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Bioethanol production from lignocellulose, whey and starch using yeasts isolated from Ethiopian traditional beverage and other sources

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DECLARATION

I, the undersigned, declare that the thesis hereby submitted for the Degree of Doctor of Philosophy (PhD) in Biology (Applied Microbiology) to the Department of Microbial, Cellular, and Molecular Biology, Addis Ababa University. This is my own work and has not previously been submitted at another University. The materials obtained from other sources have been duly acknowledged in the thesis.

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Acronym

atm	Atmospheric Pressure
BOD	Biological Oxygen Demand
CCD	Central Composite Design
CFU	Colony Forming Unit
CMC	Carboxymethylcellulose
COD	Chemical Oxygen Demand
CSL	Corn Steep Liquor
CV	Coefficient of Variation
DDGS	Distillers Dried Grains with Solubles
df	Degree of Freedom
DNS	Dinitrosalicylic Acid
EPFBF	Empty Palm Fruit Bunch Fibers
FAO	Food And Agricultural Organization
FBMAN	Fermentation Broth Medium for *A. Niger*
GRAS	Generally Regarded As Safe
GS-MS	Gas Chromatography with Mass Spectrometry
HMF	5-Hydroxymethylfurfural
HPLC	High Pressure Liquid Chromatography
MSW	Municipal Solid Waste
NCBI	National Center for Biotechnology Institute
OD	Optical Density
ORP	Oxidation Reduction Potential
RSM	Response Surface Methodology
SCP	Single Cell Protein
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
SSF_F	Solid State Fermentation
V/V	Volume by Volume
VHG	Very High Gravity
W/V	Weight by Volume
YPD	Yeast Extract Peptone Dextrose
YPDA	Yeast Extract Peptone Dextrose Agar
YPL	Yeast Extract Peptone Lactose

Abstract

Ethanol is produced by yeasts and used for different applications. The ever-increasing demand for energy necessitates the production of ethanol as biofuel to supplement fossil fuels as a source of energy and protect the environment from environmental pollution. This requires for selection

of effective ethanol producing local yeasts to produce ethanol from easily available agricultural wastes. To this end, indigenous yeasts were isolated from yeast habitats such as traditional fermented beverages, soil, flower, and compost samples collected from different part of Ethiopia. The isolates were screened for their glucose fermentation and ethanol production.. The selected yeast isolates with better ethanol production from glucose were identified using 26S rRNA sequence analysis using NL1 and NL4 primers. They were further evaluated for their ability to ferment different carbon sources, sedimentation rate, tolerance to sugar and ethanol concentration. Production of ethanol by selected yeasts was optimized based on variables such as inoculum size, temperature, pH, and incubation time using response surface methodology based on central composite design. Enzymatic, acid, and alkali hydrolysis, and coculture were employed to produce ethanol from grass pea and wild oat straws and starch. In order to produce ethanol from whey, experimental runs such as molasses and external nutrient supplementation, effect of whey pH, sterilized and non-sterilized whey were evaluated. Five isolates were identified as *Saccharomyces cerevisae* and the remaining three were grouped into *Kluveromyces marxianus*, *Pichia fermentans* and *Candida humilis*. The pattern of sugar utilization showed that only *K. marxianus* ETP87 and *P. fermentans* ETP22 were able to grow on xylose; and *K. marxianus* ETP87 was the only yeast that fermented lactose and, therefore, was selected to produce ethanol from whey. All the 8 yeasts other than *C. humilis* were able to flocculate a feature that makes the biomass separation easy for industrial applications. Regarding tolerance to ethanol, *S. cerevisae* ETP53, *K. marxianus* ETP87, *P. fermentans* ETP22 and *C. humilis* ETP122 were tolerant to 10% extraneous ethanol but the percentage of ethanol tolerance considerably decreased at 15% and 20% ethanol shock treatment. *S. cerevisae* ETP53 produced ethanol optimally at pH 5.0, 60 hours, and 34°C; whereas the optimal growth and fermentation by *K marxianus* ETP87 was at pH 4.8, temperature 36°C, and incubation time of 65 hours. Highest reducing sugar was released from 1% (v/v) H_2SO_4 and 1% (w/v) NaOH treated straws; where sulfuric acid yielded higher amount of sugars than NaOH, and more ethanol was obtained from alkali hydrolysates. Significant amount of furfural was liberated from acid hydrolyzed straw that was reduced by treatment with activated carbon and overliming. The data also showed that significant amount of sugars was released from fungi (*Pleurotus ostreatus* M2191, *Pleurotus sajor-caju* M2145, *Trichoderma reesei* JCM22676, and *Aspergillus niger* JCM22344) grown on straws solid state media than enzymatic and chemically treated straws. However, the ethanol

production from fungal-treated straws was small. It was also established that higher ethanol production was obtained from acid-hydrolyzed sorghum flour than the one produced from crude amylase enzyme treated substrate and ethanol derived from coculturing, indicating that simultaneous saccharification and fermentation is a promising method to produce ethanol from starch. The data also showed that *K. marxianus* ETP87 was capable of producing ethanol from non-sterilized and non-deproteinized substrates. The effect of nutrient supplementation to whey was variable depending on the kind of nutrient added. Generally, it could be concluded that *S. cerevisiae* ETP53, *K. marxianus* ETP87, and *P. fermentans* ETP22 are promising yeasts to produce ethanol from different substrates at acidic pH, near 35°C and late fermentation time.

Key words: ethanol fermentation, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Candida humilis*, lignocellulose, sorghum flour, whey

Chapter 1

INTRODUCTION

Yeasts are unicellular fungi that are widely distributed in a wide variety of habitats, and occupy aquatic and terrestrial environments that are moist with abundant supply of simple, soluble nutrients such as sugars and amino acids (Pozo *et al.*, 2012). Consequently, they are common on leaf and fruit surfaces, on roots and dairy products, fermented foods and beverages, different soil. They are also found in association with invertebrate animals, and even in extreme environments. A few group of yeasts are able to degrade polymers, such as starch and cellulose characteristics of many hyphal fungi (Tarr, 2004).

Yeasts are industrially important in production of bioethanol, enzymes, organic acids, and others industrially important chemicals. Besides yeasts, bacteria also produce ethanol. Bioethanol, a renewable fuel, is becoming important as one of the several alternatives to fossil fuels because of depleting oil reserves, rising crude oil prices and greenhouse effect of gases emanating from burning these fuels (Tatijarern *et al.*, 2013). The primary raw materials for ethanol production are grain starch following chemical, physico-chemical, or/and biological treatment and saccharification. In addition to grain starch, ethanol production from potato, sweet potato, and cassava is also increasing.

In Brazil, ethanol is produced from sugarcane while biodiesel (methyl or ethyl esters) is produced from soya and castor beans (Herrera, 2006). Malaysia and Indonesia produce ethanol from palm oil; whereas Canada and USA use corn, corn kernels and corn stover and wheat for the production of ethanol and the United States mostly uses corn.

However, starch based ethanol production (first generation feedstocks) are not sustainable. Consequently, these days, industrial and agricultural wastes (second generation feedstocks) such as molasses, whey, and other lignocellulosic feedstock have become attractive raw materials for the production of bio-ethanol because of their availability in large quantities at low cost (Bhattacharya and Kumar, 2010).

Molasses, which is obtained from sugar cane and sweet potato processing industries, is the cheap and most commonly employed substrate for industrial ethanol production by yeasts. Molasses serves basically as a carbon source and the composition of other nutrients from sugar cane molasses varies over the harvesting season and with cultivation condition.

Cheese whey is the liquid residue from cheese-making processing that remains after milk represents about 85–95% of the milk volume and retains 55% of milk nutrients (Guimarães *et al.*, 2010). It is composed of 92–95% w/w water and 5–8% w/w dry matter, of which around 10–20% are proteins, 60–80% are lactose and the rest are minerals, vitamins, fat, lactic acid and trace elements (Vamvakaki *et al.*, 2010). According to (Akbas and Stark, 2016) 5-6% lactose in the liquid portion and other nutrients are used for the growth of *Kluyveromyces* spp to produce ethanol.

The use of first generation feed stocks is ultimately unsustainable to meet growing demands for future biofuel production because of severe limitations to starch and sugar-based ethanol production. Second generation raw materials for bioethanol production typically refer to non-food biomass sources, mainly lignocellulosic biomass. Non-food, or second generation, feedstocks for bioethanol are, therefore, the future promising substrates due to abundance, ethical considerations and favorable economics.

In Ethiopia starch rich grains are mainly grown for food and biofuel production from these substrates leads to land competition for fuel and food and this might be more severe in the food insecure country. Alternatively, there are rich sources of agricultural wastes in the country. The country has several sugar cane industries with a large amount of molasses for ethanol production. Since the country is agrarian, there are other additional rich lignocellulosic sources such as straws of common wild oat, or simply wild oat (*Avena fatua* L) and grass pea (*Lathyrus sativus* L.), staple food teff, barley, wheat can be used for yeast ethanol production.

OBJECTIVES

General Objective

The general objective of this research project was to isolate, test strains, and evaluate their potential for ethanol production from lignocellulose, sorghum flour, and whey.

Specific objectives

The specific objectives of the this study were to

- Isolate, characterize, and identify yeasts for ethanol production.
- Optimize ethanol production by the yeast isolates through experimental variables and model.
- Produce ethanol from grass pea and wild oat straws, whey, and starch.

Chapter 2

LITERATURE REVIEW

The role of yeast in food and beverage technology is rooted back to several millennia (Mattanovich *et al.*, 2014). These days, yeasts are used for many biotechnological applications in food industries (enzymes, flavors, amino acids), beverage industries (ethanol, and organic acids), pharmaceutical industries (hormones and vaccines). Quite recently, the role of yeasts in environmental biotechnology (bioremediation and pollutant degradation), biocontrol applications (crop protection, food and feed safety, and probiotics) and biomedical research have been well documented (Johnson, 2013).

The modern drive of urbanization and industrialization has increased energy demand in the face of the increase cost of non-renewable energy such as crude oil, coal, and natural gas. The shortage of energy sources together with the increase of green house gases due to release of carbon dioxide from combustion of fossil fuel has become a major world problem (Tatijarern *et al.*, 2013). This necessitates the development of renewable energy.

Nowadays, bioethanol is considered as the most important renewable energy and its global production reached 86 billion liters (Renewable Fuels Association, 2013). According to US Department of Agriculture (2016) report, biofuels accounted for roughly 7.1 percent of total transport fuel consumption, or 13.8 billion gallons in 2012. Bioethanol is a byproduct of anaerobic respiration known as fermentation carried out by a variety of microorganisms, of which yeasts are the most dominant group.

Yeasts are unicellular fungi that are capable of reproducing by budding or fission and form spores which are not enclosed in a fruiting body. They are first classified based on its sexuality (Ascomycotina or Basidiomycotina) or the lack of sexual phase in the life cycle (Deuteromycotina). Approximately 1500 species (1% of yeast species) is currently known (Mohd Azhar *et al.*, 2017). They can be isolated from the terrestrial, aquatic and aerial environment. Plant is the preferred habitat of yeasts community since they provide simple sugar.

S. cerevisiae is one of the most studied and widely utilized yeasts in brewery, bakery and ethanol industries. It is superior to bacteria, other yeasts and filamentous fungi in various physiological characteristics related to ethanol production at industrial level. It is tolerant to a wide range of pH (Lin *et al.*, 2012) with acidic optimum which makes the process less susceptible to infection than bacteria fermentation. It also tolerates ethanol better than other ethanol producing microorganisms (Prasertwasu *et al.*, 2014). Fermented products from *S. cerevisiae* are free from toxin and other undesirable byproducts and are GRAS (generally regarded as safe) for human consumption compared to other products by others yeasts and microorganisms.

2.1. Substrates for ethanol production

Ethanol can be produced from several substrates such as starch, lignocelluloses and different wastes. Lignocellulosic biomass are preferred to starch or sugar-based crops for production of ethanol, since it is mainly agricultural wastes; it is not questionable for ethanol production as a feedstock; and it reduces environmental pollution (Gutiérrez-Rivera *et al.*, 2012; Ishola and Taherzadeh, 2014).

Ethanol production from different wastes such as molasses, sugar beet pulp, waste from cassava starch production (Akaracharanya *et al.*, 2011), food waste leachate (Le Man *et al.*, 2011), whey and waste newspapers (Xin *et al.*, 2010) has been reported. Some of the advantages of ethanol production from the waste are; to avoid competition of using grains for ethanol production with food and feed production, reduction of cost of production, and reduction cost of waste disposal.

2.1.1. Lignocellulose based substrate

Lignocellulosic substrates are the complex matrix of cellulose, hemicellulose, and lignin. They also include small amount of proteins and pectin which don’t participate significantly in the structure of the substrates.

Cellulose

Cellulose is the most abundant renewable organic resource on Earth. Cellulose is a homopolymer of glucose linked by β-1, 4 glycosidic bond (Figure 2-1) and it forms long straight chain. The

hydroxides in both sides of monomers of long chain form strong intermolecular hydrogen bonds between hydroxyl groups of adjacent molecules in the parallel chains (Figure 2-2). Cellulose is insoluble in water, dilute acidic solutions, and dilute alkaline solutions at ambient temperatures.

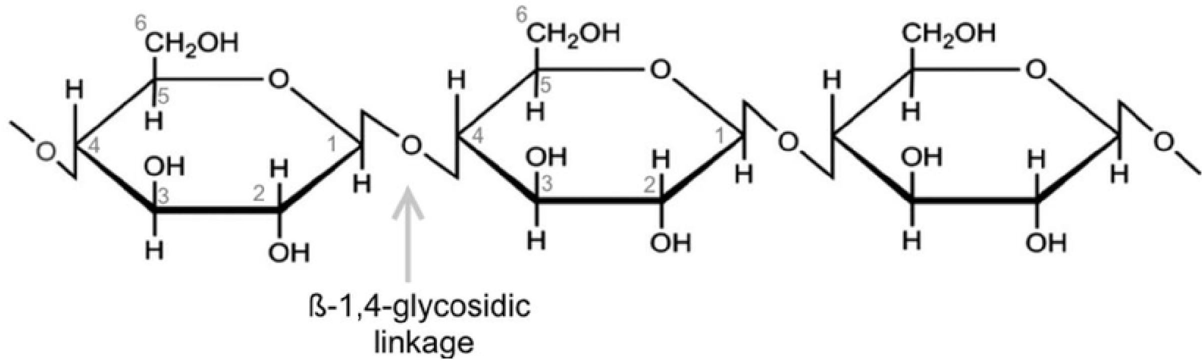

Figure 2-1 β-1, 4-glycosidic bond to synthesize cellulose in plant cells (Zhen, 2013).

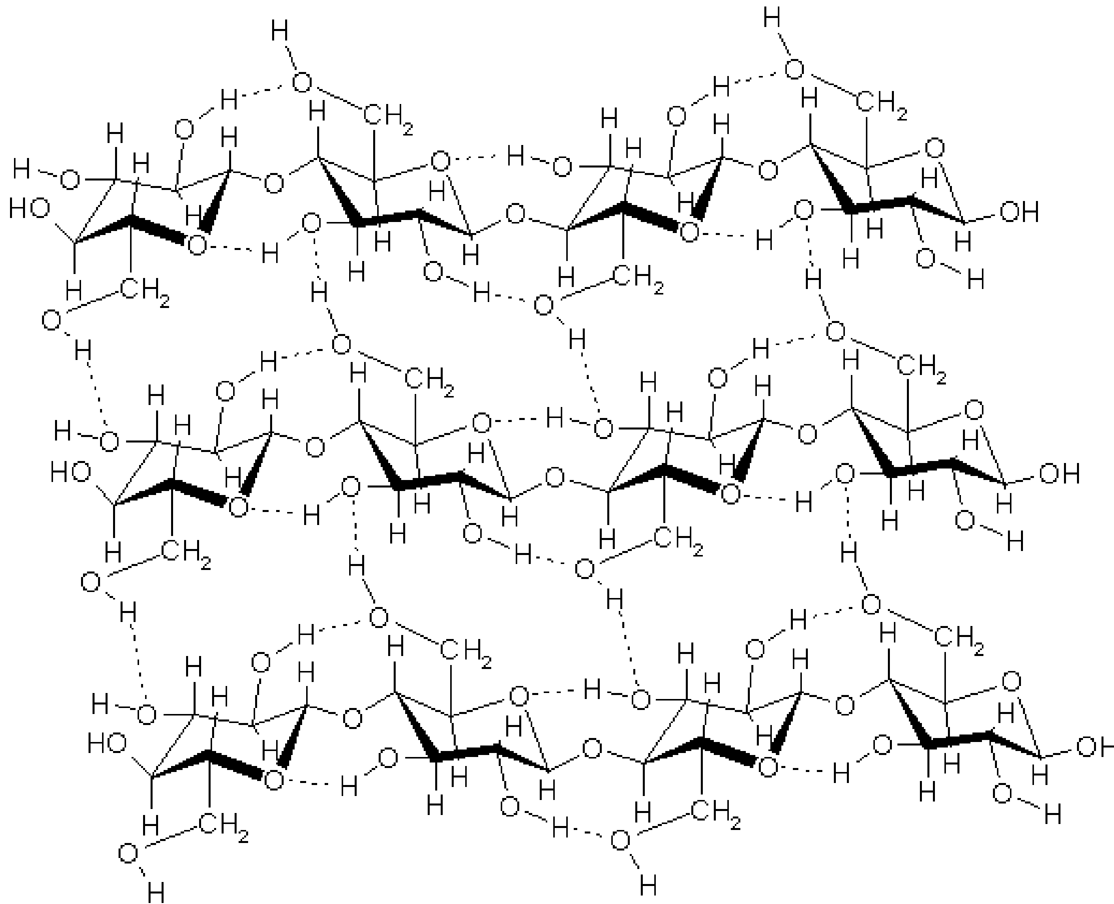

Figure 2-2 Cellulose (Potumarthi *et al.*, 2013)

Hemicellulose

Hemicellulose is the linking material between cellulose and lignin. Unlike cellulose, hemicellulose is composed of mixture of different monosaccharide units such as glucose, mannose, galactose, xylose, arabinose, 4-*O*-methyl glucuronic acid and galacturonic acid residues (Figure 2-3). In hardwood xylan, the backbone chain consists of xylose units which are linked by β-1, 4-glycosidic bonds and branched by β-1, 2-glycosidic bonds with 4-O-methyl glucuronic acid groups but softwood xylan has additional branches consisting of arabinofuranose units linked by β-(1, 3)-glycosidic bonds to the backbone (Potumarthi *et al.*, 2013).

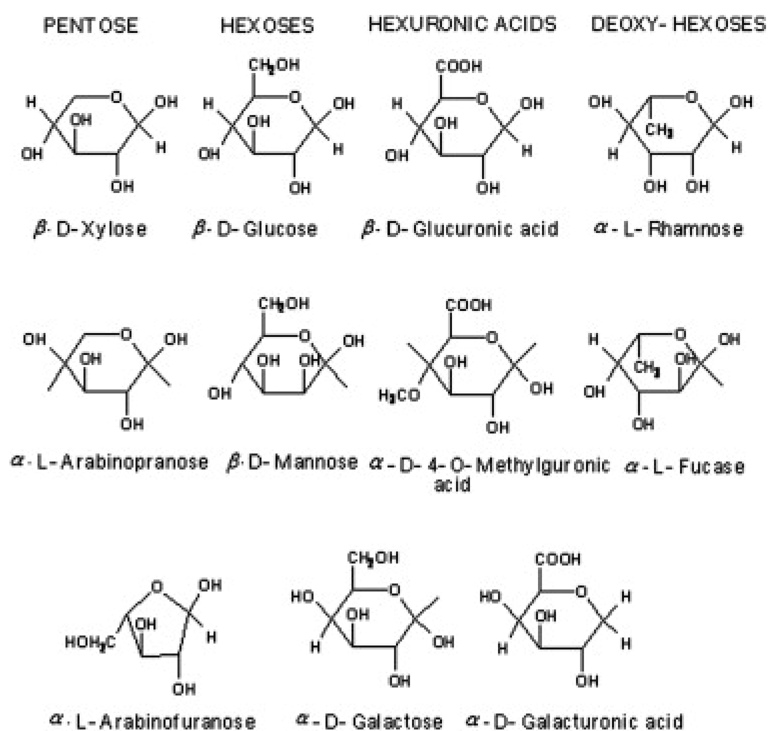

Figure 2-3 Sugar units of hemicellulose (Potumarthi *et al.*, 2013)

Lignin

Lignin is an aromatic polymer synthesized from dimethoxylated (syringyl), monomethoxylated (guaiacyl), and nonmethoxylated (p-hydroxyphenil) phenylpropanoid units (Figure 2-4 and Figure 2-5). Lignin is hydrophobic and highly resistant toward chemical and biological degradation (Potumarthi *et al.*, 2013). It is located in the middle lamella, acting as cement between the plant cells, and in the layers of the cell wall, forming, together with hemicellulose,

an amorphous matrix in which the cellulose fibrils are embedded and protected against biodegradation.

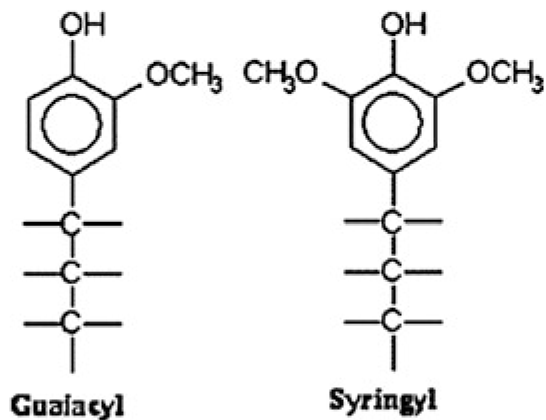

Figure 2-4 Monomeric building units of lignin (Potumarthi *et al.*, 2013)

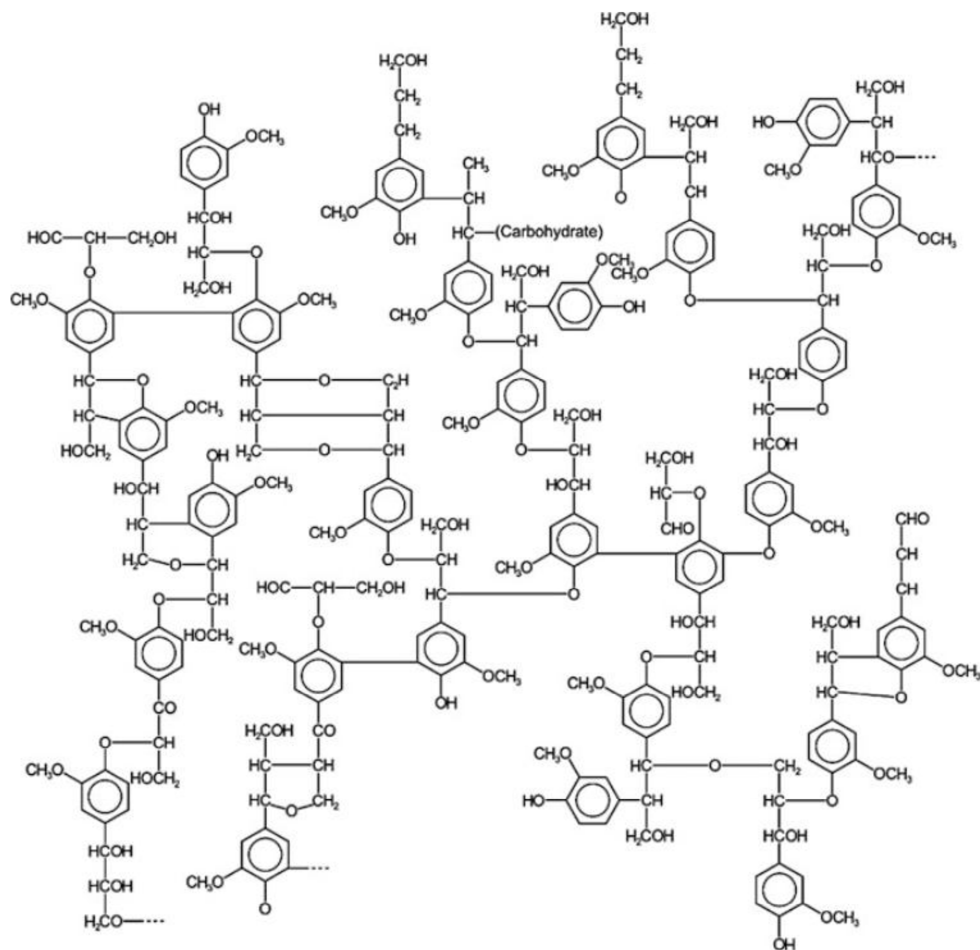

Figure 2-5 Structure of lignin (Potumarthi *et al.*, 2013)

The lignocellulosic agricultural residues such as mission grass, rice husk, wheat straw, leaf and stems of *Dendranthema grandiflora*, corn stover, sugar cane leaves, and news paper were investigated by different researchers to optimize lignocellulosic ethanol production (Table 2.1 and Table 2.2).

Table 2.1 Ethanol production by *S. cerevisiae* from different substrates at various treatment and optimization conditions

***S. cerevisiae* strain**	**Substrate**	**Pretreatment**	**Enzymatic Hydrolysis**	**Ethanol (g/L)**	**References**
MTCC 174	Rice husk	NaOH	Crude enzyme	14	(Singh *et al.*, 2014)
ATCC 26602	Wheat straw	H_2O_2	Cellulase	10	(Karagöz and Özkan, 2014)
L2524	EPFBF	NaOH	Cellulase	64.2[B]	(Park *et al.*, 2013)
Y5	Corn stover	Steam explosion	Cellulase glucosidase	50[B]	(Tian *et al.*, 2013)
Y5	Corn stover	Steam explosion	Cellulase	40	(Yun *et al.*, 2011)
TISTR 5596	cassava pulp		α-amylase glucoamylase	9.9	(Akaracharanya *et al.*, 2011)
TISTR 5596	cassava pulp	[A]H_2SO_4 or $Ca(OH)_2$	Cellulase	11.9	(Akaracharanya *et al.*, 2011)
DQ1	Corn stover [C]	Steam explosion	Cellulase	55[B]	(Bi *et al.*, 2011)
ATCC 96581	Waste newspaper	Sodium dodecyl sulphate	Cellulase glucosidase	14.29	(Xin *et al.*, 2010)

A : at 121°C and 2 atm; B: simultaneous saccharification and fermentation; and C: supplemented with dry distiller's grain and solubles

2.1.1.1. Pretreatment of Lignocellulose

Ethanol production from lignocellulose at industrial level is still a challenge due to high production cost arising from high energy consumption for the distillation of fermentation broth

with low ethanol titer when lignocellulose materials are used as feedstock (Gu *et al.*, 2014). Therefore, bioethanol production from lignocellulose requires chemical or/and biological pretreatment to increase the sugar concentration (Table 2.1 and Table 2.2).

The lignocellulose biomass can be pretreated by physical, chemical and biological methods or their combinations. Biological pretreatments involve the use of microorganisms usually among white-rot, brown-rot and soft-rot fungi or their enzyme to break down lignin barrier and alter lignocelluloses structure. Biological pretreatment requires no chemicals, low energy input and seems to be the best environmental friendly method of pretreatment (Ishola and Taherzadeh, 2014).

The chemical pretreatment of lignocellulose employs acidic, alkali, ozone and hydrogen peroxide. Acidic pretreatment make use of sulfuric, hydrochloric, phosphoric and nitric acids where as alkali treatment uses sodium hydroxide and potassium hydroxide. The most common mechanical treatments are ball milling and steam explosion.

A variety of researchers showed that chemical pretreatment change the percentage composition of cellulose, hemicellulose, and lignin of plant biomass (Table 2.2); The total lignin was found to decrease after alkali pretreatment (Table 2) and Park Park *et al.* (2013) reasoned out that alkali solublized the lignin easily.

During chemical pretreatment and treatment, complete and efficient sugar utilization is one of the prerequisites for cost effective ethanol production from biomass (Yuan *et al.*, 2012). However, five carbon sugars, cellobiose, disaccharides, and partially degraded cellulose are produced in addition to the six carbon sugars that can be fermented by most ethanol producing yeasts. Among the five carbon sugars, xylose is the most dominant sugar in the hydrolysates. Xylose cannot be utilized by *S. cerevisiae*. Genetic manipulations have been used to add key enzymes to the yeast to ferment both glucose and xylose; yet, this approach has its own limitations due to co-factor imbalances, unfavorable intracellular xylose-to-xylulose isomerization equilibrium, and decreased robustness of the yeast.

Table 2.2 Composition of selected lignocellulosic substrate (in percentage)

S. cerevisae strain	Substrate	Pretreatment	Composition (A) of substrates						Ethanol (g/L)	References
			Cellulose		Hemi-cellulose		Lignin			
			Before	**After**	**Before**	**After**	**Before**	**After**		
CBS 8066	EPFB	H_3PO_4 and fungal	39.13	53.81	23.04	9.07	34.37	37.22	23 (B)	Ishola and Taherzadeh (2014)
TISTR 5596	Mission grass	NaOH	47.2		27.3		18.2		16 (C)	Prasertwasu *et al.* (2014)
ATCC 26602	Wheat straw	H_2O_2	42.8	63.5	23.8	23.6	15.1	9.1	10	Karagöz and Özkan (2014)
MCAB-H	bagasse	H_2SO_4	20.9	27.2	16.3	5.3	33.6	50.3	9.59	Rocha *et al.* (2014)
Fermentis Ethanol	Wheat straw	Stem exploded	40.5	63	26.1	2.7	18.1	35.3	11.3	Moreno *et al.* (2013)
L3262a	EPFB	NaOH	39.8	58.0	17.3	21.1	28.8	8.8	62.5 (D)	Park *et al.* (2013)
Thermosacc®	Rice straw	NaOH	38.89	69.87	23.21	18.09	20.65	3.28	12.17	Suriyachai *et al.* (2013)
MTCC 174	Bagasse	NaOH	43	55.2	24	31.6	20	8.3	15.4	Singh *et al.* (2013)

(A) the extractives and ashes are included in compositional analysis; (B) there was additional pretreatment with white-rot fungus *Pleurotus floridanus*; (C) the hydrolysates were overlimed at pH 10; and (D) the fermentation was carried out under simultaneous saccharification and fermentation (SSF) with cellulase and yeast

To address such challenges, Yuan *et al.* (2012) used a new approach by isomerizing xylose to xylulose exogenously to obtain; 39 g/L ethanol and 86.4% xylose utilization within 10 hrs. Another approach is to coculture five carbon utilizing and ethanol producing yeast with *S. cerevisiae* (Suriyachai *et al.*, 2013; Karagöz and Özkan, 2014) so that both sugars can be efficiently utilized to optimize production process.

2.1.1.2. Inhibitor Reduction in Lignocellulosic Hydrolysates

Besides the five and six carbon sugars, other groups of inhibitors such as furans (furfural), carboxylic acids (acetic acids and formic acids), and phenolic compounds (catechol, and vanillin) (Ludwig *et al.*, 2013) and glycoaldehyde (Kim *et al.*, 2013) are produced during hydrolysis of lignocelluloses that hinders cell growth and sugar consumption by *S. cerevisiae* .

Furfural are produced as a result of dehydration of five carbon sugars such as xylose by losing three water molecules in the presence of acids and under high temperatures (Taherzadeh and Karimi, 2011).

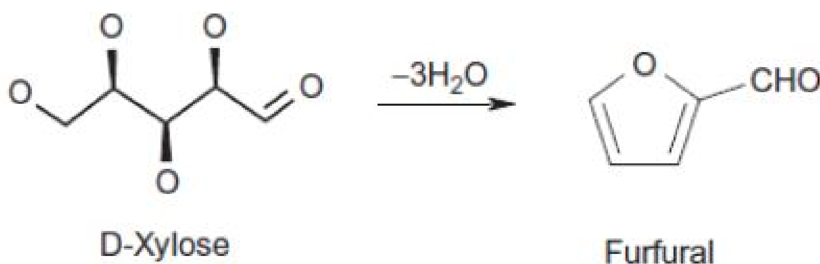

Figure 2-6 Conversion of xylose to furfural (Taherzadeh and Karimi, 2011)

Several physical, chemical and biological methods are employed to solve the inhibitory effects of these chemicals in the production process. The chemical treatment for removing inhibitory chemical include; over-liming (De Bari *et al.*, 2014; Prasertwasu *et al.*, 2014), ion exchange (Ludwig *et al.*, 2013), activated charcoal treatment (Kim *et al.*, 2013), neutralization, and solvent extraction (Zhu *et al.*, 2011).

