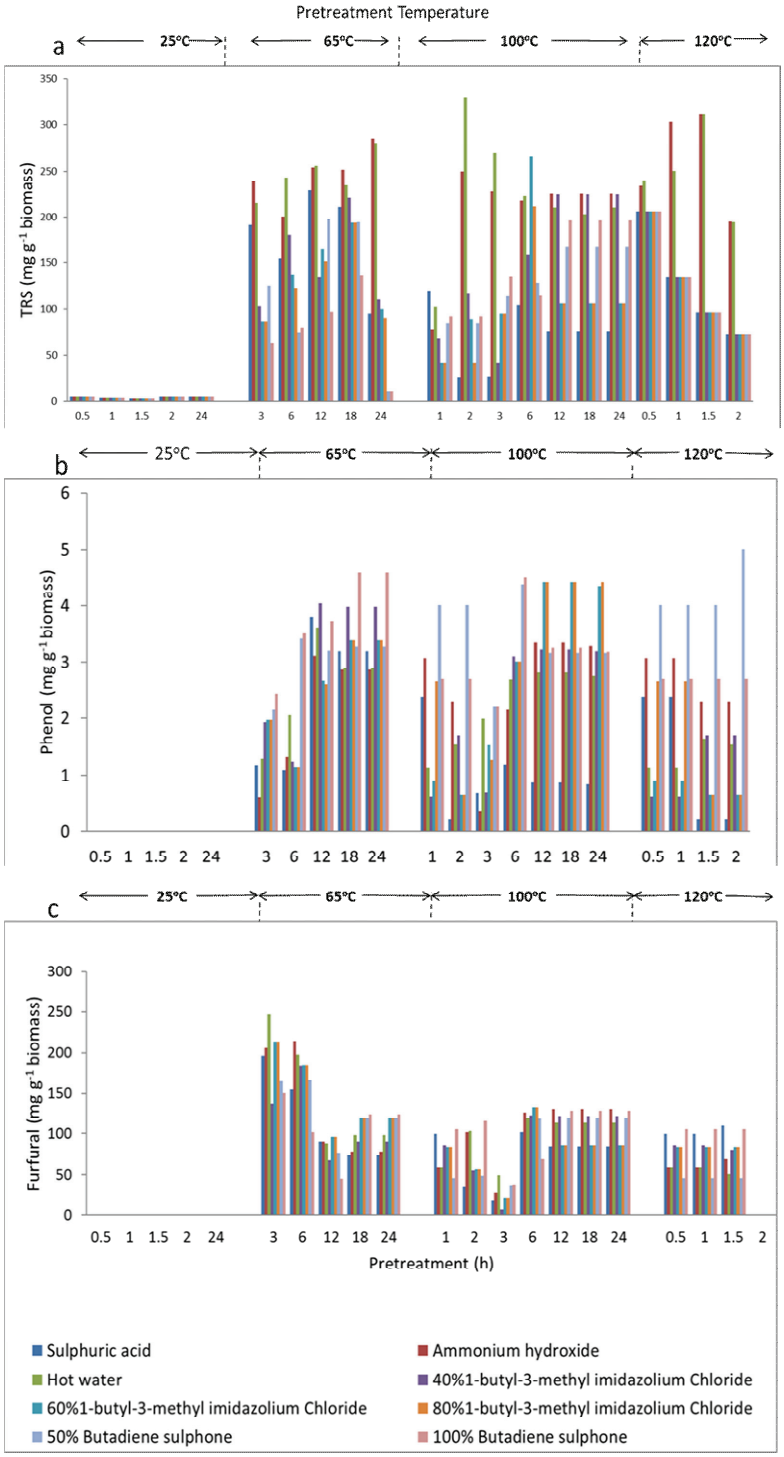


Figure.3: Product released from biomass pretreated with sulphuric acid, ammonium hydroxide, water, 1-butyl-3-methyl imidazolium chloride and butadiene sulphone by Hydrochloric acid hydrolysis. a. TRS b. Phenol c. Furfural. Hydrolysis was performed at 90°C as described in materials and methods.

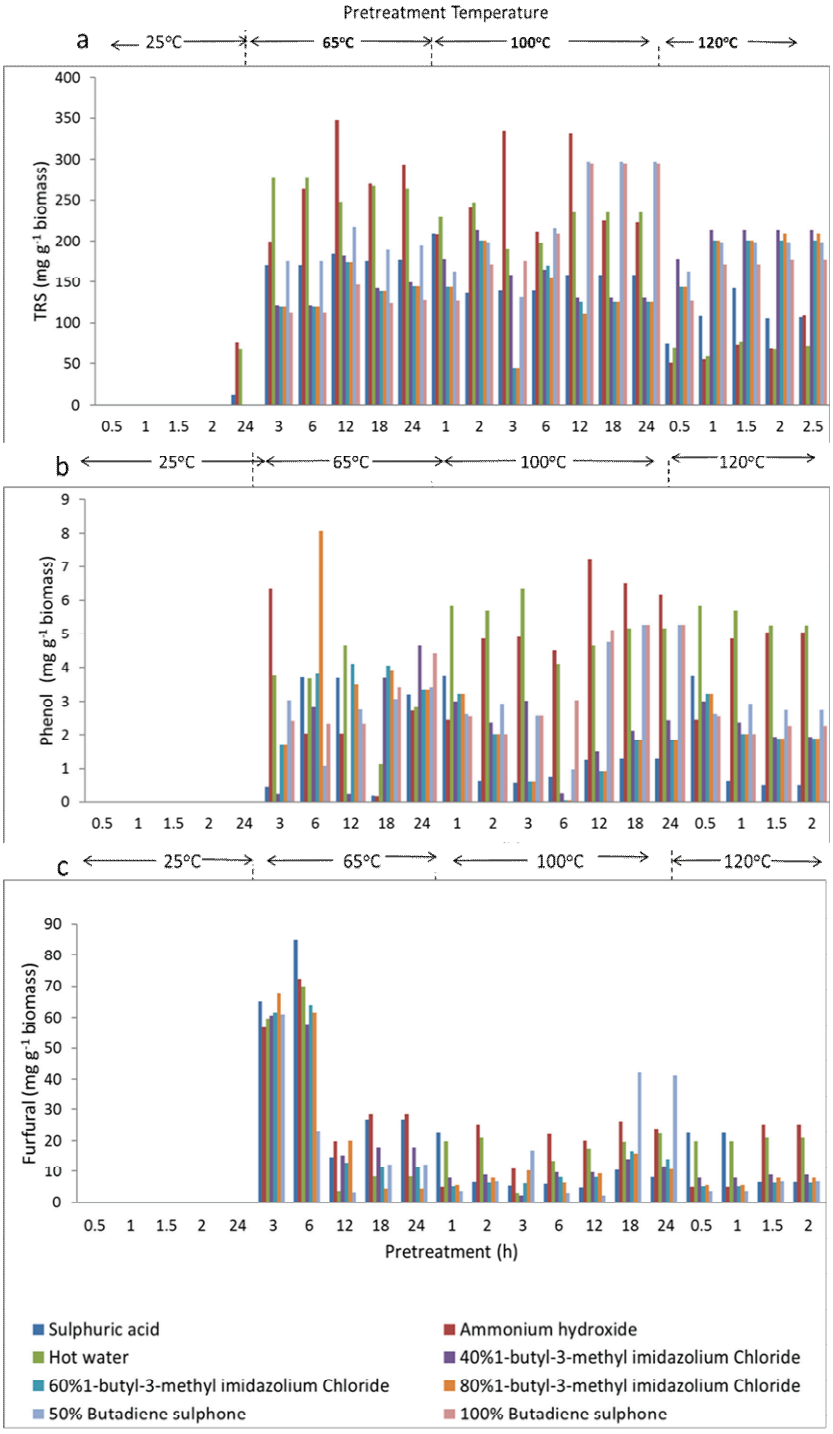


Figure.4: Product released from *S. spontaneum* biomass pretreated with sulphuric acid, ammonium hydroxide, water, 1-butyl-3-methyl imidazolium chloride and butadiene sulphone on 10 FPU cellulase hydrolysis. a. TRS b. Phenol c. Furfural. Hydrolysis was performed at 45^{o}C as described in materials and methods.

The maximum phenol concentration measured was 4.01 mg per gram when the biomass was pretreated with 50% butadiene sulphone and the residue was acid hydrolyzed. The hot water pretreatment for 2 h followed by acid hydrolysis of the residue released only 1.5 mg phenol per gram biomass. The results reported herein also reveal that temperatures above100°C are not necessarily more efficient for pretreatment of biomass. At temperatures above 100°C, the TRS levels are lower than at 150°C and this is likely due to degradation of sugars as well as poor sugar extraction. Temperatures above 160°C favor the unwanted hydrolysis of the cellulosic fraction, and the formation of toxic compounds such as furfural and hydroxyl methyl furfural. It has been shown that ionic liquids display interesting properties such as chemical inertness, good thermal stability, very low to no toxicity and unique salvation ability; accordingly they are considered a good choice of matrices for pretreatment of lignocellulosic biomass. Further, butadiene sulphone in presence of water is believed to efficiently catalyze the breakage of xylan-lignin bonds [3]. The latter could not verified in our study.

3.3 Characterization of untreated (crude) and pretreated *S. spontaneum* biomass

S. spontaneum biomass pretreated at 100°C for 2 h was found to be the best condition for obtaining higher total reducing sugar yields. The untreated and pretreated biomass (hot water at 100°C for 2h) were analyzed by various techniques described below to further characterize the biomass.

3.3.1 Thermogravimetric analysis (TGA)

The remaining (% weight) of hot water pretreated*S. spontaneum* biomass is less than that of crude biomass (Figure 5). The remaining weight is lower due to the removal of metal ions, silica and other components during pretreatment process. The decrease in hemicellulose/lignin and cellulose/lignin peak intensity at around 225 to 325°C and 200 to 500°C indicates the removal of hemicelluloses and lignin during pretreatment. Tapering of hemicellulose/ lignin peak indicates the ease of combustion of biomass due to pretreatment. At temperatures below 380°C, the pretreated biomass was found to be more stable as compared to untreated (crude) *S. spontaneum* biomass. Similarly, pretreated biomass shows a decrease in weight beyond 540°C [35]. They have shown that the pretreated biomass has more crystalline cellulose, which is difficult to degrade. Moisture content in pretreated biomass is somewhat higher (Table 2) and this may be due to increased porosity in pretreated biomass. Ash content of untreated (crude) biomass is higher than pretreated biomass because of the presence of silica and other metal contents.

Figure 5: Differential thermogravimetric curves of untreated (Crude) and pretreated (hot water at 100°C for 2 h) *S. spontaneum* biomass showing the variation in biomass weight with increasing temperature.

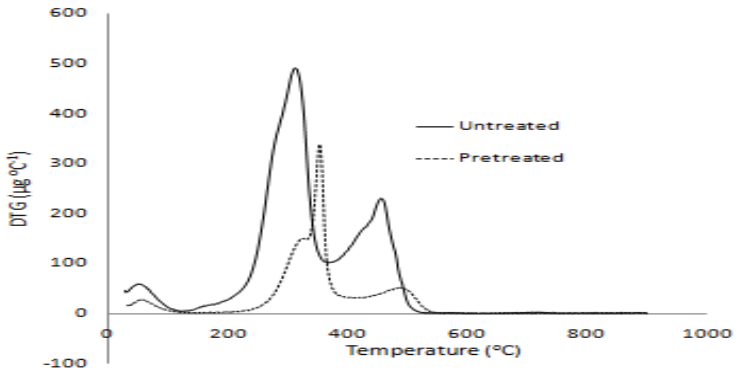


Table 2: Calorific value, moisture contents and ash contents of untreated (crude) and pretreated (hot water at 100°C for 2 h) *S. spontaneum* biomass.

S. No.	Contents	Untreated biomass	Pretreated biomass
1	Calorific value (g $^{-1}$ biomass)	4380±112 cal	4130±60 cal
2	Moisture	6.3%	6.7%
3	Ash	7.8%	4.1%

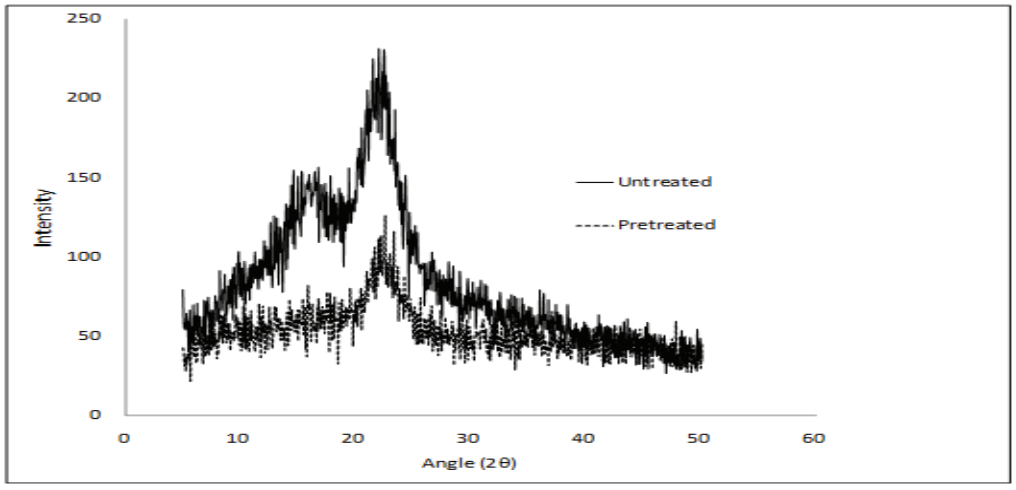
3.3.2 Calorific value determination

Calorific values of biomass before and after pretreatment were 4380±112 and 4130±60 calories respectively (Table 2). The decrease in calorific value of pretreated biomass is due to partial removal of carbohydrate and lipid content during pretreatment and increase in moisture content due to increase in porosity [29].

3.3.4 XRD analysis

The biomass before pretreatment is more amorphous and after pretreatment the amorphous character decreases and this can be investigated by XRD analysis (Figure 6). From the X-ray defractograms, it can seen that the peak at 2θ = 22.4° becomes sharper and while the height of the other peak at 15.5° disappears for the pretreated biomass. This is in turn due to decrease in amorphous nature of biomass [7]. Further, the calculated crystallinity index for the pretreated biomass increased from 41.05 to 45.9. This too is consistent with the previous reports published by Xiao *et al*., 2011 [35]. According to the paper, solubilization of hemicelluloses and lignin together with less ordered cellulose is the main cause of increasing crystallinity.

Figure 6: XRD analysis of untreated (crude) and pretreated *S. spontaneum* biomass. The biomass was pretreated with hot water at 100°C for 2 h as described in materials and methods.



3.3.5 FTIR analysis

The FTIR band at 1163 cm^{-1}corresponds to crystalline cellulose whereas the band at 1156 cm^{-1} corresponds to amorphous cellulose. The FTIR spectra (Figure 7) for untreated (crude) biomass shows a band for amorphous cellulose at 1156 cm^{-1}, whereas this peak is shifted to 1163 cm^{-1}in the pretreated lignocellulosic biomass. Further, FTIR Crystallinity index (CIFTIR) is represented by intensity ratio of 1375/1512. In our samples, the CIFTIR were found to be 0.79 and 0.92 for untreated (crude) and pretreated biomass, respectively, and this is consistent with previous reports [6].

3.3.6 Chemical analysis of untreated (crude) and pretreated biomass

The biomass (untreated and pretreated) were analyzed for cellulose and hemicellulose content using NREL method (2012) as described above which involves the determination of glucose, xylose, arabinose and their derivatives 5-HMF and furfural by HPLC (Table 3). The glucose concentration is an indicator of total cellulose concentration whereas the pentose sugar (xylose and arabinose) are an indicator of total hemicellulose concentration in the biomass [31]. Pretreatment with hot water at 100°C leads to minimal decrease in cellulose content whereas xylose content is significantly decreased, suggesting loss of hemicellulose during pretreatment. This can be further confirmed by significant increases in furfural formation during pretreatment. On the other hand, 5-HMF formation was in smaller amounts and this is inturn due to minimal release of glucose during pretreatment.