Evaporation and membrane separation are the most commonly used physical methods to reduce inhibitory substances in biomass hydrolysates (Prasertwasu *et al.*, 2014). Volatile inhibiting

compounds such as furfural, acetic acids, formic acids and other lignin degradation products were reduced by evaporation in lignocellulosic hydrolysates. Though evaporation is less costly and ease to operate, it requires lot of energy and this might make it a little uneconomical at industry level.

Synergistic effects of detoxification methods have been investigated in several studies. For instances, overliming together with sodium sulfite methods (Prasertwasu *et al.*, 2014), overliming and adsorption onto ion-exchange resins, and overliming with activated charcoal methods (Kuhad *et al.*, 2010) further reduced the inhibitor compounds than one method alone.

The biological methods of avoiding the inhibitors include enhancing the yeast to adapt to the inhibitory chemicals with repeated sequential fermentation (Gu *et al.*, 2014), enzymatic treatment with peroxidase and laccase (Zhu *et al.*, 2011), and *in situ* detoxification by fermenting microbes (biological methods) (Taherzadeh and Karimi, 2011). These authors investigated the sequential coculturing of an extreme thermophilic bacterium, *Thermoanaerobacter pentosaceus* and *S. cerevisiae* in alkaline-peroxide pretreated rapeseed straw to reduce inhibitory compounds and enhance ethanol production. The result showed that *T. pentosaceus* was able to metabolize 5-hydroxymethyl furfural and furfural up to concentration of 1 and 0.5 g/L respectively. Likewise, Ludwig *et al.* (2013) were able to detoxify phenolic compounds using immobilized enzymes laccase from *Trametes versicolor*.

2.1.2. Starch

These days, ethanol is being produced from cassava, potato, barely, maize, sorghum, and wheat starch though starch based ethanol compete with food sources in terms of land, labor and capital.

Most yeast cannot ferment starch directly to ethanol. Consequently, starch hydrolysis is the limiting step in starch based bioethanol production (Trovati *et al.*, 2009). Saccharification can be carried out by chemical, mechanical and biological methods. The chemical hydrolysis is carried out by acid or alkaline treatments where as the biological method involves the application of α-amylase and glucoamylase to catalyze the hydrolysis reaction to produce glucose or the use of amylase producing microorganisms to release the sugars. Therefore, when using starch as raw

material, the time and energy required for its prehydrolysis and the price of enzymes represent additional costs when compared to direct fermentation of the raw materials.

The direct saccharification and fermentation of starch is more efficient in comparison to lignocellulose. However, starchy raw materials are very costly and the cost of the feedstock can exceed 65% of the price of final product in order to use ethanol for fuel.

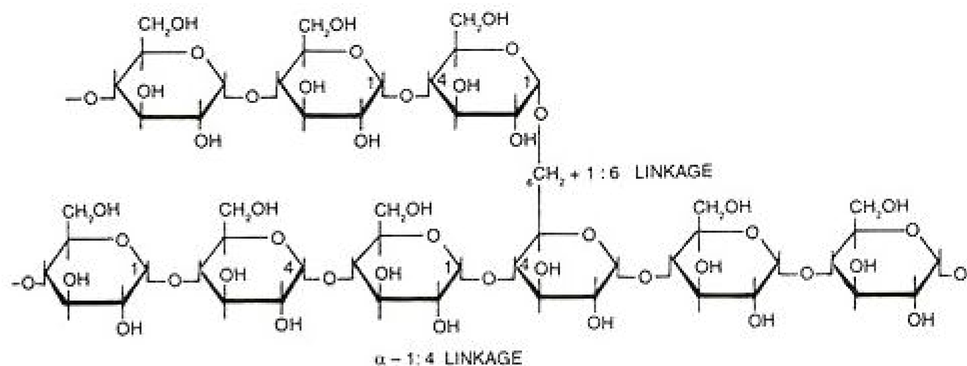

Figure 2-7 Starch (Zhen, 2013)

2.1.2.1. Pretreatments

It is established that the protein which covers the starch in the seed inhibits the starch decomposition. As a result, the entire sheath should be peeled by pretreatment for amylase activity. Starch pretreatment could be mechanical, chemical, and biological. The chemical pretreatment includes the alkali, acidic, ozone, and hydrogen peroxide pretreatment where as the biological one employ the mixture of enzymes, mainly proteases. Abu Bakar *et al.* (2015) recommended overnight soaking of sorghum grain in 0.5M NaOH before drying and grinding of the grains to degrade the protein.

The starch substrate is found together with proteins, cellulose, hemicellulose, and lignin in grains. Consequently, apart for amylases, other accessory enzymes such as proteases, cellulases, xylanases, pollulanases, and β-glucanases are commonly used to enhance starch saccharification and ethanol production (Cinelli *et al.*, 2015; Sapińska *et al.*, 2014). These accessory enzymes (the protease and xylanase enzymes) contribute to a better breakdown of the starch granules and

consequently led to improved accessibility of the starch for amylase saccharification and hence improved production.

The corn's kernel that covers the starch could be degraded by proteases to make starch more exposed to amylases. Therefore, the application of proteases from *Bacillus licheniformis* with amylases improved the ethanol production (Cinelli *et al.*, 2015). The use of commercial proteases such as Stargen™ 001, a commercial fungal acid protease and Neutrase 0.5 L, a bacterial protease from *Bacillus amyloliquefaciens* enhanced starch degradation and increased in ethanol production by 23% (Cinelli *et al.*, 2015) and 31% (Nghiem *et al.*, 2010).

Enhanced dry grind enzymatic process is a new fermentation process for ethanol production from a winter barley variety (Nghiem *et al.*, 2010). This method involves the addition of two enzyme systems. β-glucanases catalyzed the hydrolysis of β-glucans to oligomers fraction and β-glucosidases were added to complete the hydrolysis and release glucose. The cellulase increase the sugar concentration besides exposing the starch granules for amylases (Cinelli *et al.*, 2015).

The application of many accessory enzymes before amylase saccharification is strongly advisable for starch based ethanol production. Sapińska *et al.* (2014) investigated the application of mixture of xylanases, cellulases, cellobiases and pullulanases for the hydrolysis of rye and corn mashes, after pretreatment and obtained better ethanol production due to enhanced starch saccharification. Similarly, the addition of pullulanase, protease and cellulase increased ethanol concentration by 10.86% (v/v), corresponding to 95% yield indicating that the use of a proteolytic preparation accelerated starch degradation and significantly shortened both initial and main fermentation phases (Cinelli *et al.*, 2015).

2.1.3. Cheese whey

The dairy industry is one of the important food processing industries all over the world. Cheese whey is the watery portion formed during coagulation of casein in cheese making; therefore, it is a byproduct of dairy industry. Whey is often considered as a waste in view of the fact that it has high organic load. (Pescuma *et al.*, 2015). Therefore, utilizing whey to produce ethanol is biologically important to treat the wastes besides the ethanol produced.

The composition of whey varies with the composition of milk, the variety of cheese made, and the cheese-making process employed (Koushki *et al.*, 2012). The lactose content of whey lie between 4.5-5.0 % (Ariyanti and Hadiyanto, 2013; Pescuma *et al.*, 2015), and 5-6% (Christensen *et al.*, 2011). The lactose content in acidic whey is smaller than sweet whey since lactose is fermented into lactic acid in acidic whey (Pescuma *et al.*, 2015). In most whey, the protein content ranges from 0.8 to 1.0 % (Pescuma *et al.*, 2015; Christensen *et al.*, 2011). The fat present in whey vary conssiderably. Christensen *et al.* (2011) reported 0.06% of fats in whey where as Koushki *et al.* (2012) found 3% fat. The lactic acid present in sweet and acid whey was 0.00% upto 0.8% respectively (Pescuma *et al.*, 2015).

It is the lactose which is rate limiting step in ethanol production from whey and the presence of lactose in whey as the sole carbohydrate that limit the growth of other microorganisms (Ariyanti and Hadiyanto, 2013).

2.1.3.1. Ethanol producing yeasts from whey

Though the yeasts that assimilate lactose aerobically are widespread, few ferment it (Tavares and Malcata, 2016). *Kluveromyces* species have been widely employed for bioethanol production from cheese whey. They include *Kluyveromyces fragilis* (Dragone *et al.*, 2011), *K. marxianus* (Ariyanti *et al.*, 2014), *Kluyveromyces lactis* (Ariyanti and Hadiyanto, 2013), *Candida pseudotropicalis* (Ariyanti and Hadiyanto, 2013), and *Candida kefyr* (Koushki *et al.*, 2012). However, most of them are very sensitive to ethanol concentration i.e. inhibited by low ethanol concentrations with a low conversion yield (40%) (Tavares and Malcata, 2016).

K. marxianus and *K. lactis* are most commonly employed for ethanol production since they are efficient and tolerant to lower pH of whey. *K. marxianus* is a thermotolerant yeast with high growth yield and β-galactosidase activity. It is, therefore, good candidate to produce ethanol from whey even at high temperature. It was a suitable microorganism for producing ethanol from lactose fermentation, showing a maximum alcohol production efficiency of 96.5 % (Pescuma *et al.*, 2015). *K. marxianus* consumes all lactose present in whey within 16 hours (Ariyanti and Hadiyanto, 2013), indicating its efficiency in ethanol production. Another study showed that lactose utilization began within 24 hours and completed after 72 hours (Christensen *et al.*, 2011).

S. cerevisiae lack the assimilatory path way to convert lactose in whey directly into ethanol. However, ethanol can be produced by *S. cerevisiae* after the whey is treated with β-galactosidase to convert lactose into glucose and galactose (Tomaszewska and Białończyk, 2016).

2.1.3.2. Growth conditions for lactose fermenting yeasts

K. marxianus was able to produce maximum ethanol (6-7 g/L) at 30-35°C in fed batch culture. However, ethanol production significantly declined at 40°C due to the inactivity of enzymes (Ariyanti *et al.*, 2014). Ferreira *et al.* (2015) reported that optimal ethanol concentration (49.65 g/L) were obtained from *K. marxianus* on ricotta whey containing hydrolysates from sugar cane bagasse at pH 5.05, agitation of 65 rpm, and temperature of 40°C. The optimum temperature and pH for *K. marxianus* and *C. kefyr* is 30°C and 4.8 for ethanol production from cheese whey (Koushki *et al.*, 2012). Nowadays, Dairy Farmers in America (USA) and Anchor Ethanol Ltd (New Zealand) are producing eight million gallons per year of ethanol from whey fermentation. However, the yield and rate of ethanol production by lactose fermenting yeast are lower than those of glucose-fermenting *S. cerevisiae* (Pescuma *et al.*, 2015).

2.1.3.3. Ethanol fermentation

Different yeasts produce ethanol under different conditions (Table 2.3). Ethanol can be produced from non-sterilized, non-diluted and non-sterilized whey (Zafar and Owais, 2006). *K. marxianus* was capable of maintaining high productivity at low pH in non-sterilized whey. *K. marxianus* was found to be a very robust microorganism capable of producing ethanol at high temperature and low pH in whey by taking over lactic acid bacteria present in the whey (Christensen *et al.*, 2011).

Table 2.3 Bioethanol production from different type of cheese whey with varying pH, supplements, lactose concentration and fermentation condition.

Yeast	**pH**	**Supplement**	**Lactose (%)**	**Fermentation**	**Ethanol (g/L)**	**References**
K. marxianus	5.0	0.45% $(NH_4)_3SO_4$, 0.1% yeast extract	4.6	Batch	8.64	(Ariyanti and Hadiyanto, 2013)
K. marxianus	4.8	Growth supplement	4.9	Batch	17.36	(Koushki *et al.*, 2012)
K. marxianus	4.8	Without	4.9	Batch	15.78	(Koushki *et al.*, 2012)
K. marxianus	4.8	Growth supplement	9.8	Batch	36.3	(Koushki *et al.*, 2012)
K. marxianus	4.8	Without	9.8	Batch	32.35	(Koushki *et al.*, 2012)
K. marxianus	No	Without	4.8	Batch	20.0	(Christensen *et al.*, 2011)
K. marxianus	No	Without	4.8	Cont.	17.6[A]	(Christensen *et al.*, 2011)
K. marxianus	No	0.1% yeast extract	4.8	Fed-batch	7.96	(Ariyanti *et al.*, 2014)
K. marxianus	4.5	0.45%(NH4)3SO4, 0.1% yeast extract, 0.1% malt extract	3.45	Batch	2.8	(Zafar and Owais, 2006)
K. marxianus	5.05	Sugarcane bagasse		Batch	49.65	(Ferreira *et al.*, 2015)
K. fragilis	5.0	Without	20.0	Batch	80.95	(Dragone *et al.*, 2011)
Candida kefyr	4.8	Growth supplement	9.8	Batch	31.56	(Koushki *et al.*, 2012)
C. kefyr	4.8	Without	9.8	Batch	29.64	(Koushki *et al.*, 2012)
S. cerevisae	6	Sucrose and lactose	Concentrated	Cont.	70[B] g.dm3	(Tomaszewska and Białończyk, 2016)

Ethanol production from whey can be enhanced by adding supplemental sugar sources. For example, the addition of extraneous lactose and sucrose to whey increases the ethanol yield (Tomaszewska and Białończyk, 2016).

Adding growth factors to whey could also enhance ethanol production (Table 2.3). Even if the addition of extraneous sugars from different sources raises the ethanol significantly, the supplementation of yeast extract, malt extract, and ammonium sulfate doesn't increase the ethanol productivity significantly (Koushki *et al.*, 2012; Zafar and Owais, 2006). Furthermore, solid particles present in whey affects the ethanol fermentation process. The rate of lactose utilization and ethanol formation increased linearly with increasing solids in whey (Koushki *et al.*, 2012).

2.2. Simultaneous saccharification and fermentation

A conventional ethanol production process from starch materials requires separate hydrolysis and fermentation (SHF) operations. A simultaneous hydrolysis and fermentation reactions shortens the residence time. Abu Bakar *et al.* (2015) studied bioethanol production from sorghum grain with co-immobilized enzyme and yeast under SSF condition. Consequently, they obtained higher ethanol concentration under SSF than conventional methods. Trovati *et al.* (2009) found a significant improvement in ethanol production in a continuous SSF process from liquefied cassava syrup in a packed-bed reactor using glucoamylase immobilized in silica and co-immobilized with *S. cerevisiae* in pectin gel.

In addition to starch, SSF is also applicable for lignocellulosic ethanol production because enhanced kinetics, yields and economics of ethanol production were obtained in SSF than separate saccharification and fermentation (Tian *et al.*, 2013). For example, Tian *et al.* (2013) produced 50 g/L ethanol from steam-exploded corn stover using *S. cerevisiae* Y5 without detoxification and minimized the cost of ethanol production.

2.3. Coculturing of yeasts with other microbes for enhanced ethanol production

Co-culture is a potential bioprocess that mimic natural environment without competitions amongst partner microbes for substrates and without toxin production (Park *et al.*, 2012). At high glucose/xylose concentration (50/20 g/L), glucose is primarily utilized where as at low

mixture concentration (25/10), simultaneous consumption of sugars was observed (Gutiérrez-Rivera *et al.*, 2012).

The *S. cerevisiae* cell utilized its own carbohydrate reserve instead of xylose when glucose was consumed in lignocellulosic hydrolysates containing xylose. De Bari *et al.* (2014) found that isomerization of the xylose using isomerase reduced the xylose in the hydrolysates and upgraded ethanol production. On the other hand, coculturing of *S. cerevisiae* that prefer six carbon sugars with other yeasts that produce efficient ethanol from five carbon sugars is also another alternative to optimize ethanol production in hydrolysates containing xylose (Gutiérrez-Rivera *et al.*, 2012; Suriyachai *et al.*, 2013; Karagöz and Özkan, 2014; Singh *et al.*, 2014).

Gutiérrez-Rivera *et al.* (2012) investigated coculture of *S. cerevisiae* ITV-01 and *Pichia stipitis* NRRL Y-7124; they found that ethanol productivity increased fivefold compared to monocultures. Similarly, Singh *et al.* (2014) studied the coculturing of *S. cerevisiae* MTCC 174 and *Scheffersomyces stipitis* NCIM No. 3497 (formerly *P. stipitis*) using microwave alkali pretreated rice husk medium and coculture produced maximum ethanol (20.8 g/L) than *S. cerevisiae* MTCC 174 (14.0 g/L) and *S. stipitis* NCIM No. 3497 (12.2 g/L) alone. Another study showed 11% improvement with coculture of *S. cerevisiae* and *S. stipitis* compared to monocultures in alkaline pretreated rice straw (Suriyachai *et al.*, 2013). Likewise, Karagöz and Özkan (2014) found that more ethanol was produced in *S. cerevisiae* ATCC 26602 and *S. stipitis* DSM 3651 coculture (7.36 g/L) than *S. cerevisiae* monoculture (6.68 g/L) using H_2O_2 pretreated and enzyme hydrolyzed wheat straw.

Ethanol productivity improvement in coculture might be due to enhanced substrate utilization since *S. cerevisiae* uses the six carbon (glucose) source and *P. stipitis* uses the five carbon (xylose) source to produce ethanol. However, the problem in this coculture was that *P. stipitis* NRRL Y-7124 tolerated lower ethanol inhibition than *S. cerevisiae* ITV-01 and hence the ethanol concentration produced by *S. cerevisiae* ITV-01 prevented further ethanol production in *P. stipitis* NRRL Y-7124 (Gutiérrez-Rivera *et al.*, 2012). Generally, Karagöz and Özkan (2014) suggested that increased ethanol production might be contributed by the competition of *S. stipitis* for xylose.

In addition to *S. cerevisiae* and *P. stipitis* coculture, Hickert *et al.* (2013) cocultured, *Candida shehatae* HM 52.2 with *S. cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate and demonstrated the simultaneous conversion of glucose and xylose, maximizing substrate utilization rates, increasing ethanol yields.

S. cerevisiae has been cocultured with polysaccharide solublizing microorganisms to get simple sugars for ethanol fermentation. Park *et al.* (2012) studied the operational condition of cellulase producing *Acremonium cellulolyticus* with ethanol producing *S. cerevisiae* using Solka-Floc as cellulase-inducing substrate in a single reactor. They were able to maximize the ethanol to 46.3g/L indicating the possibility of efficient ethanol production without pretreatment and addition of extraneous cellulase.

Similarly, amylase producing fungi can be cocultured with the yeast to produce ethanol without purifying the amylase. For instances, highest ethanol was produced by a co-culture of *Rhizopus oryzae* and *S. cerevisiae* in the presence of 5mM NaCl (Jang *et al.*, 2015).

Effective coculturing is influenced by medium composition and growing parameters. Accordingly, in coculture of *Aspergillus niger* and *K. marxianus*, the optimum conditions for fermentation were incubation temperature of 30°C, initial pH of 5.5, and substrate concentration of 6% starch level with ethanol yield of 23.10 g/L (Manikandan and Viruthagiri, 2009). Separate reactors for amylolytic (*A. niger*) and ethanolegenic (*S. cerevisiae*) microorganisms were designed in one pool reactor system to solve different growing requirements for the two organisms.

Generally, coculturing *S. cerevisiae* with other microbes reduce inhibitory compounds in lignocellulosic hydrolysates (Taherzadeh and Karimi, 2011), increase ethanol yield and production rate (Singh *et al.*, 2014), shorten fermentation time and reduce process cost (Hickert *et al.*, 2013). Therefore, coculturing could be an alternative strategy for ethanol production besides the classical way of biofuel optimization.

2.4. Growth conditions affecting ethanol fermentation

Temperature, pH, oxygen, initial sugar concentrations, organic acids, dissolved solids and immobilization of the yeast are greatly essential parameters that influence the specific rate of yeast growth and ethanol production (Lin *et al.*, 2012).

2.4.1. Temperature

Temperature greatly affects the enzymatic activity and membrane turgidity of yeast cells. Yeasts which are active and tolerant at high temperature are ideal for industrial bioethanol production. *S. cerevisiae* ITV-01, isolated from sugar cane molasses, produced more ethanol (58.4 g/L) optimally at 30°C with pH 3.5 (Ortiz-Muñiz *et al.*, 2010), whereas Lin *et al.* (2012) reported that 30 - 40°C were optimal for *S. cerevisiae* BY4742; shortened the exponential phase of the yeast cell. They also showed that ethanol production reduced considerably at 50°C and this might be due to change in transport system which might increase accumulation of toxin including ethanol in the cell.

Although most yeasts grow optimally between 25 and 30°C (Yadav *et al.*, 2015), some yeasts like *K. marxianus* grow very well at 40-45°C (Yadav *et al.*, 2014). On the other study, ethanol production decreased when the temperature is raised to 30°C using alkali pretreated palm fruit bench fiber under fed-batch SSF condition (Park *et al.*, 2013).

2.4.2. pH

Generally, yeasts grow best in slightly acid condition though their pH preference is from acid to neutral. The optimal pH range for yeast growth is 4.5 to 5.5 (Gao *et al.*, 2012), 4 to 6 (Hashem *et al.*, 2014), and 4.5 to 5 (Joseph, 1999); however, some yeast grow better at slightly lower than 4. *S. cerevisiae* grow optimally at pH 4.5-5.0, it can tolerate a pH range of 3.6–6.0 (Joseph, 1999). Economically-sound biomass production was obtained from mixed culture of *Kluyveromyces marxianus* and *Candida krusei* on whey substrates at pH 3.5 and 40°C by reducing the potential contaminants (Yadav *et al.*, 2014).

Optimum pH for *S. cerevisiae* BY4742 was in the range of 4.0 – 5.0 (Lin *et al.*, 2012). Lin *et al.* (2012) reported the formation of acetic acid when the pH was below 4.0 and pH above 5.0 favored butyric acid production. Ortiz-Muñiz *et al.* (2010) investigated that pH 3.5 was optimal for ethanol production by *S. cerevisiae* ITV-01 at 30°C with initial glucose concentration of 150 g/L.

Currently, stillage (a waste after ethanol production) is commonly reused as a substrate to make efficient ethanol production. However, stillage contains more organic acids than expected and prolonged the ethanol fermentation time (Zhang *et al.*, 2011). Another study showed that ethanol and glycerol fermentation from cassava mash using *S. cerevisiae* was inhibited by undissociated propionic acid at low pH because of the easy entrance of undissociated acids, and the intracellular dissociation acidified the cytoplasm. Some proton must be transported by membrane ATPase to maintain the equilibrium of intracellular pH in which it increased ATP consumption and decreased biomass yield of the yeast (Zhang *et al.*, 2011).

All taken together, different acids are produced in the process by the yeast and other any exogenous factors affect the activity of *S. cerevisiae.* Other investigations showed that yeast uses organic acids as a substrate. *S. cerevisiae* NAM34-4C grew rapidly and produced ethanol (2.7 g/L) in YPDL at pH 3.5 and temperature 35°C (Vilela-Moura *et al.*, 2010). Similarly, the volatile acidity from acidic white wine was efficiently reduced by *S. cerevisiae* S26 when the acetic acid and ethanol concentration were kept below 1.0 g/L and 11% (v/v), respectively.

2.4.3. Substrate concentration

Jin *et al.* (2012) studied the effect of initial reducing sugar concentration (85-156 g/L) from sweet sorghum stalk juice on *S. cerevisiae* CICC 1308 immobilized with sodium alginate. Accordingly, the increase in sugar concentration significantly inhibited the average specific growth rate and average biomass yield of the yeast but increased average specific substrate uptake, average specific ethanol productivity and average ethanol yield. Similarly, as reducing sugar concentration from food waste leachate was increased from 45 – 75 g/L, the ethanol production by *S. cerevisiae* KCTC-7904 increased by 2.3 fold (Le Man *et al.*, 2011). On the

contrary, low ethanol (0.22 L ethanol kg^{-1}) was produced at high gravity sorghum mashes (20° Plato) than lower counterpart (13°Plato) that produced 0.22L ethanol kg^{-1} (Pérez-Carrillo *et al.*, 2011).

2.4.4. Substrate supplementation

Apart from carbohydrate (lignocellulosic) sources, ethanol production by *S. cerevisiae* was enhanced by the supplementation of exogenous nitrogen sources such as yeast extract, malt extract, peptone and $(NH_4)_2SO_4$ to the natural growing media. Supplements also enhance sugar utilization for better ethanol production (Hashem *et al.*, 2014).

The presence of important cofactors like biotin and riboflavin in yeast extract enhanced ethanol production (Ortiz-Muñiz *et al.* (2010), and Li *et al.* (2011) showed that high ethanol (44.55 g/L ethanol, corresponding to 94.5% of the theoretical value) was recorded from fermentation of enzymatic hydrolysates of corn stover supplemented with nitrogen source (corn steep liquor(CSL), yeast extract, and peptone). This is because glucose consumption was higher with yeast extract and peptone (glucose depletion in 36 hrs). .

Bi *et al.* (2011) found that the addition of DDGS (distillers dried grains with solubles) to corn stover hydrolysate enhanced ethanol production by *S. cerevisiae* DQ1 as good as the supplement of the expensive yeast extract in SSF condition.

The addition of other ingredients such as vitamins, amino acids, sterols, or yeast extract also increases higher ethanol. However, these supplements are too expensive to be used at industrial level and hence cheap additives such as sunflower, groundnut, and safflower oil seed meal cakes, wheat mash, or soy flour could be used (Li *et al.*, 2011). The authors showed that supplementation of oilseed meal (4%) from sunflower enhanced the ethanol production by 50% and improved sugar tolerance from 8 to 16%; the addition of 2% (w/v) rice husk also raised ethanol amount by 48%.

2.4.5. Inoculum size

Although many studies showed that different substrate components significantly influenced the ethanol production, the population density (inoculum size) of the yeast also affects ethanol production. Accordingly, Yun *et al.* (2011) reported that lower inoculum size (5% v/v) reduced cost of production for ethanol fermentation and population yielded almost the same result with 10% inoculum of 12 hrs old culture of *S. cerevisiae* Y5 on enzymatic hydrolysate of corn stover. Ethanol productivity by baker yeast decreased as yeast concentration increased from 3 to 4 and 5 g/L in coffee husk based substrate. However, Akaracharanya *et al.* (2011) used 10% (v/v) *S. cerevisiae* TISTR 5596 to produce high ethanol using waste from cassava starch production without nitrogen source supplementation.

2.5. Immobilization improves ethanol productivity

Immobilization is the process of putting active cells (yeasts) in the carrier materials. The most commonly used immobilizing agents are sodium or calcium alginate and agar-agar cubes (Karagöz and Özkan, 2014). Alternatively, new immobilizing agents mainly lignocellulose materials are cheap and easy to use have been investigated in several studies for various reasons. Immobilization of yeast cells has been considered as potential alternative for enhancing ethanol productivity, because it reduces risk of contamination and makes the separation of cell mass from the bulk liquid easy (Sembiring *et al.*, 2013), minimize production cost (Singh *et al.*, 2013), enables biocatalyst recycling (Sembiring *et al.*, 2013), reduce fermentation time and produced more ethanol (Karagöz and Özkan, 2014), and protect the cells from inhibitors (Kirdponpattara and Phisalaphong, 2013)

Recycling microorganisms, especially at industry level, saves time, energy, and money whenever they are applied properly. As a result, immobilized yeast cells can be reused up to 15 cycles with bacterial cellulose–alginate sponge (Kirdponpattara and Phisalaphong, 2013).

Quite recently, new, better and cheap supporting materials than the classical immobilizing agents were found to produce ethanol. For instance, lignocellulose based immobilizing materials increased ethanol production than the commonly used supporting material like sodium or calcium alginate (Singh *et al.*, 2013).

2.6. Biomass production from yeasts

Biomass protein (Single cell protein) is one of the alternative commodities that can be produced by microorganisms. Single cell protein (SCP) is a protein derived from yeast, fungi, algae and bacteria (Hashem *et al.*, 2014; Chi *et al.*, 2016; Arous *et al.*, 2015; Ravindra, 2000).

Microorganisms are an excellent source of SCP because of their rapid growth rate, their ability to use very inexpensive raw materials as carbon sources, and the uniquely high efficiency, expressed as grams of protein produced per kilogram of raw material, with which they transform these carbon sources to protein (Hashem *et al.*, 2014). The use of yeasts for SCP production is more convenient, as they can be easily propagated using cheap waste materials and easily harvested due to their bigger cell sizes and flocculation abilities (Ravindra, 2000). Some yeasts have been used widely in the manufacture of human foods; *S. cerevisiae*, *K. marxianus*, and *C. utilis* are recognized as GRAS for human consumption by the US Food and Drugs Administration (García-Garibay *et al.*, 2014; Gao *et al.*, 2012).

Yeast biomass contains significant amounts of proteins, vitamins, fats and mineral. Yadav *et al.* (2014) summarized the composition of yeast biomass and approximately dried yeast biomass contains 50–52 % protein, 30–37 % carbohydrate, 4–7 % lipids, 6–8 % nucleic acids and 7–8 % minerals. Yeasts are a good source of protein or amino acids (Hashem *et al.*, 2014). Specifically, biomass from *K. marxianus* and *Candida krusei* contained protein (43.4% w/w), carbohydrates (33.6% w/w), crude fiber (4.6% w/w), lipid (6.4% w/w) and ash (minerals) (8.4% w/w). It contains minerals such as calcium (1700 mg), phosphorus (2120 mg), potassium (1700 mg), sulfur (610 mg), sodium (200 mg), magnesium (170 mg), iron (0.15 mg), manganese (0.007 mg), copper (0.03), zinc (0.13), molybdenum (0.01 mg), and nickel (0.007). There were no heavy metals (toxic metals) detected in the minerals analysis of biomass (Yadav *et al.*, 2014).

The protein quality in yeast biomass is high (García-Garibay *et al.*, 2014; Anvari and Khayati, 2011) and it more closely resembles animal protein than plant proteins (Anvari and Khayati, 2011). Other study indicated that the yeast's protein is almost identical to soy protein (Najafpour, 2015).

SCP from *Pichia* and *C. tropicalis* (Gao et al., 2012) are rich in essential amino acids. Similarly, marine yeasts are rich in lysine, and leucine (Chi *et al.*, 2016). Biomass produced from *C. utilis* contains all essential amino acids except valine (Ibrahim Rajoka *et al.*, 2004). In addition, SCP is limited in sulfur containing amino acids such as cystine, cysteine, and methionine (Najafpour, 2015; Yadav *et al.*, 2014). In addition, Rajoka *et al.* (2012) reported the existence of sulfur containing amino acid, lysine and methionine, in biomass produced by sequential culture of *Candida utilis* and *Brevibacterium lactofermentum*. Generally, most of the essential amino acids present in yeast biomass are higher than FAO standards; with the exception of leucine, isoleucine and valine, essential amino acids in biomass by *C. tropicalis* were higher than those of soybean (Gao *et al.*, 2012). Vitamin C is dominant in yeast biomass (Chi *et al.*, 2016). Consequently, brewer's yeast has been used as a vitamin supplement for long (García-Garibay *et al.*, 2014).

Table 2.4 Biomass production from different substrates

Yeast	**Substrate**	**Dry weight (g/L)**	**Crude Protein %**	**References**
Candida utilis	Rice polishing		32.5	(Ibrahim Rajoka *et al.*, 2004)
C. utilis	Distillery wastes	9.8	43.87	(García *et al.*, 2014)
C. tropicalis	Soy molasses	10.83	56.42	(Gao *et al.*, 2012)
C. pararugosa	Olive mill waste	21.68	35.9	(Arous *et al.*, 2015)
K. marxianus	Whey	15.9	30*	(Yadav *et al.*, 2014)
K. marxianus	Whey	12.68	29.25	(Anvari and Khayati, 2011)
Schwanniomyces etchellsii	Olive mill waste water	15.11	39.5	(Arous *et al.*, 2015)
K marxianus and Candida krusei	Whey	8	50.5	(Yadav *et al.*, 2014)
Yarrowia lipolytica	Crude oils			(Johnson, 2013)

*g/L soluble protein

2.6.1. Substrates for biomass production

Yeast biomass can be produced from different substrates (Table 2.4). Yeasts grow very well in different agro- industrial wastes such as sugarcane molasses (Joseph, 1999; García-Garibay *et al.*, 2014), soy molasses (Gao *et al.*, 2012),, whey (Yadav *et al.*, 2015), sulfite liquor from paper manufacturing wastes (García-Garibay *et al.*, 2014), olive mill wastes (Arous *et al.*, 2015), date wastes (Hashem *et al.*, 2014), and distillery wastes (García *et al.*, 2014).

2.6.2. Substrate fortification

The supplements that contain different nutrients include yeast extract, peptone, corn steep liquor, and whey. Higher *Candida utilis* biomass was obtained on rice polishing fortified with corn steep liquor that was essential for rapid uptake of substrate and microbial activity; the addition of yeast extract to soy molasses enhanced the yield of *C. tropicalis* biomass (Gao *et al.*, 2012).

Minerals supplimenattion promote more biomass yield since they facilitate the enzymatic system of the yeasts. Addition of metals (Zn, Mn, Mg and Co) to wasted date fruits resulted in higher SCP from *Hanseniaspora uvarum* and *Zygosaccharomyces rouxii* than non fortified wasted fruits (Hashem *et al.*, 2014). García *et al.* (2014) showed a substantial increase in biomass of *Candida utilis on* distillery wastes supplemented with urea.

Yeast extract supplimenattion to soy molasses (Gao *et al.*, 2012) and tryptone addition to dated fruits (Hashem *et al.*, 2014) improved SCP production better than inorganic nitrogen sources. This might be due to the presence of growth factors besides nitrogen source in yeast extract and tryptone However, it increases the production cost since yeast extract and tryptone are relatively more expensive than other inorganic nitrogen supplements at industry level.

2.6.2. Limitations of yeast SCP

Besides good nutritional value, yeasts have to be evaluated for toxicology and cost of production. High cost of production limits the utilization of yeast SCP (Ravindra, 2000). The high cost is due to sterility condition and separation of yeast from growing media at industrial level (Srividya *et al.*, 2013). However, cost of yeast SCP production is lower than bacterial bound SCP. Nucleic acid degradation results in the release of uric acid. Hence, intake of high nucleic acid SCP leads to accumulation of uric acid since uricase enzymes are absent in human cells. Therefore, nucleic acids in different SCPs should be reduced to acceptable limits.

If they are to be used for human consumption, SCP content of 2 or more gram nucleic acid per day may lead to kidney stone formation and gout (Ravindra, 2000). Proteins from yeast cells are not easily released due to rigid and thick cell wall (Chi *et al.*, 2016). In addition, microbial SCP causes allergies and gastrointestinal reactions resulting in nausea and vomiting which can be considered as limitations in utilizing yeast biomass (Chi *et al.*, 2016; Ravindra, 2000).When cheap wastes are utilized as substrate for yeast growth, the existence of toxic or/and carcinogenic substances should be evaluated. The growth of yeasts on industrial wastes for food create psychological and social complications (Srividya *et al.*, 2013) since most people perceive wastes contain nontreatable toxic compounds.

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Chapter 3

Isolation, Identification and Characterization of Ethanolegenic Yeasts in Ethiopia

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Abstract

Yeasts are important microorganisms used for ethanol and biomass production. However, they are not equally efficient in the amount of ethanol and biomass production under different environmental conditions. It is, therefore, necessary to screen for elite strains to utilize them for commercial production of these commodities. In this study, yeasts were isolated from different Ethiopian traditional fermented alcoholic beverages (teji, tella, shamiata, and areqe tinisis), milk and ergo, teff and maize dough, soil and compost, flowers, and fruits using yeasts extract peptone dextrose agar (YPDA). They were screened for efficient ethanol production, and the selected isolates were characterized using phenotypic and genetic characters using D1/D2 region of LSU rDNA sequence analysis. The yeast isolates were evaluated to grow and ferment different carbon sources. A total of 211 yeasts colonies were isolated of which 60% ethanologenic yeasts (ethanol producers) and 40% were non-ethanol producers. The yeast population detected from various sources was in the range of CFU 10^5 *from traditional foods and beverages to that of CFU* 10^3 *from fruits and soil samples. The data also showed the number of colony types (diversity) did not corroborate with population density in that tella with CFU*10^5 *contained 34 colony types similar number of colony types (33) from soil with CFU* 10^3 *density of and categorized into different colony types. The highly fermentative isolates were taxonomically characterized into four genera, of which 65% of the isolates (ETP37, ETP50; ETP53, ETP89, ETP94) were categorized under* Saccharomyces cerevisiae, *and the remaining were* Pichia fermentans *ETP22,* Kluveromyces marxianus *ETP87, and Candida humilis ETP122. The* S. cerevisiae *isolates produced ethanol (7.6-9.0g/L) similar with* K. marxianus *ETP87 producing 7.97g/L; comparable to the ethanol produced from commercial baker's yeast (8.43g/L); whereas* C. humilis *ETP122 and* P. fermentans *ETP22, produced 5.37g/L and 6.43 g/L ethanol respectively. The sedimentation rate of all 8 yeasts other than* C. humilis *ETP122 was higher than 70%.* S. cerevisae *ETP53,* K. marxianus *ETP87,* P. fermentans *ETP22 and* C. humilis *ETP122 tolerated 10% extraneous ethanol but the percentage of ethanol tolerance considerably decreased upon 15%. Therefore,* S. cerevisiae *ETP53,* K. marxianus *and* P. fermentans *ETP22 are good candidates for ethanol production.*

Key words/phrases: Colony types; *Candida humilis, Kluveromyces marxianus,* sedimentation, *P. fermentans*

3.1. INTRODUCTION

Yeasts are cosmopolitan microorganisms that are mostly found in natural ecosystems rich in sugar. Mohd Azhar *et al.* (2017) estimated that over 150, 0000 yeast species are distributed on earth of which only1% of yeasts (1,500 species) are known.

Plants are the preferable habitats for yeasts and they include nectars, flowers, fruits, decaying tissues, and tree saps. (de Azeredo *et al.*, 2010). These plant parts also attract insects that help the distribution of yeasts to new different habitats (de Azeredo *et al.*, 2010; Glushakova *et al.*, 2014). The authors also showed that insects also play a great role in diversifying the yeast communities in flowers; therefore, the yeast diversity is seasonal since the pollinating insects are mostly seasonal. According to Mittelbach *et al.*, (2015), flower visitor birds are also important to determine yeast diversity in flowers.

Traditionally, yeasts from the genus *Saccharomyces* are used in bakery and brewery industries for ethanol production of high sprit and industrial grade ethanol for human consumption from simple sugars. However, simple sugars are relatively expensive substrate for economical ethanol production. They can also produce ethanol from agricultural crush (starch and lignocellulose), sugar industry wastes (sugar cane and beet molasses), and dairy industry waste (whey) with a dual purpose of producing energy from cheap sources and alleviation environmental pollution. However, the use of different agricultural wastes require the selection of yeasts that are capable of utilizing substrates derived from hydrolysis of complex carbohydrates.

Yeasts are among the essential microorganisms found in traditional fermented foods and beverages in Ethiopia. The yeasts that dominate the Ethiopian dairy products are *Kluyveromyces* (46.8%), *Sporobolomyces* (31.5%), *Candida* (12.5%), *Torulopsis* (6%) and *Leucosporidium* (3.2%) (Ashenafi, 2006). The author also showed that *Candida milleri*, *Rhodotorula mucilaginosa*, *K. marxianus*, *Pichia naganishii*, *Rhodotorula glutinis*, *K. marxianus* and *Pichia membranefaciens* are isolated from staple fermented food injera and kocho. Several studies also showed that *S. cerevisiae*, *Kluyveromyces bulgaricus*, *Debaromyces phaffi* and *Kluyveromyces veronae* are found in *teji tella*, *shamita*, and *borde* (Ashenafi, 2006; Bahiru *et al.*, 2001; Bahiru *et al.*, 2006; Bacha *et al.*, 1999).

This shows that the Ethiopian fermented drinks and food could be good sources of yeast for ethanol production. Most of the hitherto studies focused on the yeast profile of the different food and beverage sources. However, there is a dearth of information on the efficiency of these isolates on ethanol production. The aim of this study was, therefore, to isolate, characterize and identify ethanologenic yeast for bioethanol production.

3.2. MATERIALS AND METHODS

3.2.1. Sampling

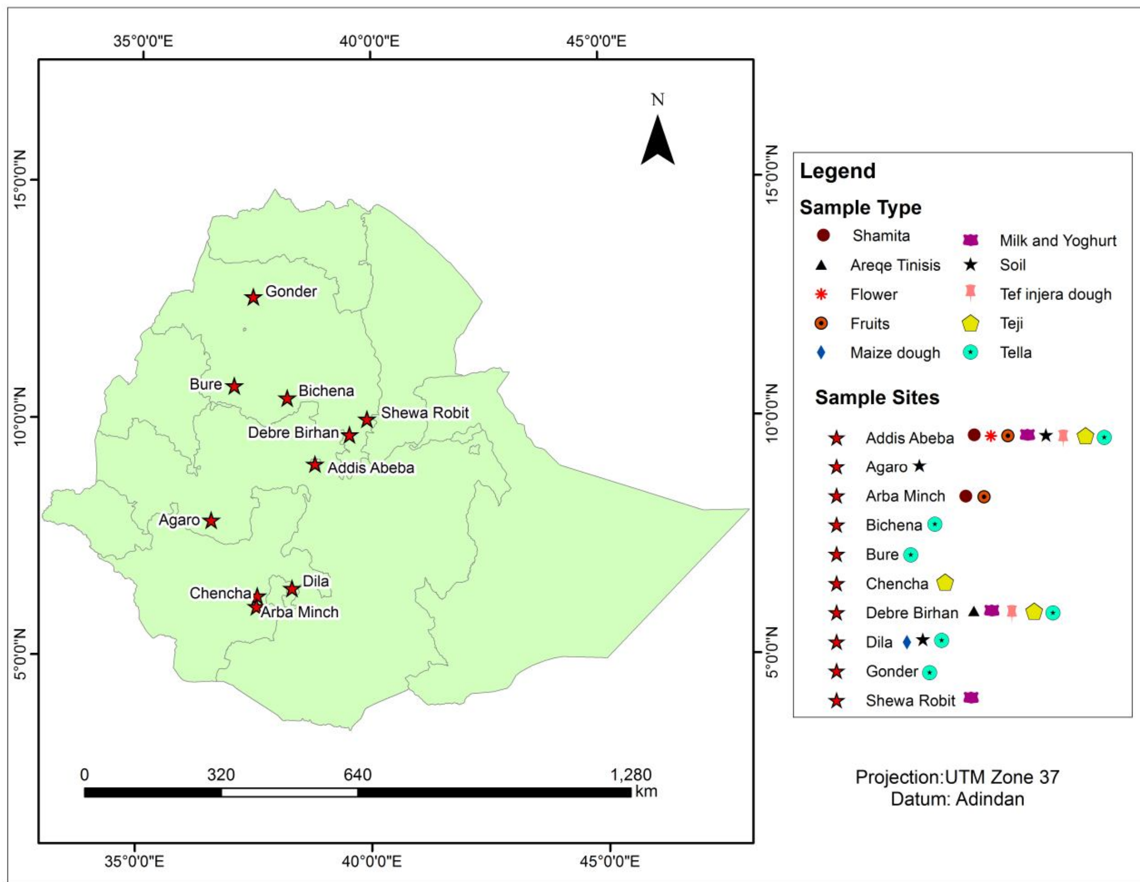

Figure 3-1 Sampling sites for yeast isolation

Samples were collected from traditional foods and beverages such as *tella*, *teji*, *shamiata, areqe tinisis,* teff *injera* dough, maize dough, milk and ergo, soil, fruits, compost, and nectar of

different flowers from different sampling sites in Addis Ababa, Debre Berhan, Dilla, Bure, Bichena, Gonder, Chencha-Dorze, Shoa Robit, Agaro, Dilla and Sebeta.

3.2.2. Isolation

The yeasts were isolated on YPDA medium (Yeast extract, 10; peptone, 20; dextrose, 20; and agar, 20 g/L) containing chloramphenicol (0.01 g/L) to inhibit bacterial growth (Hong and Park, 2013). In the case of isolation of five carbon-utilizing yeasts, dextrose was substituted by ribose (20 g/L). Aliquots (100 μL) of samples from processed samples were prepared to appropriate dilutions. From the last dilution (100 μL) of the suspension was poured and spread plated on YPDA and incubated at 30°C for 3 days (Hong and Park, 2013).

3.2.3. Screening of ethanol producing yeasts

The isolates were screened for ethanol production using standard methods (Kurtman and Fell, 1998). For screening of 5-carbon utilizing yeasts, dextrose was replaced by ribose as before. Then, 0.5 mL (approximately number 10^6) of active yeast cells for each isolates were inoculated into the media in test tubes with Durham tube. They were incubated at 30°C for 10 days without agitation. The fermenting yeasts were further screened based on time that completely displace 1 mL Durham tube with gas in 12, 24, 48 and 72 hours. They were, then, screened based on ethanol concentration that they produced (Kurtman and Fell, 1998). Finally, they were compared with instant baker yeast.

3.2.4. Growth and fermentation at different carbon sources

The effects of different carbon sources (xylose, arabinose, raffinose, trehalose, mannitol, cellobiose, galactose, fructose, maltose, lactose, mannose, sucrose, and starch (each 2% W/V)) on ethanol production in 100 mL media containing 1g yeast extract and 2g peptone were investigated (Barnett *et al.*, 2000). During solid media preparation, ethanol was added after sterilization before pouring and mixed very well. Solid media were used for growth evaluation where as the fermentation was tested using inverted 1mL Durham tube in test tube containing the same media.

3.2.5. Identification of yeast species

DNA extraction

Isolates ETP53, ETP37, ETP87, ETP94, ETP50, ETP89, ETP22, and ETP122 that showed relatively good ethanol production were selected and identified using standard genetic methods. Accordingly, DNA was extracted by sub-culturing yeast cells on YM agar plate at 20˚C for 5-7 days; then 50 µL volume of cell mass was harvested in a microtube suspended in 200 µL lysis solution (1% [w/v] Yatalase™ (TAKARA Bio Inc.), 1% [v/v] RNase A solution (Qiagen), 10 mM potassium phosphate, 10 mM EDTA, 0.8 M sodium chloride, pH 7.0), and incubated at 37˚C for 1.5 hours. Approximately, 50 µL of Φ 0.8 mm glass beads and 67 µL of SDS/ProK solution (8% [w/v] sodium dodecylsulfate, 300 U of Proteinase K (Nacalai Tesque), 5 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, pH 8.0) were added to each tube. The tube was vortex-mixed for 1.5 minutes, and incubated at 60˚C for 10 min. After this, 87 µl of 3 M sodium acetate (pH 5.2) solution was added, vortex-mixed, and chilled on ice.

The tube was centrifuged at 15000 rpm for 5 minutes at 4˚C from which seventy microliter of the supernatant was transferred to a well of AcroPrep™ 96 Multi-Well Filter Plate with 3.0 µM glass fiber media/0.2 µM Bio-Inert® membrane, natural housing (PALL Life Science), to which 110 µL of isopropanol was added and mixed well by pipetting. After incubation for 3 min at room temperature (15-20˚C), the filter plate was vacuumed with a vacuum manifold device. The well was rinsed with 200 µL of 70% [w/v] ethanol twice in vacuum. After the filter plate was air-dried, 60 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was poured in the well, and incubated for 3 min. The filter plate was placed onto a new 96-well plastic plate. Two plates were centrifuged at 3000 rpm for 5 min at room temperature. The DNA was resuspended with TE buffer, centrifuged twice, and kept at -20˚C for further use.

Sequencing of LSU rRNA

The DNA was sequenced using NL1 and NL4 as PCR primers for amplification of D1/D2 region of LSU rDNA. PCR amplification were performed in 20 µL reaction, containing 10 µL of GoTaq® Green Master Mix (Promega), 10 pmol of each primer, and 2 µL of 1-20 ng/µL extracted DNA, on GeneAmp® PCR System 9700 (Applied Biosystems) or iCycler (BioLad). The PCR program was as follows; an initial denaturation at 94˚C for 5 min, followed by 36 cycles of 30 sec at 94˚C, 30 sec at 52˚C, 1 min at 72˚C, and a final extension of 5 min at 72˚C. The PCR-amplified fragment was visualized by electrophoresis on agarose and staining with ethidium bromide. Purification of the fragment was performed using MinElute® 96 UF PCR

Purification Kit (Qiagen) according to the manufacturer's instructions. The purified fragment was resuspended in 50 µL of 10-fold diluted TE buffer. The nucleotide sequences of PCR-amplified fragment were determined by Sanger-sequencing using the ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems) following the manufacturer's instruction.

Sequence data were corrected by manual inspection whenever needed, and aligned using BioEdit Sequence Alignment Editor version 7.1.3.0 (Hall, 1999).

The partial sequence (D1/D2 region) was edited by BioEdit. Genetic identification of yeast isolates were done by blasting isolates' D1/D2 sequences against GenBanks such as National Center for Biotechnology Institute (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and CBS database, Westerdijk Institute (http://www.westerdijkinstitute.nl/). The percentage of similarity between partial sequence result of the yeast isolates was compared with sequences similar to isolates accessed from GenBanks (NCBI and CBS) and created by free Mega6 and BioEdit software.

3.2.6. Yeasts sedimentation rate

One mL of 24hr culture grown on YPD was transferred in 1.5mL Eppendroff tubes and centrifuged at 14000×g (eppendorf centrifuge 5418 R, Germany) for 10 minutes and the pellets were resuspended in 1mL NaCl (0.89%) solution for 2 hours. The optical density was measured using Jenway 6405 UV/Vis Spectrophotometer (United Kingdom). The sedimentation rate was expressed according to Moneke *et al.* (2008).

$$\% \text{ of sedimentation} = \left(1 - \frac{\text{total drop in OD reading after 2 hour}}{\text{OD reading at 0 hour}}\right) \times 100\% \qquad \text{Equation 3.1}$$

3.2.7. Measurement of ethanol tolerance

Measurement of cell viability after ethanol shock treatment was employed to evaluate ethanol tolerance (Xue *et al.*, 2008). Accordingly, overnight yeast cultures on YPD were harvested and washed two times with de-ionized water. After centrifugation at 13000×g (eppendorf centrifuge 5418 R, Germany) the yeast pellets were diluted with acetate buffer (pH 5.0) and exposed to 10, 15 and 20% (v/v) ethanol in the same acetate buffer. They were incubated at 30°C in water bath (Clifton, England) at 150 forth and back shakings per minute for 2 hours. The samples were serially diluted to 10^{-5} using acetate buffer (pH 5.0) from which 100µL portion of diluent were

spread to YPD agar plate and incubated at 30°C for 4 days to count colonies. The percentage of colonies was taken as a measure for ethanol tolerance (Xue *et al.*, 2008).

$$\text{Survived percentage} = \frac{\text{Number of survived cells after exposure}}{\text{Unstressed (unexposed) control}} \times 100\% \qquad \text{Equation 3.2}$$

3.2.8. Ethanol quantification by pycnometer method

Fermented broth samples (100 mL) were transferred in 500mL distillation flask containing glass beads and fitted with diagonally assembled condenser (Quickfit CX7/33/SC, England). The joints were standard taper and the heat source was gas operated system.

Distillation of sample: A100mL volume of fermented broth was transferred to 500mL distillation flask containing glass beads. The pycnometer was rinsed 3 times (13mL each) with 25mL cold distilled water and the rinsed water was transferred back to the flask containing 100mL sample. The distillation was run for 40 minutes from which distillates were collected to pycnometer and stopped when distillates volume reached graduation mark. The pycnometer neck was dried by tissue paper and the dry weight (DW) distillates were weighed using the formula

$$DW = DPW - PW \qquad \text{Equation 3.3}$$

Where

DW= weight of distillate

DPW= weight of distillate and pycnometer

PW= weight of pycnometer

Finally, the specific gravity (SG) was calculated as

$$SG = \frac{DW}{WW} \qquad \text{Equation 3.4}$$

Where

SG= specific gravity

DW= weight of distillate

WW= weight of recently distilled water

Ethanol determination: The ethanol concentration was determined from tables that shows ethyl alcohol percentage corresponding to apparent specific gravity at various temperatures (Patricia, 1995). The percentage of volume was converted into g/L using density of ethyl alcohol.

3.3. RESULTS

3.3.1. Yeast Isolation

A total of 211 yeast colonies were collected from traditional alcoholic beverages, *enjera* and maize dough, *ergo* and milk, soil and compost, flowers and fruits. The highest yeast counts were recorded from beverages *tella*, *teji*, and fermented enjera-maize dough with population of 4.6×10^5, 2.4×10^5, and 2.7×10^5, followed by population density of 9.9×10^4, 6.6×10^4, 1.4×10^4, 1.2×10^4, and 1.1×10^4 yeast cells from *areki tensis*, *shamita*, flowers, milk and *ergo*, and compost, respectively (Table 3.1). .

Table 3.1 Yeasts density and number of glucose fermenters

Samples	CFU	Number of colony types	Number of glucose fermenters	Percentage of positive
Tella	4.6×10^5**	34	21	62
Tej	2.4×10^5**	24	19	79
Araki tensis	9.9×10^4**	19	16	84
Milk and *ergo*	1.2×10^4**	13	6	46
Enjera and maize dough	2.7×10^5**	11	9	82
Shamita	6.6×10^4**	8	6	75
Fruits	8.9×10^3*	30	10	33
Flowers	1.4×10^4*	24	9	38
Soil	9.8×10^3*	33	22	67
Compost	1.1×10^4*	15	8	53
			126	60

*CFU per gram whereas ** CFU per milliliter

The number of the different types of yeasts (diversity) was in the range of 8 colony types from compost to that of 34 colony types obtained from tella (Table 2.1). Thus, the number of colony types can be categorized into low diversity (8-20 different colony types) recorded from s*hamita, areki tinisis,* Milk and *ergo,* compost, and enjera and maize dough, and medium (20-30 colony types) recorded from samples of Teji, flowers, and fruits and high diversity colony types (>30) from tella and soil (Table 3.1).

Accordingly, the number of isolates (34 colonies) detected from tella was similar to the number of isolates detected from soil (33 isolates) and fruits (30 isolates) (Table 3.1). Similarly, the higher population density exhibited from maize dough showed less diversity (11 colonies) Although the number of yeast colonies was higher from *tella*, *shamita*, *teji*, flowers and maize dough, and lower in fruits and soil than other sources, they did not match the number of colony types (diversity) indicating that population density did not necessarily corroborate with species diversity.

3.3.2. Yeast screening by glucose fermentation

Among 211 yeasts isolated from all samples, 126 yeasts (60%) were able to produce gas from glucose (Table 3.1) which was the confirmatory test for ethanol production by yeasts**.** The existence of high glucose fermentative yeasts (60-80%) were recorded from *tella*, *teji*, *areqe tinisis* and *shamita, enjera* and maize dough, and soil; whereas 30-50% ethanol producing isolates were recorded from other samples. It was interesting to note that although fruits, and flowers contained diverse groups of yeasts, the ethanol producers were relatively lower (<40%) than the other yeasts founds from beverages (Table 3.1).

3.3.3. Ethanol production by local isolates and commercial baker yeast

The different isolates were screened for efficient ethanol production, of which 8 isolates were selected and characterized (Table 2.2). These yeasts isolates were categorized into four genera *Saccharomyces cerevisiae* containing four isolates; ETP37, ETP50, ETP53, ETP89 isolated from beverages tella, teji, and shamita and ETP94 isolated from flower sample. The others were identified as *Kluyveromyces marxianus,* ETP87 isolated from the local milk product in Ethiopia called ergo, *Pichia fermentans,* ETP22 isolated from compost, and *Candida humilis* (*milleri*), ETP122 isolated from enjera dough. These isolates produced ethanol ranging from 5.37g/L (ETP122) to 9.0g/L (ETP53) (Table 3.2). Thus, the *Saccharomyces cerevisae* isolates were dominant both in terms of number (65%) and production of alcohol (7.67g/L-9.0g/L).

It is interesting to note that the non-Saccharomyces yeast, *Kluyveromyces marxianus* was as equally efficient in alcohol production (7.97gL) as that of *Saccharomyces cerevisae* isolates

Table 3.2 Ethanol from 2% dextrose (w/v) by yeasts isolated from different sources

Yeast isolates	Isolated from	Molecularly identified as	Ethanol produced (g/L)
Baker yeast	Instant Baking powder	*Saccharomyces cerevisae*	8.43
ETP22	Compost	*Pichia fermentans*	6.43
ETP37	Teji	*Saccharomyces cerevisae*	8.10
ETP50	Teji	*Saccharomyces cerevisae*	7.67
ETP53	Tella	*Saccharomyces cerevisae*	9.00
ETP87	Ergo	*Kluyveromyces marxianus*	7.97
ETP89	Shamita	*Saccharomyces cerevisae*	7.60
ETP94	Flower	*Saccharomyces cerevisae*	7.80
ETP122	Enjera Dough	*Candida humilis* (*milleri*)	5.37

3.3.4. Growth and fermentation of different carbon sources selected yeasts isolates

Almost all of the selected isolates were capable of growing and vigorously fermenting glucose, fructose and galactose, and majority of them utilized mannose, raffinose, maltose and sucrose (Table 3.3). One or two isolates weekly grew on one of xylose, arabinose, trehalose, mannitol, and starch with or mild fermentation.

Out of the isolates, *K. marxianus* ETP87 was capable of utilizing the maximum number of sugars (78%) and vigorously fermented glucose, galactose, fructose, and raffinose, lactose and sucrose unlike the other isolates that were limited to grow on and ferment fewer substrates. The different *Saccharomyces cerevisae* strains were consistent in their growth and fermentation of the majority of the sugars (50%-60%).

K. marxianus ETP87 was the only yeast that ferments lactose besides glucose, galactose, fructose, maltose and sucrose within 24 hours. It could be good candidate for ethanol production from whey.

Table 3.3 Growth and fermentation of yeast isolates in different sugar sources

Sugar source	Yeast Isolates															
	ETP22		ETP37		ETP50		ETP53		ETP87		ETP89		ETP94		ETP122	
	Growth	**Fermentation**	**Growth**	**Fermentation**	**Growth**	**Fermentation**	**Growth**	**Fermentation**	**Growth**	**Fermentation**	**Growth**	**Fermentation**	**Growth**	**Fermentation**	**Growth**	**Fermentation**
Glucose	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+++	+	++
Galactose	+	-	+	++	+	++	+	+++	+	++	+	+++	+	+++	+	+
Fructose	+	++	+	++	+	+++	+	+++	+	+++	+	+++	+	+++	+	+
Mannose	+	+++	+	++	+	++	+	++	-	+++	+	+++	+	+++	-	-
Xylose	+	-	-	-	-	-	-	-	+	-	-	-	-	-	_	-
Arabinose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Trehalose	-	-	W	D	W	D	W	D	W	-	-	-	-	-	+	D
Raffinose	+	-	+	++	+	++	+	++	+	++	+	-	+	-	+	++
Cellobiose	+	-	-	-	+	D	-	-	+	-	-	-	W	-	+	-
Mannitol	-	-	W	-	W	-	W	-	W	D	W	D	-	-	+	+
Maltose	+	-	+	+++	+	+++	+	+++	+	+	+	+++	+	D	+	-
Lactose	-	-	-	-	-	-	-	-	+	+++	-	-	-	-	+	-
Sucrose	-	-	+	++	+	++	+	++	+	+++	+	+++	+	-	-	_
Starch	-	-	W	-	W	-	W	-	-	-	W	-	W	-	+	D
Ethanol	+	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-

Fermentation: +++ fermentation less than 24 hours; ++, fermentation within 24-48 hours; +, 48 hrs to 7 days; hrs; D, Delayed (>7 days) positive response

Growth: + positive response; W, weak positive response

The other non-Saccharomyces yeast *P. fermentans* ETP22, and *Candida humilis* (*milleri*) ETP122 were capable of growing on the majority of the sugar substrates (60-65%), but fermented fewer (20-30%) of the substrates indicting that they were not good alcohol fermenters. This isolate was able to grow on xylose even if it didn’t ferment it and can be good candidate to

produce biomass from acid hydrolysate of lignocellulose since five carbon sugars are dominant in acid hydrolysates of such substrates.

3.3.5. Yeast flocculation and sedimentation

The sedimentation rate of the yeast species was higher than 85-90% under natural condition, except *C. humilis* ETP122 with sedimentation rate of 6.8 (Figure 3-2). Generally, all the 7 yeast isolates can be good in terms of cell separation for industrial production of yeast biomass and alcohol.

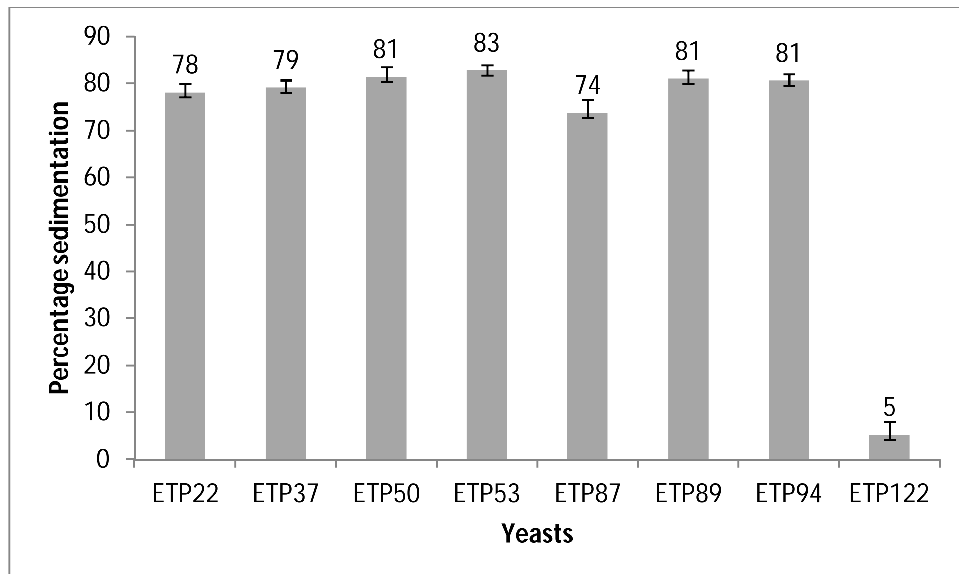

Figure 3-2 Percentage of sedimentation rate for yeasts grown in YPD

3.3.6. Ethanol tolerance

Four selected yeast strains from each genus were evaluated for their ethanol tolerance (shock treatment) on YPD medium containing 10, 15, and 20% extraneous ethanol (Figure 3-3). The highest cell viability of 68% was recorded from *S. cerevisae* ETP53 at 10% ethanol concentration, followed by *K. marxianus* ETP87 *P. fermentans* ETP22 and *C. humilis* ETP122

strains with survival rate of 65%, 60%, and 40%, respectively. However, the viability of the strains decreased with the same pattern at 15% ethanol concentration within the range of 10-20%. Most yeasts died at 20% ethanol concentration.