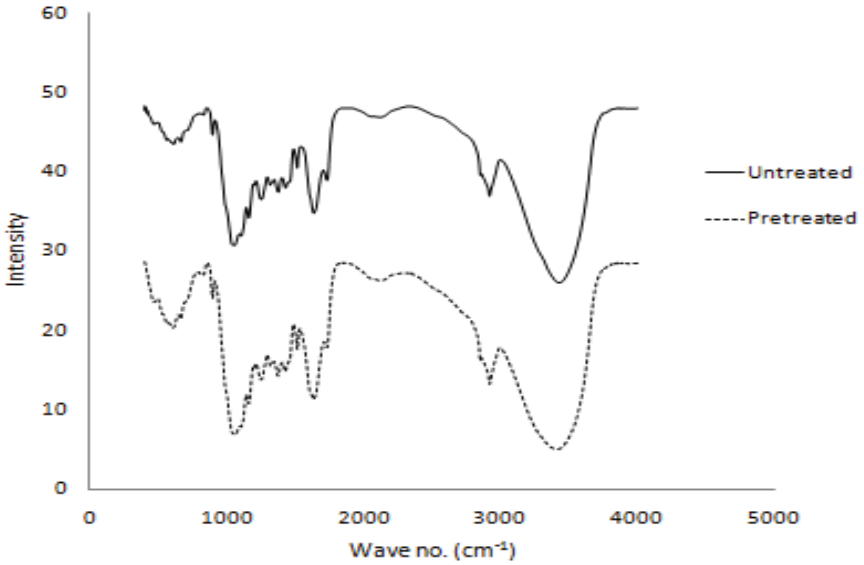


Figure 7: FTIR analysis of untreated (crude) and pretreated *S. spontaneum* biomass. The biomass was pretreated with hot water at 100°C for 2 h as described in materials and methods.

Table 3: Sugar and fermentation inhibitory substance compositions in untreated and pretreated *S. spontaneum* biomass.

Biomass types	Glucose	Xylose	Arabinose	5-HMF	Furfural
Untreated (mg g^{-1})	246.7±4.0	86.6±3.9	6.5±1.0	72.9±3.9	5.7±0.8
Pretreated (mg g^{-1})	235.1±5.0	62.5±3.0	6.6±0.8	58.6±3.9	9.9±1.0

4. CONCLUSION

Among the four different fast growing and easily available lignocellulosic biomass in Nepal, *Ipomoea cornea, Phragmites karka, Saccharum spontanum* and *Zea mays* cobs, for their use in bioethanol production, *S. spontaneum* is the best given its thermal degradability, calorific content and fermentable sugar levels. *S. spontaneum* biomass when pretreated with hot water followed by of hydrochloric acid hydrolysis can release sufficient amount of total reducing sugar which can be used for ethanol production. The biomass characterization methods suggest that pretreatment with hot water results significant loss of hemicellulose whereas the loss of cellulose content is minimal. Through this study, we suggest simpler but more economical pretreatment procedure for *S. spontaneum* biomass. Further evaluation of this pretreated biomass showed

significant change in physical and chemical structure of biomass after pretreatment.

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Improvement of ethanol production by electrochemical redox combination of yeast cells

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Abstract
Electrolytic cell are used for enhancement in production of microbial products. Ethanol is produced as one of the renewable source of energy for which one can use even waste products like lignocellulosic biomass. Selection of best ethanol producing yeast strain and best ethanol tolerant yeast cell is first priority for optimized production. Out of eight yeast strains isolated in Central Department of Biotechnology (CDBT). strain CDBT 2 showed good ethanol tolerance with maximum growth even at 8% ethanol. Thebest ethanol tolerant strain CDBT 2 found to grow till 18% ethanol. Total ethanol production by CDBT 2 was found to enhance by about 15±0.12% than control without supply of 4V external energy when glucose was used as carbohydrate source. Drastic increase in ethanol production by 129±0.877% was seen when external voltage was supplied in electrochemical cell with alginate immobilized yeast cells on porous graphite cathode with or withput external voltage supply. However total enhancement is 7.0±0.056% than control w/o immobilization of yeast strain in cathode. 9.0±0.225% increament was observed when cathode was immobilized with neutral red with and without supply of external voltage. 30.64±0.30% and 28.67±0.344% increament in ethanol were observed when cathode were immobilized with neutral red only and neutral red followed by yeast immobilization than with normal graphite felt cathode without external voltage supply. The best ethanol tolerant yeast strain, CDBT 2 was grown in electrolytic cell at best electrode combination with lignocellulosic biomass, *Saccharum spontanum*, pretreated with ammonium hydroxide followed by hydrolysis with hydrochloric acid gave the ethanol production of 16.67% greater than without external voltage supply.

1. Introduction

A microbial electrolytic cell is like a biological system in which microbes do not directly transfer their produced electrons to their characteristic electron acceptors instead, they are subsequently transferred through a resistance or power source to cathode (Korneel Rabaey *et al*, 2003). Current technologies to produce ethanol largely depend upon sugarcane and/or starch based grains and tubers, however, the increasing stress on food prices and food security has evolved lignocellulosic materials as a source of ethanol. Significant efforts on the development of second

generation processes of ethanol production from lignocellulosic materials rich in their lignin, cellulose, hemicelluloses and pectin contents are being made. Lignocellulosic materials for this purpose includes lignocellulosic biomass like corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corn fiber, cotton stalk; energy crops such as switch grass and Alfa-Alfa and various weeds such *Saccharum spontaneum, Lantana camara, Eichhornia crassipies* etc. Cellulose is the major component of lignocellulosic biomass and its concentration ranges from 40-50% of dry weight (Zhang *et al*, 2004), hemicelluloses is present at a concentration of 25-35% (Saha *et al,* 2007), lignin being the third major component is present at a concentration of 20-35% (Joshi et al, 2011) and pectin in trace amounts of 1-2% . Pretreatment is the key technology to reduce the cost of cellulosic ethanol (Yang B., 2008)

As compared to the use of prokaryotes in the biofuel cell, much less research have been carried out on the use of eukaryotes such as yeasts as biocatalysts in the electrolytic cell. Yeasts such as *Saccharomyces cerevisiae* and *Candida melibiosica* have the electrochemical capabilities to use as biocatalysts in a biofuel cell (Hubenova.Y and Mitov .M, 2008). *S. cerevisiae* was genetically modified for enhancement in production of ethanol in normal fermentation, Result showed very less improvement [David et al, 2010]. However, *S. cerevisiae* found to enhance ethanol production when grown in cathode with 1.5V supply [Shin et al, 2002].

Most microorganisms are not anodophiles i.e.; cannot transfer electrons directly to the electrodes, electron mediators to enhance the power output are used. Various endogenous and synthetic electron mediators are commonly used in cells. Neutral red, methylene blue, thionine, iron (III) EDTA, Meldola's blue, Mn^{4+} are the synthetic mediators and humic acids, Anthraquinone, the oxyanions of sulphur (sulphate and thiosulphate) are the endogenous mediators (Park *et al*, 2000). The endogenous mediators transfer electrons from inside the cell membrane to the anode but there are only a limited number of organisms that can transfer electrons across the membrane by themselves to the anode. Microorganisms majorly bacteria such as *Geobacteraceae sulferreducens, Geobacter metallireducens, Shewanella putrefaciens* (Kim *et al,* 2010) and *Rhodoferax ferrireducens* (Chaudhari and Lovely, 2003) are known to efficiently form film on the anode surface and transfer electrons directly to the electrode across the membrane minimizing the use of electron mediators that are known to be toxic (Hahn-Hägerdal et al., 2006). This paper studied the improvement in ethanol production by electrochemical redox combination of yeast cells.

2. Materials and Methods

2.1. Isolation and Characterization of Yeast Strains

All the chemicals were purchased from HiMedia unless stated. Murcha samples (locally used Fermentation starter ie; the local yeast sources specifically used for ethanol production) were collected from Bhaktapur and Lubu area of Kathmandu valley, Central Nepal. They were serially diluted and used pour plate for isolation in Yeast Maltose Agar (YMA) media with composition Yeast extract (3 gm/l), Malt extract (3 gm/l), Peptone (5 gm/l) and Glucose (10 gm/l). Media adjusted to pH 4.5. The isolated strains were sub-cultured for getting pure strains [T. Karki et al 1999; Middelhoven et al 2002; Middelhoven et al 1998]. Pure cultures were stored as glycerol (15%) stock for further use.

2.2. Study of Ethanol Tolerance of Yeast Cells

All isolated yeast cells were cultured in Peptone Yeast Extract Nitrogen (PYN) media supplemented with Peptone 3.5 gm/l, Yeast extract 3 gm/l, KH2PO4 2 gm /l, MgSO4 1 gm/l, $(NH_4)_2SO_4$ 1 gm/l and Glucose 50 gm/l with variable concentrations of ethanol ranges from 0 to 22%. Culture conditions were maintained at 28^{o}C, pH 4.5 for 3 days to see effect on growth pattern. Microbial growth pattern were observed spectrophotometrically [Genesis] at 615 nm against medium blank. The best ethanol tolerant strain was sent for molecular characterization.

2.3. Effect of pH on Ethanol Production

The effect of pH on the fermentation process was determined by adjusting the pH of the PYN media. The pH of the media were adjusted in the range from 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 to 7.0 with 1N HCl and 1N NaOH. The media was then inoculated with 4% (18 hrs culture) of the inoculum and incubated at optimum temperature for 72 hrs. Ethanol concentration was measured after solvent extraction with tri- n-butyl phosphate and treating with acidified potassium dichromate as described by Seo et al, 2009.

2.4. Effect of Temperature on Ethanol Production

The effect of temperature on the fermentation process was determined by inoculating the inoculum to PYN medium and incubating at different temperature. The culture was incubated at temperatures 24^{o}C, 26^{o}C, 28^{o}C, 30^{o}C and 32^{o}C respectively for 72 hrs. The temperature at which maximum ethanol production was observed as described by Seo et al, 2009.

2.5. Effect of Different Nitrogen Supplements on Ethanol Production

The effect of various nitrogen sources on fermentation was determined by incorporating various nitrogen sources in the fermentation media. The nitrogen sources used were ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium oxalate, ammonium acetate, yeast extract, peptone and urea. The media were then inoculated and incubated at optimum temperature for 72 hrs. The best source of nitrogen for ethanol production was then determined.

2.6. Ethanol Production in Electrochemical Cell

Immobilization of Neutral red to Graphite Electrode

The graphite felt soaked in methanol was dipped in 1% polyvinyl alcohol solution for 3 to 4 hours. Dry in oven at about 80^{o}C for 24 hours. The completely dried graphite felt was then soaked in pure chloroform containing 10% thionylchloride and 0.01% neutral red (Sigma Co.) for 6 hours. The graphite felt was then left for 12 hours for air dry. It was then autoclaved and washed in running water till color persist. Finally dried at 60^{o}C for 1 day and used as NR graphite electrode (Jeon et al, 2009).

Immoblization of Yeast Cells on Graphite Electrode

2.4 ml of 18 hrs. culture of yeast cells were centrifuged and pellet mixed with 10 ml of 25 mM phosphate buffer (pH 7.0) containing 4% sodium alginate and absorbed into the graphite electrode for 30 mins. The NR-graphite electrode containing alginate and yeast cells was then soaked in a chilled 100 mM $CaCl_2$ solution for 30 mins to induce calcium alginate coagulation and washed with a 25 mM phosphate buffer.

Construction of Electrolytic Cell

A two compartment electrolytic cell was designed to induce the electrochemical oxidation and reduction reaction simultaneously. The anode and cathode compartments were separated by a porcelian membrane and cellulose acetate film. Anode is made from Platinum electrode (0.2 mm diameter, Sigma) and cathode is made specifically from graphite fiber (Immoblized and unimmoblized). Small scale working volume of the cathode compartment was adjusted to 60 ml each and observed the product concentration at optimized condition.

Ethanol Production in Electrochemical Cell Using Glucose as Carbohydrate Source

Potent yeast strain was observed for improvement in ethanol production in electrochemical cell with PYN media. Electrochemical cell without electrical connection was taken as control.

Ethanol Production in Electrochemical Cell Using Pretreated Lignocellulosic Biomass Hydrolysate as Substrate

Lignocellulosic biomass was pretreated with 0.5M ammonium hydroxide for 24 hours at 65^{o}C. The pretreated biomass was then hydrolyzed with 0.5M hydrochloric acid. The hydrolysate was adjusted to optimized pH and used as substrate for ethanol fermentation by CDBT 2 in electrically enhanced cell.

3. Results and Discussions

Isolation and Characterization of Yeast Isolates

Yeast strains were isolated from Murcha samples collected from Lubhu and Bhaktapur area using PYN media. Eight yeast isolates were selected and maintained pure cultures.

Fig. 1. *Yeast strains isolated from Murcha samples in PYN media. Left: CDBT 1 and right: CDBT 2.*

All the yeasts were creamy white, round or irregular margin with smooth raised or flat surface (Fig.1). Most of the yeast showed budding characteristics.

Ethanol Tolerance of Yeast Cells

All the eight yeast strains found to grow well till 4 % (Fig. 2). However CDBT 2 showed regular growth till 6% ethanol concentration. According to the report by Kyung, 2003, ethanol tolerance of 5% is considered as good yeast. Almost all yeasts tolerated ethanol up to 14%. Beyond this there was sharp decrease in the growth ie; almost nil, which was similar to the ethanol tolerance showed by most of *Saccharomyces cerevisiae* i.e. 14.5 % (v/v) (Teramoto et al., 2005).

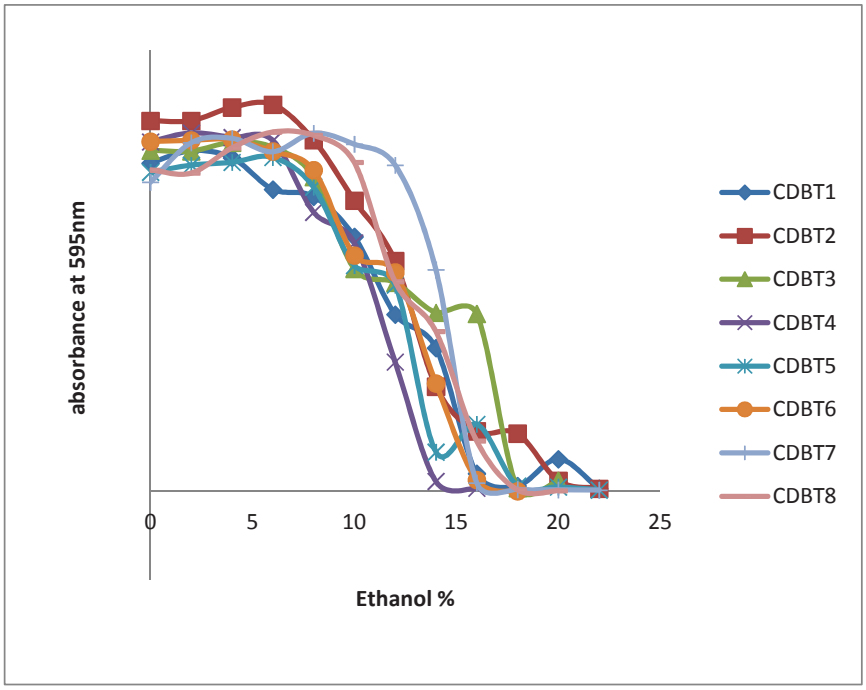

Fig. 2. *Strains CDBT 1 to CDBT 8 grown at different initial concentration of ethanol ranges from 0 to 22% in PYN media for 3 days at pH 4.5 and temperature 28°C. [The data is average of three repeated results]*

Yeast strain CDBT 2 found to grow till 18% ethanol. Ethanol tolerance is an advantage when yeast is being used for the industrial production of ethanol (Ekunsanmi and Odunfa, 1990). During fermentation it is difficult to avoid the ethanol tolerance but substrate inhibition can be overcome by the stepwise addition of substrate. However, temperature (Casey and Ingledew, 1986; D'Amore and Stewart, 1987), natural habitat and the origination of isolation area (Torija et al, 2003) may be the factor for the resistance of strain to ethanol. The ethanol tolerance may be contributed by the physiological factors such as medium composition and the mode of substrate feeding intracellular ethanol accumulation, temperature and osmotic pressure (D'Amore and Stewart 1987). Gonzalez et al 2002 verified that the changes in the expression of several genes concerned with the synthesis of cell envelop components may contribute to increased ethanol tolerance of yeasts. According to Logothetis et al, 2007, water molecules due to its hydrophilic nature can penetrate the lipid bilayer and form hydrogen bonds with the polar groups of phospholipids maintaining the structure of the biological membrane. Ethanol displaces water molecules altering the position of molecules on the membrane affecting the interaction between the lipids and proteins, ultimately damage the structure and function of the membrane. Trehalose has the ability to retain water in the yeast membranes and hence stabilizes the membrane.