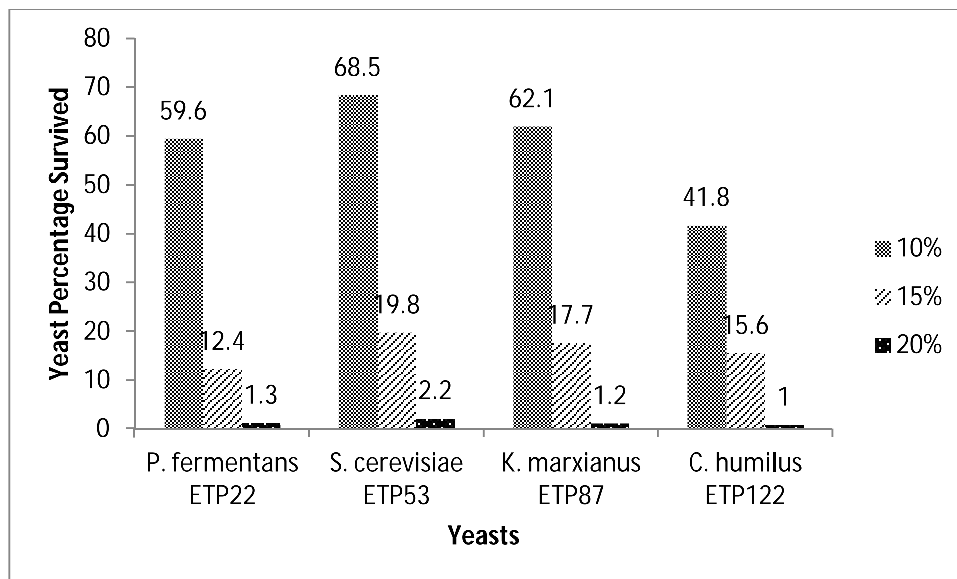

Figure 3-3 Ethanol tolerance test for 10, 15, and 20% (v/v) ethanol concentration

3.4. DISCUSSION

3.4.1. Yeast Isolation

Yeasts are cosmopolitan and hence they could be isolated from different habitats (Mohd Azhar *et al.*, 2017). In this study, yeasts were isolated from fermented beverages, fruits, soils, compost, and flowers. Most of the yeast fermenters were detected (60-80%) from fermented food and beverages indicating the ethanol fermenters dominated by *S. cerevisiae* (Sanni and Lönner, 1993) and that is why most of the yeasts (>50) isolated from fermented products are fermentation positive (Table 3.1) and hence they could be good candidate for ethanol production since the yeasts are adapted to ethanol content which ranges from 1.8 to 5.0% (Ashenafi, 2006). This suggests that the probability of getting good ethanol-producing yeast is higher in these samples (Alemu *et al.*, 1991).

Tella fermentation was dominated by *S. cerevisae* (Samuel and Berhanu, 1991; Ashenafi, 2006; Lee *et al.*, 2015); therefore, the dominant yeasts isolated from *tella* samples might be *S. cerevisae*. Similarly, the dominant yeast flora in *shamita* fermentation was *Saccharomyces* spp. though *Rhodotorula* spp. was found in different fermentation stages of *shamita* (Bacha *et al.*, 1999; Samuel and Berhanu, 1991).

The number of ethanol producing yeasts obtained from compost, soil and fruits were small compared to other samples; however, they were more diverse as it was depicted yeast culture morphology difference. Fruits contain enough sugar exudates that yeasts need to grow and ferment; nevertheless, the number of yeast numbers obtained was lower. This might be due to high sugar content that reduce the water activity for the yeast growth. Even if fruits are rich in sugars, *S. cerevisae* was rare in fruits (Lee *et al.*, 2011).

The most dominant yeast in enjera dough was isolated and found to be *Candida milleri* (Table 3.1). Ashenafi (1994) studied teff dough fermentation and it was found that *Candida milleri, Rhodotorula mucilaginosa, Kluyveromyces marxianus, Pichia naganishii* and *Debaromyces hansenii* were the yeast flora in late teff fermentation and it was used as *ersho*, starter culture for next teff fermentation; *D. hansenii* was in low frequency. *Candida* spp. are found frequently in grain based fermented products (Ashenafi, 1994; Greppi *et al.*, 2013). *C. milleri* (Ashenafi, 1994) and *Candida krusei* (Greppi *et al.*, 2013) were the yeasts most commonly isolated in teff dough and maize based *ogi* and *mawè* (traditionally fermented products widely consumed by the population of Benin, West Africa) fermentation, respectively.

3.4.2. Yeast screening by glucose fermentation

The gas production by the yeast could be considered as confirmatory for yeasts to be ethanol producing (Ashenafi and Mehari, 1995).The existence of high glucose fermentative yeasts in *tella*, *teji*, *areqe tinisis* and *shamita* was noticeable since they are ethanol containing beverage. The ethanol content of *tella*, *teji*, and *shamita* were in the range 2.8-5.0 (Alemu *et al.*, 1991), 6.95 - 10.9 (Ashenafi, 2006), and low (Ashenafi and Mehari, 1995) respectively. Therefore, yeasts isolated from traditionally produced alcoholic beverages might be good candidate for ethanol production.

3.4.3. Growth and fermentation of different carbon sources selected yeasts isolates

K. marxianus would only ferment glucose and assimilate glucose and xylose among carbon sources tested (Kurtman and Fell, 1998); however, *K. marxianus* ETP87 was not able to ferment five carbon sugars tested in this study. This makes it less significant to produce ethanol from the dominant xylose and arabinose in acid hydrolysates of straws. Barnett *et al.* (2000) also reported that *K. marxianus* had a capability to grow on and ferment glucose, galactose, maltose, sucrose, lactose, trehalose and raffinose despite variation among different strains.

P. fermentans ETP22 which was isolated from compost, fermented glucose, mannose and fructose though glucose fermentation was rarely reported before (Kurtman and Fell, 1998; Barnett *et al.*, 2000).

Candida humilis ETP122 also grew better on starch than others and it might be due to long time adaptation since it was isolated from *enjera* dough. Some *Candida* spp produce ethanol from simple sugars and lactose and are good candidate for industrial ethanol productions (Barnett *et al.*, 2000). All the isolates except *C. humilis* ETP122 were grown on ethanol and hence they might decrease the ethanol titer produced when the sugar in the growing media is depleted.

3.4.4. Yeast flocculation and sedimentation

The percentage of sedimentation rate of yeast isolates other than *C. humilis* ETP122 ranged from 74% to 83%. This was much better than the sedimentation rate of 70% recorded in wine yeast by applying external flocculating agent such as sucrose and sorbitol to the growing media (Moneke *et al.*, 2008). *C. humilis* ETP122 might be due to inability to express of *FLO* genes since *FLO* gene is present among industrial yeast strains though its expression varies among strains (Van Mulders *et al.*, 2010).

Yeast sedimentation was correlated to cell surface hydrophobicity and affected by different factors. Cations (Ca^{2+}, Rb^{+}, Cs^{+}, Fe^{2+}, Co^{2+},Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+}, Mg^{2+} and Mn^{2+}), lower pH (3-5), moderate aeration, agitation, and high cells load (>4 X10^7 cells per mL) induce and

promote yeast flocculation; however, higher pH, higher temperature, fermentable sugars, EDTA and high ethanol facilitate loss of flocculation (Soares, 2011).

3.4.5. Ethanol tolerance

Osmotolerance at the beginning of the fermentation and ethanol tolerance in late fermentation is the pre-requisite for ethanol production. Most of the yeast isolates died at 15 and 20% extraneous ethanol. Similarly, Matsumoto *et al.* (2004) showed most of *S. cerevisiae* strains died at 12.5% ethanol after 3 hours of incubation. The higher ethanol concentration affects hydrophobic proteins present in cell, vacuolar, lysosomal, mitochondrial, nuclear membranes and endoplasmic reticulum besides hydrophilic proteins in cytoplasm and nucleoplasm and hence it influences the integrity membranes and their functions (Pina *et al.*, 2004).

This study showed that non-Saccharomyces yeasts, *K. marxianus* ETP87 and *C. humilis* ETP122 showed similar pattern of tolerance with *S. cerevisiae* strain to 10% ethanol which was higher than their tolerance to 6% reported by Pina *et al.* (2004). Costa *et al.* (2014) also showed that non-Saccharomyces *K. marxianus* tolerated relatively lower (5-7% v/v) ethanol concentration than *S. cerevisae* (8-10%). However, few studies also indicated that non-*Saccharomyces* species had similar ethanol tolerance with *S. cerevisiae* and a stronger resistance to fermentation conditions than *S. cerevisiae* (Hong and Park, 2013).

Even though viable yeast cells were found in the medium containing 15% exogenous ethanol in this study, Hack and Marchant (1998) concluded that yeast viability at increased ethanol concentrations may not necessarily lead to the ability to produce ethanol at these conditions. This should be substantiated by additional studies on different substrates and at different incubation time, and concentration of ethanol to use non-Saccharomyces yeasts for large scale production of ethanol.

3.5. CONCLUSIONS AND RECOMMENDATIONS

Ethiopian fermented beverages and foods could be good sources of ethanologenic yeasts. Since the yeast isolates could grow and ferment different monosaccharides and disaccharides, these could make them good candidates for ethanol production from different carbon sources, whey,

and lignocellulosic hydrolysates. Biomass production from *C. humilis* ETP122 at industrial level could be expensive than *P. fermentans* ETP22, *K. marxianus* ETP87, and *S. cerevisiae* ETP37, ETP50, ETP53, ETP94 because *C. humilis* is not able to flocculate and hence it needs the addition of chemical to flocculate and separate the biomass which increases the cost. The yeasts capability to tolerate 10% extraneous ethanol make them good candidate for ethanol production.

In order to apply the yeast isolates for beverage production, it needs to assess other parameters specially safety issues and sensory evaluation. The yeasts isolates have to be improved by different techniques in order to optimize the ethanol concentrations.

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Chapter 4

Optimization of ethanol production of local isolates by *Saccharomyces cerevisae* ETP53 and non-Saccharomyces *Pichia fermentans* ETP22 and *Kluyveromyces marxianus* ETP87 through experimental variables and model

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Abstract

Response surface methodology (RSM) that employ both mathematical and statistical model is being applied to optimize microbial products and growth variable in Applied M5icrobiology. RSM was applied to optimize temperature, pH and incubation time using central composite design (CCD) in Design-Expert 7.0.0. Effects of sugar concentrations and inoculum sizes were studied to explore optimal concentration for higher ethanol titer using one factor at a time. Yeasts exhibited different response to varying inoculum sizes and sugar concentrations. S. cerevisae *ETP53 produced ethanol optimally at pH 5.0, 60 hours, and 34°C. pH 4.8, temperature 36°C, and time 65 hours were optimal growth conditions of ethanol fermentation by* K marxianus *ETP87. The ethanol fermentation conditions of* P. fermentans *ETP22 was similar to S. cerevisae ETP53 though the ethanol titer of S. cerevisiae ETP53 was higher than P. fermentans ETP22. Although their optimal condition varied, the three yeasts produced high ethanol concentrations when the inoculum size ranged from* $2.75 \times 10^6\ to\ 5.5 \times 10^6$. S. cerevisiae *ETP53,* K. marxianus *ETP87, and P. fermentans ETP22 produced maximal ethanol at 100, 80, and 60g/L glucose, respectively. Despite its lower ethanol production capability,* P. fermentans *ETP22 was able to yield more dry biomass than* K. marxianus *ETP87, and* S. cerevisae *ETP53.*

Key words: Response surface methodology, *Saccharomyces cerevisiae*, *Pichia fermentans*, *Kluyveromyces marxianus*, central composite design

4.1. INTRODUCTION

It is established that yeast growth and ethanol production are influenced by different nutritional and environmental factors such as temperature, pH, oxygen, and initial sugar concentrations. Temperature and pH affect membrane turgidity, enzymatic activity and metabolism of yeast cells. They usually prefer acidic pH that enables to control bacterial growth at industrial level. Consequently, yeasts which are active and tolerant at high temperature and low pH are ideal for industrial bioethanol production.

Yeasts also require different nutrients of which sugar mostly limit their growth and activity. According to Costa *et al.* (2014), initial sugar concentration reduces the average specific growth rate of yeasts but enhances their substrate uptake. For this reason, yeast osmo-tolerance at the beginning of fermentation and ethanol-tolerance in late fermentation is the pre-requisite for very high gravity (VHG). Hoondee *et al.* (2016) showed that high sugar concentration commonly greater than 200 g/L produced high ethanol titer from VHG fermentation. This requires the evaluation of glucose concentration on yeast isolates for their potential for (VHG) ethanol fermentation.

Ethanol also inhibits yeast growth and viability of yeast cells. It affects various transport systems inhibits glycolytic enzymes, damages mitochondrial DNA, modifies the fluidity of plasma membrane, and stimulates ATPase activity (Aguilera *et al.*, 2006). Apart from that different, environmental stresses reduce yeast tolerance to ethanol (Costa *et al.*, 2014). The yeast which tolerant ethanol is a prerequisite for high fermentation efficiency and high ethanol yield (De la Torre-González *et al.*, 2016)..

Apart from nutritional and ecological factors, inoculum concentrations of yeast (population density) significantly influence ethanol production. Accordingly, Yun *et al.* (2011) reported that lower inoculum size reduces cost of production in ethanol fermentation; 5% (v/v) inoculum size incubated for 24 hours produced almost the same ethanol concentration with 10% (v/v) that was incubated for 72 hours using *S. cerevisiae* Y5 in enzymatic hydrolysate of corn stover. Therefore, reducing the inoculum size elongates the fermentation time.

Optimization of multiple variables using the conventional method employs one factor at a time, i.e. carrying out many experiments separately, and cannot evaluate the interaction among different variables that could not enable to derive statistical conclusion can be done regarding alternative effects between components. Recently, statistical experimental methods are employed using mathematical models in bioprocesses.

Among these methods, response surface methodology (RSM) is suitable for optimization in different disciplines (Ercan *et al.*, 2013). It enables to design experiments, build model, evaluate

interactions, look for optimum conditions for responses, and reduce the number of experiments (Wang *et al.*, 2008). This method has been used to optimize various chemical production including bio-ethanol production (Ercan *et al.*, 2013).

The objective of this research is to optimize the newly isolated *S. cerevisiae* ETP53, *P. fermentans* ETP22, and *K. marxianus* ETP87 using response surface methodology and conventional methods.

4.2. MATERIALS AND METHODS

4.2.1. Optimizing growth variables

Effects of temperature (30, 35, and 40°C) and pH (4, 5, and 6) on ethanol production were run using YPD (yeast extract, 1%; peptone, 2%; and dextrose 2 and 4 % W/V) under batch fermentation for 48 hours (Wang *et al.*, 2008). Samples were taken at 5, 20, 27 and 48 hours for ethanol analysis for yeasts inoculated in YPD containing 2% Dextrose. Samples were also taken for detection and quantification of butanol and furfural. On the other experimental run, 24, 48 and 72 hours were considered as sampling time for yeasts grown in YPD containing 4% dextrose.

The response surface methodology experiments were undertaken by cultivating the yeasts in 250 mL Erlenmeyer flask containing 100 mL YPD (1% yeast extract, 2% peptone, and 4% dextrose (W/V)) by applying the Central Composite Design (CCD) using Design Expert 7.0.0, Trial version (Stat Ease, Inc., Minneapolis, USA). The number of experiments generated by CCD was 20 from 3 factors at 3 levels with six replications at center point to evaluate the pure error (Table 4.1). The performance of the system was assessed by the response (ethanol produced in g/L). The optimization process employed both quadratic and linear model; the model was given as (Ercan *et al.*, 2013).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1\beta_2 X_1 X_2 + \beta_1\beta_3 X_1 X_3 + \beta_2\beta_3 X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \varepsilon \qquad \text{Equation 4.1}$$

Where

Y = ethanol produced in g/L (dependent output)

β_0 = intercept (the constant process effect in total)

β_1, β_2, and β_3= Linear, quadratic and interaction regression coefficients for temperature, pH and time respectively (parameters)

X_1, X_2, and X_3 = independent variable for temperature (degree centigrade), pH, and time (hours) respectively

ε = random experimental error assumed to have a zero mean

Table 4.1 Experimental design generated by Expert Design for optimization of growth variable for ethanol production

Std	Run	Factor 1 (Temperature, ^{o}C)	Factor 2 (pH)	Factor 3 (time, hours)	Response (Ethanol produced, g/L)
19	1	35.00	5.00	48.00	
3	2	30.00	6.00	24.00	
7	3	30.00	6.00	72.00	
9	4	26.59	5.00	48.00	
15	5	35.00	5.00	48.00	
16	6	35.00	5.00	48.00	
6	7	40.00	4.00	72.00	
17	8	35.00	5.00	48.00	
14	9	35.00	5.00	88.36	
1	10	30.00	4.00	24.00	
10	11	43.41	5.00	48.00	
2	12	40.00	4.00	24.00	
18	13	35.00	5.00	48.00	
8	14	40.00	6.00	72.00	
11	15	35.00	3.32	48.00	
4	16	40.00	6.00	24.00	
5	17	30.00	4.00	72.00	
12	18	35.00	6.68	48.00	
13	19	35.00	5.00	7.64	
20	20	35.00	5.00	48.00	

The analysis of variance (ANOVA) and the significance of the model equation were determined by the coefficient of determination (R^2), p-value and F-test using Design Expert. The response surface was optimized for the maximum ethanol production (Wang *et al.*, 2008).

4.2.2. Sugar utilization during ethanol fermentation

The yeasts (approximately 6.6×20^6) were inoculated to 100mL YPD that contained 2% dextrose and it was incubated at 30°C for 24 hours. Samples were taken at 5, 10, 13, 16, 19, and 22 hours for ethanol and sugar analysis.

4.2.3. Effect of sugar concentration

Yeasts were inoculated (inoculum size 7.8×10^7) for very high gravity (VHG) fermentations using different dextrose concentrations at2, 4, 6, 8, 10, and 12% W/V) into YPD containing 1% yeast extract and 2% peptone, and incubated at 30oC for 4 days (Costa *et al.*, 2014).

4.2.4. Inoculum size

The effect of inoculum size on ethanol fermentation was done by inoculating $9.12 \times 10^5, 2.75 \times 10^6, 5.5 \times 10^6, 9.12 \times 10^6, 1.62 \times 10^7$ (CFU in 100mL YPD) of *P. fermentans* ETP22, *S. cerevisae* ETP53, *K. marxianus* ETP87, and *C. humilis* 122 to YPD containing 4% dextrose and were incubated at 30°C for 48 hours (Laluce *et al.*, 2009)**.**

4.2.5. Aerated shaking versus closed non-shaking fermenting for biomass production

Oxygen is required to produce ergosterol required for membrane rigidity and hence most yeast couldn't grow in strict anaerobic environment. On the first hand, the flasks containing YPD (2% w/v glucose) inoculated with yeasts were plugged with cotton and incubated in bath shaker at 30°C and 200 forth and back shaking per minute to investigate oxygen effect via shaking. On the other hand, the flasks containing yeasts growing media were closed by aluminum foil with pin hole and they were incubated in incubator at 30°C under static condition.

4.2.6. Biomass determination

Yeast biomass was determined as yeasts dry weight using a standard curve. Yeasts grown in YPD for 24 hours were diluted to make different OD readings (Jenway 6405 UV/Vis Spectrophotometer, United Kingdom) and 1mL of yeast broth was transferred into 1.5mL Eppendroff tube and centrifuged at 13000×g (eppendorf centrifuge 5418 R, Germany). The

pellets were carefully transferred and dried in dry oven at 70°C for 3 days and weighed using precision balance (Radwag WAS220/C/2, Poland).

4.2.7. Estimation of reducing sugars

The reducing sugars were estimated by DNS (Miller, 1959). Appropriately diluted solution sample (0.5mL) was added to 1.5mL of DNS reagent in test tubes. The tubes were boiled in a boiling water bath for 15 minutes. Rochelle salt (0.5mL) was added to the test tubes taken from boiling water bath. The mixture optical density (Jenway 6405 UV/Vis Spectrophotometer, United Kingdom) was measured at 540 nm after solution in test tubes was cooled to room temperature. A standard curve of glucose was prepared by using 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 g/L concentrations.

4.2.8. Ethanol quantification by pycnometer method

Fermented broth samples (100 mL) were transferred in 500mL distillation flask containing glass beads and it was fitted with diagonally assembled condenser (Quickfit CX7/33/SC, England). The joints were standard taper and the heat source was gas operated system.

Distillation of sample: A100mL volume of fermented broth was transferred to 500mL distillation flask containing glass beads. The pycnometer was rinsed 3 times (13mL each) with 25mL cold distilled water and the rinsed water was transferred back to the flask containing 100mL sample. The distillation was run for 40 minutes from which distillates were collected to pycnometer and stopped when distillates volume was reached to graduation mark. The pycnometer neck was dried by tissue paper and the dry weight (DW) distillates were weighed using the formula

$$DW = DPW - PW \qquad \text{Equation 4.2}$$

Where

DW= weight of distillate

DPW= weight of distillate and pycnometer

PW= weight of pycnometer

Finally, the specific gravity (SG) was calculated as

$$SG = \frac{DW}{WW}$$ Equation 4.3

Where

SG= specific gravity

DW= weight of distillate

WW= weight of recently distilled water

Ethanol determination: The ethanol concentration was determined from a table that shows ethyl alcohol percentage corresponding to apparent specific gravity at various temperatures (Patricia, 1995). The percentage of volume was converted into g/L using density of ethyl alcohol.

4.2.9. Gas chromatography

In order to determine the ethanol, the fermented products were centrifuged at 14000×g for 5 minutes. The supernatant was analyzed by Gas Chromatography (Agilent 6890N) coupled with a mass spectrometer (MS) with headspace autosampler (Agilent7694E). The GC-MS was equipped with Mass Selective Detector (Agilent5973Network) and a polar polyethylene glycol (PEG) phase DB-wax122-7032 capillary column. Helium was used as a carrier gas. The flow rate for the column was 1mL/min. The column temperature was held at 40°C for 4 min, and then raised to 240°C at 40°C/min; the inlet temperature was 220°C.The GC was operated with 20:1 split injection of the headspace.

The mass-to-charge ratios (m/z) for ethanol was 31-45 m/z range with the retention time of 3.36 min and for furfural 32-95 m/z range with the retention time of 7.64 min. The MS Quadrupole, MS source and transfer line temperature were 150°C, 230°C, and 250°C respectively. The conditions of the headspace autosampler were 25min for the GC cycle time, 10 min for the vial equilibration time, 0.5min for the pressurization time, 1min for the injection time and a constant vial pressure of 14.0 psi. The temperatures at 110°C for the transfer line to the column and at 90°C for the loop. The equilibration temperature was 80°C for 10 min. Fermentation sample (200µL) were put in 10mL headspace vials.

4.2.10. Ethanol productivity, yield, and efficiency

Ethanol productivity, yield, and efficiency was determined using 16g/L glucose in YPD according to Hoondee *et al.* (2016). Samples were taken at 5, 20, 27 and 48 hours to quantify ethanol in g/L. Ethanol productivity was calculated as

$$\text{Ethanol productivity} = \frac{\text{Ethanol produced (g/L)}}{\text{Incubation time (hours)}} \qquad \text{Equation 4.4}$$

The yield was computed as

$$\text{Ethanol yield} = \frac{\text{Ethanol produced (g/L)}}{\text{Intial dextrose concentration (g/L)}} \qquad \text{Equation 4.5}$$

Fermentation (ethanol) efficiency was calculated as the percentage of experimental to maximum theoretical yield .

$$\text{Ethanol efficiency} = \frac{\text{Experimental Yield}}{\text{Theoretical Yield (0.51)}} \times 100\% \qquad \text{Equation 4.6}$$

4.3. RESULTS

4.3.1. Ethanol productivity, yield and efficiency

In this study, *S. cerevisae* ETP53 (92%) and *K. marxianus* ETP87 (84%) were more efficient for ethanol production from glucose than *P. fermentans* ETP22 (62%) within 5-20 hrs at 2% (w/v) glucose. The highest ethanol productivity was observed in *S. cerevisae* ETP53 (1.38 g/L/hr) within 5 hours which was 14 times higher than *P. fermentans* ETP53 and *C. humilis* ETP122 within the same incubation time. Thus, only *S. cerevisae* ETP53 produced economical ethanol within 5 hours with 84% efficiency (Table 4.2). The pattern of ethanol concentration, yield, and efficiency was the same since yield and efficiency calculations depends on the concentration of ethanol.

Table 4.2 Ethanol productivity, yield and efficiency of the three isolates grown in YPD at 2% glucose

Isolate	Time (hrs)	Ethanol conc. g/L	Productivity (g/L/hr)	Yield (g/g)	Efficiency (%)
	5	0.52	0.1	0.03	0.06
	20	1.13	0.06	0.07	13.73
	27	5.15	0.19	0.32	62.75
P. fermentans ETP22	48	4.19	0.09	0.26	50.98
	5	6.9	1.38	0.43	84.13
	20	7.5	0.38	0.47	92.16
	27	5.88	0.22	0.37	72.55
S. cerevisiae ETP53	48	5.9	0.12	0.37	72.55
	5	0.67	0.13	0.04	0.08
	20	6.88	0.34	0.43	84.13
	27	6.95	0.26	0.43	84.13
K. marxianus ETP87	48	3.44	0.07	0.22	43.14
Theoretical				0.51	100

4.3.2. Response surface analysis for temperature, pH and time optimization

4.3.2.1. S. cerevisae ETP53

The actual yield and predicted value generated by the model is given in Table 4.3. The correlation between actual and predicted value for *S. cerevisae* ETP53 was 0.9846. Therefore, the deviation between the actual and predicted vale was low. The maximum deviation of predicted value from the actual value was 2.5 whereas the minimum was 0.03. The average deviation for all the data was 0.81 which was low to consider the mode for optimization prediction.

Table 4.3 Central composite design matrix for three independent variables with actual and predicted values of ethanol produced from 40g/L dextrose by *S. cerevisae* ETP53

Standard order	Run order	Temperature (^{o}C)	pH	Time (hours)	Actual value (g/L)	Predicted value (g/L)	Residual
1	8	30	4	24	10.1	8.95	1.15
2	3	40	4	24	1.4	1.34	0.06
3	11	30	6	24	9.2	8.97	0.23
4	5	40	6	24	1.5	2.21	-0.71
5	18	30	4	72	19.4	17.14	2.26
6	15	40	4	72	2.3	0.98	1.32
7	19	30	6	72	18.9	17.42	1.48
8	6	40	6	72	2.5	2.11	0.39
9	9	26.59	5	48	17.36	19.66	-2.30
10	17	43.41	5	48	0.5	0.38	0.12
11	4	35	3.32	48	3.2	5.30	-2.10
12	2	35	6.68	48	6.18	6.26	-0.08
13	12	35	5	7.64	4.8	4.48	0.32
14	14	35	5	88.36	8.79	11.29	-2.50
15	16	35	5	48	16.46	16.19	0.27
16	20	35	5	48	15.99	16.19	-0.20
17	13	35	5	48	16.22	16.19	0.03
18	7	35	5	48	16	16.19	-0.19
19	10	35	5	48	16.32	16.19	0.13
20	1	35	5	48	16.54	16.19	0.35

Table 4.4 shows that the quadratic model employed was fit (p <0.00001). The degree of significance (Table 4.4) showed that temperature had greatest effect where as pH was the lowest. However, pH played significant role in the interactions even if it was the lowest compared to temperature and time.

Though the model was sufficient (p<0.0001) to explain the interactions, not all effects of interactions were significant (p<0.05) for ethanol production (Table 4.4); nevertheless, all were included in the model equation because adding insignificant value to a number will not change it significantly.

Table 4.4 ANOVA for response surface (temperature, pH and time) quadratic model of *S. cerevisae* ETP53

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	870.71	9	96.75	35.35	< 0.0001
A-Temperature	448.41	1	448.41	163.86	< 0.0001
B-pH	1.12	1	1.12	0.41	0.5366
C-Time	55.82	1	55.82	20.40	0.0011
AB	0.36	1	0.36	0.13	0.7239
AC	36.55	1	36.55	13.36	0.0044
BC	0.03	1	0.03	0.01	0.9170
A^2	68.64	1	68.64	25.08	0.0005
B^2	195.31	1	195.31	71.37	< 0.0001
C^2	124.33	1	124.33	45.43	< 0.0001
Residual	27.37	10	2.74		
Lack of Fit	27.10	5	5.42	102.68	< 0.0001
Pure Error	0.26	5	0.05		
Cor Total	898.07	19			
Std. Dev.	1.65				
Mean	10.18				
C.V. %	16.25				

The second order polynomial equation to produce ethanol (Y) as a function of temperature (X_1), pH (X_2) and time (X_3) was obtained as

$$Y = -181.73 + 5.61X_1 + 35.49X_2 + 1.18X_3 + 0.04X_1X_2 - 0.02X_1X_3 + 2.6X_2X_3 - 0.09X_1^2 - 3.68X_2^2 - 5.1X_3^2$$ Equation 4.7

Interactions among temperature, pH and time

Figure 4-1 shows the response surface curve with contour plots for optimization of ethanol production as a function of temperature, pH and time. Maximum ethanol was produced at the acidic pH (5.5) and lower temperature (less than 33°C) (Figure 4-1 A).

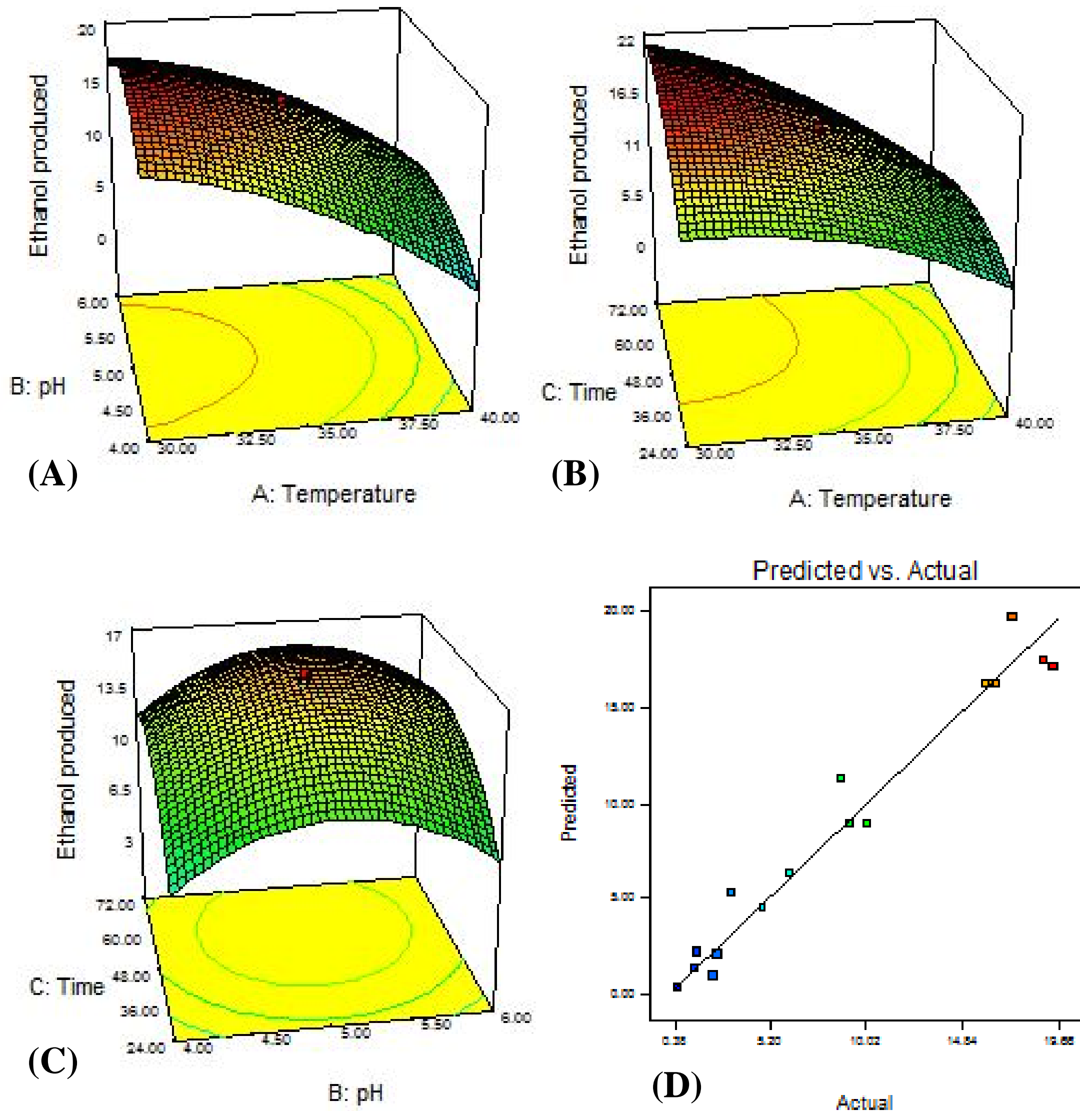

Figure 4-1 *S. cerevisae* ETP53's response surface and contour plate of temperature vs. pH (A), temperature vs. time (B) , pH vs. time (C), and correlation plot showing the distribution of actual (observed) and predicted values of ethanol (g/L) produced from 40g/L dextrose.