According to Gunclave lab, the D1D2 sequencing of CDBT 2 strain was found to resemble *Saccharomyces cereviceae.*

Optimization of Ethanol Production

Effect of pH on Ethanol Production

Fig. 3 showed the effect of pH on ethanol production. Maximum production of ethanol was found at pH 5.5 with 12.37±0.44 mg/ml.

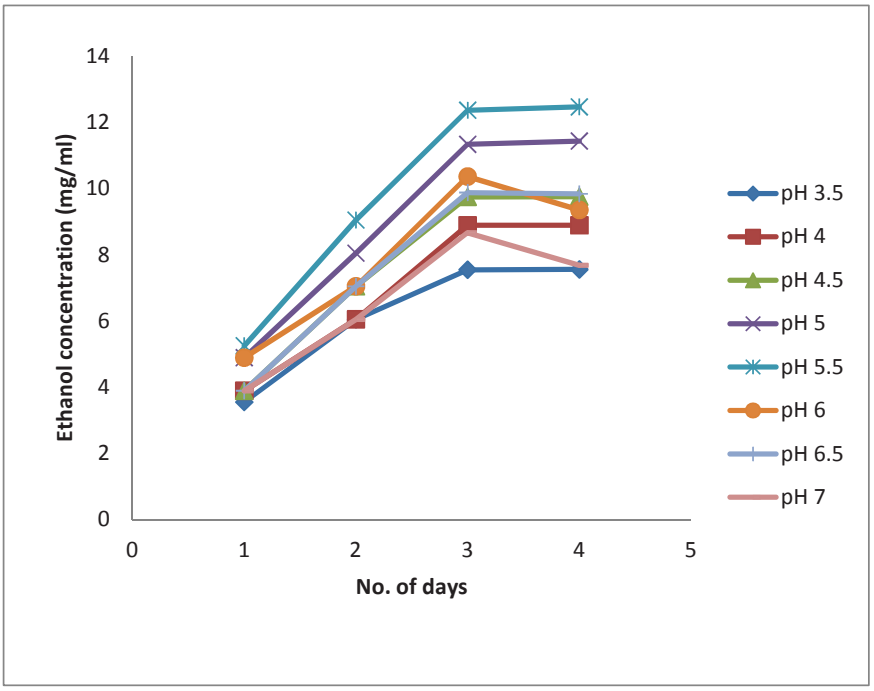

Fig. 3. *Ethanol production by strain CDBT 2 grown in PYN media adjusted at different pH ranges from 3.5 to 7.0 at 28°C . [The data is average of three repeated results]*

Effect of Temperature on Ethanol Production

Ethanol production was found to be optimum at 30°C with 12.55± 0.70 mg/ml yield.

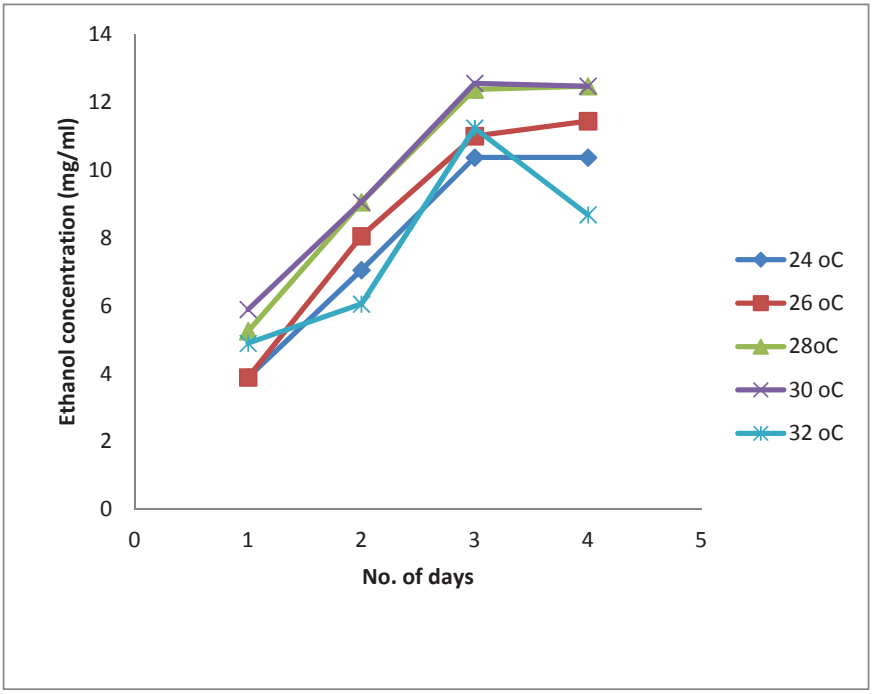

Fig. 4. *Ethanol production by strain CDBT 2 grown in PYN media adjusted at different temperature ranges from 24 to 32°C at pH 5.5. [The data is average of three repeated results]*

Effect of Different Nitrogen Supplements on Ethanol Production

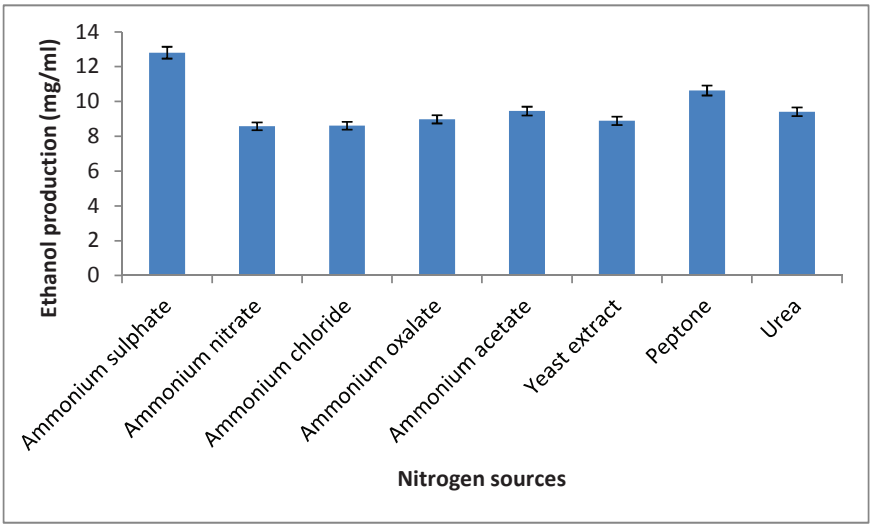

Fig. 5. *Ethanol production by strain CDBT 2 grown in YNB media supplemented with different nitrogen sources adjusted at pH 5.5 and temperature 308°C .*

Out of different nitrogen supliments, ammonium sulphate was found to be the best nitrogen source for ethanol production. The efficiency of ethanol production was found to be in the order 12.82 ± 0.34 mg/ml.

Ethanol Production in Electrochemical Cell

Several types of Electrochemical cells were designed for studying ethanol production. PYN media with 5% glucose adjusted at optimized condition of pH 5.5, temperature 30°C were inoculated with yeast cells and cultured for 3 days. Platinum wire (Sigma) with diameter 0.2 mm were used as anode. Cathodes were made with graphite fibre of thickness 10 mm (Nippon company, Japan) with several modifications. Graphite fibre without immobilization of yeast strain CDBT2 ie; *S. cereviceae*, yeast strain immobilized graphite fibre, neutral red immobilized graphite fibre and neutral red and *S. cereviceae* both immobilized graphite fibre respectively and were separately used as cathode electrode in combination with Pt anode. Graphite felt has more surface area to use as electrode and neutral red immobilized graphite is a good electron donor for reduction of NAD to NADH in vitro, hence to produce more ethanol [Jeon Bo, 2009, Park et al, 1999]. Ethanol production efficiencies were studied for all possible cathode arrangements.

Ethanol Production in Electrochemical Cell with PYN Media Using Different Combination of Electrode

Ethanol was allowed to produce in 4V external energy supply which is optimum for *S. cereviceae* to produce ethanol [Jeon Bo et al, 2009]. When ethanol was allowed to produce in electrochemical cell with 4V external electricity supply, using normal graphite felt as cathode and Platinum as anode, total production was found to enhance by about 15±0.12% than control (without supply of electricity) which was quiet near to 12% enhancement in ethanol production of *S. cereviceae* as reported by Shin et al, 2002 when cultured in cathode. Ethanol production was 15.5±0.124 mg/ml and 13.5± 0.108 mg/ml in anode and cathode respectively where as total ethanol production in control was 12.63±0.076 mg/ml only (Fig 6). Drastic increase in ethanol production from 5.12±0.378 mg/ml to 15.0± 0.09 mg/ml was seen when external voltage was supplied in electrochemical cell with alginate immobilized yeast cells on cathode than ethanol production by control system with yeast cell immobilization on graphite electrode which was around 129±0.877% increment. However total enhancement is 7±0.056% less than control without yeast immobilization.

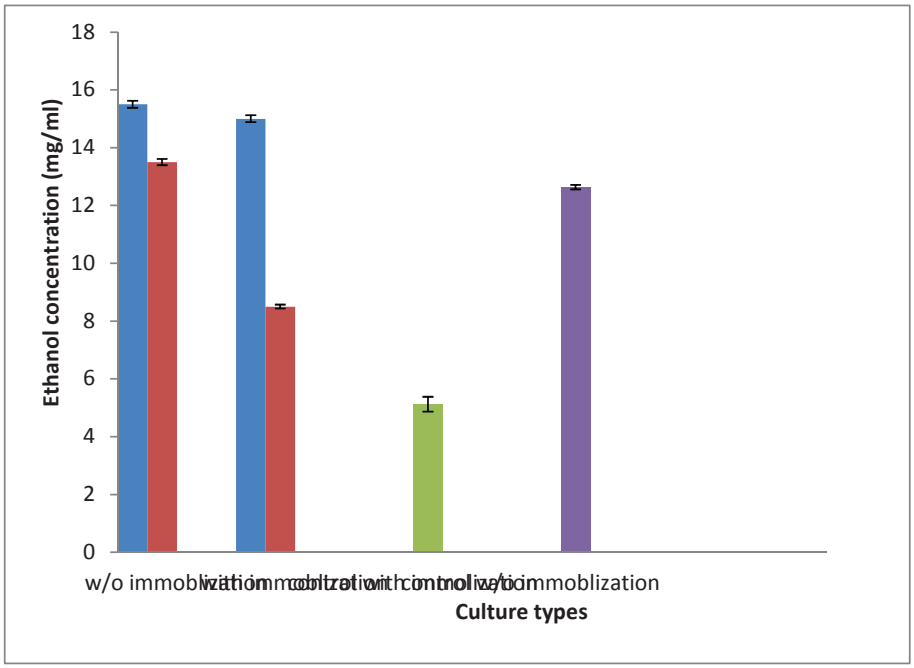

Fig 6. Ethanol production by strain CDBT 2 in electrochemical cell with or without immobilization of yeast cells in graphite felt cathode. Pt wire was used as anode. Culture was incubated in PYN media adjusted at pH 5.5, temperature 30 °C for 3 days . Blue: ethanol produced at cathode, Red ethanol produced at anode and green and purple: control without electricity supply respectively.

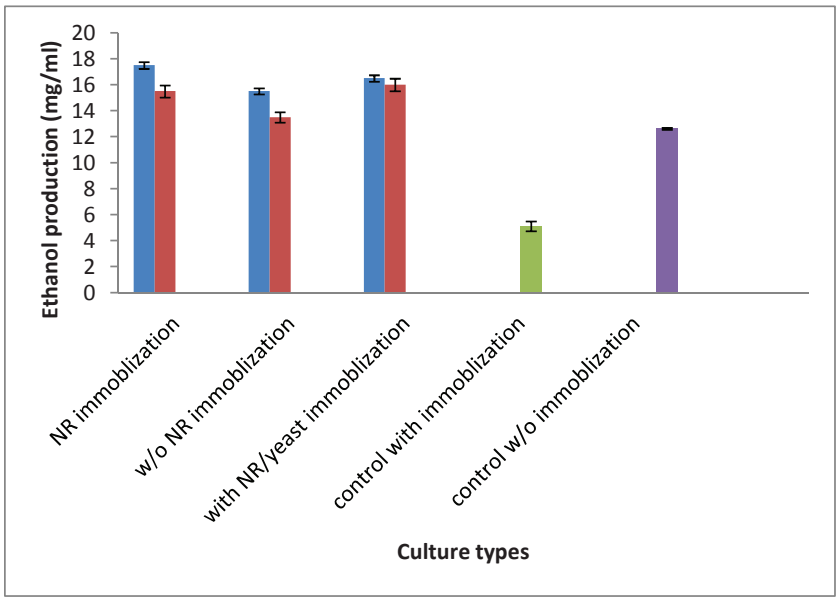

Fig. 7. Ethanol production by strain CDBT 2 in electrochemical cell with or without immobilization of yeast cells, with or without immobilization of neutral red or with or without immobilization of neutral red followed by yeast cells in graphite felt cathode. Pt wire was used as anode. Culture was incubated in PYN media adjusted at pH 5.5, temperature 30 °C for 3 days . Blue: ethanol produced at cathode, Red ethanol produced at anode and green and purple: control without electricity supply respectively.

Fig.7 showed the effect of neutral red and followed by yeast cell immobilization on ethanol production. Total ethanol production in anode (17.51± 0.343 mg/ml) and cathode (15.50 ± 0.465 mg/ml) were found to be enhanced by about 9 ± 0.225 % when cathode was immobilized with neutral red in comparison to 15.12± 0.378 mg/ml without voltage supply. While the total ethanol production was enhanced by 30.64±0.30% than control without neutral red immobilization and without supply of external voltage. Whereas the average total ethanol production in anode

(16.51± 0.066 mg/ml) and cathode (16.0±0.128 mg/ml) was enhanced by only 28.67±0.344% than normal fermentation ie control when NR and yeast both were immoblized in cathode which showed that yeast cell immobilization in cathode electrode is not necessary in electrochemical cell. According to Jeon Bo et al. immobilization of yeast cells on cathode does not enhance ethanol production. In the two compartment electrochemical bioreactor, the anode functions as working electrode and cathode functions as counter electrode. There was drastic improvement in ethanol production found in anode compartment than in cathode compartment when cultured at 4V. This indicates that oxygen is absolutely required for normal growth and fermentatively metabolism [Sablayrolles et al, 1996]. At this voltage anaerobic condition have been reported to cause the NADH/NAD+ ratio to be imbalance and limit sugar in the fermentative metabolism of *Saccharomyces* spps.[Sims et al, 1978]

Ethanol Production in Electrochemical Cell Using Pretreated Lignocellulosic Biomass Hydrolysate

The washed and oven dried biomass was pretreated with 0.5M Ammonium hydroxide followed by 0.5M Hydrochloric acid hydrolysis. The hydrolysate was adjusted to optimized pH of 5.5 and kept in electrochemical cell with neutral red immobilized cathode. Fermentable sugar converted to ethanol by *S. cerevisiae* found be 11.10± 0.067 mg/ml and 10.0 ± 0.04mg/ml in anode and cathode respectively which was 16.66±0.0833% more than non electrolytic condition (Fig. 8). The decrease in ethanol production may be due to generation of salt during neutralization. However use of electrically enhanced method found to enhance lipid biosynthesis hence increases salt tolerancy [Rosenfeld et al, 2003]

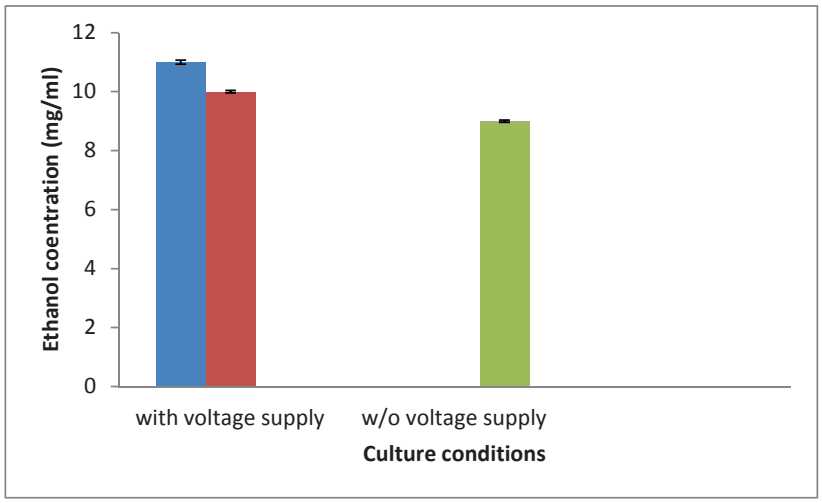

Fig. 8. *Ethanol production by Saccharum spontanum hydrolysate using strain CDBT 2 in electrochemical cell with or without supply of external voltage. Graphite felt immobilization of neutral red was used as cathode and Platinumt was used as anode. Blue: ethanol produced at cathode, Red ethanol produced at anode respectively.Green:control w/o voltage supply.*

Acknowledgement

We would like to express our sincere gratitude to University Grants Commission for supporting us financially and Central Department of biotechnology for creating environment to accomplish work. We are thankful for Prof. Paula Goncalves laboratory, Portugal for helping us identifying yeast strain. We are also thankful to Prof. Doo Hun Park, Department of Biological Engineering, Seokyeong University, Seoul, South Korea; Dr. Amar Yadav, Central Department of Chemistry, Tribhuvan University, Nepal and Renewable Nepal, Kathmandu University, NORAD, SINTEF Programme for providing additional help.