The increase in temperature above 35°C significantly reduced the ethanol yield (Figure 4-1 B). The data also showed that higher incubation time (greater than 38 hours) and lower temperature (less than 33°C) resulted in optimal yield (Figure 4-1 B). In pH and time interaction, best ethanol was produced nearly (a little higher time) at the center (Figure 4-1 C) and then the yield decreased at any direction in the model. The maximum ethanol was attained (20.93 g/L) was obtained at a temperature (30.1°C), pH (5.13) and incubation time (58.97 hours) (Figure 4-1).

4.3.2.2. K. marxianus ETP87

A 3-level 3-factors central composite design (CCD) was performed with different combinations of temperature, pH and time to upgrade ethanol production by the yeast. Table 4.5 shows the strong correlation value between observed and predicted ethanol production generated by the model.

Table 4.5 Central composite design matrix for three independent variable with actual and predicted values of ethanol produced from 40g/L dextrose by *K. marxianus* ETP87.

Standard order	**Run order**	**Temperature (^{o}C)**	**pH**	**Time (hours)**	**Actual value (g/L)**	**Predicted value (g/L)**	**Residual**
1	13	30	4	24	7.85	6.60	1.25
2	11	40	4	24	9.3	8.65	0.65
3	2	30	6	24	7.65	6.84	0.81
4	15	40	6	24	8.56	7.38	1.18
5	1	30	4	72	16.98	16.10	0.88
6	12	40	4	72	16.05	14.80	1.25
7	6	30	6	72	13.62	12.22	1.40
8	5	40	6	72	10.22	9.41	0.81
9	10	26.59	5	48	9.84	11.43	-1.59
10	19	43.41	5	48	9.47	10.79	-1.32
11	16	35	3.32	48	11.13	12.53	-1.40
12	17	35	6.68	48	6.7	8.20	-1.50
13	14	35	5	7.64	4	5.32	-1.32
14	9	35	5	88.36	13.43	15.01	-1.58
15	4	35	5	48	15.78	15.38	0.40
16	20	35	5	48	15.52	15.38	0.14
17	3	35	5	48	15.63	15.38	0.25
18	18	35	5	48	15.11	15.38	-0.27
19	7	35	5	48	15.83	15.38	0.45
20	8	35	5	48	14.9	15.38	-0.48

Unlike *P. fermentans* ETP22 and *S. cerevisae* ETP53, time and temperature had the highest and lowest impact on ethanol production, respectively. This shows that yeast was thermophilic and acidophilic.

The regression analysis of the model (Table 4.6) showed that 92.02% of the variation could be explained. The analysis of ANOVA and multiple regression resulted second order polynomial equation. In the equation, Y (ethanol produced) was the function of temperature (X_1), pH (X_2), and time (X_3) and it was given as

$$Y = -143.51 + 4.9X_1 + 21.13X_2 + 0.89X_3 - 0.08X_1X_2 - 6.97X_1X_3 - 0.04X_2X_3 - 0.06X_1^2 - 1.77X_2^2 - 3.2X_3^2 \quad \text{Equation 4.8}$$

All the factors and interactions selected by the quadratic model were included in the equation (unreduced equation) even if the *p*-value for temperature, temperature-pH interaction, and temperature-time interaction was higher than 0.05. The importance of the variables and their effects on the production could be elucidated by the magnitude and the sign of regression coefficient of the actual or coded values generated by Design-Expert.

Table 4.6 ANOVA for response surface (temperature, pH and time) quadratic model of *K. marxianus* ETP87

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	258.14	9	28.68	12.81	0.0002
A-Temperature	0.49	1	0.49	0.22	0.6493
B-pH	22.63	1	22.63	10.10	0.0098
C-Time	113.49	1	113.49	50.67	< 0.0001
AB	1.13	1	1.13	0.51	0.4933
AC	5.59	1	5.59	2.50	0.1451
BC	8.51	1	8.51	3.80	0.0799
A^2	32.89	1	32.89	14.68	0.0033
B^2	45.27	1	45.27	20.21	0.0012
C^2	48.95	1	48.95	21.85	0.0009
Residual	22.40	10	2.24		
Lack of Fit	21.69	5	4.34	30.64	0.0009
Pure Error	0.71	5	0.14		
Cor Total	280.53	19			
Std. Dev.	1.50				
Mean	11.88				
C.V. %	12.60				

Interactions among temperature, pH and time

The data showed that ethanol production did not significantly vary with interaction between pH and temperature between 30 and 40°C (Figure 4-2 A) which was the optimal temperature range for ethanol yield.

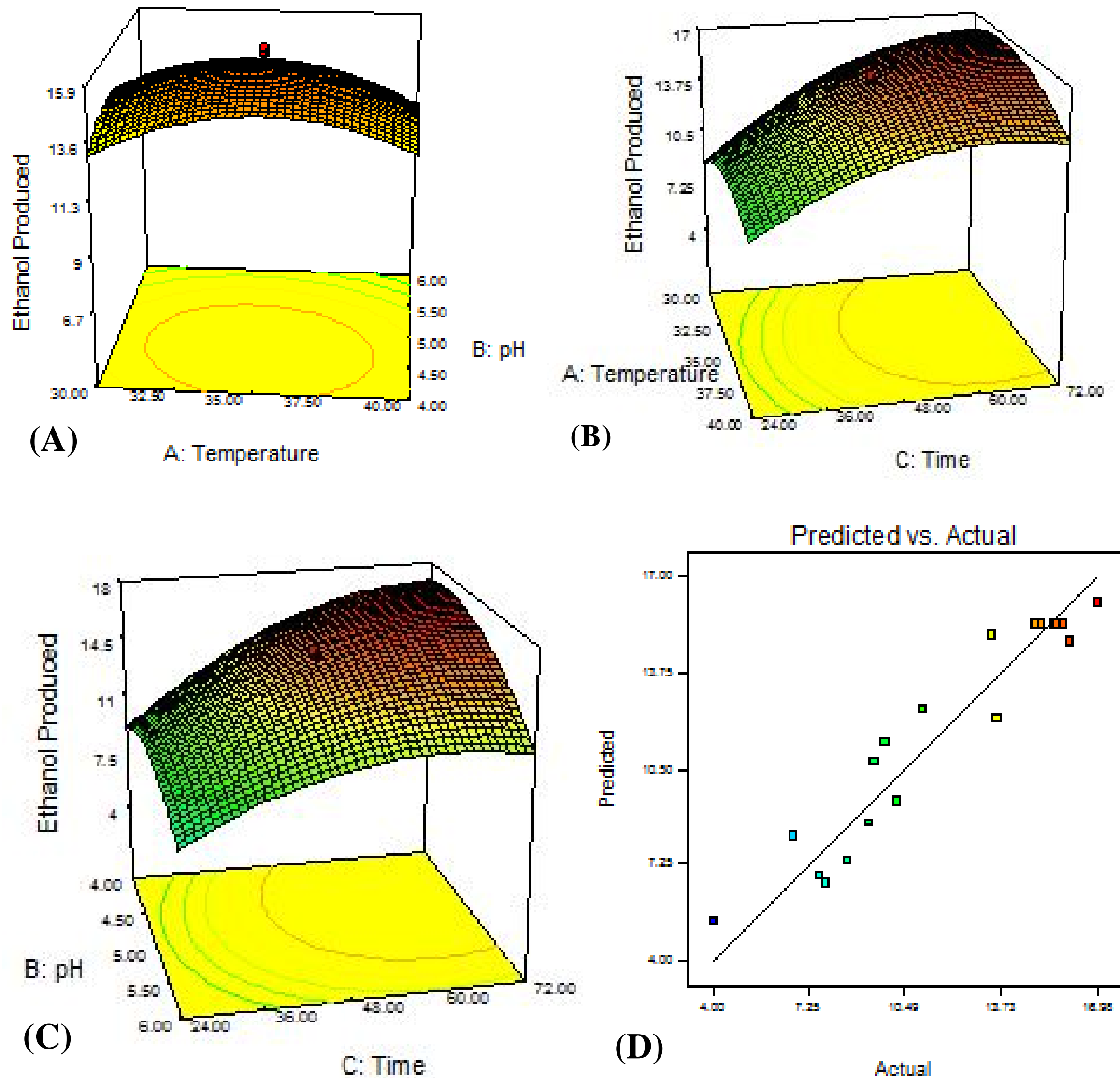

Figure 4-2 *K. marxianus* ETP87's response surface and contour plate of temperature vs. pH (A), temperature vs. time (B), pH vs. time (C), and correlation plot showing the distribution of actual (observed) and predicted values of ethanol produced from 40g/L.

The interaction between temperature and incubation time showed that optimal ethanol production was detected at higher than 40 hours and in nearly all temperature range (Figure 4-2 B). Unlike *P. fermentans* ETP22 and *S. cerevisae* ETP53, optimal location was not at the center when pH was interacted with time (Figure 4-2 C). The optimum production was attained at pH lower than 5.5 and incubation time higher than 48 hours. The interaction of temperature (34.42^oC), pH (4.24) and incubation time (71.93) was able to produce optimal ethanol (17.22 g/L).

The adjusted R-squared value was 0.8851which indicated that the variations in ethanol production were contributed by the three factors at 88.51% confidence level hours) resulted maximum ethanol (17.22 g/L).

4.3.2.3. P. fermentans ETP22

Table 4.7 Central composite design matrix for three independent variable with actual and predicted values of ethanol produced from 40 g/L dextrose by *P. fermentans* ETP22

Standard order	Run order	Temperature (^oC)	pH	Time (hours)	Actual value (g/L)	Predicted value (g/L)	Residual
1	5	30	4	24	6.72	5.38	1.34
2	16	40	4	24	4.18	3.00	1.18
3	9	30	6	24	5.76	4.52	1.24
4	19	40	6	24	0.22	0.07	0.15
5	15	30	4	72	9.23	8.19	1.04
6	18	40	4	72	0.42	0.46	-0.04
7	7	30	6	72	10.52	10.50	0.02
8	2	40	6	72	0.55	0.70	-0.15
9	10	26.59	5	48	8.78	10.37	-1.59
10	3	43.41	5	48	0.02	0.12	-0.10
11	20	35	3.32	48	2.73	4.25	-1.52
12	8	35	6.68	48	3.55	3.72	-0.17
13	12	35	5	7.64	0.6	2.35	-1.75
14	11	35	5	88.36	5.29	5.23	0.06
15	13	35	5	48	8.81	8.25	0.56
16	6	35	5	48	8.02	8.25	-0.23
17	14	35	5	48	7.75	8.25	-0.50
18	17	35	5	48	8.64	8.25	0.39
19	4	35	5	48	7.91	8.25	-0.34
20	1	35	5	48	8.67	8.25	0.42

Table 4.7 shows the combination of three interacting variables with actual and predicted value randomized by Expert Design. The predicted value was strongly correlated with actual value; therefore, the model was fit to predict by employing independent variables.

Table 4.8 ANOVA for response surface (temperature, pH and time) quadratic model of *P. fermentans* ETP22

Source	**Sum of Squares**	**Df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	230.06	9	25.56	17.26	< 0.0001
A-Temperature	126.67	1	126.67	85.54	< 0.0001
B-pH	0.33	1	0.33	0.22	0.6473
C-Time	10.07	1	10.07	6.80	0.0261
AB	2.16	1	2.16	1.46	0.2546
AC	14.31	1	14.31	9.66	0.0111
BC	5.02	1	5.02	3.39	0.0953
A^2	16.29	1	16.29	11.00	0.0078
B^2	32.80	1	32.80	22.15	0.0008
C^2	35.86	1	35.86	24.22	0.0006
Residual	14.81	10	1.48		
Lack of Fit	13.76	5	2.75	13.16	0.0067
Pure Error	1.05	5	0.21		
Cor Total	244.87	19			
Std. Dev.	1.22				
Mean	5.42				
C.V. %	22.46				

The experimental responses (ethanol produced, g/L) were analyzed using ANOVA to estimate the impact of temperature, pH and time. The F-value analysis in ANOVA table exhibits that temperature had the highest impact where as pH had the lowest contribution. In fit summary analysis, the quadratic model was suggested by the software (Design-Expert). The empirical model in terms of actual factors was given as

$$Y = -96.48 + 3.24X_1 + 16.99X_2 + 0.52X_3 - 0.1X_1X_2 - 0.01X_1X_3 + 0.03X_2X_3 - 0.04X_1^2 - 1.51X_2^2 - 2.74X_3^2$$ Equation 4.9

where Y was the ethanol produced (g/L) and X_1, X_2, and X_3 were temperature, pH and incubation time respectively.

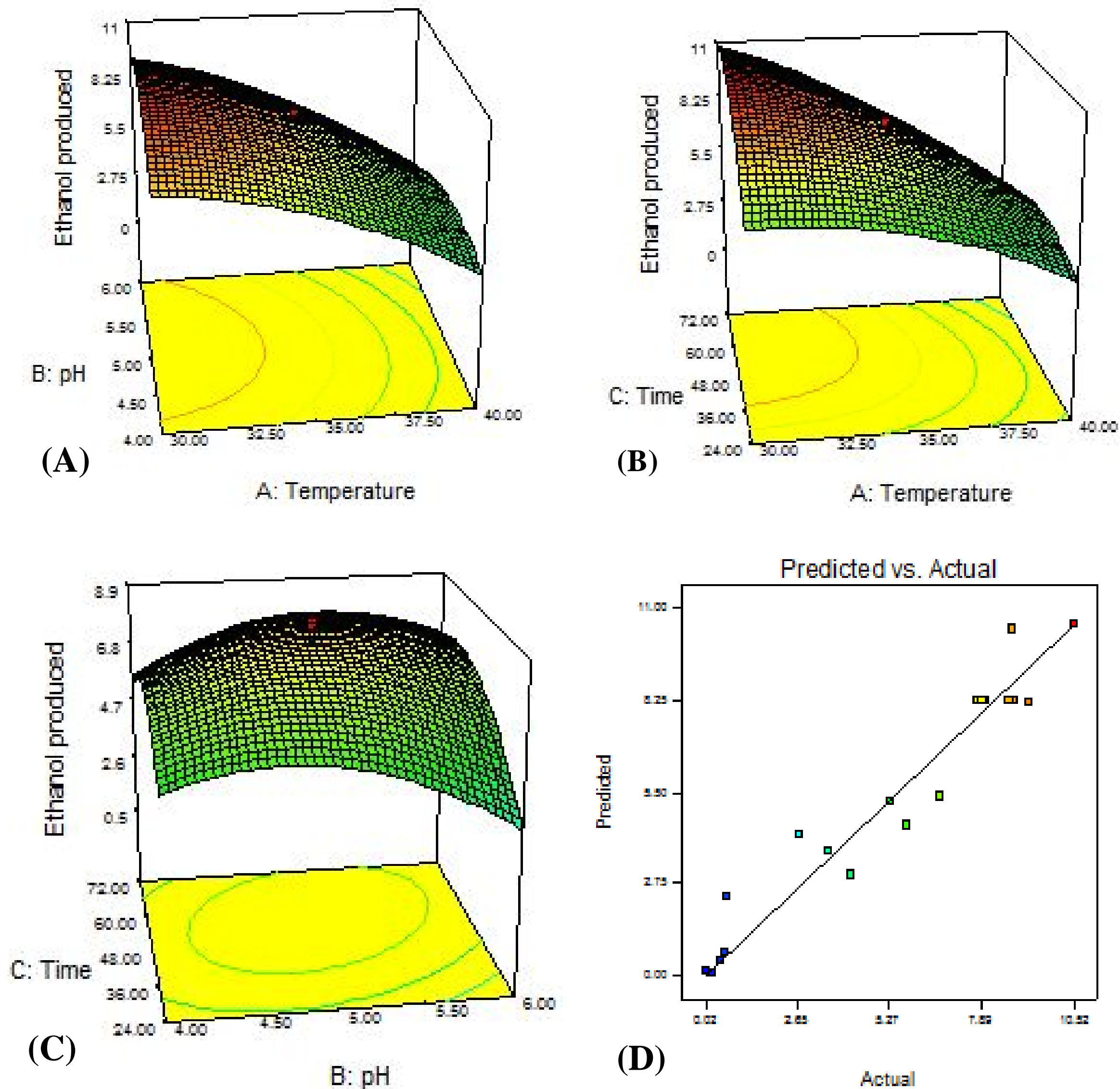

Figure 4-3 *P. fermentans* ETP22's response surface and contour plate of temperature vs. pH (A), temperature vs. time (B), pH vs. time (C), and correlation plot showing the distribution of actual (observed) and predicted values of ethanol produced from 40g/L.

Though the p-value for pH, temperature-pH, and pH-time was higher than 0.05, all factors and interactions were considered in the equation. The p-value of the model was 0.0001 and this value intensified the significance of the model. A smaller p-value inferred a significant influence to the interaction than the higher one.

Interaction among temperature, pH and time

The interaction effect of temperature, pH and time on the ethanol yield from dextrose was illustrated graphically by plotting the three dimensional response surfaces and the two dimensional isoresponse contour (Figure 4-3). The optimum ethanol production was achieved at higher incubation time (61.3 hours), mild acidic pH (5.4), and lower temperature (30.3^{o}C). The ethanol yield was significantly reduced at the temperature higher than 34°C (Figure 4-3 A and B). pH-time interaction was weaker than temperature-pH and temperature-time interaction to maximize ethanol titer.

4.3.3. Effect of glucose concentration

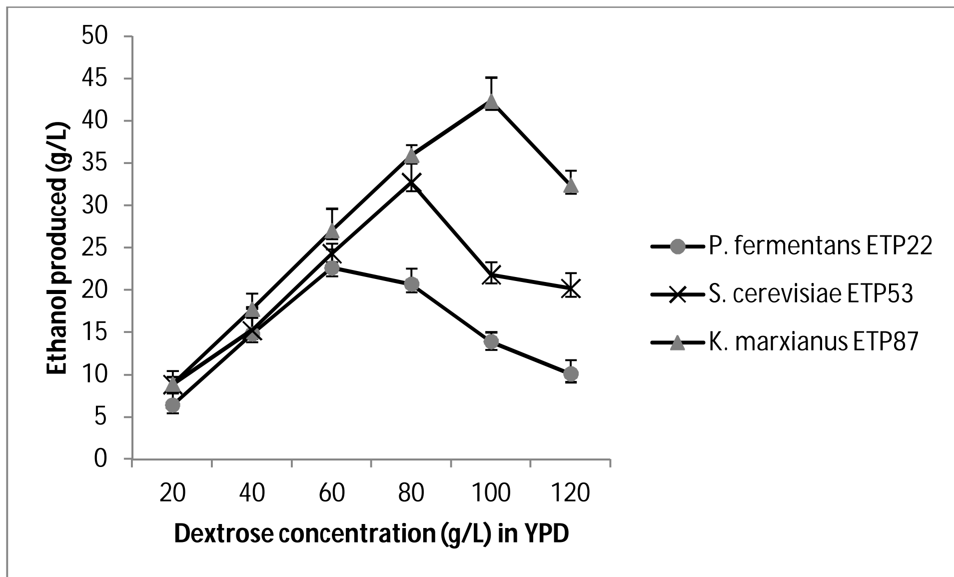

Figure 4-4 Effect of sugar concentration on ethanol production incubated at 30°C for 4 days

The effect of glucose concentration on yeast isolates were investigated to evaluate their potential for very high gravity (VHG) ethanol fermentation. *S. cerevisae* ETP53 and *K. marxianus* ETP87 produced optimum ethanol at 10 and 8% (w/v) glucose concentration, respectively at 30°C and then ethanol concentration was decreased when the glucose concentration was higher than 10% (w/v) (Figure 4-4). *P. fermentans* ETP22 was less osmotolerant than *S. cerevisae* ETP53 and *K. marxianus* ETP87 in ethanol fermentation (Figure 4-4). The pattern of raising in ethanol concentration when the sugar concentration increased up to optimal was the same for the three yeasts; nevertheless, the manner of declining was different for the three yeasts after optimal ethanol was produced.

4.3.4. Yeasts' inoculum size

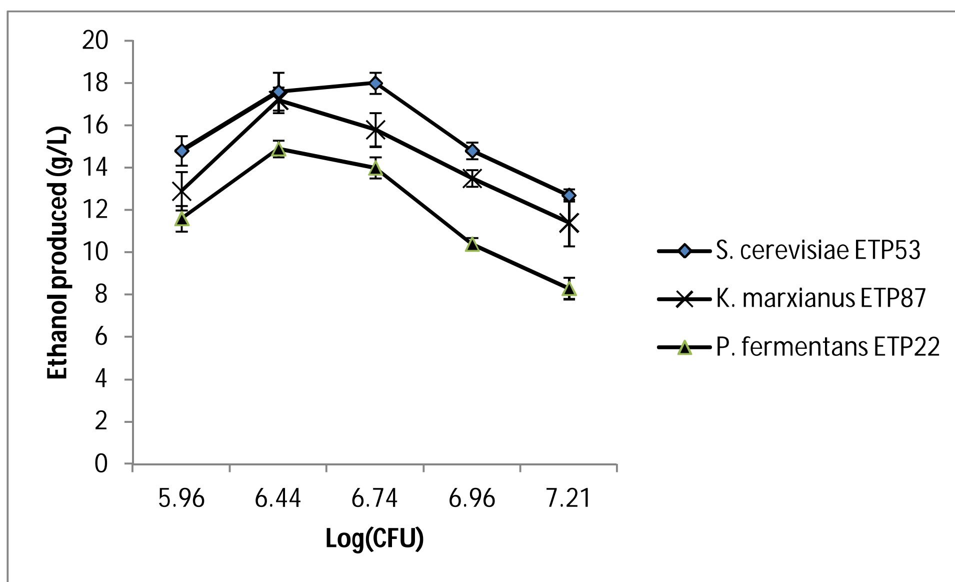

Figure 4-5 Effect of inoculum size on ethanol produced from 40g/L dextrose which was incubated at 30°C for 48 hours

C. humilis ETP122 and *S. cerevisiae* ETP53 produced maximum ethanol with inoculum size of 3-6% (2.75-5.5 $\times 10^6$ yeast cells per mL) in YPD at 30°C then the production declined with inoculum of 10 and 12%. However, *K. marxianus* ETP87 was able to produce optimum ethanol at 3% v/v (2.75$\times 10^6$ yeast cells per mL) yeast inoculum. The pattern of raising and declining

due to inoculum size effect in the three yeasts were the same though *K. marxianus* ETP87 declined at relatively lower yeast concentration.

4.3.5. Sugar utilization during ethanol fermentation

The ability of the yeast strains to utilize glucose and produce ethanol is shown in Figure 4-6. The glucose was depleted quickly at the beginning of the fermentation at rates of 44.44%, 60%, and 48.33% by *P. fermentans* ETP22, *S. cerevisae* ETP53, and *K. marxianus* ETP87, respectively within 5 hours of growth and ethanol production. However, only *S. cerevisae* ETP53 produced sufficient ethanol (12 g/L) during five hours (Figure 4-6). The ethanol producing capability of *P. fermentans* ETP22 was minimal in 19 hours. The trend of sugar consumption and ethanol production by *S. cerevisae* ETP53 and *K. marxianus* ETP87 was almost the same except that ETP53 produced more ethanol than ETP87.

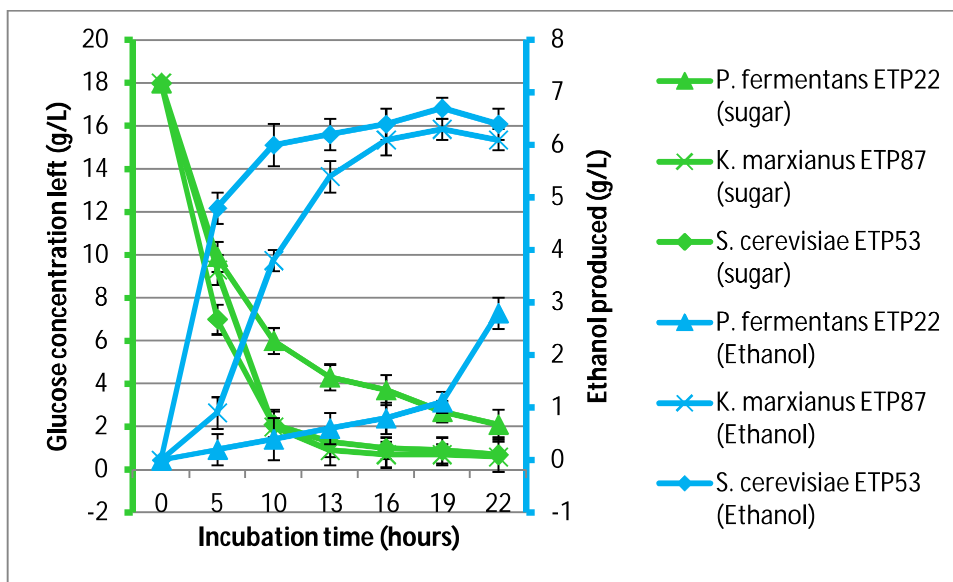

Figure 4-6 Sugar utilization during ethanol fermentation

4.3.6. Bioethanol versus Biomass Production

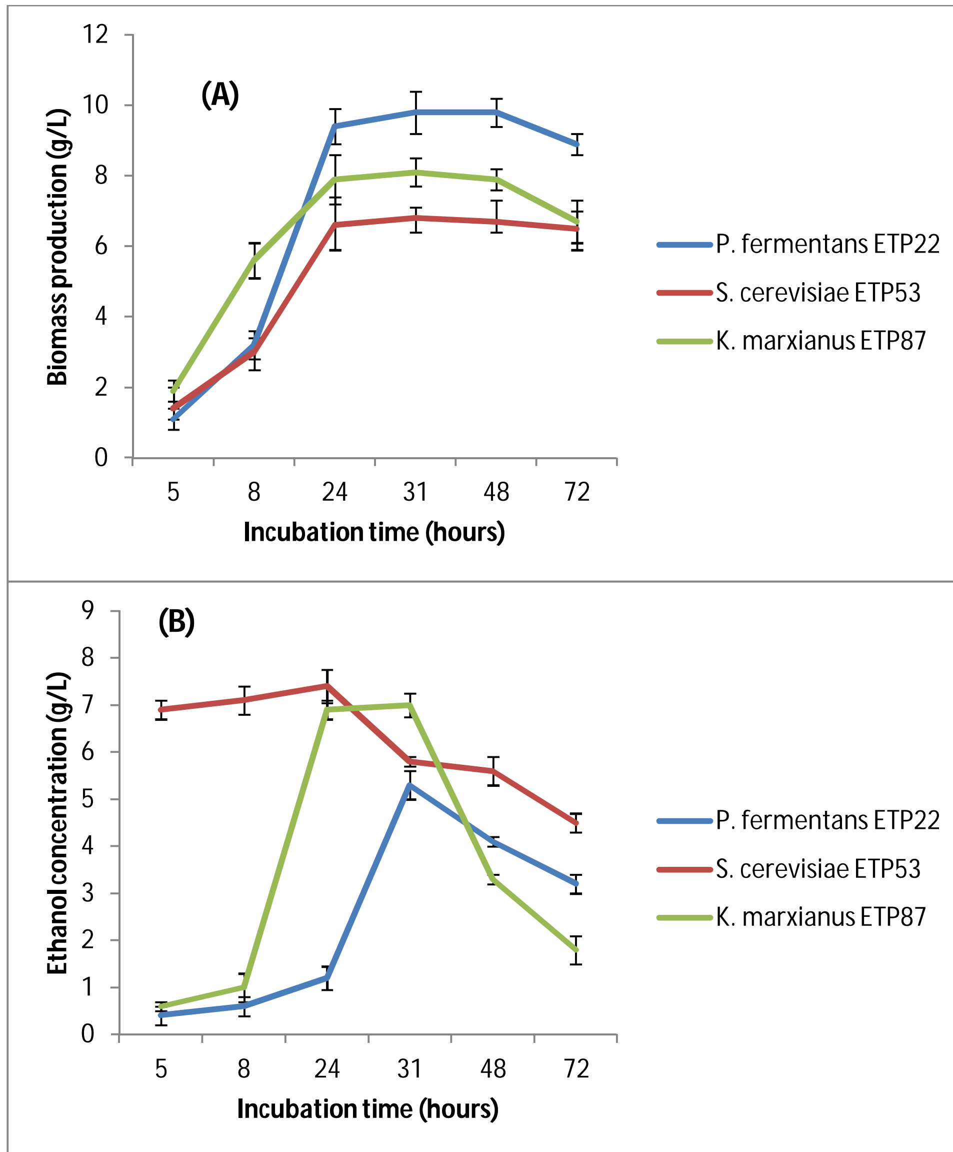

Figure 4-7 Biomass (A) and bioethanol (B) production using *P. fermentans* ETP22, *S. cerevisae* ETP53, and *K. marxianus* ETP87 grown on YPD containing 4% (w/v) under ethanol production condition.

The performance of the yeast strains for biomass and ethanol production is presented in Figure 4-7. Accordingly, *P. fermentans* ETP22 was the highest biomass and lowest ethanol producer from glucose. This corroborates the previous result of sugar utilization with less ethanol production (Figure 4-7). This might be due to utilization of glucose for biomass building rather than fermenting. All yeast isolates produced maximum dry biomass after 24 hours despite

varying optimal time for ethanol production. *K. marxianus* ETP87 was good candidate for both ethanol and biomass producer.

4.3.7. Aerated shaking versus closed non-shaking fermenting on biomass

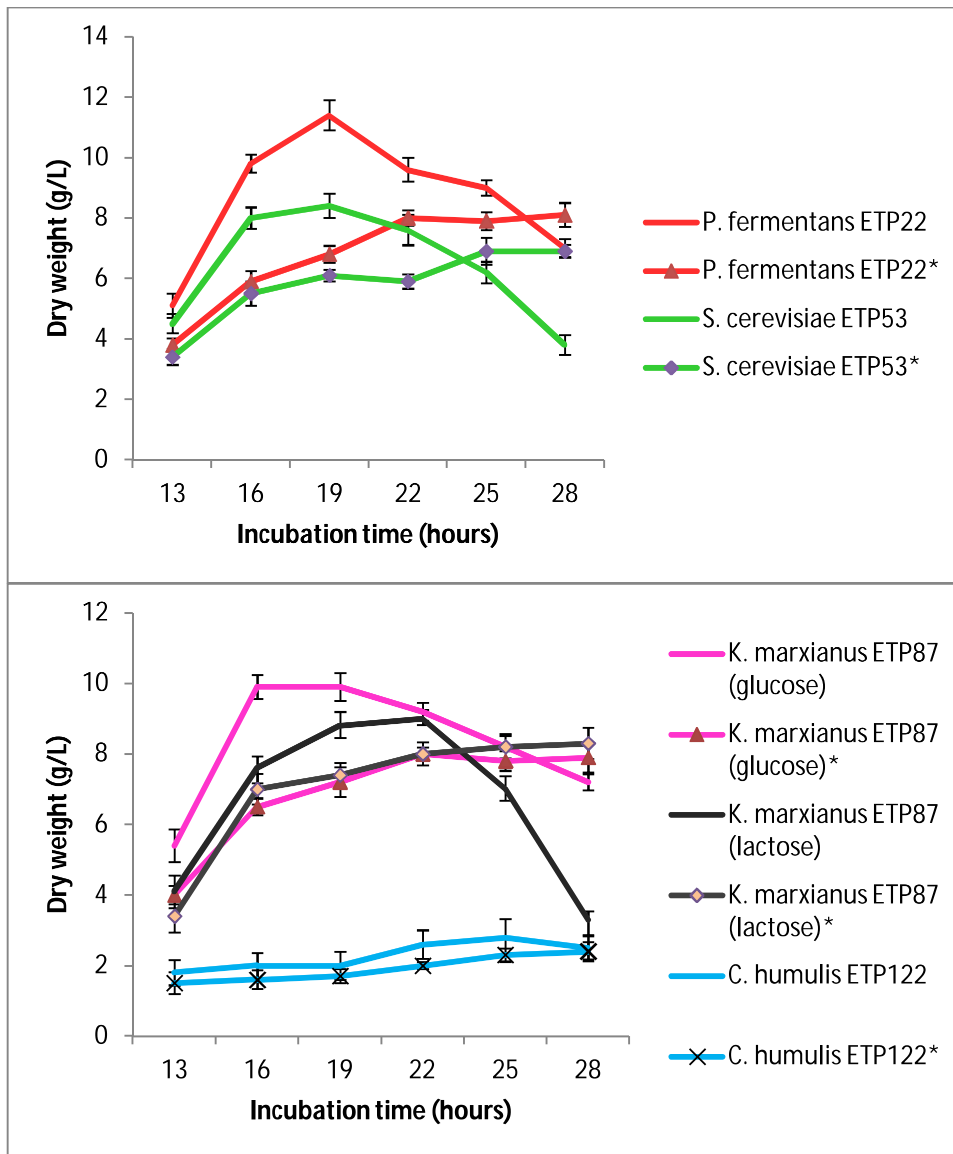

Figure 4-8 Biomass production comparison between shaking and non-shaking (*static condition) on YPD containing 2% glucose (w/v) at 30°C with different incubation time.

The effect of shaking on biomass is displayed in Figure 4-8. All yeasts showed higher biomass under cotton plugged shaking fermentation conditions than closed and static fermentation although the biomass yield in *C. humilis* ETP122 was not as significant as *S. cerevisae* ETP53 and *K. marxianus* ETP87.

The dry biomass of *S. cerevisae* ETP53 and *K. marxianus* ETP87 started to decline around 24 hours when the yeasts were grown under aerobic via shaking conditions. However, the growth of all yeast strains did not start to decline within 28 hours when the static cultures were incubated at 30°C.

4.4. DISCUSSION

4.4.1. Ethanol productivity, yield and efficiency

Ethanol concentration refers to the amount of ethanol (weight mainly gram) per volume (commonly liter) and ethanol productivity shows the ethanol concentration per certain specified period. The amount of ethanol in terms of weight per weight of substrate utilized is termed to be yield and efficiency refers to yield that is changed into percentage. Ethanol concentration, yield and efficiency are correlated. However, ethanol production (concentration), productivity, and yield cannot be used interchangeably to describe ethanol quantification.

Ethanol production is mostly measured in ethanol concentration (g/L); nevertheless yield or efficiency is better if the interest of the researcher is to know how much substrate is converted into ethanol. Ethanol concentration in g/L does not show the effectiveness of the ethanol production process. High g/L does not necessarily mean high yield or efficiency.

4.4.2. Response surface analysis for temperature, pH and time optimization

4.4.2.1. *S. cerevisae* ETP53

Improved ethanol fermentation activity can be achieved by controlling several parameters. pH and temperature are key factor for ethanol production (Lin *et al.*, 2012). The produced ethanol may be utilized by the yeast as a carbon source when sugars are depleted in the media during

decline phase. Therefore, appropriate fermentation time is very important to harvest higher ethanol from yeast fermentation.

The pH affects the transport of molecules including glucose and other sugars and hence it also affects the ethanol production. the *S. cerevisae* ETP53 had wide pH preference range (4.3-5.8) though the optimum was closer to 5.0. Similarly, Ercan *et al.* (2013) reported that optimum ethanol production using *S. cerevisae* was obtained at pH 5.5. Most favorable pH of *S. cerevisae* for ethanol production is ranged from 4.0 to 5.0 (Lin *et al.*, 2012).

Ethanol is a primary metabolites and hence it is mainly produced during log phase. The time of growth curve of the yeast is controlled by the concentration of simple sugars. Therefore, it needs to know the appropriate time for ethanol production depending on the simple sugar amount in the media. The optimum incubation time for ethanol production by the *S. cerevisiae* ETP53 ranged from 48 to 72 hours in the medium containing 40g/L. Then, the ethanol production reduced after 76 hours. Like this study, Lin *et al.* (2012) investigated the fermentation time effect on ethanol production and they found that best yield was attained at 48 to 72 hours; however, it remained almost constant until 144 hours.

The temperature affects the membrane permeability and enzymatic activity in yeast cells. In addition, high temperature reduced the log phase significantly (Lin *et al.*, 2012). In this study, the optimum ethanol was produced at 28-32°C. Similarly, Lin et al., (2012) reported that ethanol concentration was found peak (18.3 g/L) at 30°C within 48 hours, but higher temperature reduced both ethanol production as well as biomass. The temperature range between 30 and 35°C was best for ethanol production by *S. cerevisae* (Park *et al.*, 2013). Costa *et al.* (2014) showed that the fermentation efficiency of *S. cerevisae* at a temperature more than 35°C was significantly low.

4.4.2.2. K. marxianus ETP87

Positive coefficients indicate a linear effect on the responses while negative coefficients reveal the opposite influence (Ruangmee and Sangwichien, 2013). The higher magnitude influences the yield strongly to reduce or maximize it depending on the sign of the coefficients. In a single factor (temperature study alone), *K. marxianus* ETP87 grew up to 50°C with significant reduction in ethanol production after 45°C, and was able to produce higher ethanol at 30-35°C

(Ariyanti *et al.*, 2014) and 40°C (Ferreira *et al.*, 2015). However, highest ethanol concentration was produced by K. marxianus ETP87 at temperature ranged from 32.5 to 40°C while RSM was employed.

K marxianus ETP87 grew on and yielded highest ethanol at pH lower than 5.5. Since the yeast was isolated from acidic *ergo* (pH, 3.7), it could adapt the lower pH. Similarly in other studies, the optimal ethanol was produced by *K. marxianus* at pH 5.05 (Ferreira *et al.*, 2015) and 4.8 (Koushki *et al.*, 2012). In a single factor at a time study, maximum ethanol yield was obtained within 75 hours of incubation using thermotolerant *K marxianus* IMB3 under batch condition (Hack and Marchant, 1998).

4.4.2.3. P. fermentans ETP22

High ethanol concentration (10.86 g/L) was produced by *Pichia fermentans* ETP22 at 30.39^{o}C, pH 5.13, and 59.38 hours with unreduced model. In a single factor study by other researcher, *P. fermentans* which was isolated from marine environment was able to produce 13 g/L ethanol at pH 6.5 and temperature of 30°C (Senthilraja *et al.*, 2011). Nakayama *et al.* (2008) reported a production of 55 g/L ethanol by *P. fermentans* NBRC 1164 using medium that contained high glucose (150g/L) and corn steep liquor under aerobic condition and at 30°C within 24 hours. Therefore, the same yeast species may need different pH requirement if they are isolated from environments which vary strongly in pH.

4.4.3. Effect of glucose concentration

The biofuel industries need the technology that enables the production of high ethanol titer to compute fossil fuel. These days great attention is given to very high gravity (VHG) fermentation technology because VHG increases ethanol titer, saves energy consumption for ethanol distillation, and reduces waste distillage (Liu *et al.*, 2012). Yeast osmo-tolerance at the beginning of fermentation and ethanol-tolerance in late fermentation is the pre-requisite for VHG since VHG fermentation produces high ethanol titer from high sugar concentration commonly greater than 200 g/L. For this reason, the effects of glucose concentration on yeast isolates were investigated to evaluate their potential for VHG ethanol fermentation.

The optimum dextrose concentration of *P. fermentans* ETP22, *K. marxianus* ETP87 and *S. cerevisae* ETP53 was 60, 80, and 100 g/L glucose, respectively and hence the newly isolated yeasts are less osmotolerant than yeasts studied by others. For example Hoondee *et al.* (2016) found that optimal ethanol was found to be 280 g/L sugar; increasing the glucose concentration above 280 (g/L) led to a significant and corresponding increase in the residual glucose levels and to a decrease in ethanol titer, with essentially the same net amount of glucose utilization. Therefore, the efficiency or the yield of ethanol production decreases as the sugar concentration increases after certain points. Unlike Hoondee *et al*. (2016) and like this study, the growth of *S. cerevisiae* and *K. marxianus* were optimal and constant at 8% w/v glucose at 30 -37°C and 30-42°C respectively (Costa *et al.*, 2014).

4.4.4. Yeasts' inoculum size

The lower inoculum size reduces the cost of production in ethanol fermentation; therefore, yeast that produce ethanol at lower inoculum size are needed for industrial ethanol production. High ethanol concentration (14.9-17.6 g/L) was produced by the three yeasts when YPD was inoculated with $2.25\text{-}5.5\times 10^6$ yeast cells. Five percent (v/v) and 12 hrs old inoculum size yielded almost the same result with 10% *S. cerevisiae* Y5 in enzymatic hydrolysate of non-detoxified steam-exploded corn stover supplemented with CSL (Yun *et al.*, 2011). The higher yeast load shortens the lag phase (Park *et al.*, 2013) and hence the appropriate time should be investigated when high yeast load was added for ethanol production. Therefore, the low ethanol yield at large inoculum size might be due to inappropriate time of harvest.

4.4.5. Sugar utilization during ethanol fermentation

The trend of sugar utilization by *S. cerevisiae* ETP53 and *K. marxianus* ETP87 was similar and higher than *P. fermentans* ETP22. *S. cerevisiae* ETP53, *K. marxianus* ETP87, and *P. fermentans* ETP22 were able to utilize 88, 89 and 67% of the dextrose within 10 hours. The *K. marxianus* UFV-3 took 8 hours to consume 100% of glucose present in yeast nitrogen base media that contain a mixture of glucose (20 g/L) and xylose (20 g/L) (dos Santos *et al.*, 2013). The fast utilization of glucose in *S. cerevisae* and *K. marxianus* was directly proportional to ethanol yield in this study as well as elsewhere (dos Santos *et al.* (2013). However, the ethanol production remained minimal (2 g/L) for *P. fermentans* ETP22 although 67% of glucose was utilized within 8 hours.

4.4.6. Bioethanol versus Biomass Production

The least ethanol producer yeast (*P. fermentans* ETP22) yielded high dry yeast biomass than the *S. cerevisiae* ETP53 and *K. marxianus* ETP87. Though both *S. cerevisae* ETP53 and *K. marxianus* ETP87 were efficient in ethanol production, isolate ETP87 produced more biomass than *S. cerevisae* ETP53 indicating the high growth rate of *K. marxianus* than that of *S. cerevisiae* (Costa *et al.*, 2014).

The fermentation conditions for ethanol and biomass production differ regarding oxygen saturation; biomass production requires more oxygen than ethanol production. Alcoholic fermentation condition decreases the biomass yield since biomass is directly proportional to ATP yield (Vieira *et al.*, 2013) and alcoholic fermentation condition is anaerobic that tends to reduce ATP yield. However, *S. cerevisae* produce ethanol even in full aerobic conditions due to Crabtree effect (Vieira *et al.*, 2013) leading to low biomass yield. This might be one reason for low biomass production from *S. cerevisae* ETP53 under all conditions since this yeast is the best ethanol producer of all yeasts under study.

Therefore, the industry needs yeast that produce economical biomass under ethanol fermentation condition since this makes the production of ethanol and biomass feasible in one fermenting system. *K. marxianus* ETP87 is a good candidate for such operation. Ariyanti *et al.* (2014) produced 8-12 g/L dry biomass from whey under fed-batch fermentation using *K. marxianus*.

4.4.7. Aerated shaking versus closed non-shaking fermenting on biomass

The dry biomass yeast has been increasing until 24 hours and then it declined. The dry biomass of aerated experimental condition was found to be higher than non-shaked experimental condition. Constant aeration triggered a quick biomass formation and fast glucose utilization with short log and stationary phase within 28 hours (Liu *et al.*, 2016). The availability of oxygen is an important environmental factor on glucose fermentation by *K. marxianus* NRRLY-6860, determining the carbon flux for ethanol or biomass production (Cunha *et al.*, 2017). However, the higher the agitation, the higher likelihood to shift to biomass production rather than ethanol production.

K. marxianus ETP87 grew actively in glucose rather than lactose at both shaking and static conditions. The low biomass in lactose might be due the energy requirement for production of β–galactosidase and lactose catalysis and glucose preference in the metabolism. Rocha *et al.* (2011) showed that *K. marxianus* strains CBS 6556, CBS 397 and CBS 712 preferred glucose, galactose, and sucrose to lactose in defined media.

4.5. CONCLUSION AND RECOMMENDATIONS

K. marxianus ETP87 is a good candidate to produce ethanol from acidic and warm substrates since it was able to grow and produce ethanol at pH lower than 5 and warm temperature (optimal temperature 30-40°C). It is possible to predict the possible concentration of ethanol produced by *K. marxianus* ETP87, *S. cerevisiae* ETP53, and *P. fermentans* ETP22 if the incubation time, pH, and temperature is given at 4% glucose. These three yeasts are less important for high gravity ethanol production since they produced optimal ethanol from less than 100g/L dextrose concentration. In ethanol producing yeast, yeast utilization does not necessarily correspond with ethanol concentration produced from the utilized sugars. Biomass and bioethanol productions need different oxygen concentration for the same yeast; it is, therefore, difficult to produce ethanol and biomass optimally from one pot fermentation system.

The optimization, in this study, was performed using artificial defined media, YPDA. It is more appropriate if it is going to be done in the actual media in which ethanol is produced in industry. In addition, including effect sugar concentrations to the RSM model is highly recommendable. The sugar utilization was done only for 2% (w/v) dextrose. Therefore, it is recommended to carry out sugar utilization at higher dextrose concentration.

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Chapter 5

Bioethanol Production from Grass Pea and Wild Oat Hydrolysates

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Abstract

Conversion of lignocellulosic biomass into bioethanol is essential to reduce dependency on fossil fuels. After the grass pea and wild oat straws were characterized, they were hydrolyzed by live fungi (in situ), crude fungal enzymes, and chemical methods (sodium hydroxide and sulfuric acids at 0.5, 1, 2, and 3%). The fungi used for in situ degradation and crude lignocellulosic enzymes were Aspergillus niger *JMC22344,* Trichoderma reesei *JMC22,* Pleorotus ostreatus *M2191, and* Pleorotus sajor-caju *M2145. Furfural content of acid and alkali hydrolysates were analyzed by GC-MS. The potential of activated charcoal, overliming, and sequential activated charcoal-overliming potential to reduce furfural in the acid hydrolysates were evaluated. The hydrolysates which were supplemented with yeast extract (0.1g/L) and peptone (0.5g/L) were fermented by* S. cerevisae *ETP53,* K. marxianus *ETP87, and* P. fermentans *ETP22. The highest reducing sugar was found in 1% (v/v)* H_2SO_4 *and 1% (w/v) NaOH hydrolyzed straws; sulfuric acid yielded higher sugars than NaOH. In both acid and alkali hydrolysis, wild oat was superior interns of sugars released.* P. ostreatus *M2191 liberated the highest sugar extracts than* T. reesei *JMC22676,* A. niger *ETP22344 and* P. sajor-caju *M2145 while straws were degraded by in situ degradation mechanism. Crude enzymatic hydrolysis of straws resulted in a lower sugar than either acid and alkali hydrolysis or in situ degradation. Overliming, activated charcoal, and the sequential activated charcoal-overliming reduced significantly the furfural in acid hydrolysates. Yeasts produced optimal ethanol from straws hydrolyzed by crude enzymes from* A. niger *JMC22344. However, the maximum ethanol concentration was produced by the yeast fermentation of sugars released by* P. ostreatus *M219 from straws. Generally, the ethanol titer was directly proportional to the amount of sugars released except acid and alkali hydrolysis.*

Key words: Bioethanol, cellulase, simultaneous saccharification and fermentation, separate saccharification and fermentation, lignocellulose

5.1. INTRODUCTION

The ever-increasing demand for energy and the problem of environmental pollution necessitate the search for renewable energy from various alternatives (Singhania *et al.*, 2014). The biofuels obtained from agricultural residues, industrial wastes, and other lignocellulose sources are alternative cheap and sustainable sources of energy supply.

Agricultural residues are important sources of biomass for ethanol production in Africa and the estimated total ethanol from agricultural residues and municipal solid waste (excluding paper) is 20.2 and 1.8 Billion liters per year respectively (Bensah *et al.*, 2015). The highest available biomass was estimated to be 136 million tonnes per year in Western Africa followed by 123 million tonnes per year in Eastern Africa (Bensah *et al.*, 2015). According to a World Bank report in 2012, Ethiopia has the potential to generate up to 14 million litter of ethanol that can be produced from its lignocellulosic agricultural and municipal residues.

Lignocellulosic materials are rigid structures consisting of mainly cellulose and hemicelluloses which are cross-linked and strongly bound to lignin (Pu *et al.*, 2013). This structural complexity make them recalcitrant and they must be saccharifed physically, chemically or/and biologically in order to release simple sugars to enhance ethanol fermentation. The biological saccharification includes enzymatic treatment and in situ degradation by cellulosic microorganisms where as the chemical methods include acid, alkali, salt, solvent, ozone, and hydrogen peroxide treatment of which acid and alkali treatments are relatively cheap and efficient in hydrolyzing lignocelluloses (Bensah and Mensah, 2013).

The degradation of lignocellulosic residues release phenolic compounds and furan derivatives as by products that are inhibitors of microbial growth and ethanol fermentation (Kim *et al.*, 2013; Jung *et al.*, 2013). Therefore, the detoxification of the lignocellulosic hydrolysates using physical, chemical and biological methods is important for successful hydrolysate fermentation process (Huang *et al.*, 2009).

A variety of methods are developed to remove the inhibitory substances of which the most inexpensive ones are over-liming (De Bari *et al.*, 2014; Prasertwasu *et al.*, 2014) and activated charcoal treatment (Jung *et al.*, 2013; Kim *et al.*, 2013),

Apart from physical and chemical treatments, co-culturing and enzyme treatment of hydrolysates can enhance ethanol production. Accordingly, Jang *et al.*, (2015) cocultured amylase producing fungus *Rhizopus oryzae* and *S. cerevisae* in the presence of 5mM NaCl to produce ethanol and coculture produced the highest amount of ethanol compared to monoculture control. Tantipaibulvut *et al.* (2015) produced economically viable ethanol for fuel by direct fermentation of starch (from desizing waste) by *Bacillus subtilis* and *S. cerevisiae*. A coculture of *Saccharomyces diastaticus* (amylolytic yeasts) and *S. cerevisiae* 21 produced higher (48%) ethanol than the yield obtained with the monoculture (Verma *et al.*, 2000).

Effective coculturing of *Aspergillus niger* and *K. marxianus* resulted in the maximum ethanol yield of 23.10 g/L (Manikandan and Viruthagiri, 2009). In some studies, separate reactors for amylolytic (*A. niger*) and ethanolegenic (*S. cerevisae*) microorganisms were designed in one pool reactor system to solve different growing requirements for the two organisms (Jeon *et al.*, 2007).

All taken together, in order to produce ethanol from lignocellulosic residues, there is a need to select yeasts that can ferment both hexose and pentose sugars, tolerate inhibitors in the hydrolysate and give high ethanol yield (Chandel *et al.*, 2013).

The aim of this study is to evaluate the potential of using *S. cerevisae* ETP53, *K. marxianus* ETP87, and *P. fermentans* ETP22 to ferment lime, activated carbon and cellulase enzyme treated acid/alkali hydrolysates of grass pea and wild oat to ethanol production.

5.2. MATERIALS AND METHODS

5.2.1. Sources and characterization of lignocelluloses

Two lignocellulosic substrates (wild oat and grass pea) were collected from different sources. Wild oat, also called common wild oat, (*Avena fatua* L.) straw was obtained from North Shoa Agricultural Research Center, Amhara Regional State and grass pea (*Lathyrus sativus* L.) straw was collected from Enemay Woreda, East Gojjam, Amhara Regional State.

Acid soluble lignin, alkali soluble lignin, moisture content and total solid of wild oat and grass pea were characterized using the National Renewable Energy Laboratory (NREL) method (Scarlata *et al.*, 2011).

Table 5.1 Wild oat and grass pea straws characterization

Variable	Wild oat	Grass pea
Total solid (%)	87.77±0.96	86.89±0.72
Moisture content (%)	12.23±0.96	13.11±0.72
Acid (1% v/v H_2SO_4) soluble lignin (%)	3.87±0.49	5.38±0.41
Alkali (1% w/v NaOH) soluble lignin (%)	4.99±0.35	7.17±0.37
Protein (%)	0.91±0.075	3.74±0.062

5.2.2. Sources of microorganisms

P. fermentans ETP22, *S. cerevisiae* ETP53, and *K. marxianus* ETP87 which were isolated from different sources were used for ethanol production. *Pleurotus ostreatus* M2191 and *Pleurotus sajor-caju* M2345 were obtained from MYCELIA, Belgium where as *Aspergillus niger* JCM22344 and *Trichoderma reesei* JCM22676 were obtained from Japan Collection of Microorganisms, Japan. were used as source for cellulase enzyme .

5.2.3. Treatment of straws

Wild oat (3.13g) and 3.11g grass pea straws were transferred to screwed 250mL bottles containing100mL of 0.5%, 1% and 3% sulfuric acid or sodium hydroxide (Jung *et al.*, 2013). The screw was closed very well but not tightly. The biomass was autoclaved at 121°C for 90 minutes. After cooling, the pretreated biomass was filtered with filter paper to analyze the liquid portion for total reducing sugars and acid soluble lignin using spectrophotometer whereas glucose, cellobiose, xylose, arabinose, and galactose were analyzed by high pressure liquid chorography (HPLC). The furfural was quantified by GC-MS. The oven dried solid portion of

previously treated straws was retreated with the same condition to evaluate for further possible saccharification.

5.2.4. Crude protein of grass pea and wild oat

Pre-dried grass pea (1g) and wild oat (1g) were put in 500mL Kjeldahl digestion flask. A mixture of copper sulfate and potassium sulphate (10g) and 25mL of 0.1N sulfuric acid was added. The flask was placed in inclined position and heated gently. It was boiled for 30 minutes after the solution cleared. After cooling for 15 minutes, 250mL of distilled water and then 90mL of 40% NaOH solution were added. The distillation apparatus was connected with loops and 300mL Erlenmeyer flask containing 30mL boric acid indicator solution (methyl red plus methyl blue). Then it was distilled and titrated with 0.1N HCl until colorless endpoint was reached.

$$\%\mathbf{N} = \frac{\mathbf{1.4 \times (ml\ sample\ titrant - ml\ blank\ titrant) \times N\ of\ Sulferic\ acid}}{\mathbf{weight\ of\ sample}} \qquad \text{Equation 5.1}$$

$$\%\mathbf{CP} = \mathbf{N} \times \mathbf{8.25} \qquad \text{Equation 5.2}$$

5.2.5. Furfural reduction in acid hydrolysates

The detoxification of acid hydrolysate was run by employing the method of Kuhad *et al.* (2010) . First, the acid hydrolysates (0.5 and 1% H_2SO_4) of grass pea and wild oat were overlimed by mixing with calcium hydroxide until pH reached 10 (Kuhad *et al.*, 2010). Second, activated charcoal (1.5%, w/v) was added to acid hydrolysate according to Kuhad *et al.* (2010). The two mixtures were stirred with magnetic stirrer at higher speed for 30 minutes and 60 minutes to see also the effects of retention time on inhibitor reduction. Third, the overlimed hydrolysate was further mixed with activated charcoal (1.5%, w/v) and continuously stirred to investigate the synergetic effect of overliming and activated charcoal. Then, overlimed, activated charcoal, and overlimed-activated charcoal treated acid hydrolysates were centrifuged at 8,000 revolution per minutes (eppendorf, Centrifuge 5418 R) to separate the precipitated calcium sulphate and activated charcoal from hydrolysates. The furfural was measured before and after treatment using GC-MS. The percentage of furfural reduction due to overliming and adsorbing with activated charcoal was calculated as

$$\text{FR\%} = [1 - \frac{\text{FAT}}{\text{FBT}}] \times 100\% \qquad \text{Equation 5.3}$$

Where

FR = Percentage of furfural reduction

FAT = Furfural (g/L) after treatment with overliming and activated charcoal

FBT = Furfural (g/L) before treatment with overliming and activated charcoal

Then, yeasts extract (0.1g/L) and peptone (0.5g/L), which were separately sterilized, was added and yeasts were inoculated and incubated.

5.2.6. Solid state fermentation (SSF) of cellulase producing fungi

Grass pea and wild oat straws were used as a substrate for solid state fermentation of *P. ostreatus* M2191, *P. sajor-caju* M2345, *A. niger* JCM22344 and *T. reesei* JCM22676. The fungi were grown for 8 days on PDA agar from which 1mm×mm cube was transferred into 250-mL Erlenmeyer flasks containing the medium and 10g of treated straw (at 3% (w/v) loading rate with 1% sulfuric acid for 1 hour at 121°C) moistened with 15mL of modified mineral salt solution in SSF (Trivedi *et al.*, 2015). The modified mineral salt solution contained (g/L): $(NH_4)_2SO_4$, 1.86; KH_2PO_4, 2.0; urea, 0.3; $CaCl_2$, 0.03; $MgSO_4·7H_2O$, 0.3; yeast extract, 2.0; $FeSO_4·7H_2O$, 0.005; $MnSO_4·H_2O$, 0.0016; $ZnSO_4·7H_2O$, 0.0014; $CoCl_2$, 0.002; (w/v) and peptone, 3.0. Flasks with untreated grass pea and wild oat straws were used as control. They were incubated at 25°C for 8 days. After 8 days growth, 100mL distilled water was added and shaken over night at 100 rpm and room temperature to extract the sugar and enzymes. The solid particles were filtered first with cheese cloth and then by suction through Whatman No. 1 filter paper. The reducing sugars released to the substrates were quantified by DNS (Miller, 1959).

5.2.7. Crude cellulase enzyme preparation and hydrolysis

One mL filtrate from above was centrifuged at 13000×g for 10 minutes to remove cell debris and fungal spores and 3mL cold acetone which was put at -18°C over night was added to supernatant in acetone compatible tube. After vortexing, the acetone-supernatant mixture was incubated at -18°C for 2 hours and centrifuged at 13000×g (eppendorf, Centrifuge 5418 R) for 10 minutes to separate crude enzymes from the mixture. The supernatant was carefully decanted. Finally, 0.5mL acetate buffer at pH 5.0 was added to dissolve the enzyme pellet. Crude enzymes obtained from 10 mL of each culture filtrate was used to hydrolyze 1% (v/v) sulfuric acid

pretreated straws (6% w/v on dry weight bases). As a positive control and comparison, carboxymethylcellulose (CMC) was hydrolyzed by crude enzymes from *A. niger* JMC22344. The mixture was incubated at 50°C for 12 hours. The sugar released was quantified by DNS (Miller, 1959). The absorbance was measured at 540 nm (JENWAY 6405 UV/Vis).

5.2.8. Yeast fermentation of acid or alkali hydrolysate

Bioethanol production from straw hydrolysate was carried out using three local yeast isolates; *P. fermentans* ETP22, *S. cerevisiae* ETP53, and *K. marxianus* ETP87 isolated from compost, tella, and traditional yoghurt, respectively. The procedure was according to Jung *et al.* (2013) with a little modification. The pH of acid and alkali treated hydrolysate were 0.9 and 13.2 respectively. The 0.9 pH was adjusted near to 5 using first solid sodium hydroxide and then 3N sodium hydroxide drop wise. Similarly, 13.2 pH was adjusted to 5.5 using concentrated hydrochloric acid (45%) then to 5.0 by adding 3N HCl drop wise. The solid NaOH and concentrated HCl were employed to avoid sugar dilution.

The hydrolysates were centrifuged at 10000×g for 15 minutes and the filtrates were separated from solid particles by decanting. All these were done aseptically to prevent contaminations. Then 50mL of the hydrolysates were supplemented with sterilized peptone (0.5g/L) and yeast extract (0.1g/L) and transferred into 250-mL flask, and inoculated with (approximately 7.5×10^6) *P. fermentans* ETP22, *S. cerevisiae* ETP53, and *K. marxianus* ETP87. They were incubated in shaker incubator at 30°C and 130 rpm. Samples were taken at 8 and 24 hours for yeast growth, sugar consumption, furfural content, butanol, isoamyl alcohol and ethanol analysis (Jung *et al.*, 2013).

5.2.9. Ethanol fermentation of enzymatically degraded straws

The hydrolysates from enzymatic hydrolysis were boiled to reduce the volume by half and increase the sugar concentration 2 times. The hydrolysate (100 mL) was transferred to 250 Erlenmeyer flask and supplemented with yeast extract 1% and peptone 2% for ethanol production. Then it was inoculated with 7.5×10^6 cells of each yeast and incubated at 30°C for 24 hours.

5.2.10. Analytical Methods

5.2.10.1. High Pressure Liquid Chromatography

The analysis of sugar contents of hydrolysates (such as cellobiose, glucose, galactose, xylose, and arabinose) was performed by high pressure liquid chromatography (HPLC) (Agilent 1100Series).The hydrolysates were centrifuged at 14500×g (eppendorf, Centrifuge 5418 R) two times for 10 minutes to remove any unsolublized materials or debris. The HPLC (Agilent 1100Series) was equipped with refractive index (RI) detector (G1362A) and quaternary pump (G1311A). MetaCarb Pb Plus (300 mm x 7.8mm, Agilent A5241) column was operated at 80°C and the flow rate was 0.1mL/min. Injection volume was adjusted at 20µL. The mobile phase consisted of 100% HPLC grade water.

5.2.10.2. Ethanol and furfural determination using gas chromatography

In order to determine the ethanol and furfural, the fermented products and hydrolysates were centrifuged at 14000×g for 5 minutes. The supernatant was analyzed by Gas Chromatography (Agilent 6890N) coupled with a mass spectrometer (MS) with headspace autosampler (Agilent7694E). The GC-MS was equipped with Mass Selective Detector (Agilent5973Network) and a polar polyethylene glycol (PEG) phase DB-wax122-7032 capillary column. Helium was used as a carrier gas. The flow rate for the column was 1mL/min. The column temperature was held at 40°C for 4 min, and then raised to 240°C at 40°C/min; the inlet temperature was 220°C.The GC was operated with 20:1 split injection of the headspace. The mass-to-charge ratios (m/z) for ethanol was 31-45 m/z range with the retention time of 3.36 min and for furfural 32-95 m/z range with the retention time of 7.64 min. The MS Quadrupole, MS source and transfer line temperature were 150°C, 230°C, and 250°C respectively. The conditions of the headspace autosampler were 25min for the GC cycle time, 10 min for the vial equilibration time, 0.5min for the pressurization time, 1min for the injection time and a constant vial pressure of 14.0 psi. The temperatures at 110°C for the transfer line to the column and at 90°C for the loop. The equilibration temperature was 80°C for 10 min. Fermentation sample (200µL) were put in 10mL headspace vials.

5.2.10.3. Biomass determination

The biomass was determined as dry weight (g/L) using standard curve (see appendix)

5.3. RESULTS

5.3.1. Straw hydrolysis by dilute acid and alkali

The amount of reducing sugars released from acid-and alkali-treated lignocellulosic residues is shown in Table 5.2. The sugar yield of acid treated was between 5.3-8.9 g/L and 7.3-13.9 g/L from wild oat and grass pea, compared with 3.4-4.2 g/L and 6.5-8.g/L obtained from the same

Table 5.2 Reducing sugars released by dilute acid and alkali hydrolysis at 121^oC for 90 minutes

Straw	Hydrolyzing chemical	Treating chemical concentration (%)	Reducing sugars (g/L)
Common wild oat (*Avena fatua* L.)	H_2SO_4	0.5	8.36
		1	8.91
		2	5.32
		3	0.42
	NaOH	0.5	3.42
		1	3.88
		2	4.26
		3	1.53
Grass pea (*Lathyrus sativus* L.)	H_2SO_4	0.5	13.03
		1	13.9
		2	7.48
		3	0.73
	NaOH	0.5	6.45
		1	7.64
		2	8..19
		3	1.42

alkali treated substrates, indicating that the sugar released from acid hydrolysis was 1.5 times higher than alkali hydrolysis. The sugar contents were drastically decreased using more than 2% concentrations of acid/alkali for the treatment.