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Optimization of External Potential for Ethanol Production by yeasts in electrochemical Cell

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Abstract **-** Ethanol was produced in electrochemical with combination of Saccharomyces cerevisiae and Wikerhamomyces anomalous . A combination of electrochemical cell with Saccharomyces cerevisiae grown in anode and Wikerhamomyces anomalous grown in cathode gave better ethanol production. Electrochemical cell with this combination of yeast was studied at different external voltage supply to see the effect in ethanol production. A supply of 4V was found to be best for ethanol production with total ethanol production of 379.76±9.52 mg/gm of glucose. When Platinum wire in anode coated with Platinum fine powder (Sigma) along with Nafion membrane in place of cellulose acetate was used, a drastic decrease in overall ethanol production was observed. The ethanol production was 138.68±1.54 mg/gm of glucose in anode and 243.85±34.76 mg/gm of glucose in cathode.

Index Terms **-** Electrochemical Cell, Ethanol, External Voltage

I. INTRODUCTION

An electrochemical cell behaves like the whole cell metabolism on which oxidation and reduction occurs due to transfer of electrons through external circuit. As compared to the use of prokaryotes in the electrolytic cell, much less research have been carried out on the use of eukaryotes such as yeasts as biocatalysts in the electrolytic cell. Yeasts such as *Saccharomyces cerevisiae* and *Candida melibiosica* have the electrochemical capabilities to use as biocatalysts in such electrolytic cells (Hubenova.Y and Mitov .M, 2008). *S. cerevisiae* and few other yeasts are found to enhance ethanol production when grown in cathode with external voltage supply [Shin et al, 2002].

Some bacteria like *Geobacteraceae sulferreducens, Geobacter metallireducens, Shewanella putrefaciens* (Kim *et al,* 2010) and *Rhodoferax ferrireducens* (Chaudhari and Lovely, 2003) are known to form film on the anode surface and transfer electrons directly to the electrode across the membrane. Most microorganisms cannot transfer electrons directly to the electrodes. Neutral red, methylene blue, thionine, iron (III) EDTA, Meldola's blue, Mn^{4+} as synthetic mediators and humic acids, Anthraquinone, the oxyanions of sulphur (sulphate and thiosulphate) as endogenous mediators (Park *et al*, 2000) are commonly used as external electron transport mediators (Hahn-Hägerdal et al., 2006) efficiently when external voltage is supplied. However very few study was conducted to see the effect on eukaryotic cells. This paper studied few possible parameters for optimized production of ethanol from yeasts in electrochemical cell.

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II. MATERIALS AND METHODS

All the chemicals were purchased from HiMedia except few stated chemicals. Yeast strains *Saccharomyces cereviseae* (CDBT2) and *Wikerhamomyces anomalous* (CDBT7) were used as yeast strains. The yeasts were collected from culture collection of Central Department of Biotechnology, Tribhuvan University, Nepal. Yeast Maltose Agar (YMA) media with composition Yeast extract (3 gm/l), Malt extract (3 gm/l), Peptone (5 gm/l) and Glucose (10 gm/l), pH 4.5 were used for revival of culture from glycerol stock. [Middelhoven et al 1998; Middelhoven et al 2002]. Ethanol concentration was measured after solvent extraction with tri- n-butyl phosphate and treating with acidified potassium dichromate as described by Seo et al, 2009.

2.1 Ethanol Production in Electrochemical Cell

2.1.1 Immobilization of Neutral red to Graphite Electrode

Porous graphite felt initially soaked in methanol was dipped in 1% polyvinyl alcohol solution for 3 to 4 hours. It was then dried in oven at about 80^{o}C for 24 hours. Completely dried graphite felt was then soaked in pure chloroform containing 10% thionylchloride and 0.01% neutral red (Sigma Co.) for 6 hours. Graphite felt was then left for overnight for air dry. It was then autoclaved and washed in running water till color persist. Finally, it was dried at 60^{o}C for 24 hrs and used as NR immobilized graphite electrode (Jeon et al, 2010).

2.2 Electrochemical Cell construction

A two compartment electrolytic cell was designed to induce the electrochemical reaction. The anode and cathode compartments were initially separated by cellulose acetate film guarded by whatmann no.1 filter paper. Anode is made from Platinum electrode (0.2 mm diameter, Sigma) and cathode is made from neutral red immobilized graphite fiber. Working volume of the electrolytic compartments were adjusted to 60 ml each.

2.3 Ethanol Production in Electrochemical Cell

Saccharomyces cerevisiae and *Wikcerhamomyces anomalous* were cultivated with either combination in anode and cathode alternatively. Once *S. cerevisiae* in anode and *W. anomalous* in cathode then vice versa and see the effect on ethanol production.

Ethanol was allowed to produce in electrochemical cell with different external voltage supply. PYN media supplemented with Peptone 3.5 gm/L, yeast extract 3.0 g/L, KH_2PO_4 2 g/L, $MgSO_4$ 1 g/L, $(NH4)_2SO_4$ 1 g/L and glucose 100 g/L, pH 5.5, 30°C was used for culture. Electrochemical cell without electrical connection was taken as control.

2.4 Ethanol Production in Electrochemical Cell Using Nafion membrane and platinum coated platinum electrode

Ethanol was produced in electrochemical cell at optimized external voltage supply using Nafion membrane (Dupont, Sigma) in place of cellulose acetate membrane. Further effect in production of ethanol was studied using Platinum electrode coated with fine platinum powder.

III. RESULTS AND DISCUSSION

Yeast strains *Saccharomyces cereviseae* (CDBT2) and *Wikerhamomyces anomalous* (CDBT7) were selected because they were the best ethanol tolerant strain and best xylose utilizing strain respectively as observed by Rejeena M.Sc. thesis, 2013. Selection of ethanol tolerance strain is must when yeast is used for the industrial production of ethanol (Ekunsanmi and Odunfa, 1990). Different factors effect for ethanol tolerancy. Temperature (Casey and Ingledew, 1986; D'Amore and Stewart, 1987), natural habitat and the origination of isolation area (Torija et al, 2003) may be the factor for the resistance of strain to ethanol. The ethanol tolerance may be effected by medium composition, mode of substrate feeding intracellular ethanol accumulation, temperature and osmotic pressure (D'Amore and Stewart 1987). Xylose utilizing strain is advantageous when it is used for ethanol production from lignocellulosic hydrolysates.

3.1 Ethanol Production in Electrochemical Cell

Electrochemical cells were designed for studying ethanol production with different combinations. PYN media with 10% glucose adjusted to pH 5.5, temperature 30°C were inoculated with overnight culture of yeast cells and incubated for 3 days. Platinum wire (Sigma) with diameter 0.2 mm was used as anode. Cathode was made with graphite fibre of thickness 10mm (Nippon company, Japan) immobilized with neutral red. Graphite felt is porous and has more workable surface area. Neutral red immobilized graphite is a good electron donor for reduction of NAD to NADH for efficient ethanol production [Jeon Bo, 2009, Park et al, 1999].

3.2 Effect of Yeast combination in ethanol production

Saccharomyces cerevisiae and *Wikcerhamomyces anomalous* when cultivated with either combination in anode and cathode alternatively, *S. cerevisiae* in anode and *W. anomalous* in cathode combination resulted 175.58±2.99 mg/gm glucose where as opposite combination gave only 145.0±2.20 mg/gm glucose (Fig 1). *S. cerevisiae* from anode resulted 190.6 ± 2.72 mg/g and *W. anomalous* at cathode resulted 165.6±3.26 mg/gm of glucose respectively. (Fig.2.).

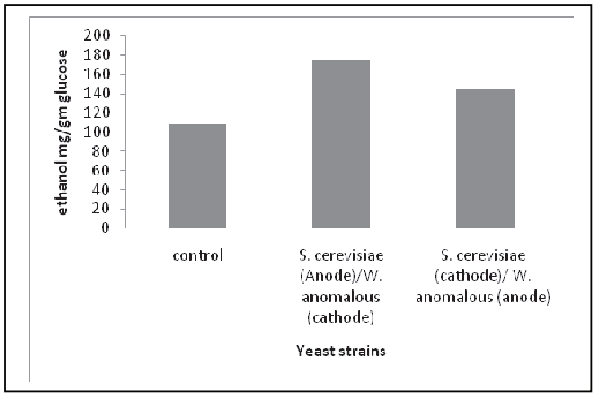

Fig. 1. Ethanol production by Yeast strains Saccharomyces cereviseae (CDBT2) and Wikerhamomyces anomalous (CDBT7) grown in PYN media adjusted at pH 5.5 and temperature 30°C to study the effect of culture condition on ethanol production .

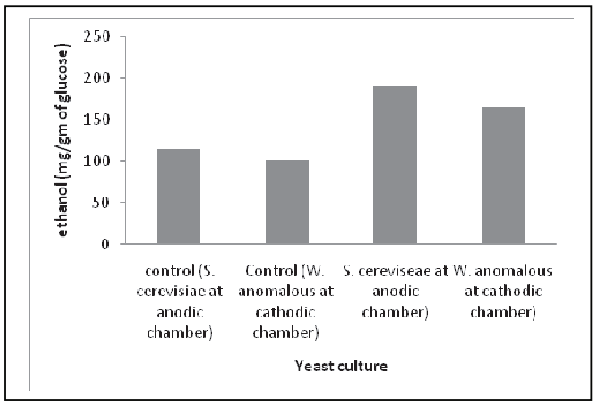

Fig. 2. Ethanol production by Yeast strains Saccharomyces cereviseae (CDBT2) grown on anode and Wikerhamomyces anomalous (CDBT7) grown on cathode in PYN media adjusted at pH 5.5 and temperature 30°C .

3.3 Optimization of external voltage in ethanol production

Ethanol production was studied at different external voltage supply ranges from 0V to 5V respectively (Fig.3) with *Saccharomyces cerevisiae* and *Wikcerhamomyces anomalous* cultivated in anode and cathode respectively. Supply of 4V was found to give good ethanol yield in total of about 379.76±9.52 mg/gm of glucose. The cathodic portion found to contain 555.79±17.19 mg/gm of glucose and anodic portion found to contain 203.7±2.67 mg/gm of glucose individually. Whereas remaining glucose in anode and cathode respectively were 79.86±2.51 mg/gm and 170.23±22.66 mg/gm of glucose.

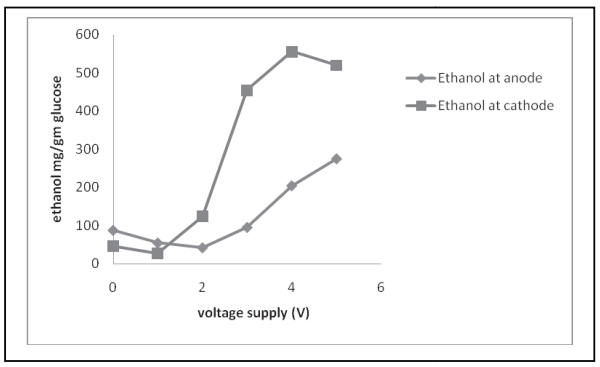

Fig. 3. Ethanol production by Yeast strains *Saccharomyces cereviseae* (CDBT2) and *Wikerhamomyces anomalous* (CDBT7) grown in PYN media adjusted at pH 5.5 and temperature 30°C to study the effect of external voltage supply on ethanol production .

3.4 Ethanol Production in Electrochemical Cell Using Nafion membrane and platinum coated platinum electrode

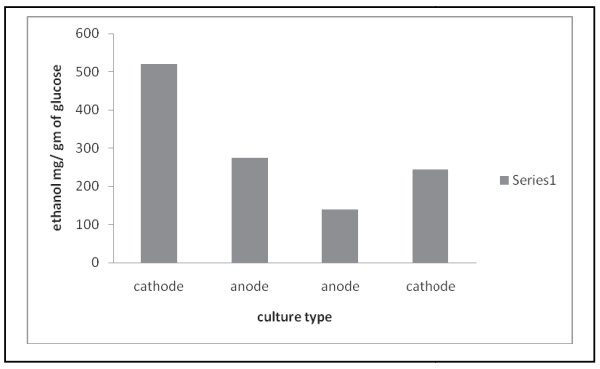

Fig. 4. Ethanol production by Yeast strains *Saccharomyces cereviseae* (CDBT2) and *Wikerhamomyces anomalous* (CDBT7) grown in PYN media adjusted at pH 5.5 and temperature 30°C to study the effect of increasing surface area and use of Nafion membrane for ethanol production. W. anomalous on cathode S. cerevisiae *on anode*. [1 and 2: *without Platinum coating and Nafion membrane; 3 and 4: with platinum coating and Nafion membrane]*

When Platinum wire was coated with Platinum fine powder (Sigma) and used as anode along with Nafion membrane in place of cellulose acetate was used there was seen overall decrease in ethanol production ie 138.68±1.54 mg/gm in anode and 243.85±34.76 mg/gm of glucose in cathode which resulted that a small external only can be used for improvement (Fig.4).

IV. CONCLUSION

W. anomalous on cathode and *S. cerevisiae on anode* is the best combination for ethanol production . Ethanol production can be increased in electrochemical cell with supply of minimum amount of voltage. Enhancement in ethanol production is comparatively lesser when high surface area of platinum is used.

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CHAPTER

15

Currently Used Microbes and Advantages of Using Genetically Modified Microbes for Ethanol Production

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15.1 INTRODUCTION

Ethanol is a high-octane liquid transportation fuel that can be produced either by fermentation or via synthetic routes. Ethanol is produced presently by fermentation of sugar rich biomass, mainly starchy grains (mainly corn), tubers (cassava, potato), and/or saccharides (sugarcane/sugar beet), whereas in synthetic processes ethanol is produced by catalytic hydration of ethylene. Ethanol (ethyl alcohol, CH_3–CH_2–OH) is an oxygenated fuel with high octane and low cetane number than that of petroleum fuels (Lynd et al., 1991). Cetane index is a value derived from fuel density and its volatility that will give a reasonably close approximation to cetane number. Besides high octane and low cetane value, ethanol possesses high heat of evaporation, higher flammable temperature, which consequently increases the overall performance of engines with high compression ratios (Wheals et al., 1999). Bioethanol qualifies to be an alternative fuel, given it is environmentally friendly and mitigates the challenges associated with rising energy demands and cost of procuring them (Perlack et al., 2005). Ethanol is blended with petroleum in certain percentage (5%–15%), which will significantly decreases in petroleum product usage and at the same time reduces the overall greenhouse gas emissions.