5.3.2. Hydrolysate sugar composition

The composition of the different types of sugars in the acid and alkali treated hydrolysates is shown in Table 5.3. In all cases, the concentration of xylose was higher than glucose, except

from 1% NaOH hydrolyzed grass pea and this suggests that the acid treatment might attack the hemicellulose portion of lignocellulose.

Table 5.3 Chemical components of acid and alkali hydrolysates (g/L)

Substrate	Treatment	Cellobiose	Glucose	Xylose	Arabinose	Galactose
wild oat	1% H_2SO_4	1	1.48	2.68	1.16	0.26
wild oat	1% NaOH	0.28	0.46	0.84	0.9	0.16
grass pea	1% H_2SO_4	0.98	2.24	5.34	1.44	0.32
grass pea	1% NaOH	0.2	1.22	0.24	0.38	0.22

5.3.3. Ethanol production from acid and alkali hydrolysates

It is interesting to note that, although the acid-treated hydrolysates contained higher sugar contents than the alkali-treated ones, more ethanol was produced from the alkali treated ones (Table 4.4). Accordingly, the ethanol content from alkali-treated wild oat and grass pea hydrolysates was in the range of 0.8-4.6gL and 0.3-3.3g/L, respectively. The higher ethanol was produced by *S. cerevisae* ETP53 (4.6 g/L ethanol) (8 hrs) and *P. fermentans* ETP22 (4.2 g/L ethanol (24hrs) and 3.3g/L (8 hrs). The lowest ethanol (1.8g/L) was detected from *K. marxianus* ETP87 (24hrs). *P. fermentans* ETP22 was able to grow on xylose, the dominant sugar in hydrolysates and it might use xylose for growth and leave the glucose for more ethanol production.

Table 5.4 Ethanol production (g/L) from alkali and acid hydrolysate incubated at 30°C in bath shaker

		***K. marxianus* ETP87**		***S. cerevisae* ETP53**		***P. fermentans* ETP22**	
Straw	**Treatment**	**8hrs**	**24 hrs**	**8hrs**	**24 hrs**	**8hrs**	**24 hrs**
wild oat	H_2SO_4 (1%)	0.8*	0.2*	1.7	0.4	0.8	0.2
wild oat	NaOH (2%)	1.3	1.4	4.6	0.8	1.3	4.2
grass pea	H_2SO_4 (1%)	0.4	0.6*	0.9	0.4	0.4	0.5
grass pea	NaOH (2%)	1.1	1.8	1.4	0.3	3.3	0.7

*Isoamyl alcohol was detected even if its amount was very small

In general, the yeast species showed variations in ethanol production upon different incubation periods. Thus *S. cerevisae* ETP53 attained the highest concentration of ethanol(4.6g/L) in 8 hrs; whereas *P. fermentans* ETP22 achieved optimum production of 4.2 g/L and 3.3 g/L in 24 hrs and 8 hrs on alkali treated hydrolysates. On the contrary, *K. marxianus* ETP87 did not show significant pattern in ethanol production at different incubation times.

In this study, however, *K. marxianus* ETP87 was able to grow and ferment sugars both in acid and alkali hydrolysates but ethanol amount was lower than *S. cerevisae* ETP53 (Table 5.4). Isoamyl alcohol was produced by only *K. marxianus* ETP87 that grew in acid treated wild oat and grass pea straws.

5.3.4. Detoxification of hydrolysates

The effect of treatment with liming and active carbon and their combination on the possible removal of the inhibitory by products (furfurals) was tested on the acid treated hydrolysates (Table 5.5). The data showed that the combination of overliming and activated charcoal removed 77% of the furfurals from wild oat and 70 % from grass pea, followed by treatment with overliming with 53% removal efficiency of furfural (wild oat) and 40% (grass pea) in almost 30 minutes. However, treatment of the hydrolysates with activated carbon removed furfural by 35% and 43% from grass pea and 23% and 30% from wild oat upon 30 and 60 minutes, respectively, indicating that raising the adsorption time from 30 to 60 minutes slightly, but not significantly, decreased the furfural removed by activated charcoal and overliming but raising time had no effect on the synergetic application of overliming and activated charcoal (Table 5.5).

The potential of the yeasts to produce ethanol from detoxified and non-detoxified hydrolysates is shown on table 4-5. The three yeasts produced higher ethanol in detoxified hydrolysates than non-detoxified counter parts. The yeast *S. cerevisae* ETP53 and *K. marxianus* ETP87 produced more ethanol from wild oat hydrolysate than the grass pea. On the contrary, *P. fermentans* ETP22 produced relatively high ethanol on grass pea hydrolysate than wild oat. Likewise, *S. cerevisae* ETP53 produced the highest ethanol (2.86 g/L) in wild oat hydrolysate overlimed

Table 5.5 Ethanol production before and after hydrolysate detoxification by activated charcoal and overliming (Incubation time was 24 hours)

substrate	**H_2SO_4**	**Detoxification method**	**Furfural reduction**		***K. marxianus* ETP87 (g/L)**		***S. cerevisae* ETP53 (g/L)**		***P. fermentans* ETP22 (g/L)**	
			30 min	**60 min**	**Before**	**After***	**Before**	**After***	**Before**	**After***
Grass pea	0.5%	Overliming	52.94%	53.22%	0.91	1.46	1.11	1.69	0.84	0.92
Grass pea	0.5%	Activated carbon	35.29%	42.67%	0.91	1.23	1.11	1.41	0.84	0.91
Grass pea	0.5%	Overliming-activated charcoal	70.59%	68.62%	0.91	1.57	1.11	1.73	0.84	1.22
Wild oat	1%	Overliming	40.00%	38.89%	0.53	1.12	1.67	2.86	0.46	0.86
Wild oat	1%	Activated carbon	23.08%	31.53%	0.53	1.08	1.67	2.52	0.46	0.51
Wild oat	1%	Overliming-activated charcoal	76.92%	77.45%	0.53	1.18	1.67	2.73	0.46	0.83

*After 30 minutes detoxification time

with $Ca(OH)_2$ whereas *P. fermentans* ETP22 produced the lowest (0.51 g/L) ethanol in wild oat hydrolysates treated with activated charcoal (Table 5.5).

5.3.5. Ethanol production by employing sugars extracted from solid state grown fungi

The effect of enzymatically treated hydrolysates from *T. reesei* JMC22676, *A. niger* JMC22344, *P. ostreatus* M2191, and *P. sajor-caju* M2345 in wild oat and grass pea straw is indicated in Table 5.6.

Table 5.6 Ethanol production from sugars released by fungi in solid state fermentation of treated and untreated straws.

Straws	Acid treatment condition	Cellulose degrading fungi	Reducing Sugar (g/L)	Ethanol (g/L) produced by *K. marxianus* ETP87	*S. cerevisae* ETP53	*P. fermentans* ETP22
wild oat	treated	*T. reesei* JMC22676	16.14±0.34b	6.32±0.20c	6.05±0.13b	5.23±0.08d
wild oat	untreated	*T. reesei* JMC22676	14.25±0.26d	4.79±0.17e	5.20±0.04c	3.50±0.06g
wild oat	treated	*A. niger* JMC22344	15.37±0.25c	5.17±0.21d	4.69±0.15d	4.75±0.05e
wild oat	untreated	*A. niger* JMC22344	14.62±0.36d	3.49±0.36f	3.32±0.18e	2.69±0.02i
wild oat	treated	*P. ostreatus* M2191	15.34±0.14c	6.47±0.35c	6.16±0.05b	5.71±0.05c
wild oat	untreated	*P. ostreatus* M2191	18.63±0.49a	8.25±0.25a	7.76±0.15a	6.95±0.06a
wild oat	treated	*P. sajor-caju* M2345	12.72±0.40e	6.20±0.20c	6.11±0.02b	5.29±0.05d
wild oat	untreated	*P. sajor-caju* M2345	16.35±0.50b	7.45±0.35b	7.45±0.15a	6.79±0.13a
Average			15.4	6.01	5.84	5.11
grass pea	treated	*T. reesei* JMC22676	9.38±0.22g	3.58±0.35f	3.30±0.17e	3.56±0.06g
grass pea	untreated	*T. reesei* JMC22676	8.77±0.35h	2.85±0.17g	2.40±0.10f	2.08±0.07j
grass pea	treated	*A. niger* JMC22344	8.79±0.12h	2.62±0.20g	2.54±0.05f	1.86±0.03k
grass pea	untreated	*A. niger* JMC22344	8.56±0.28h	2.00±0.20g	1.86±0.04g	1.42±0.08l
grass pea	treated	*P. ostreatus* M2191	8.69±0.17h	2.34±0.05g	2.36±0.05f	1.78±0.03k
grass pea	untreated	*P. ostreatus* M2191	10.41±0.34f	3.53±0.08f	3.43±0.05e	2.45±0.11j
grass pea	treated	*P. sajor-caju* M2345	7.25±0.26i	2.14±0.15g	1.91±0.11g	1.28±0.01l
grass pea	untreated	*P. sajor-caju* M2345	9.39±0.25g	3.40±0.10f	2.97±0.15f	2.86±0.06l
Average			8.9	2.81	2.6	2.16

The data indicated that the amount of sugar produced in the hydrolysates of the two treated and untreated substrates was in the range of 7.25g/L to 18.63g/L. The highest reducing sugar was derived from oat straw ranging from 12.72mg/L to 18.63mg/L (average 15.42) compared to the 7.25-10.41mg/L (average = 8.91) released from grass pea straw indicating twice as much as reducing sugars released from oat. The quantity of reducing sugar showed a pattern in that untreated substrates contained more sugars in their hydrolysates than the treated ones. The lower sugar in treated straw might be associated with loss of sugars by treatment before the fungus was inoculated. The hydrolysate derived from *P. ostreatus* on oat straw was the highest (18.63) followed by *T. reesei* and *P. sajor-caju.*

Table 5.7 Ethanol production from acid pre-treated straws hydrolyzed by fungi crude enzymes

straw	**Crude cellulase source**	**Reducing sugars**	***K. marxianus* ETP87**	***S. cerevisae* ETP53**	***P. fermentans* ETP22**
Wild oat	*T. reesei* JMC22676	3.51±0.29	1.21±0.01	1.49±0.07	1.38±0.02
Wild oat	*A. niger* JMC22344	4.42±0.19	1.66±0.04	1.84±0.06	1.51±0.04
Wild oat	*P. ostreatus* M2191	2.70±0.10	0.48±0.02	0.50±0.05	0.48±0.10
Wild oat	*P. sajor-caju* M2345	2.39±0.29	0.49±0.03	0.45±0.02	0.40±0.04
Grass pea	*T. reesei* JMC22676	2.53±0.25	0.69±0.02	0.74±0.05	0.52±0.03
Grass pea	*A. niger* JMC22344	2.87±0.22	0.97±0.03	0.94±0.05	0.46±0.03
Grass pea	*P. ostreatus* M2191	2.04±0.19	0.43±0.03	0.51±0.03	0.48±0.03
Grass pea	*P. sajor-caju* M2345	2.04±0.31	0.29±0.02	0.46±0.04	0.30±0.02
Wild oat	Cocktail (25%, each)	4.13±0.15	1.49±0.07	1.76±0.06	1.48±0.08
CMC	*A. niger* JMC22344	6.58±0.21	1.17±0.06	1.93±0.04	0.97±0.06

The amount of ethanol produced from the hydrolysate also showed variations amongst the fermenting yeast (Table 5.6). Accordingly, *K. marxianus* ETP87 produced the highest amount of ethanol from oat hydrolysate by (3.49-8.25g/L) with average ethanol of 6.02g/L, followed by *S. cerevisae* ETP53 with 3.32-7.76 g/L with average ethanol production of 5.84; and *P. fermentans* ETP22, with a range of 2.69-6.95g/L and mean of 5.11g/L.

The efficiency of ethanol production by all the yeasts corresponded with the amount of sugar released in acid treated/untreated but enzymatically treated wild oat with *P. ostreatus* M2191; followed by the same substrate treated with by *P. sajor-caju*. The data showed that all the yeast strains produced the highest concentration of ethanol derived from the hydrolysates of *P. ostreatus* grown on wild oat (6.95-8.25g/L) which was almost twice higher than the ones treated with *A. niger* although the difference in the amount of sugar released by the latter was only 21%.

5.3.6. Biomass production from acid and alkali hydrolysates

The biomass obtained from yeasts grown in acid hydrolyzed straws was higher than alkali hydrolyzed straws (Figure 5-1). The yeast dry weight obtained from *P. fermentans* ETP22 was higher on 1% acid treated grass pea with 7.0g/L (biomass) followed by 6 g/L from *K. marxianus* and 4-5g/L with *S. cerevisae* on the same substrate, respectively. The biomass recovered from acid-hydrolysates was higher than alkali-hydrolysates (Figure 5-1). Minimum yeast dry biomass was obtained in all yeasts grown on grass pea treated with 1% (w/v) sodium hydroxide.

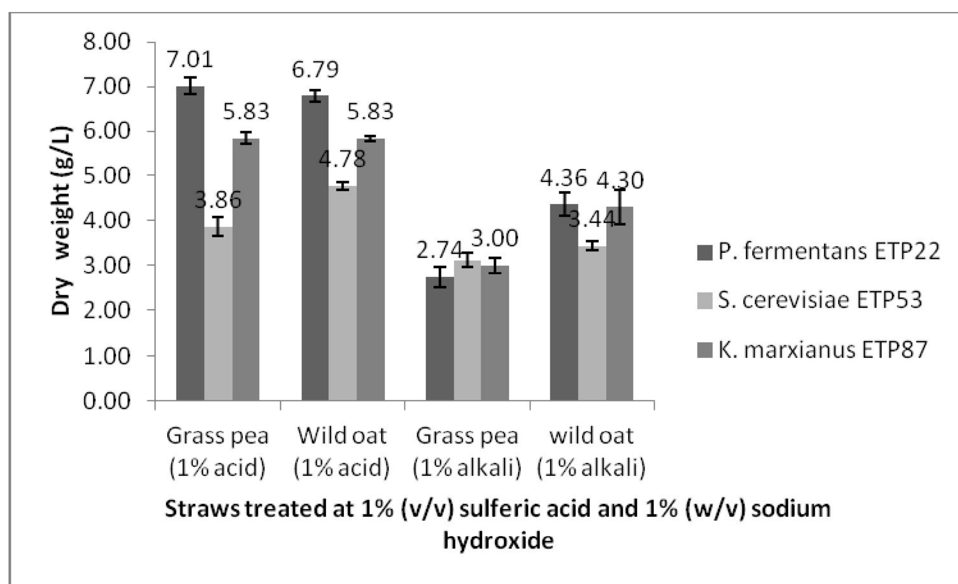

Figure 5-1Biomass production by yeasts in acid and alkali hydrolysates incubated at 30°C for 24 hours.

5.3.7. Biomass production from in situ degraded straws

The effect of fungal treated hydrolysates on biomass production by the three test yeasts is shown in Figure 5-2. Accordingly, *K. marxianus* ETP87 and *P. fermentans* ETP22 gave higher

biomass than the *S. cerevisiae* ETP53; except on *P. sajor-caju* and *T. reesei* treated grass pea, the biomass production ranged from 5.0g/L to 8.0g/L. The pattern showed that more biomass was recovered from wild oat than grass pea.

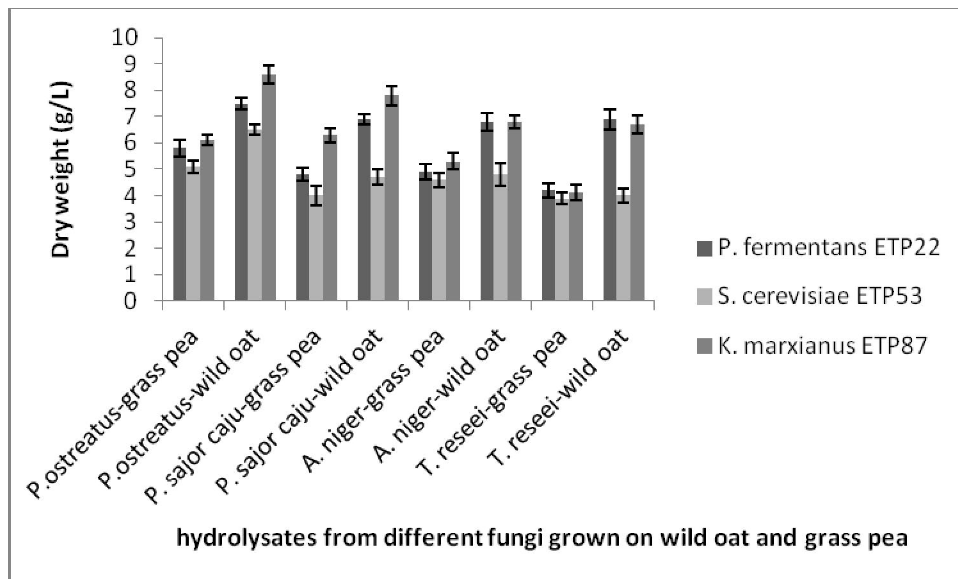

Figure 5-2 Yeast dry weight from straws degraded by in situ growing fungi

5.4. DISCUSSION

5.4.1. Straw hydrolysis by dilute acid and alkali

Straws can be hydrolyzed by physical, chemical and biological methods. Among the biological methods, enzymes are commonly employed to hydrolyze straws; however, enzymatic hydrolysis required economical consideration(Bujang *et al.*, 2013). On the other hand, chemical hydrolysis such as acidic, alkali, ozone, and other are used up for hydrolysis of straws. Dilute acid hydrolysis has received considerable researched attention as it is inexpensive and effective (Bujang *et al.*, 2013). Therefore, dilute sulfuric acid and sodium hydroxide hydrolysis was utilized to hydrolyze common wild oat and grass pea straws.

Like Bujang *et al.* (2013) study, 0.5 -1% of sulfuric acid was able to produce higher reducing sugar in common wild oat (8.9 g/L) and grass pea (13.9 g/L). However, Jung *et al.* (2013) attained optimal sugars (14.5g per 100g straw) from Thai Mission grass (*Pennisetum polystachyon*) which was hydrolyzed by 1% H_2SO_4 (w/v).

The sugar yield of 4.26-8.19g/L obtained from 2% alkali treated substrates in this study is much lower than 6.6g/100g straw released at 3% (w/v) NaOH hydrolyzed mission grass pretreated with microwave (Tatijarern *et al.* (2013), and the application of 4-7% NaOH resulted in the degradation of monomeric sugars released from switch grass (Hu and Wen, 2008). Therefore, the lower sugar at higher alkali and acid treating chemical might be due to degradation of the reducing sugar at higher temperature after they were released from lignocellulose.

5.4.2. Hydrolysate sugar composition

Cellobiose, galactose, glucose, xylose, and arabinose were released during dilute acid and alkali hydrolysis and xylose concentration was higher than all sugars detected in acid and alkali hydrolysates. This shows that most of the xylose (75–90%) present in hemicellulose could be extracted by dilute acid pretreatment technology (Huang *et al.*, 2009). Similarly, Rita de Cássia *et al.* (2010) reported that D-xylose (19.19 g/L) was the major product released from acid hydrolysates of sugarcane bagasse hemicellulosic whereas the concentrations of D-glucose (0.981 g/L) and L-arabinose (1.82 g/L) were very low. The authors showed that the mild hydrolysis condition was not enough to release D-glucose from the cellulose fraction. In addition, almost 100% of hemicelluloses are completely removed when sulfuric acid is mixing up with cellulosic biomass (Bujang *et al.*, 2013) and hemicellulose is composed of mainly five carbon sugars and few six carbon sugars but cellulose is not entirely released with dilute acid hydrolysis at 121°C.

5.4.3. Ethanol production from acid and alkali hydrolysates

Huang *et al.* (2011) suggested that xylose fermenting yeasts are highly needed to produce ethanol from acid hydrolysates that contains higher percentage of xylose than glucose. *Candida tropicalis* JH030 which was grown in non-detoxified rice straw acid hydrolysates containing 4.5 g/L glucose, 35.9 g/L xylose, and 4.3 g/L arabinose resulted 3.3 g/L ethanol (Huang *et al.*, 2011). The ethanol produced by the *C. tropicalis* JH030 was higher (3.3 g/L) than half of the glucose ($1/2 \times 4.3 = 2.15$) and this shows that *C. tropicalis* JH030 utilize xylose to produce ethanol. However, none of the yeasts in this study was able to ferment xylose the dominant sugar

in dilute acid hydrolysates. As a result, the ethanol concentration produced from acid and alkali hydrolysates was very small.

Different studies have demonstrated variation on *K. marxianus* and *S. cerevisae* potential to tolerate inhibitors and produce ethanol depending upon treatments, types of substrates and strains (Moreno *et al.*, 2013). Therefore, the inhibitors contribution to reduce ethanol production may be very small.

5.4.4. Detoxification of hydrolysates

Growth and ethanol fermentation inhibitors such as furans, phenolic compounds, carboxylic acids, and glycoaldehyde are also produced besides five and six carbon sugars during acid hydrolysis (Ludwig *et al.*, 2013). Therefore, it needs to reduce the concentration of the inhibitors to the level that has less effect on growth and ethanol fermentation. The biological methods (sequential fermentation and enzymatic treatment), physical methods (evaporation and membrane separation), and chemical methods (over-liming, ion exchange, activated charcoal treatment, neutralization, and solvent extraction) were potentially reduced the inhibitors. Among all these methods activated carbon and overliming were investigated to reduce furfural; activated carbon and overliming potentially reduced the furfural content of acid hydrolysates of grass pea and common wild oat.

The removal efficiency of furfural by overliming, therefore, was higher than the 41.75% of furfural removal from acid hydrolysate (1.5% H_2SO_4) of *S. spontaneum* (Chandel *et al.*, 2009) and 38% removal from mission grass hydrolysates (Prasertwasu *et al.*, 2014). Kuhad *et al.* (2010) reported that overliming reduced furfurals (by 34.28%) present in dilute acid hydrolysates of red sage (*Lantana camara*) while subsequent detoxification with activated charcoal resulted in further reduction of furfural (65.0%).

Combined detoxification through overliming followed by activated charcoal strongly reduced furfural content by 77.45% and 68.62% for grass pea and wild oat hydrolysate respectively where as overliming and activated charcoal separately detoxified the furfural to a less extent. Activated charcoal treatment and ammonia neutralization of acid hydrolyzed oil palm empty fruit bunches removed 90% of the furfural (Jung *et al.*, 2013).

Fermentation of dilute acid hydrolysates of red sage by *Pichia stipitis* resulted in 5.16 g/L ethanol (Kuhad *et al.*, 2010), which was higher than the present study. The maximum ethanol concentration produced by *P. stipitis* grown in over-limed acid hydrolysates of rice straw was 9.29 g/L ethanol which may be associated with xylose utilization (Huang *et al.*, 2009). In this study, however, even if *P. fermentans* ETP22 metabolized xylose, it did not produce enough ethanol. In general, the production of more ethanol in detoxified hydrolysates is due to increased cell growth and sugar consumption when inhibitors were reduced by different methods (Kim *et al.*, 2013; Kumar *et al.*, 2009; Moreno *et al.*, 2013)

5.4.5. Ethanol production by employing sugars extracted from solid state grown fungi

When lignocellulose degrading fungi such as *T. reesei* JMC22676, *A. niger* IMC22344, *P. ostreatus* M2191, and *P. sajor-caju* M2345 grow on grass pea and common wild oat, the sugars can be released into the media. However, different researchers suggest that the released sugars may not be sufficient to produce economical ethanol. In this study, 2-8g/L ethanol was produced from 9-16 g/L reducing sugars librated from grass pea and common wild oat straws directly by the fungi grown on the solid state media. Singhania *et al.* (2014) reported that 10.5 g/L ethanol was generated by *K. marxianus* MTCC 4136 from 27 g/L glucose released by crude enzymatic activity from acid pre-treated and crude cellulases from *Penicillium janthinellum.*

In this study, the ethanol concentration in sugars released by crude enzymes obtained from treated substrates was lower than acid or alkali hydrolysates and fungi hydrolysate. This is due to 10% of enzymes extracted from 15g of solid state fermented fungi were used to degrade 12 g of pretreated straws. Cunha *et al.* (2017) suggested that due to complex structure and recalcitrant nature of lignocellulose, high crude enzyme loadings are needed in the production of ethanol from biomass.

5.4.6. Biomass production from acid and alkali hydrolysates

The chemical composition of the acid and alkali hydrolysates are dominated by five carbon sugars (Huang *et al.*, 2011; Bujang *et al.*, 2013) and hence five carbon utilizing yeasts should be applied in order to get high dry biomass from lignocellulosic material. *S. cerevisiae* ETP53 and *K. marxianus* ETP87 were not able to grow on xylose and arabinose and hence the dry biomass

from acid and alkali hydrolysates was lower than *P. fermentans* ETP22 which was able to utilize xylose even if it couldn't ferment it. In addition, the yeast dry weight from acid hydrolysates may be minimal due to low growth rate of yeast by inhibitors in the hydrolysates. In this study, the maximum *P. fermentans* ETP22 dry weight was 7 g/L which is lower than dry biomass from *Debaryomyces hansenii*, *Kluyveromyces marxianus*, and *Pichia stipitis* grew on hemicellulose hydrolysates from spent grain (Duarte *et al.*, 2008).

5.4.7. Biomass production from in situ degraded straws

The dry yeast biomass from in situ degraded straws was higher than acid hydrolysates. Unlike acid hydrolysates, the in situ degraded straws contains no furfural or other inhibitors (Jang *et al.*, 2015; Huang *et al.*, 2009) and hence it could give higher biomass. The highest *P. fermentans* ETP22, *S. cerevisiae* ETP53 and *K. marxianus* ETP87 dry weight which grew on in situ degraded grass pea and common wild oat was 7.5, 6.5, and 8.6 g/L, respectively. This was much higher than 2g/L biomass obtained from *S. cerevisiae* grown on the hydrolysates of Chrysanthemum waste by *P. ostreatus* degradation (Quevedo-Hidalgo *et al.*, 2013).

5.5. CONCLUSION AND RECOMMENDATIONS

Dilute acid and alkali hydrolysis, in situ straw degradation by fungi, and enzymatic degradation of straws are all possible means of converting lignocellulose into sugars for bioethanol production. Enzymatic degradation is less cost effective since microbial fermentation and enzyme purification process are expensive and it needs skilled manpower. The low ethanol concentration produced by *S. cerevisiae* ETP53, *K. marxianus* ETP87, and *P. fermentans* ETP22 from acid hydrolyzed straws are attributed by high furfural concentration, high xylose concentration, and less glucose concentration. Biological degradations are more important for bioethanol production since it does not release and inhibitors.

All the detoxifying methods such as overliming, activated carbon, and overliming followed by activated charcoal reduced the furfural content of acid hydrolysates. However, making the adsorption and reaction time more than 30 minutes did not further reduce the furfural

significantly. The overliming and biological detoxification cannot be run since the overliming pH was 13 which is unsuitable for enzymic and microbial catalysis.

This study was limited to ethanol production from treated hydrolysates under specific controlled conditions However, there is a need to optimize the acid and alkali concentration, temperature, and retention time to get more reducing sugars since maximum concentration of acid and alkali degrades the reducing sugars released by the chemical hydrolysis. Biological detoxification of acid hydrolysates and sequential adaptation of *S. cerevisiae* ETP53, *K. marxianus* ETP87, and *P. fermentans* ETP22 to furfural are open for research.

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Chapter 6

Bioethanol production from sorghum flour using locally isolated *Saccharomyces cerevisae* ETP53, *Kluyveromyces marxianus* ETP87 and *Pichia fermentans* ETP22.

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Abstract

Ethanol is one of the main biobased fuel produced mainly from corn and other starch materials. In order to produce ethanol from sorghum flour, sorghum raw starch was hydrolyzed by sulfuric acid, crude enzyme extract from A. niger *JCM22344, and in situ degradation by* A. niger *JCM22344 via submerged fermentation. Different ethanol production methods such as separate saccharification and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and coculturing were investigated. Sorghum hydrolysates, which were supplemented with yeast extract and peptone, were fermented by locally isolated* S. cerevisae *ETP53,* K. marxianus *ETP87 and* P. fermentans *ETP22. Yeasts were also cocultured with the fungus in order to produce ethanol directly from raw sorghum flour in one pot system. The ethanol produced by* K. marxianus *was higher than the ethanol produced by other yeasts in acid hydrolysate. However, ethanol produced by* S. cerevisiae *was higher than those produced by* K. marxianus *and* P. fermentans *when SHF, SSF and coculturing was employed as ethanol production means from sorghum flour. The order of method that produced ethanol on raw sorghum flour from maximum to minimum was SSF, coculturing, and SHF where as on soluble starch, maximal, intermediate, and minimal ethanol contents were produced via occulting, SSF, and SHF respectively. Therefore, coculturing and simultaneous saccharification and fermentation are promising methods to produce ethanol directly from raw starch by making use of amylolytic fungus and yeast.*

Key words: Bioethanol, amylase, simultaneous saccharification and fermentation, separate saccharification and fermentation, coculture, sorghum flour

6.1. INTRODUCTION

Bioethanol is one of the most promising renewable liquid fuels that could replace oil-derived fossil fuels in the future. Demand for biodegradable and renewable resources for bioethanol production has been increasing due to the necessity to rely less on petroleum and develop sustainable, eco-friendly materials and products. Nowadays, bioethanol is derived primarily from sugar crops (sugar cane) and crop starch such as corn, wheat, and sorghum (Diaz *et al.*, 2014). Starch is the main carbohydrate reserve in plants and one of the most abundant renewable

carbohydrate next to lignocellulose in a large variety of higher plants (Izmirlioglu and Demirci, 2017; Xu *et al.*, 2016).

Like other cereal crops, sorghum kernels contain high starch content ranging from 72.3 to 75.1% depending on their varieties (Ai *et al.*, 2011). Animal feeding studies have shown that sorghum has a lower starch digestibility because of the presence of highly cross-linked prolamine protein matrices surrounding starch granules and tannin (Ai *et al.*, 2011).

Conversion of starch to bioethanol and biomass involves conventional methods, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and coculture. Basically, sugars are produced from starch through chemical (acid and alkali) and enzymatic hydrolysis. This requires gelatinization, liquefaction and saccharification before fermentation. They are usually performed at elevated temperatures (60^oC to 90^oC) that demand high energy and cost (Xu *et al.*, 2016).

Separate hydrolysis of starch by enzymes and fermentation is one of the conventional methods used for bioethanol production (Abu Bakar *et al.*, 2015). Accordingly, Alpha-amylase is employed for liquefaction, while glucoamylase is used for saccharification step to produce glucose monomers (Izmirlioglu and Demirci, 2017).

In addition, utilization of commercial enzymes for saccharification is an expensive process for the production of alcohol from starchy materials. Enzyme saccharification or SHF is limited by the accumulation of high concentration of sugar that inhibits the enzyme activity (Xu *et al.*, 2016; Izmirlioglu and Demirci, 2017; Diaz *et al.*, 2014).

The use of pure or crude enzyme and coculturing are being practiced in SSF in order to overcome the challenges that SHF and other conventional methods face (Diaz *et al.*, 2014). It employs the simultaneously saccharification of substrates by crude enzymes and ethanol production by yeasts. In SSF method, starch is hydrolyzed by glucoamylase to form glucose and simultaneously converted to ethanol and carbon dioxide by ethanologenic yeasts..

Coculturing is the synergistic method that involves the mixing of ethanologenic yeasts with amylolytic fungi. During coculturing, amylolytic fungi provide the sugar to the yeasts that produce ethanol and remove inhibitory effects of sugars for efficient enzyme activity. Therefore, coculturing and SSF can be alternatives for efficient ethanol production by shortening processing times, decreasing energy demand, and eventually reducing production costs (Izmirlioglu and Demirci, 2017). According to Cinelli *et al.* (2015), coculturing enables the direct conversion of starch to ethanol and reduces the capital and operational cost by approximately 41% and 51%, respectively.

Like coculturing, SSF has various advantageous over SHF. The SSF is also less energy demanding, less time consuming and cost efficient than SHF because no need of separate starch saccharification step and two processes (saccharification and fermentation) acts is one reactor so the investment cost is lower (Pietrzak and Kawa-Rygielska, 2015). The yeasts consume the glucose as soon as it is librated and hence it removes the end product inhibition (Azmi *et al.*, 2009; Diaz *et al.*, 2014). Therefore, it facilitates the liberation of more sugars as a result higher ethanol is produced in SSF than SHF. The SSF serves as fed-batch system for carbon sources since it supplies glucose as far as starch is present in the media.