Bioethanol Production From Food Crops
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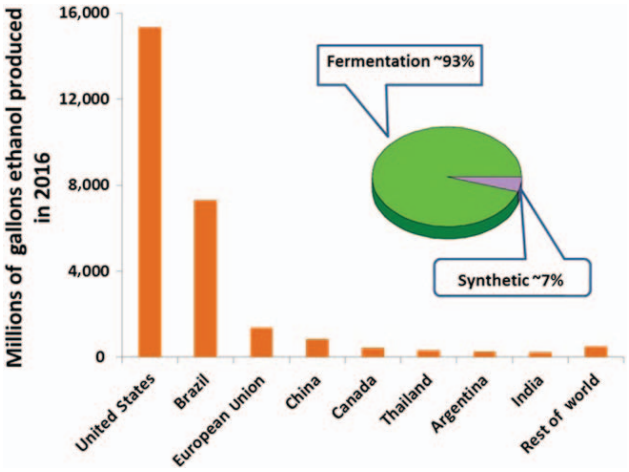

FIGURE 15.1 **World's leading ethanol producers.** *Source: Renewable Fuel Association. Available from: http://www.ethanolrfa.org/resources/industry/statistics.*

15.2 WORLD FUEL ETHANOL PRODUCTION AND CHALLENGES

As per 2016 statistics, the annual worldwide ethanol production (Fig. 15.1) was about 32 billion L (RFA, 2017). About 7% of it corresponds to the synthetic alcohol derived from ethylene or coal, and the rest is bioethanol (Smeets et al., 2005). Brazil and USA are the leading countries in ethanol production and they use sugarcane and corn as the raw material, respectively. Sucrose from sugar beet is also used by growing ethanol industries of Western Europe (Fig. 15.1). The advantages of using sugar beet include a shorter crop production cycle, higher yield, and higher tolerance to wide range of climatic variations, and low water and fertilizer requirement.

Owing to global industrialization, increasing population and other factors, the demand of transportation fuels is increasing every year. In this scenario, bioethanol production by first-generation methodologies as well as synthetic processes is not enough to meet the global demand. Further, ethanol produced by first-generation technologies directly competes with food security and its use in biofuel production is often criticized.

Starch-rich feedstocks are a common source of sugar needed for ethanol production. Starch is branched homopolymer of D-glucose units that is broken down to D-glucose using a variety of enzymes, that is, α-amylases break drown starch to limited dextrin, β-amylases break down starch and limited dextrin to maltose and glucoamylase breaks down starch, dextrin, and maltose to D-glucose. Debranching enzymes play an important role in breaking α (1–6) linkages in starch and dextrin. This process generates fermentable sugars. The factors that affect the enzymatic hydrolysis of starch include substrate used, enzyme activity, and the reaction conditions (temperature, pH, and other parameters). Dilute hydrochloric acid (~0.5 M) may also be used for breakdown of starch, however, the enzymatic process is most commonly used.

The starch- and sugarcane-based industry for bioethanol production has been commercially viable for decades. However, the cost of

bioethanol production is higher in first-generation technologies. Yeast cannot utilize starchy materials directly and the processing requires a large amount of amylolytic enzymes. The process also needs the starchy material to be cooked at higher temperature (140–180°C) (Bai et al., 2008; Balat et al., 2008).

Current ongoing research in the area of bioethanol production is focused on utilizing lignocellulosic biomass (LCB) as source of sugar for fermentation. In this process (also known as second-generation technology), LCB is used as cheap feedstock to produce ethanol. LCB is cheap, renewable, and widely available (Lynd et al., 1996; Mosier et al., 2005; Sun and Cheng, 2002). In the past decade, tremendous progress has been made to utilize LCB for the commercial production of liquid biofuels, including bioethanol. This process of ethanol production generally involves pretreatment and hydrolysis of LCB to sugars followed by fermentation of such sugars to ethanol (Fig. 15.2) (Joshi et al., 2011).

The main bottleneck in commercial ethanol production from second generation technologies is mainly due to the cost associated with chemical and/or enzymatic pretreatment processes involved to generate fermentable sugars. The commonly used pretreatment processes, and their advantages and disadvantages are summarized in Table 15.1.

The main reason for pretreatment processes is to assist in lignin removal, cellulose/hemicellulose decrystalization and partial depolymerization, and to increase the surface area to make cellulose/hemicellulose accessible for the enzymatic hydrolysis. Biological pretreatment methods are more ecofriendly than chemical pretreatment methods because nature has bestowed us with tremendous microorganisms that are a potential source of valuable enzymes, which could be utilized rationally to hydrolyze recalcitrant biomass, such as cellulose and lignin (Sindhu et al., 2016). In this process, mostly wood-feeding termites have been explored for isolating cellulolytic microbes. The most well-studied species in this regard include *Amycolatopsis*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, and various species of *Enterobacteriaceae* and *Bacillaceae* families (Pourramezan et al., 2012; Sharma et al., 2015). Filamentous fungi (*Trichoderma reesei*, *Trich. viride*, *Aspergillus niger*, *A. oryzae*, and *Fusarium oxysporum*) and *basidiomycetes* (*Phanerochaete chrysosporium*, *Fomitopsis* sp.) have also shown promising cellulase production abilities under different cultivation conditions using a number of carbon sources (Chandel et al., 2012; Mathew et al., 2008). To date, *Phan. chrysosporium* and *Gloeophyllum trabeum* are considered as very competent cellulase producers. Similarly, *Trich. reesei* and their mutant strain lineages have been reported to produce 40–100 g cellulase enzyme per liter of culture broth (Vitikainen et al., 2010).

Cellulase system of many fungi, including *Trich. reesei* is deficient in β-glucosidase and sensitive to feedback inhibition by glucose. Considering this aspect, attempts have been made to increase the copy number and introduce glucose tolerant β-glucosidase gene in *Trich. reesei* (Nakari-Setälä and Penttilä, 1995). The most productive cellulolytic bacteria produce only a few grams cellulase per liter (Xu et al., 2009). Highly cellulolytic anaerobic hyperthermophiles are found in the genera *Thermotoga* and *Caldicellulosiruptor bescii* (formerly *Anaerocellum thermophilum*, isolated from a geothermally heated pool) and some of its relatives in the same genus, such as *Caldicellulosiruptor saccharolyticus* (Bok et al., 1998; Rainey et al., 1994). From the genome sequences of *Caldicellulosiruptor* sp., it has been known that ~20% of the total open reading frames (ORF) are involved in the degradation, transport, and metabolism of carbohydrates, such as cellulose, xylan, and starch (Lee et al., 2017). Hydrolases of *Caldicellulosiruptor* are considered as "megazymes" due to their multidomain functions and multicatalytic

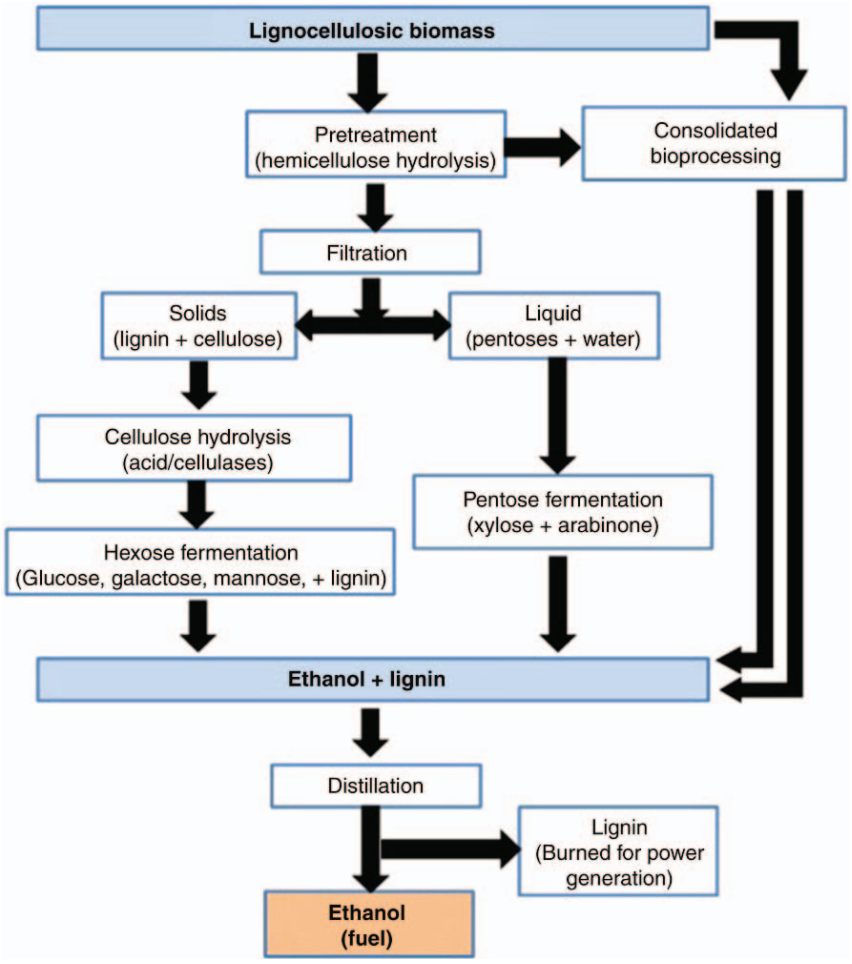

FIGURE 15.2 **Schematic of various processes used to utilize lignocellulosic biomass to produce bioethanol.** *Source: Adapted with permission from Joshi, B., Bhatt, M.R., Sharma, D., Joshi, J., Malla, R., Sreerama, L., 2011. Lignocellulosic ethanol production: current practices and recent developments. Biotechnol. Mol. Biol. Rev. 6, 172–182.*

nature. Many of these megazymes contain five or more domains, which can include a variety of cellulases, hemicellulases, and carbohydrate binding modules (Bergquist et al., 1999). One of the enzymes for cellulose degradation termed CelA from *Cald. bescii* has been characterized (Zverlov et al., 1998), however its physiochemical properties, such as cellulose and hemicellulose degrading ability at elevated temperatures were only reported very recently (Kanafusa-Shinkai et al., 2013). *Cald. bescii* has the capability of not only solubilizing cellulose and hemicellulose in unpretreated biomass but also much of the lignin in unpretreated switchgrass (Kataeva et al., 2013).

Likewise, fungi belonging to Ascomycota and Basidiomycota, such as brown-rot and white-rot fungi, respectively (Dashtban et al., 2010), are also efficient degraders of LCB. From a productive perspective, brown-rot fungi appear

TABLE 15.1 Biomass Pretreatment Methods: Advantages and Disadvantages

Pretreatment method	Process	Advantages	Disadvantages	Sources
Physical pretreatment methods	Milling • Ball milling • Two row milling • Colloid milling	Reduces cellulose crystallinity Increases surface area for enzymatic hydrolysis	High power and energy consumption	Sun and Cheng (2002)
	Irradiation • Gamma-ray irradiation • Microwave irradiation	Decreases cellulose crystallinity and decrease degree of polymerization	Lignin removal is inefficient	Saini et al. (2015)
	Others • Extrusion • Pyrolysis	80%–85% conversion of cellulose to reducing sugars with more than 50% glucose	Temperature >300°C is required	Taherzadeh and Karimi (2008)
Chemical pretreatment methods	Acids • Sulfuric acid • Hydrochloric acid • Phosphoric acid Dilute vs. Concentrated acids	Partial or nearly complete delignification High glucose yields Ambient temperature Less corrosion problem than concentrated acid and dilute acids forms lower concentrations of inhibitors, for example, furfural	High cost of acids and need to be recovered/ neutralization Reactor corrosion problems Acid pretreatment leads to formation of degradation products	Mosier et al. (2005), Sun and Cheng (2002), Yang and Wyman (2008)
	Alkalis Organic solvents	Effective lignin removal and hemicellulose solubilization Causes lignin and hemicellulose hydrolysis	Long exposure times and requires alkali removal High cost; solvent needs to be drained and recycled	Taherzadeh and Karimi (2008)
Physico chemical methods	Ammonia fiber expansion (AFEX) Stem explosion	Increase accessible surface area Minimal inhibitor formation Causes lignin transformation and Hemicellulose solubilization	Not efficient for raw material with high lignin content. High cost and requires large amount of ammonia Generation of toxic compounds	Chiaramonti et al. (2012)
Biological methods	Use of lignin degrading microorganisms, mainly rot fungi and actinomycetes Use of cellulolytic organisms from termite gut and soil	Degrade lignin and hemicelluloses Low energy consumption Minimal environmental impact	Requires long incubation time Requires careful control of growth condition	Ali et al. (2016)
Ionic liquid pretreatment methods	Biomass hydrolysis in ionic liquids with different mineral acids as catalysts	Achieves ~ 81% liberation of total reducing sugars initially present in the biomass with 1-N-butyl-3-methylimidazolium chloride and hydrochloric acid	Acids are corrosive	Li et al. (2008)

to contribute very little toward lignin degradation. For example, Hatakka (1983) have shown that 35% of the straw was converted to sugars by *Pleurotus ostreatus*. On the contrary, white-rot fungi are very efficient for lignin breakdown and some of them can even exhibit high selectivity, thus leaving the cellulose and hemicellulose fractions almost intact (Mäkelä et al., 2014). The lignin degradation is due to the presence of efficient laccase enzymes in these fungi. In our earlier studies, we have successfully purified laccase enzyme from *Ganoderma* sp. having molecular mass of 43 kDa, exhibiting optimal activity at 30°C and pH 5.0. The isolated laccase was thermally stable (up to 70°C for 1 h) and exhibited broad pH stability (Shrestha et al., 2016). The microorganisms used in the fermentation process to date are inefficient/incompatible in the cofermenting the variety of sugars released from LCB. To economize the process of ethanol production, there is an urgent need to identify robust microoraganisms that can ferment both hexose (C_6) and pentose (C_5) sugars, as well as compatible with process ingeration, for example, consodilated bioprocessing (CBP) in which pretreatment, saccharification, and fermentation are all carried out in a single bioreactor using genetically modified microorganisms.

15.3 HETEROGENITY IN CARBOHYDRATE COMPOSITION OF LIGNOCELLULOSIC BIOMASS

Many countries and laboratories are working toward the development of technologies to exploit the potential of LCB as a substrate for the production of bioethanol. This process generally involves pretreatment and hydrolysis of LCB to fermentable sugars (see earlier) followed by fermentation of such sugars to ethanol. Achieving fermentable levels of sugars from LCB requires relatively harsh pretreatment processes, costly enzymes and design of different processing tanks for the saccharification, hydrolysis and fermentation processes (Banerjee et al., 2010). All these steps make the cost of production of ethanol from LCB to be prohibitive, for example, pretreatment processes takes up to 33% of the overall cost of producing lignocellulosic bioethanol (Behera et al., 2014). Given this, the current research focus is on the genetic manipulation of the lignin biosynthetic pathway in plants in order to improve bioethanol yields from LCB (Fu et al., 2011). An alternative strategy could be CBP scheme (Fig. 15.2) in which lignin degradation, cellulase/hemicellulase production, substrate hydrolysis, and fermentation are accomplished in a single step and in one reactor (Lynd et al., 2017). It is important to note that in CBP only one microbial consortium is employed for simultaneous biodegradation, fermentation, and distillation, a three-in-one process, which eventually help in lowering ethanol production costs due to simpler LCB processing, lower energy inputs and higher conversion efficiencies than simultaneous saccharification fermentation (SSF) or simultaneous saccharification cofermentation (SSCF) based processes. Efforts are being made to engineer ethanol producing yeast (*Saccharomyces cerevisiae*) capable of producing lacase/cellulases/hemicellulases and lignocellulose degrading bacteria (*Clostridium thermocellum*) to be efficient ethanol producers (Chen, 2017; Khatun et al., 2017; Kricka et al., 2014; Lynd et al., 2017).

In nature, LCB is in abundance and it is renewable. LCB is a complex matrix with three main components; lignin, cellulose (polymer of β-glucose units), and hemicellulose (polymer of C5/C6 sugars). More than 70% of the dry mass LCB is made of sugar polymers. If these sugar polymers can be efficiently hydrolyzed and fermented, bioethanol production from LCB holds tremendous potential in meeting energy needs of the world providing many environmental benefits. Suitable substrates for LCB includes corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corncob, oat hull, corn

TABLE 15.2 Compositional Analysis of Carbohydrate Present in Various Weed Species and Their Carbohydrate Composition

Biomass source	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Moisture (%)	Ash (%)	Sources
Eichhornia crassipes	36.5	22.0	15.2	92.8	NR	Mishima et al. (2008)
E. crassipes	18.2	48.7	3.5	NR	NR	Kumar et al. (2009)
E. crassipes	25.61	18.4	9.9	NR	NR	Poddar et al. (1991)
E. crassipes	33.97	18.0	26.4	NR	NR	Chanakya et al. (1993)
E. crassipes	36.5	22.0	5.2	NR	NR	Mukherjee and Nandi (2004)
Saccharum spontaneum	45.1	22.75	24.5	4.9	2.5	Chandel et al. (2011)
Sacchar. spontaneum	36.8	2.2 – 21.5	20.1	5.4	NR	Scordia et al. (2010)
Lantana camara	44.1	17.0	32.3	4.4	2.3	Kuhad et al. (2010)
L. camara	45.1	17.0	27.3	6.5	2.3	Pasha et al. (2007)

NR, Not reported.

fiber, wood chips, saw dust, and cotton stalk, energy crops, such as switch grass and alfa alfa, and various agricultural and forest weeds, for example, *Saccharum spontaneum*, *Lantana camara*, *Eichhornia crassipes* (water hyacinth) (Chandel and Singh, 2011; Joshi et al., 2011). The amount of biomass (cellulose, hemicellulose, and lignin) present in these feedstocks also depends on the region from where the plant has been harvested. Studies have shown of obtaining varying amounts of cellulose from the same species obtained from different places/regions, accordingly various weeds have been evaluated for their carbohydrate content with goal of using them for bioethanol production (Table 15.2).

15.4 INHIBITORS GENERATED DURING PRETREATMENT/HYDROLYSIS AND THEIR EFFECTS ON FERMENTING MICROORGANISMS

Use of concentrated acids and higher temperatures in the pretreatment process generates not only large amounts of fermentable sugars but also generates intermediates that act as inhibitors later in the fermentation process. These inhibitors either inhibit the enzymes responsible for saccharification and fermentation and/or growth of the organisms used in pretreatment/fermentation process. These fermentation inhibitors (Fig. 15.3 and Table 15.3) can be divided into three major groups: (1) organic acids, such as acetic, formic, and levulinic acids, (2) furan derivatives, such as furfural and 5-hydroxymethylfurfural (5-HMF), and (3) phenolic compounds (Chandel et al., 2010). These intermediates slow down or even completely inhibit fermentation process (Frazer and McCaskey, 1989; Larsson et al., 1999). Among the inhibitors produced furfural (a pentose sugar byproduct) and hydroxy methyl furfural (a hexose sugar by-product) are the most potent toxic compounds/inhibitors among compounds listed earlier (Taherzadeh et al., 1999). The effects of these compounds may be overcome via (1) overliming fermentation broths, (2) use of ion exchange resins to selectively remove them from the broth, and (3) use enzymes to mitigate the effect of toxins/inhibitors by converting them to nontoxic products. More recently, the researchers (Chong et al., 2013; Yuan et al., 2017; Zhang et al., 2017; Zhu et al., 2013) are moving toward

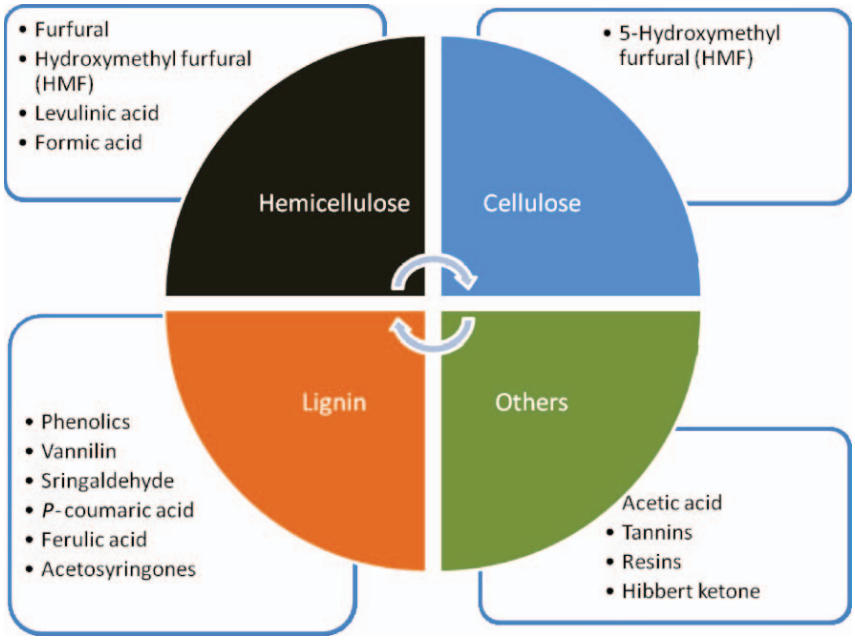

FIGURE 15.3 **Inhibitors of fermentation process generated during pretreatment process.**

TABLE 15.3 Effects of Fermentation Inhibitors Aliphatic Acid, Furan Derivative, and Aromatics

Fermentation inhibitors	Effect	Detoxification	Sources
Aliphatic acids • Acetic acid • Levulinic acid • Formic acid	Uncoupling and intracellular anion accumulation Inhibit the growth and metabolism allowing the higher permeation of cell membrane in microorganisms.	Activated charcoal	Zaldivar and Ingram (1999)
Furan derivatives • Furfural • 5-HMF	ROS accumulation causes damage to mitochondria and vacuole membrane Cell wall damage, DNA breakdown, inhibition of protein and RNA synthesis	Use of calcium hydroxide	Sanchez and Bautista (1988)
Aromatics • Vanillin • 4 hydroxy-benzaldehyde	Loss of integrity of biological membrane, diminished cell growth and sugar assimilation	Use of stress (inhibitor) tolerant yeast	Palmqvist et al. (1999)

metabolic engineering of microbes (yeast/bacteria) that can tolerate high concentration of alcohol as well as inhibitors. Tolerant ethanologenic yeast strains have been found to convert furfural and 5-(hydroxymethyl)-2-furaldehyde to less toxic compounds, such as furan methanol (FM) and furan-2,5-dimethanol (FDM; 2,5-bis-hydroxymethylfuran) and at the same time produce normal yields of ethanol (Liu et al., 2004; Liu and Slininger, 2005). The enzymes that have shown to catalyze detoxification of fermentation inhibitors (earlier) include alcohol dehydrogenases (ADH1, ALD4, ADH6, and ADH7), and methylglyoxal reductases (GRE2

and GRE3) (Liu, 2011, 2012). Systems biology approaches, including disruptome screening, transcriptomics, and metabolomics have been recently exploited to gain insight into the molecular and genetic traits involved in tolerance and adaptation to the fermentation inhibitors in the above organisms (Cheng et al., 2017; Kasavi et al., 2016).

15.5 ENGINEERING BACTERIA FOR ETHANOL PRODUCTION

The following group of microorganisms are genetically engineered for higher ethanol productivity.

15.5.1 Escherichia coli

Escherichia coli is considered as an attractive host for the conversion of LCB into ethanol because of the useful characteristics, such as (1) metabolism of a wide variety of substrates (pentose and hexose sugars as well as uronic acid from in pectin) found in pretreated LCB, (2) sustained high glycolytic flux (both aerobic and anaerobic), and (3) high tolerance to ethanol (up to 50 g/L). Other advantages of using *E. coli* for ethanol production are the extensive understanding of its physiology and metabolism and the ease of genetic manipulation. Therefore, *E. coli* has been an obvious choice for metabolic engineering towards ethanol production. An *E.coli* strain, ATCC 11303 (pLOI297), is reported to be the most promising strain for xylose fermentation under a wide variety of culture conditions. Under the optimum fermentation conditions (≤42°C, pH > 6, xylose 80 g/L), xylose fermenting cultures showed maximum ethanol tolerance (53–56 g/L) and an average productivity 0.72 g/L convert the pyruvate to ethanol has been unsuccessful due to low level of ADH expression in native *E. coli*. To overcome this shortcoming, *E. coli* W strain KO11 was constructed by introducing *pdc* (pyruvate decarboxylase) and *adhB* (ADH) genes isolated from *Zymomonas mobilis* under the control PET operon and deletion of *frd* gene (fumarate reductase) to prevent succinate production (Ingram et al., 1987). In this strain, the PET operon was stably integrated into the chromosome at the *pfl* locus along with an antibiotic resistance marker. Spontaneous mutants exhibiting high ADH activity and high antibiotic resistance were selected to ensure high PET operon activity. The resulting strain KO11 produced ethanol at a yield of 95% in complex media (Ohta et al., 1991a). However, recombinant *E. coli* has several drawbacks as it requires high nutrient supply, weak ability of secreting active cellulase, and sensitivity to end product inhibition. Recombinant *E. coli* W KO11 has been used for production of ethanol from biomass using multiple substrates including, rice hull (Moniruzzaman and Ingram, 1998), sugarcane bagasse, corncobs, hull and agricultural residues (Asghari et al., 1996), pectin-rich beet pulp (Doran et al., 2000), and sweet whey (Leite et al., 2000). Similarly, feedback resistant strains have been developed using *pfl* and *ldh* mutant strains in which the conversion of pyruvate to lactate and recycling of the NADH generated from glycolysis was blocked. Transforming the strains with plasmid pLOI297, which encodes the PET operon, restored fermentative growth. The transformed strains selectively produced ethanol from arabinose, glucose and/or xylose. These strains have been used to ferment hydrolysates prepared from corn hull and germ meal. Ethanol production by the best strain, FBR5, was 0.46–0.51 g/g, and fermentation was complete within 36–60 h (Dien et al., 2000).

Recombinant *E. coli* DH5α (pEglABglA) expressing endophytic endoglucanase A (*EglA*) from *Bacillus pumilus* (Lima et al., 2005) and hyperthermophilic β-glucosidase A (*BglA*) from *Fervidobacterium* sp. (Lima et al., 2009) has been shown to grow in carboxymethyl cellulose (CMC) as sole source of carbon and energy. The expression of the *Bgl*A and *Egl*A genes enabled

E. coli to partially degrade CMC. Among the five strains of *E. coli* transfected with pEglABglA, when cultured in CMC media, specific growth rates of recombinant *E. coli* strains BL21, JM101, and Top 10 were higher than those of DH5α and DH10B strains. The recombinant *E. coli* DH5α reduced the viscosity of the CMC medium by 5-fold and increased the reducing sugar released from CMC by 30-fold. The pEglABglA plasmid can be used as backbone for further cellulase gene addition, which may enhance the *E. coli*'s cellulolytic capacity even more (Rodrigues et al., 2010).

The gene for cellulase from *Ruminococcus albus* F-40 when cloned in *E. coli* HB101 with pBR322 vector and cultured in LB broth the cellulase production was remarkably enhanced (up to ~170 U/L after 8 h). Further, maintaining pH at ~6.5 and NaCl concentration at ~ 80 mM lead to stable cellulase expression for several hours (Ohmiya et al., 1988).

The success of using any microorganism for industrial production of fuels depends on its ability to quickly convert renewable raw material into fuel with high productivity at a low price without being toxic to the organism itself. Availability of genetic and molecular tools to engineer existing native pathways or to create a synthetic new pathway has made *E. coli* as the microorganism of best choice in order to produce biofuels from renewable energy sources. Although significant work has been done, some challenges still exist to consider using *E. coli* as a cost-efficient strategy for commercial production of bioethanol, higher chain alcohols, and biodiesel.

To date, the state-of-the-art bioethanol-producing *E. coli* strains have shown titers in the range of 40–55 g/L and yield of ~100% theoretical maximum from various cellulosic and hemicellulosic feed sources and are similar to bioethanol produced through *Sacch. cerevisiae*. However, it is important to note that, although the cellulosic and hemicellulosic materials are cheap, their hydrolysates contain toxic compounds, such as organic acids, furan derivatives, and phenolic compounds that inhibit the growth of *E. coli* more as compared to *Sacch. cerevisiae*. Improved pretreatment technology and genetic engineering approaches to improve tolerance to these compounds are useful to mitigate the challenges stated earlier.

15.5.2 Ethanol Production Using Actinomycetes

Among the actinomycetes, *Thermomonospora fusca* (Deng and Fong, 2010), *Streptomyces thermodiastaticus* (Crawford and McCoy, 1972), *Thermomonospora curvata*, *Streptomyces viridosporus*, *Streptomyces setonii*, and other strains (Godden et al., 1989) have been shown to contain cellulolytic activity. Arora et al. (2005) studied the role of *Streptomyces griseus* B1 isolated from leaf litter in ethanol production. These studies used hardwood as well as softwood as substrates in fermentation at 37°C and *Strep. griseus* B1 caused much higher loss of Klason lignin content compared to the control. The control experiments showed hardly any loss of Klason lignin when treated with 0.1 M alkali for 2 h. *Strep. griseus* B1 preferred hardwood substrates (23.4% lignin loss) as compared to softwood substrates (10.5% lignin loss). Lee et al. (2013) have recently heterologously expressed *pdc* and *Adh II* genes in the Gram-positive *Strep. lividans* TK24. The recombinant *Strep. lividans* TK24 produced ethanol from glucose with a yield of 23.7%. The recombinant *Strep. lividans* TK24 was also able to produce ethanol from xylose, L-arabinose, mannose, L-rhamnose, galactose, ribose, and cellobiose with yields of 16.0, 25.6, 21.5, 33.6, 30.6, 14.6, and 33.3%, respectively.

15.5.3 Thermophilic Bacteria

Production of ethanol from thermophilic organisms have distinctive advantages over mesophiles in terms of their ability to (1) increase solubility of substrates, (2) improved mass

transfer due to decreased viscosity, (3) increased diffusion rates, (4) high bioconversion rates, (5) their ability to use variety of inexpensive biomass feedstocks, (6) low risk of contamination, (7) reduced cost of bioreactor operation (avoid cooling costs of bioreactors), and (8) facilitated product recovery (high vapor pressure of the volatile compounds). Given the aforementioned, thermophilic bacteria are currently of interest in bioethanol production because they have the ability to ferment biomass to ethanol without the addition of external hydrolytic enzymes, a substantial cost advantage. These bacteria also metabolize both pentose and hexose sugars found in lignocellulosic hydrolysates (Lynd et al., 2005).

Several anaerobic thermophiles have been shown to utilize cellulose, hemicellulose, and starch. These organisms include *Clost. thermocellum*, *Clost. raminisolvens*, *Clost. stercorarium*, *Cald. saccharolyticus*, and *Cald. obsidiansis* (Freier et al., 1988; Hamilton-Brehm et al., 2010; Wiegel and Dykstra, 1984). The noncellulolytic thermophiles, such as *Clost. thermohydrosulphuricum, Clost. themosaccharolyticus* and *Thermoanaerobacter ethanolicus* utilize xylose and other pentose sugars at comparable levels to that of hexose. On the other hand, cellulolytic thermophile *Clost. thermocellum* does not utilize pentoses (Lynd et al., 2005).

Thermo. saccharolyticum JW/SL-YS485, a thermophilic anaerobic bacterium, grows in a temperature range of 45–65°C and a pH range of 4.0–6.5 and is able to ferment hemicellulose and xylan polymers directly. It also ferments primary sugars found in cellulosic biomass, including cellobiose, glucose, xylose, mannose, galactose, and arabinose (Shaw et al., 2008).

Therm. ethanolicus is a nonspore-forming extreme thermophilic anaerobic bacterium that produces ethanol at 69°C (Kannan and Mutharasan, 1985). The thermophilic anaerobic bacterial strain *Thermoanaerobacter* BG1L1 was able to ferment undetoxified corn stover hydrolysate in a continuous immobilized reactor system at 70°C with ethanol yield of 0.39–0.42 g/g sugars consumed. Nearly 89%–98% xylose in corn stover hydrolysate was utilized to produce ethanol by this bacterium (Angelidaki et al., 2012). Researchers have explored the coculture of *Clost. thermocellum* paired with *Clost. thermosaccharolyticus*, *Clost. themohydrosulphuricum* for simultaneous utilization of pentose and hexose sugars to produce ethanol and these studies do show that the coculture yield higher ethanol concentrations (Angelidaki et al., 2012).

Clost. thermocellum exhibits a high growth rate on crystalline cellulose (Zhang and Lynd, 2005). Specific growth rates reported for thermophiles, such as *Clost. thermohydrosulfuricum*, *Clost. theromocellum*, *Clost. thermosaccharolyticum*, and *Thermo. ethanolicus* on soluble substrates generally range between 0.4 and 0.6/h (Zeikus et al., 1981), which are comparable to 0.43/h to 0.46/h for the yeast (*Sacch. cerevisiae*) growing on glucose (Laopaiboon et al., 2008). *Clost. thermocellum* has been reported to directly ferment cellulosic biomass without pretreatment process and produce 0.28–0.29 g ethanol/g glucose fermented (Ram and Seenayya, 1989). It is not exploited industrially due to low ethanol tolerance [tolerates only up to 1.5% (v/v) ethanol]. Research to improve ethanol tolerance by these organisms is ongoing. Rani and Seenayya (1999) have isolated *Clost. thermocellum* strain SS21 and SS22, producing high yields of ethanol, that are tolerant to 4.0% and 5.0% (v/v) ethanol, respectively. These strains are also tolerant to various solvents and acetic acid. Similarly, Brown et al. (2011) have reported that mutated bifunctional acetaldehyde-CoA/alcohol dehydrogenase gene (adhE), leads to improved ethanol tolerance in *Clost. thermocellum.* Extremely thermophilic cellulolytic *Cald. saccharolyticus* can coutilize glucose and xylose and its close relative *Cald. bescii* DSM 6725 has been found to degrade xylan and xylose (Yang et al., 2010).

Several mesophilic *Clostridium* strains have also been reported to utilize both cellulose

and xylan, including strains of *Clost. phytofermentans* and *Clost. cellulovorans* (Warnick et al., 2002). *Clost. phytofermentans* is an obligate anaerobe, mesophilic, Gram-negative, cellulolytic, motile, straight rod, spherical terminal spore former that can utilize cellulose, pectin, polygalacturonic acid, starch, xylan, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, ribose, and xylose in growth medium in the temperature range of 35–37°C. The major end products of this fermentation are ethanol, acetate, CO_2, and H_2 and minor products include formate and lactate (Warnick et al., 2002). The genome of *Clost. phytofermentans* (ATCC 700394) encodes for the highest number of enzymes for degradation of lignocellulosic biomass among the sequenced *Clostridia* genomes (Weber et al., 2010). In one of our laboratories, this species has been used to ferment LCB with and without pretreatment and observed production of ethanol under laboratory conditions. Others have also observed that this species can ferment LCB directly without any pretreatment of biomass and the efficiency of ethanol production remains the same with or without pretreatment of biomass. Thus, it has become a promising native anaerobic CBP microorganism. Recently published work shows that it secretes individual enzymes, which not only degrade cellulose and hemicellulose to fermentable sugars but also can consume almost all the sugars present in LCB and produce ethanol and acetate as the major product (Warnick et al., 2002; Weber et al., 2010). In a CBP scheme, *Clost. phytofermentans* can be the best microorganism for the production of ethanol in a cost-effective and viable way. In this regard, Jin et al. (2011) have evaluated the performance of *Clost. phytofermentans* (ATCC 700394) on ammonia fiber expansion-treated corn stover (AFEX-CS). Under optimal conditions with 0.5% (w/w) glucan load of AFEX-CS, *Clost. phytofermentans* hydrolyzed 76% of glucan and 88.6% of xylan in 10 days. These values reached 87% and 102% of those obtained by SSCF using commercial enzymes and *Sacch. cerevisiae* 424A. Ethanol titer for CBP was found to be 2.8 g/L which was about 72% of that yielded by SSCF (3.9 g/L) procedure.

Z. mobilis is an anaerobic, Gram-negative bacterium, which produces ethanol from glucose via the Entner–Doudoroff (ED) pathway in conjunction with the enzymes PDC and ADH (Conway, 1992). It is reported that the ethanol yield in *Z. mobilis* cultures could be as high as 97% of the theoretical yield of ethanol from glucose (Sprenger, 1996). When compared to yeast, *Z. mobilis* exhibits 5%–10% higher yields and up to 5-fold greater volumetric productivity while tolerating upto 120 g/L ethanol. Also as a consequence of the low ATP yield, *Z. mobilis* maintains a higher glucose metabolic flux, and correspondingly, higher ethanol productivity (3–5-fold higher as compared to *Sacch. cerevisiae*) (Gunasekaran and Raj, 1999; Sprenger, 1996). *Z. mobilis* also has simple nutritional needs with the exception that some strains require the adition of pantothenate and biotin for their growth (Rogers et al., 1982). However, it is not exploited industrially because of narrow substrate range, that is, it can only utilize three sugars: D-glucose, D-fructose, and sucrose, and is unable to ferment widely available pentose sugars. Second, although *Z. mobilis* is generally regarded as safe (GRAS organism) (Lin and Tanaka, 2006), the postfermentation biomass is not acceptable to be used as animal feed. The later inevitably generates the problem for biomass disposal if it replaces *Sacch. cerevisiae* in the industrial ethanol production. Finally, the continuous ethanol fermentation with *Z. mobilis* tends to be oscillatory (McLellan et al., 1999).

15.5.4 Engineering *Z. mobilis* for Ethanol Production

Z. mobilis is the first recombinant strain engineered to ferment xylose (Zhang et al., 1995a). To achieve this, four *E. coli* genes: xylose isomerase

(xylA), xylulose kinase (xylB), transketolase (tktA), and transaldolase (talB) were introduced to this strain (Zhang et al., 1995a). Xylose isomerase and xylulose kinase convert xylose into xylulose-5-phosphate, an important pentose that can enter the pentose phosphate pathway. Xylulose-5-phosphate is next converted to intermediates of the ED pathway by transketolase and transaldolase. The genes clonned into a plasmid (pZB5) using strong constitutive promoters of either enolase or glyceraldhyde-3-phosphate dehydrogenase from *Z. mobilis*. The transformed strain CP4 with pZB5 grew on xylose, and the ethanol yield was 86% of theoretical yield. This strain also simultaneously fermented glucose and xylose. Xylose uptake depends on the native glucose permease; *Z. mobilis* does not have active sugar transport systems (Parker et al., 1995).

For arabinose fermentation, pZB206 plasmid containing five genes isolated from *E. coli* coding for (1) L-arabinose isomerase (*araA*), (2) L-ribulose kinase (*araB*), (3) L-ribulose-5-phosphate-4-epimerase (*araD*), (4) transketolase (*tktA*), and (5) transaldolase (*talB*) has been contructed and transforend into *Z. mobilis*. The first three enzymes are responsible for converting arabinose to xylulose-5-phosphate, which is converted to ED pathway intermediate xylulose-5-phosphate via transketolase and transaldolase. The *Z. mobilis* strain transformed, namely, ATCC 39676, with the genes coding for the five enzymes earlier, successfully fermented arabinose to ethanol (25 g/L) and displayed a very high yield (98% of theoretical). But the rate of arabinose fermentation was much lower compared to that observed for the xylose. This may be due to low affinity of the glucose permease transporter for arabinose (Parker et al., 1995). Yet another strain of *Z. mobilis*, ATCC 39767, carrying pZB5 containing cellulase genes was able to convert cellulose substrate to ethanol efficiently (Zhang et al., 1995b), however, this strain was sensitive to inhibitors. The strain has previously been used for industrial scale trails (up to 586,000 L) for fermenting ground milo (sorghum), corn or wheat (Doelle and Doelle, 1990).

Lawford and Rousseau (1999) successfully adapted xylose-fermenting ATCC 39767 strain to tolerate higher concentrations of acetic acid, as well as other inhibitors, by culturing the strain continuously in higher levels of lignocellulosic hydrolysates. The continuous culture was run for 149 days and the level of hydrolysate in the medium increased from 10% to 50% (v/v). Isolates recovered at the end of the fermentation demonstrated significantly improved ethanol productivity in the presence of acetic acid compared to the unadapted strain. The adapted strain was subsequently evaluated for converting poplar wood hydrolysate to ethanol in SSF. The hydrolysate was prepared by steam explosion of poplar wood chips followed by overliming to reduce inhibitor levels. The fermentation was performed at 34°C and pH 5.5, which represents a compromise between the optimal conditions for the cellulase enzymes and typical culture conditions for *Z. mobilis*. After 7 days, the fermentation reached an ethanol concentration of 30 g/L, a yield of 54% based on total initial carbohydrate content (McMillan et al., 1999).

Recently, Zhang et al. (2017) have made a number improvements to their *Z. mobilis* strains. The new stain (AX101, parental strain ATCC 39676) ferments both arbinose and xylose and carries the seven important heterologous genes. When AX101 was used to ferment a mixture of sugars (40 g/L glucose, 40 g/L xylose, and 20 g/L arbinose) it fermented all of the glucose and xylose, and 75% of the arabinose in 50 h (Lawford and Rousseau, 2002; Mohagheghi et al., 2002). Overall, ethanol yield was 0.43 to 0.46 g/g with by product of xylitol (3.35 g/L), lactic acid (0.21 g/L), and acetic acid (0.84 g/L). Lactic acid production was reduced in this strain compared to previous pentose utilizing strains, possibly because the arabinose fermentation genes were integrated at the site of the putative *ldh* gene.

15.5.5 Engineering *Klebsiella* for Ethanol Production

Klebsiella oxytoca is an enteric bacterium found growing in paper and pulp streams as well as around other sources of wood. This microorganism is capable of growing at pH as low as 5.0 and temperatures as warm as 35°C. *K. oxytoca* can utilize a wide variety of sugars, including hexoses and pentoses, as well as cellobiose and cellotriose (Doran and Ingram, 1993). The strain is especially appealing for cellulose fermentation by eliminating the need for β-glucosidadase and xylosidases. Another difference between *K. oxytoca* and *E. coli* is ability of *K. oxytoca* to use urea as a sole source of nitrogen. Urea costs roughly half of the ammonium hydroxide on an equivalent nitrogen basis. Urea metabolism does not contribute to media acidification (rather reduces it) and therefore reduces the production costs (Jarboe et al., 2007). In mineral salt medium, *K. oxytoca* starin BW21 produces more than 40 g/L of ethanol from 90 g/L of glucose within 48 h. As in *E. coli*, the PET operon has also been expressed in *K. oxytoca* (Ohta et al., 1991a) and later chromosomally integrated resulting in strain P2 (Wood and Ingram, 1992). *K. oxytoca* strain BW21 derived from strain P2 by eliminating butanediol pathway, produces over 40 g/liter ethanol in 48 h in optimized urea medium, a medium designed specifically for *K. oxytoca*. In addition to the urea as the sole nitrogen source, this medium contains corn steep liquor, mineral salts, and glucose (Wood et al., 2005).

K. oxytoca P2 strains in which *Z. mobilis* ethanol genes have been integrated into the chromosome, have some distinct characteristics (Ohta et al., 1991a). They have advantage over *E. coli* based constructs, for example, (1) they contain transport systems and enzymes that allow the intracellular metabolism of short oligosaccharides from cellulose and xylan (Burchhardt and Ingram, 1992), eliminating the need for β-glucosidase and xylosidases, (2) contain chromosomally integrated *pdc* and *adh* genes (Wood and Ingram, 1992). These strains are designed specifically for their use in SSF process, but their ethanol tolerance is inferior to that of most other yeasts (Doran and Ingram, 1993), however, they rapidly produce ethanol at high yield (30 g/L in 71 h) from both glucose and cellobiose (Golias et al., 2002; Wood and Ingram, 1992).

K. oxytoca strains expressing two endoglucanases from *Erwiniachrys anthemi* and genes for ethanol production from *Z. mobilis* (Zhou and Ingram, 2001) also have high demand for bioethanol production. A native strain of *K. oxytoca* THLC0409 was isolated from a lignocelluloses-degrading microflora. Preliminary investigations were conducted to obtain the optimal growth conditions for this strain. Batch operations were applied for producing ethanol from various lignocellulose sources via direct microbial conversion. *K. oxytoca* THLC0409 has been shown to utilize cellulose (avicel, α-cellulose) as well as natural LCB (corncob, Napier grass, purified bamboo, raw bamboo, rice straw) as substrates. On the other hand, under the optimal conditions, ethanol yields on corncob and Napier grass were 0.0623 and 0.0475 g/g, respectively. This was far higher when compared to other substrates, such as avicel (0.019 g/g), α-cellulose (0.02 g/g), purified bamboo (0.02 g/g), raw bamboo (0.018 g/g), and rice straw (0.016 g/g). Ethanol yields on oat-extracted xylan and corncob-extracted xylan were 0.015 and 0.04 g/g, respectively (Tran et al., 2011).

15.5.6 Engineering *Bacillus* for Ethanol Production

Recombinant cellulolytic *Bacillus subtilis* strains are under construction these days, because of their numerous advantages, for example, (1) it is industrially safe (GRAS microorganism by the Food and Drug Administration), (2) has a very high-protein-secreting capability, and (3) fast

growing with very low nutrient requirements. It has native properties of utilizing soluble pentose, hexose sugars including glucose, xylose, mannose, cellobiose, and so on, with tolerance to very high concentrations of salts and solvents. Further, its genome has been sequenced. Given it is an industrially safe microorganism, the biomass after fermentation can be used as animal feed (Bien et al., 2014). Further, *B. subtilis* is considered a perfect host for the production of secretary protein (Yamane et al., 2004). It can naturally produce several polysaccharide degrading enzymes. Besides carbohydrate active enzymes, *B. subtilis* can also produce metabolites, which can enhance the enzymatic hydrolysis of cellulose (Kim et al., 2009).

A family of 48 cellobiohydrolases from *Clost. phytofermentans* have been successfully expressed and secreted as major extracellular protein in *B. subtilis* using high-level expression and secretion system (Zhang et al., 2010). Doi (2008) has constructed a cellulosome producing *B. substilis* strain from a protease deficient *B. subtilis*. However, for making good cellulolytic strain, coexpression of many cellulases in the same strain remains to be challenging. Plasmid and chromosome integration expression has been proposed to be a better alternative for minimizing complex expression (Nguyen et al., 2005) as the chromosome of *B. subtilis* has a large number of dispensable regions (Westers et al., 2003). Cellulase productivity of *B. subtilis* may be enhanced by reducing its genome size by eliminating spore-formation-related genes, protease genes, sigma factors, genes related to catabolite inhibition, glucose metabolism, and intercellular signaling mechanism (Ara et al., 2007). In this regard, Morimoto et al. (2008) have enhanced recombinant protein productivity by genome reduction by depleting of 874 kb (20%) of genomic sequence and named the new stain as *B. subtilis* MBG 874. One-step production of ethanol or lactate from cellulose or pretreated biomass from recombinant *B. subtilis* is also possible (Zhang and Zhang, 2010).

15.5.7 Yeasts and Their Role in Fermentation

Sacch. cerevisiae is a facultative anaerobe that can ferment glucose into ethanol efficiently. It can tolerate temperatures up to 40°C with an optimum temperature of about 30–35°C. It is a preferred organism in the industrial scale for ethanol production from sugar or starch rich substrates because fermentation of sugar and starch generally reaches 90%–95% of the theoretical yield, equivalent to 0.45–0.48 g/g sugar in the raw material. The theoretical specific productivity for *Sacch. cerevisiae* is around 2 g ethanol/g cells/h. This makes it a much faster ethanol producing organism compared to filamentous fungi (Skoog and Hahn-Hägerdal, 1988). Lately *Sacch. cerevisiae* is also being employed in ethanol production from LCB because it can tolerate ethanol up to 105 g/liter (Busche et al., 1992). This organism can also tolerate lignocellulose derived metabolic inhibitors (organic acids, phenolics and furan derivatives) (Hahn-Hägerdal et al., 2006) as well as a wide range of pH (although acidic pH preferred to avoid the problem of contamination by pathogenic bacteria). The main challenge associated with ethanol production from lignocellulosic hydrolysate using *Sacch. cerevisiae* is its inability to ferment a wide range of sugars (mono-, di-, and trisaccharides) derived from cellulose and hemicellulose thus making the ethanol production cost-prohibitive. Neither it can readily utilize starch, cellulose, and hemicellulose, nor the disaccharides cellobiose and xylobiose (Lynd et al., 2002; Zhang and Lynd, 2005)]. Further, *Sacch. cerevisiae* is also unable to ferment pentose sugars (xylose and arabinose). Given the earlier challenges, extensive research has been focused on engineering *Sacch. cerevisiae* for efficient utilization of sugars in commercial settings.

Yeasts, other than *Sacch. cerevisiae*, also referred to as nonconventional yeast, are important in alcohol fermentation. They include the species of *Zygosaccharomyces* and *Hanensula*

polymorpha. These species can utilize pentose sugars, such as xylose and arabinose, as well as starch and lignocellulose derived di- and trisaccharides (Skoog and Hahn-Hägerdal, 1988). However, their use in industrial settings to produce bioethanol from LCB has been unsuccessful due to lack of robustness.

So far, more than 2000 yeast species have been described and some of these could provide a solution to these limitations mostly encountered by native *Saach. cerevisiae*, that is, unable to ferment pentose sugars (xylose and arabinose). Utilization of pentose sugars is a predominant cost-reducing factor in second-generation bioethanol reactors. The nonconventional yeast species showing unusual tolerance to stresses include *Zygosaccharomyces rouxii* (osmotolerance), *Kluyveromyces marxianus* (Ogataea; methylotrophic yeast), and *H. polymorpha* (thermotolerance), *Dekkera bruxellensis* (ethanol tolerance), *Pichia kudriavzevii* (furan derivatives tolerance), and *Z. bailii* (acetic acid tolerance). These organisms could be potentially used as model organisms to study the molecular basis for the tolerances as well as to further develop robust *Sacch. cerevisiae* (Radecka et al., 2015).

The hemicellulosic hydrolysate consists pentose sugar mainly xylose and arabinose, which consistitute about 25% of the total ferentable sugar present in the lignocellulosic hydrolysates. However, depending on the raw material the arabinose fraction may be substantial. The natural xylose fermenting organisms are very limited; for example, *Scheffersomyces stipitis* (*Pichia*) and its anamorphs *Candida shehatae* and *Pachysolen tanophilus* have been isolated that can ferment xylose to ethanol under oxygen-limited conditions. *Pich. stipitis* is also able to produce hemicellulolytic enzymes (Njoku et al., 2013) and its unique ability to ferment xylose with less production of xyliol (a poor substrate for pentose sugar fermentation) (Nigam, 2001). Furthermore, it will not require vitamins for growth. It also possesses both high-affinity and low-affinity proton symport that operate simultaneously. These yeasts are less tolerant to pH, ethanol, and hydrolysate inhibitors when compared to *Sacch. cerevisiae* (Koti et al., 2016; Ma et al., 2013). Nonetheless, they can be utilized as the source of genes to be introduced into *Sacch. cerevisiae* for improvement of ethanol production in industrial processes.

15.5.8 Metabolic Engineering of *Sacch. cerevisiae* for Pentose Fermentation

Sacch. cerevisiae cannot utilize xylose but it can use its keto isomer xylulose and ferment it to ethanol (Chiang et al., 1981). Although it harbors all the genes (xylose reductase, xylose dehydrogenase, xylulokinase) required for the xylose utilization (Deng and Ho, 1990), it cannot grow in xylose medium or ferment it to ethanol. It might be due to low level of expression of these genes. Attempts to upregulate endogenous xylose utilization pathways through an adaptation protocol have been positive, the rate of xylose utilization and its fermentation to ethanol by such strains have not reached the industrially competitive levels yet (Attfield and Bell, 2006).

Initial attempts to introduce the xylose isomerization pathway genes (Fig. 15.4) into *Sacch. cerevisiae* employing bacterial xylose isomerization pathway genes have been unsuccessful due to the difficulties in expressing these genes functionally in yeast (Amore et al., 1989; Moes et al., 1996; Sarthy et al., 1987). There have been attempts to identify novel xylose isomerization pathway genes from soil metagenomics libraries based on protein sequences and activities in *E. coli*, however, the identified xylose isomerization pathway genes could not perform efficiently in *Sacch. cerevisiae* (Parachin and Gorwa-Grauslund, 2011). Discovery and application of eukaryotic xylose isomerization coding genes from anaerobic fungi is ongoing (Harhangi et al., 2003; Madhavan et al., 2009). Kuyper et al. (2003) have reported that *Sacch. cerevisiae* can utilize xylose when heterologous genes XYL1 and XYL2 encoding xylose reductase and xylitol

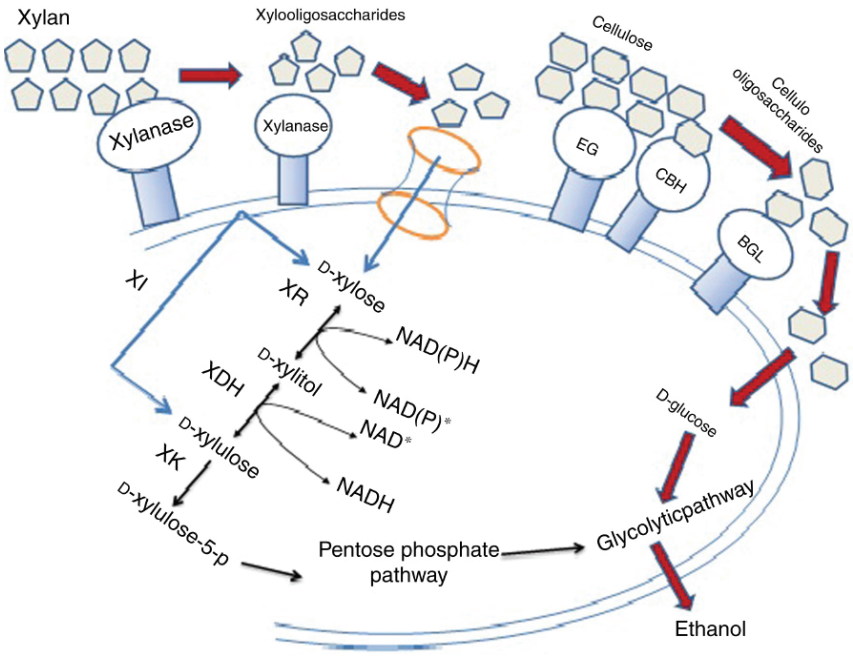

FIGURE 15.4 **Schematic representation of xylose utilization pathway.** BGL: β-Glucosidases, CBH: cellobiohydrolase EG: Endogluconase, *XDH*, Xylose dehydrogenase; *XI*, xylose isomerase; *XK*, xylose kinase; *XR*, xylose reductase.

dehydrogenase from *P. stipites* were introduced and overexpressed in *Sacch. cerevisiae*. The ethanol yields obtained in such engineered *Sacch. cerevisiae* was found to be low due to cofactor imbalance. Xylose reductase has a higher specificity for NADPH than for NADH (*Km* = 3.2 μmol./L for NADPH and *Km* = 40 μmol./L for NADH) and xylose dehydrogenase uses only NAD. In yeast, there is a disparity between the amount and accessibility to intracellular NADPH and NADH for the xylose pathway enzymes (Fernandes et al., 2009), an impairment in the redox balance resulting from the different coenzyme specificities of xylose reductase and xylitol dehydrogenase. This disparity could be due to competition for NAD by other endogenous metabolic enzymes or inefficiency of other xylose pathway enzymes (Fernandes et al., 2009; Krahulec et al., 2010). A severe cofactor imbalance can also lead to poor cell growth or little ethanol production even though the enzymes are actively expressed (Bruinenberg et al., 1983; Hahn-Hägerdal et al., 2007). Various approaches to alleviate the cofactor imbalance have been reported and these efforts include (1) controlling xylose reductase/xylose dehydrogenase expression ratio (Walfridsson et al., 1996), (2) mutations to reduce the affinity of xylose reductase for NADPH (Jeppsson et al., 2006), (3) reduce the affinity of xylose dehydrogenase for NAD (Metzger and Hollenberg, 1995), and (4) shifting the cofactor specificity of xylose dehydrogenase from NAD to NADP (Hou et al., 2007). Similarly, to avoid xylitol accumulation and cofactor imbalances xylose isomerization pathway genes that directly converts D-xylose into D-xylulose without redox reactions have also been attempted (Jeffries, 2006).

Heterologous expression of bacterial xylose isomeraization genes, which was initially from bacterial strains (Amore et al., 1989), anaerobic fungi assimilating xylose, for example,

Piromyces (Kuyper et al., 2003) and *Orpinomyces* (Madhavan et al., 2009) have also been introduced into *Sacch. cerevisiae* but expression of an active prokaryotic xylose isomerase in *Sacch. cerevisiae* has been a challenge. Efforts to express an active prokaryotic xylose isomerase from *E. coli* (Sarthy et al., 1987), *B. subtilis* (Amore et al., 1989), and *Clost. thermosulfurogens* (Moes et al., 1996) in *Sacch. cerevisiae* have failed. The first successful attempt at expression of *xylaA* gene encoding for xylose isomerase from thermophilic bacterium *Thermus thermophilus* into *Sacch. cerevisiae* was performed by Walfridsson et al. (1996), however, the activity of thermophilic enzymes (temperature optima of 85°C) was very low at 30°C. More recently, a xylose isomerase from obligate anaerobic rumen fungus, that is, *Piromyces*, has been expressed in *Saach. cerevisiae* with an activity of ~1 U/mg protein at 30°C and produced a high yield of ethanol (0.42 g/g xylose). One of the disadvantages has been that the xylose isomerase is strongly inhibited by xylitol. Bacterial xylose isomerase with high sequence similarity to the *Piromyces* xylose isomerase, such as those from *Bacteroides thetaiotaomicron* and *Xanthomonas campestris*, have also expressed in *Sacch. cerevisiae*, but the enzyme expression levels in *Sacch. cerevisiae* are lower than that of *Piromyces*. Similarly, Brat et al. (2009) have reported successful cloning and expression of xylose isomerase from *Clost. phytofermentans* in *Sacch. cerevisiae* with ethanol yield of 0.42 g/g of xylose consumed.

15.6 CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, much research has gone into identifying many organisms that are capable of producing industrially important enzymes for ethanol production. Isolation of genes, heterologous cloning of these genes into suitable organisms and metabolic engineering has been largely successful. Some of recombinant organisms have been very robust, however, challenges to use them in commercial settings remain to be addressed, although the potential of using these organisms in commercial settings for ethanol production remains high and optimistic.

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Further Readings

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ISMET
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Certificate

This is to certify that Mr./ Ms. /Dr. Jasina Joshi.
has participated in the *"4th Asia Pacific – International Society of Microbial Electrochemistry and Technology (AP-ISMET) Meeting with special focus on "Bioelectrochemical And Electrochemical Approaches for Decentralized Sanitation"* during November 13th -16th, 2018 held at Birla Institute of Technology & Science - Pilani, K K Birla Goa Campus, Goa, India.

15th November, 2018
Date

Convener

"REGIONAL CO-OPERATION FOR BIOTECHNOLOGY"

SABC-2017 Kathmandu, Nepal

3RD INTERNATIONAL SOUTH ASIAN BIOTECHNOLOGY CONFERENCE

March 16-18, 2017

Certificate of Presentation

This is to certify that Jarina Joshi

has presented Research (Oral / Poster) entitled

at the **3rd International South Asian Biotechnology Conference**

held at Kathmandu, Nepal.

Sarbajit Pd. Mahato Secretary Ministry of Science & Technology Nepal	**Prof. Krishna D Manandhar** Head Central Department of Biotechnology Tribhuvan University	**Dr. Mukunda Ranjit** President Nepal Biotechnology Association	**Sameer M Dixit, PhD** Convenor SABC-2017

Ministry of Science and Technology | Central Department of Biotechnology | Nepal Biotechnology Association | Nepal Academy of Science & Technology | South Asian University

Nepal Biotechnology Association
Kathmandu, Nepal

CERTIFICATE

This is to certify that

Prof./Dr./Mr./Ms. Tasina Joshi

has Chaired a session/ Presented/ Participated at the

National Conference on Biotechnology entitled, Plant Biotechnology for Economic Development

being held on 22-23 Nov 2014, Kathmandu, Nepal.

Dr. Mukunda Ranjit
President, NBA
Principal, SANN International College

Prof. Jiba Raj Pokharel
Vice Chancellor,
Nepal Academy of Science and Technology

Prof. Pranab Goswami
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CENTRE FOR ENERGY

TO WHOM IT MAY CONCERN

This is to certify that **Mrs. Jarina Joshi Rajbhandari**, Lecturer, Central Department of Biotechnology, Tribhuvan University, Nepal, has completed a short term visit and training in my laboratory at Centre for Energy, IIT Guwahati for the period of 20th September to 19th November, 2015. The main purpose of this visit was to acquire advance knowledge on Microbial Fuel Cell (MFC) with an aim of utilizing the knowledgebase in her future work. During the period, she had interacted with my research group and presented a seminar with an idea to exchange her views with my group. She had undertaken an intensive experiment on MFC using the existing fuelcell setup designed by my group where a mixed microbial consortium initially isolated in the lab as anodic catalyst and a chemical catalyst in the cathodic compartment was used to perform the experiment. The cell was assembled with nafion membrane for compartmentalize the half-cells. She investigated the voltage generation in response to glucose as anodic substrate and recorded the data through a data logger periodically. The fuel cell was run for one week to get stable Open Circuit Voltage (OCV). The voltage generated by MFC was observed by supplying an external load of 1KΩ and the corresponding current was measured. Additionally, the redox behavior of the bioelectrode was also examined by her in cyclic voltammetry. Finally, MFC electrodes were coated with Carbon Nano Tubules/ Polyaniline (CNT/PANI) and observed the enhancement in OCV generation. She also got an opportunity to operate advance equipment such as, FTIR, HPLC and XRD instrument available in IIT Guwahati. I wish all success for her future carrier.

19/11/15

Professor Pranab Goswami, Ph.D.
Department of Biotechnology
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Trained Certification Letter

Trainee

Name: Jarina Joshi

Position: Lecturer

Affiliation: Central Department of Biotechnology, Tribhuwan University

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Trainer:

Doo Hyun Park, Professor, Ph.D.

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As above aforementioned, Jarina Jorshi trained in Seokyeong University for 15 days from July 29, 2013 to August 14, 2013. She studied about alcohol fermentation and tried to apply electrochemical bioreactor for cultivation of Zymomonas mobilis. She learned and practiced successfully to work by herself after returning to her country (Nepal). She is bringing two electrochemical bioreactors that were used for learning and practicing in Doo Hyun Park's lab. Doo Hyun Park gave Jarina Joshi the electrochemical bioreactors for self-practicing because she didn't have enough time to practice in Korea.

August 14, 2013

Doo Hyun Park

D. H. Park

National Institute of Environmental Health Sciences

Nepal Progressive Sustainable Developers

Certificate of Participation

in the 2-day workshop

Scientific Writing and Publishing

awarded to

Jarina Joshi

on 4th November 2014

Banalata Sen, PhD, MPH
Lead, Training and Capacity Building
WHO - NIEHS Collaborating Center
National Institute of Environmental Health Sciences, USA

Govinda Bhandari, MSc
President, Progressive Sustainable Developers Nepal (PSD-Nepal)
Editor-in-Chief, International Journal of Environment (IJE)