However, the challenge in SSF is the different optimal conditions for yeasts and enzymes. The pH and temperature greatly affects the optimal activity of the enzyme as well as the yeasts. For example, the pH of the media for SSF was 5.0 and it was incubated at 30°C. However, fungal amylase hydrolyzes optimally at 50°C and pH 4.5 (Lin *et al.*, 2011). The pH was almost closer to the optimal condition but the temperature (30°C) was lower than its optimality (50°C). Furthermore, the reaction kinetics of SSF process is less favored because it is run at temperatures below the enzymes optimum temperature (Cinelli *et al.*, 2015).

However, SSF and coculturing require an enzyme that hydrolyzes raw starch. Even if amylases are produced by many filamentous fungi and bacteria, raw starch-digesting glucoamylase (RSDG) is produced by only few microorganisms such as *A. niger* which is the efficient producer of RSDG and α-amylase (Xu *et al.*, 2016; Izmirlioglu and Demirci, 2017). RSDG can hydrolyze raw starch directly to produce glucose as the sole product in a single step, which

would simplify starch processing and reduce energy consumption during the industrial production of starch-based products (Izmirlioglu and Demirci, 2017; Xu *et al.*, 2016).

Amylolytic fungi like *A. niger* van Tieghem also produces α-amylase, glucoamylase, tannase, protease, laccase, xylanase and cellulase (Ramos *et al.*, 2011; Aguilar *et al.*, 2002; Izmirlioglu and Demirci, 2017). Therefore, the crude enzymes from *A. niger* van Tieghem could degrade protein and tannin that surrounds the starch granules. Consequently, applying *A. niger* van Tieghem for SHF, SSF, and coculturing facilitates the efficient degradation of starch . The aim of this study was to produce bioethanol from sorghum flour that were hydrolyzed by different methods using locally isolated *Saccharomyces cerevisae* ETP53, *Kluyveromyces marxianus* ETP87 and *Pichia fermentans* ETP22.

6.2. MATERIALS AND METHODS

6.2.1. Microorganisms

Amylase producing fungus, *Aspergillus niger* JCM22344, was obtained from Japan Collection of Microorganism, Japan. The newly isolated ethanol producing yeasts such as *S. cerevisiae* ETP53, *K. marxianus* ETP87, and *P. fermentans* ETP22 were used to ferment sugars released by *A. niger* JCM22344 and acid hydrolysis.

6.2.2. Sorghum flour

Red sorghum grain was obtained from local market in Debre Berhan and it was grinded by a local mill in Debre Berhan.

6.2.3. Media preparation for *A. niger* JCM22344 submerged fermentation

The fermentation broth medium for *A. niger* (FBMAN) in percentage (w/v) consisted of soluble starch, 2; peptone, 0.3; yeast extract, 0.1; $(NH_4)_2SO_4$, 0.6; $CaCl.2H_2O$, 0.2; NaCl, 0.5; KH_2PO_4, 0.35; $FeSO_4.7H_2O$, 0.015; $MnSO_4$, 0.14; $ZnSO_4$, 0.14; and $MgSO_4.H_2O$ (Hernández *et al.*, 2006). The percentage of soluble starch or sorghum flour during SHF, SSF and coculturing was 8%. Broth media (50 mL) was added to 500-mL Erlenmeyer flask to run the fermentation. After sterilizing and cooling, fungal spores (1.64×10^5 CFU/mL) were inoculated aseptically to broth

and they were incubated in bath shaker at 28°C and 100 shake per minutes. Samples were taken every day for released sugar quantification and enzyme activities evaluation for 9 days.

6.2.4. Crude amylase preparation

Samples, taken every day for 9 days, were centrifuged at 10000×g (eppendorf Centrifuge 5418 R, Germany) for 10 minutes to separate fungal biomass and the supernatant was taken to further separate the enzymes. Like cellulase enzyme procedure, the crude enzyme was precipitated by cold acetone and the precipitate was recovered by centrifugation and dissolved by 0.1M acetate buffer at pH 5 and this was considered as crude enzyme.

6.2.5. Ethanol production from sorghum flour hydrolyzed by acid

In order to hydrolyze the starch, sorghum flour (8g) that was dried over night at 105°C was added to 100mL sulfuric acids (1% v/v) and autoclaved at 121°C for 90 minutes. After cooling the pH of the mixture was adjusted to 5, filtered with cheese cloth, and followed by centrifugation at 10000×g (eppendorf Centrifuge 5418 R, Germany) to separate unhydrolyzed sorghum flour from the hydrolysates. The sugars released was quantified by DNS (Miller, 1959). Yeast extract (1% w/v) and peptone (2% w/v) were added to the hydrolysates. *S. cerevisae* ETP53, *P. fermentans* ETP22 and *K marxianus* ETP87 (7.4×10^7 for each yeasts) were inoculated and incubated in bath shaker at 30°C for 36 hours.

6.2.6. Coculturing yeasts with *A. niger* JCM22344.

To produce ethanol by coculture, *S. cerevisiae* ETP53, *P. fermentans* ETP22 and *K. marxianus* ETP87 (7.4×10^7 for each yeasts) were inoculated to 0, 24, 48, and 72 hours grown *A. niger* JCM22344 on FBMAN containing 8% sorghum flour. During *A. niger* JCM22344 cultivation, 1 mL of spore suspension in saline water, which had 4.6×10^5 CFU/mL, was inoculated to FBMAN. The same procedure was repeated for soluble starch. The ethanol produced and yeast biomass were analyzed after the yeasts were grown for 24 hours. On the other coculturing condition, the yeast added simultaneously with the fungus and samples were analyzed at 12, 24, 48, 72 and 96 hours. This experiment was run for soluble starch and sorghum flour independently. The fermentation was conducted in 500mL Erlenmeyer flask with 100 mL working volume in bath shaker (Clifton, England) at 28°C and 100 strokes per minutes.

6.2.7. Separate hydrolysis and fermentation

Sorghum flour (8% w/v) was added to 100mL distilled water and boiled for 15 minutes with Bunsen burner. The liquefaction was conducted by using 6mL crude enzyme in boiling water bath (Kottormann 3043, Germany) at 90°C. In order to hydrolyze sorghum flour, additional crude amylase (6mL) was put in and the enzyme was absorbed by gently stirring with magnetic stirrer at higher speed for 30 minutes. The mixture was incubated in bath shaker (Clifton, England) at 50°C for 36 hours and samples were taken at 12, 24, and 36 hours to quantify reducing sugars released. The yeasts were inoculated to saccharifed sorghum that was fortified with yeast extract (1% w/v) and peptone (2% w/v); after incubation at 30°C for 36 hours in bath shaker, samples were analyzed for ethanol produced.

6.2.8. Simultaneous saccharification and fermentation

The simultaneous saccharification and fermentation was run using soluble starch and sorghum flour.

Soluble starch: Enzyme was precipitated from cell free *A. niger* broth (9 mL) using cold acetone method and it was solublized by 6mL 0.1 M acetate buffer at pH 5. The 6mL crude enzyme was added to sterilized 100mL YPS (yeast extract, 1%; peptone, 2%; and soluble starch, 8%) in 250 Erlenmeyer flask and incubated for 12 hours at 50°C. The yeast were inoculated and incubated in bath shaker at 30°C with speed of 100 back and forth movement per minutes. The ethanol produced was determined after 36 hours incubation. The biomass was determined by viable count methods that corresponds to specific dry weight in YPD.

Sorghum flour: This was done according to Prajapati *et al.* (2015) method. The sorghum suspension was prepared by mixing up 8g flour with 100mL distilled water in 250-mL Erlenmeyer flask. The gelatinization was conducted by heating the mixture at 95°C for 15 minutes. The liquefaction was run with the addition of 6mL crude enzyme that was produced in the same procedure with above for soluble starch. The crude enzymes (6mL) was added as a source of α-amylase. The liquefaction was maintained at 70°C for 90 minutes. After cooling to 50°C, another crude amylase (6mL) was added as source of glucoamylase and incubated for 12 hours at 50°C. Separately sterilized yeast extract and peptone was supplemented to liquefied sorghum; yeast inoculation was conducted and incubated in bath shaker 100 shake per minute at 30°C for 36 hours. The ethanol and biomass was quantified .

6.2.9. Biomass Determination

Yeast cells which were grown for 48 hours were taken to analyze dry weight. Yeast biomass in coculturing, separate hydrolysis and fermentation, simultaneous saccharification and fermentation conditions was determined by counting CFU (colony forming unit) and it was converted into dry weight using standards made by yeasts grown in dextrose. In order to determine yeast dry weight from coculturing, the fungal biomass was first filtered by cheese cloth and CFU were made from the filtrate. The direct dry weight measurement was not employed since starch granules and fungal biomass contribute to yeast weight increment. However, the biomass of yeasts grown in acid hydrolysates were determined as a dry weight (see appendix).

6.3. RESULTS

6.3.1. Ethanol production from sorghum flour hydrolyzed by acid

The amount of reducing sugars from acid hydrolysates (1% sulphuric acid) was 39.96g/L which was almost twice higher than the enzyme treated starch (Figure 6-2) indicating acid degradation

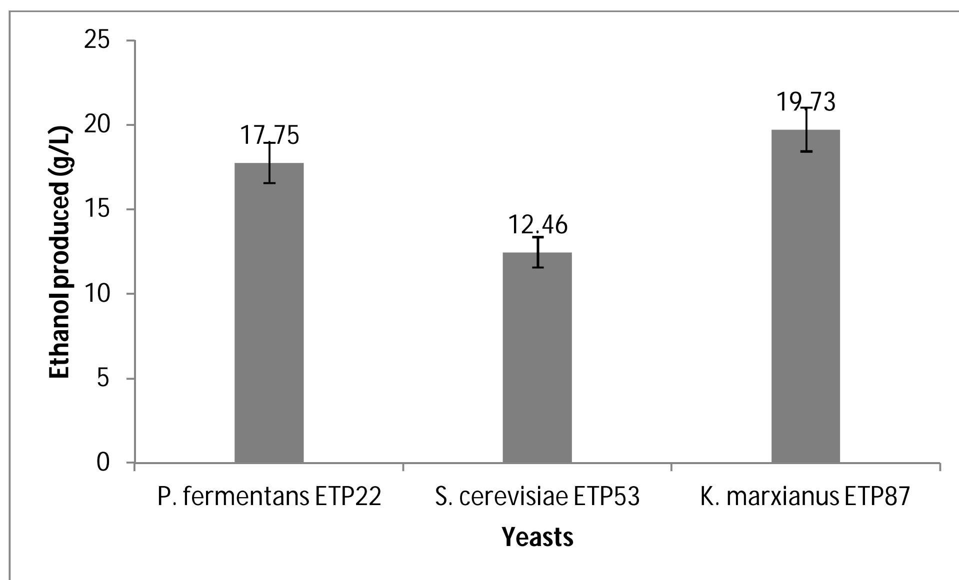

Figure 6-1 Ethanol produced from acid hydrolysates supplemented with yeast extract and peptone incubated at 30°C in water bath for 36 hours.

was more efficient than crude amylase degradation in order to liberate sugars for ethanol fermentation, and amount of ethanol produced using this hydrolysate is shown in (Figure 6-1). Accordingly, *K. marxianus* ETP87 produced 19.73 g/L ethanol followed by *P. fermentans* ETP22 (17.75 g/L) and *S. cerevisae* ETP53 (12.46 g/L) from acid hydrolysates supplemented with yeast extract and peptone. *K. marxianus* ETP87 produced higher than *S. cerevisiae* ETP53 only when they were grown on acid hydrolysates of sorghum flour. Unlike substrates in chapter 3, 4, and 5, *S. cerevisiae* ETP53 produced minimal ethanol in acid hydrolyzed sorghum starch only.

6.3.2. Sorghum flour hydrolysis by crude enzyme and in situ degradation

The sugars liberated by in situ degradation with *A. niger* JCM22344 and its crude enzymes was investigated and the results are displayed in Figure 6-2. Crude amylase produced in 2 days showed optimal activity toward sorghum flour with the release of up to 20g/ L; the enzymatic activity declined after 2 days with a sharp decrease on 4th day of incubation (Figure 6-2). The slow decline in the crude enzymatic activity and the elevated sugar production might be due to inhibition of the enzyme by high sugar content and decline of the population in the batch culture

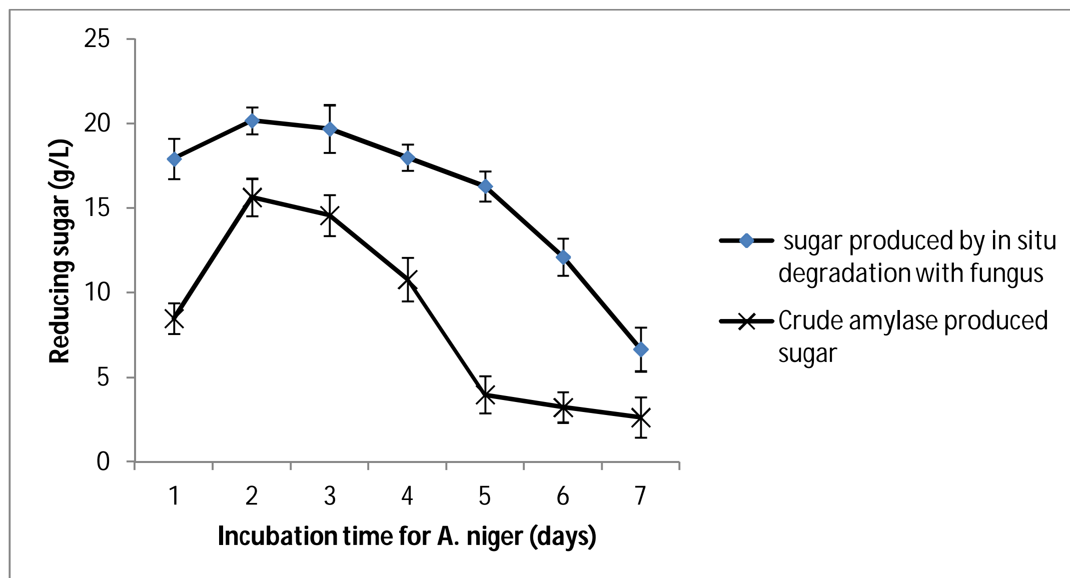

Figure 6-2 Sugar released by in situ and crude amylase degradation from *A. niger* JCM22344 grown on broth at 28°C.

6.3.3. Separate hydrolysis and fermentation (SHF): Enzymes

The performance of the yeasts in SHF system was used to establish a baseline for ethanol production by the yeast strains. The result showed that yeasts yielded lower ethanol when SHF (Figure 6-3) was employed on YPS containing both soluble and sorghum flour than SSF (Figure 6-4). *S. cerevisae* ETP53 produced the highest (10.57 and 8.14 g/L) ethanol where as P. fermentans ETP22 produced the least (7.19 and 6.23 g/L) from both soluble starch and sorghum flour under SHF condition.

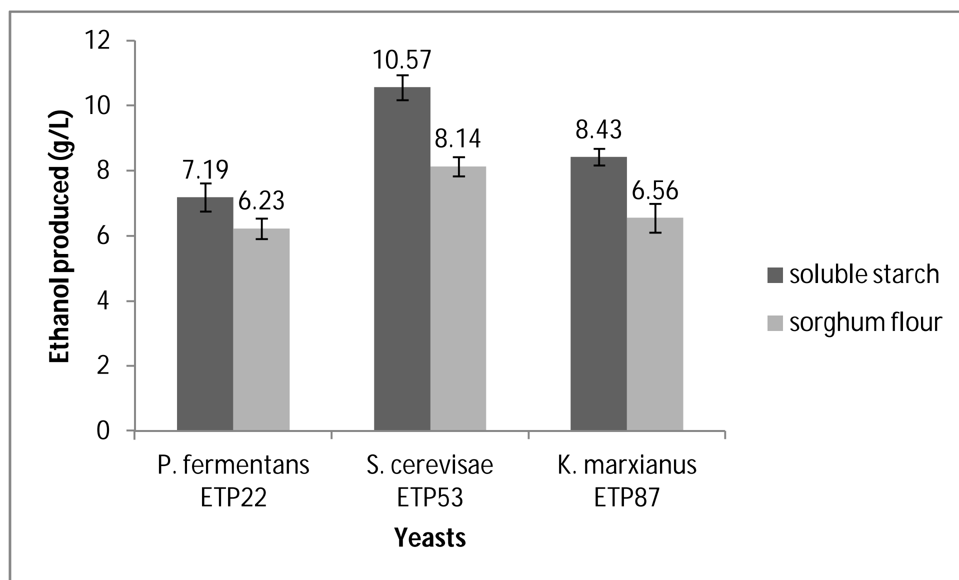

Figure 6-3 Separate hydrolysis and fermentation by yeasts on YPS containing soluble starch and sorghum starch at 30°C for 36 hours.

6.3.4. Simultaneous Saccharification and Fermentation (SSF): Enzymes

During SSF, the starch present in red sorghum was hydrolyzed by crude enzymes from *A. niger* JCM22344 and the sugars were fermented by yeasts simultaneously. The highest concentration of ethanol (9.12 g/L) was produced by *S. cerevisiae* ETP53 from crude amylase (Figure 6-4). Unlike ethanol production from dextrose (industrially produced) and lignocellulose (wild oat and grass pea), the ethanol produced by *P. fermentans* ETP22 was higher than *K. marxianus* ETP87

in YPS containing red sorghum flour. SSF with soluble starch substrate provided higher ethanol yield than SSF containing sorghum flour (Figure 6-4). Figure 6-4 also reveals that the ethanol generated by *S. cerevisiae* (9-12g/L) was greater than that of produced by *K. marxianus* (5-6g/L) and *P. fermentans* (7-8.5g/L).

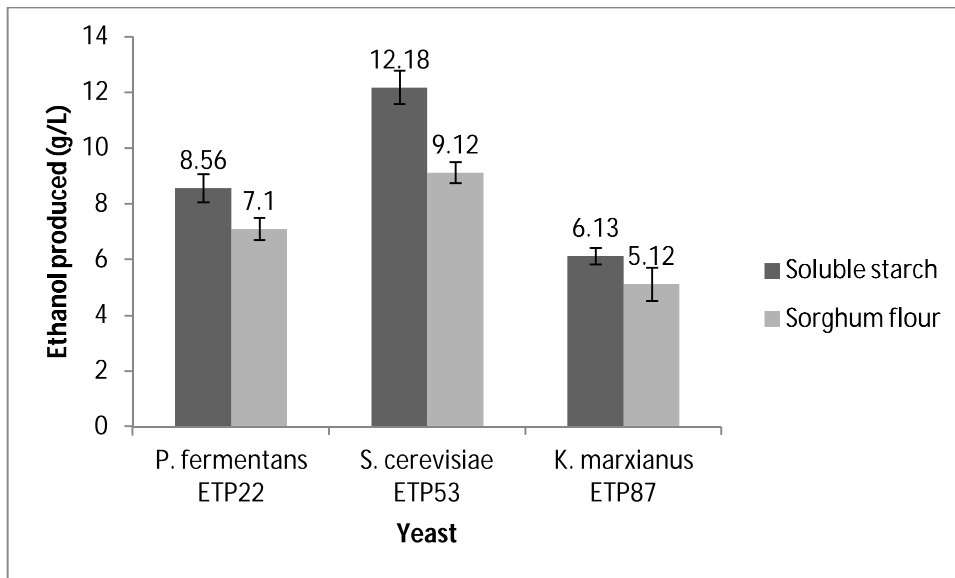

Figure 6-4 SSF by yeasts on YPS containing soluble starch and sorghum flour at 30°C for 36 hours

6.3.5. Yeast inoculation time to fungus during coculturing

The effect of time on the co-inoculants on growth of yeasts and optimum production of ethanol was tested at 0, 24, 48, and 72 hours old fungal cultures and the results is illustrated on Figure 6-5. The data showed that *S. cerevisiae* ETP53 yield the highest ethanol yield of 13-14 g/L with 24, and 48 hrs grown fungal cultures of *A niger* JCM 22344 which was much higher than the ethanol yield of 6-7g/L by *K. marxianus* inoculated with the same old fungal culture (24hr, 48hr) . The ethanol yield from the yeasts was slightly, but not significantly higher in 48 hrs than 24 hrs old co-cultured fungus yeast species with the fungus. Within the economics perspective, the production process using *S. cerevisiae* ETP53 could be shortened to 24 hours without loss in the product efficiency.

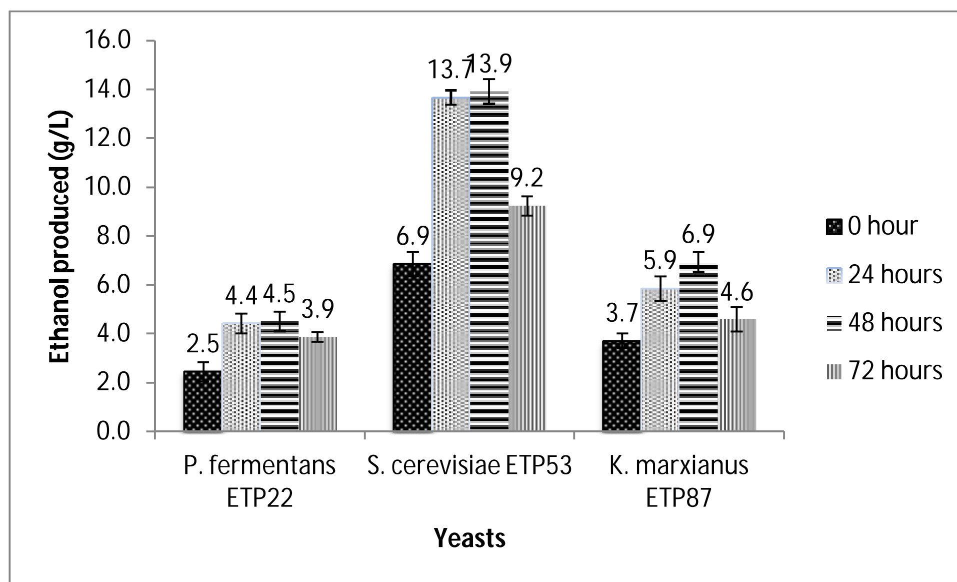

Figure 6-5 Ethanol produced by yeasts cocultured at different time to *A. niger* JCM22344 grown on FBMAN

6.3.6. Coculturing of yeasts with amylolytic fungus on soluble and sorghum flour

The simultaneous coculturing results of yeasts and *A. niger* on soluble starch and sorghum flour based substrates was investigated to produce ethanol and the result is shown in Table 6.1. Accordingly, co-culturing with sorghum flour (8.43 g/L) based coculturing resulted lower ethanol than soluble starch (13.24 g/L). Among yeasts, the ethanol produced by *S. cerevisiae* ETP53 (13.24 and 8.43 g/L) was by far higher than *K. marxianus* ETP87 (6.1 and 5.81 g/L) and *P. fermentans* ETP22 (4.56 and 4.34 g/L) both in soluble starch and sorghum flour. The ethanol produced by *S. cerevisiae* ETP53 sharply increased and reached maximum within 24-48 hours both in sorghum flour and soluble starch.

Table 6.1Ethanol production (g/L) by coculturing *A. niger* JCM22344 and yeasts simultaneously on FBMAN containing soluble and sorghum flour incubated at 28 for different time

Yeasts	Substrate	Incubation time (hours)				
		12	**24**	**48**	**72**	**96**
S. cerevisiae ETP53	Soluble starch	4.86	12.39	13.24	10.86	7.86
	Sorghum flour	2.86	7.79	8.43	8.32	5.38
K. marxianus ETP87	Soluble starch	2.71	5.28	6.1	4.22	1.94
	Sorghum flour	1.88	3.95	5.81	3.67	1.44
P. fermentans ETP22	Soluble starch	1.86	5.69	4.56	1.87	0.78
	Sorghum flour	1.21	4.48	4.34	1.63	1.27

6.3.7. Biomass production

The yeast dry biomass obtained from yeasts grown in sugars released from sorghum flour by acid hydrolysates, SHF, SSF, and coculturing were analyzed and the result is shown in Figure 6-6. The acid hydrolysate substrate gave more yeast dry weight than SHF, SSF and coculturing. The strongly concentrated reducing sugars released during acid hydrolysis might contribute to increased dry weight (Figure 6-1).

Biomass produced by *P. fermentans* ETP22 (4-7.8 g/L) was higher than *K. marxianus* ETP87 (3.3-7.2 g/L) and *S. cerevisiae* ETP53 (3.9-6.5 g/L) in all growth conditions such as acid hydrolysate, SHF, SSF, and coculturing. *S. cerevisiae* ETP53 was the lowest biomass producer in SSF, SHF, and acid hydrolysate growth. However, *K. marxianus* ETP87 (3.3 g/L) resulted the lowest biomass compared with *S. cerevisiae* ETP53 (3.9 g/L) and *P. fermentans* ETP22 (4 g/L) during coculturing with *A. niger* JCM22344 suggesting that *K. marxianus* might be less competitive for fungus than *S. cerevisiae* ETP53 and *P. fermentans* ETP22. Nevertheless, like *P. fermentans*, *K. marxianus* produced higher biomass when it grew under SHF and SSF conditions.

The average of the three yeast biomass from acid hydrolysates was 18.15%, 13.63%, and 93.26% higher than SHF, SSF and coculturing, respectively. The lowest yeast cell dry weight was attained in yeasts cocultured with the fungus (average 3.7 g/L) (Figure 6-6). In addition, lower yeast biomass yield might be attributed by the competition between the yeast and the fungus for sugar and other resources.

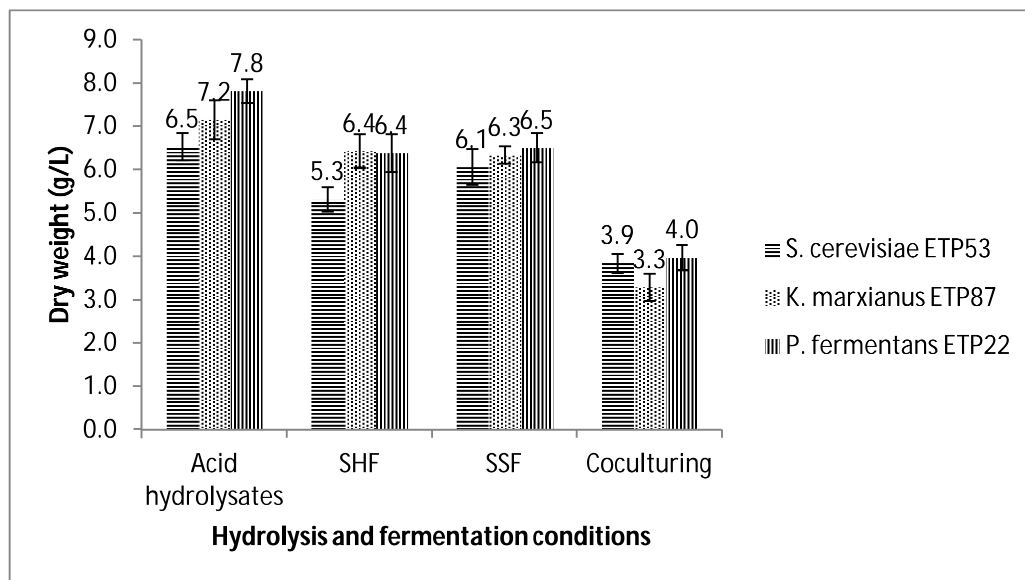

Figure 6-6 Yeast dry weight from different hydrolysis and fermentation condition

6.4. DISCUSSION

6.4.1. Ethanol production from sorghum flour hydrolyzed by acid

The ethanol produced by the tree yeasts from acid hydrolysates of sorghum flour ranged from 12.46 to 19.73 g/L. This was much higher than the ethanol produced (7.6 g/L) from hydrochloric acid hydrolyzed potato peel waste using *S. cerevisae var. bayanus* after 48 hours fermentation (Arapoglou *et al.*, 2010), but lower than ethanol produced (20.77g/L from acid hydrolyzed potato starch by *S. cerevisiae* after 24 hours (Scholz *et al.*, 2013).

Scholz *et al.* (2013) reported that dilute sulfuric acid hydrolysis of algal starch at 121°C converted much of the starch into glucose without detectable production of inhibitors. Therefore,

the minimal inhibitors in starch hydrolysis might enhance the ethanol titer than inhibitor rich acid hydrolysates from straws.

6.4.2. Sorghum flour hydrolysis by crude enzyme and in situ degradation

The sugars released by in situ degradation was higher than crude amylase. During in situ degradation, the librated sugars are consumed by the fungi and hence it reduces the simple sugars that brings the feedback inhibition of the amylase. Optimal sugar was released by both in situ degradation and crude amylase within 24 and 48 hours. Similar studies showed the maximum glucose concentration released by *A. niger* MTCC1349 from wheat flour was found between 6-48 hours (Manikandan and Viruthagiri, 2009). Izmirlioglu and Demirci (2017) studied the glucoamylase activity of *A. niger* on potato starch sharply increased between 12 and 24 hours; it remained constant during 24 to 48 hours incubation and then the enzymatic activity declined after 48 hours.

6.4.3. Separate hydrolysis and fermentation (SHF): Enzymes

Separate hydrolysis and fermentation was less efficient than simultaneous saccharification and fermentation to produce ethanol from sorghum flour. Pietrzak and Kawa-Rygielska (2015) proved that processing of waste wheat–rye bread to ethanol in the SSF conditions was significantly higher in comparison to SHF. Removal of feedback inhibition of starch hydrolysis in SSF might enhance ethanol production by increasing sugar releasing.

It is interesting to note that *S. cerevisiae* ETP53 was more effective (30%) in ethanol production, than *P. fermentans* ETP22 and *K. marxianus* ETP87 on both soluble starch and sorghum flour. Diaz *et al.* (2014) studied SHF of industrial α-amylase and glucoamylase with *S. cerevisiae* and *K. marxianus* on sweet potato starch and the ethanol yield by *S. cerevisiae* during SHF was 1.1-fold increase more than that produced by *K. marxianus*.

In general, ethanol production from soluble starch was 15-30% higher than the one produced from sorghum flour. The cellulose, hemicellulose and protein that are present in the seed coat reduces the enzymatic hydrolysis of sorghum flour or flour (Abu Bakar *et al.*, 2015). Therefore, this might reduce the ethanol production from sorghum flour.

6.4.4. Simultaneous Saccharification and Fermentation (SSF): Enzymes

S. cerevisiae ETP53 was able to produce 12% higher ethanol in SSF than SHF. Previous study also showed that *S. cerevisiae* produced more ethanol than *K. marxianus* on SSF that used sweet potato starch as a substrate (Diaz *et al.*, 2014).

Abu Bakar *et al.* (2015) SSF study showed that alkali treated white sorghum flour liquefied and hydrolyzed by pure α-amylase and glucoamylase and fermented by *S. cerevisiae* increased ethanol by 11.5% (87.97g/L), which was higher than the ethanol yield in this study. This because the previous work involved removal of protein content using 0.1M sodium hydroxide to accelerate hydrolysis of starch and treated with 35% of glucoamylase enzyme concentration immobilized using Na-alginate and 9% of yeast concentration. In other study, 53.3 g/L ethanol was obtained at flour concentration of 150 g/L and 92% efficiency was achieved after 48 hours of SSF (Lin *et al.*, 2011).

In general, the ethanol produced by SSF in this study was much lower than previous studies (Lin *et al.*, 2011; Abu Bakar *et al.*, 2015). This might be due to high enzyme and substrate load, seed coat removal, yeast immobilizations, and enzyme immobilization that they employed.

6.4.5. Yeast inoculation time to fungus during coculturing

Yeasts produced highest ethanol when the yeasts were inoculated to the fungi on 2nd or/and 3rd day and *S. cerevisiae* ETP53 produced 3 times more than *P. fermentans* ETP22. This suggest that *P. fermentans* ETP22 less competent to the fungi than *S. cerevisiae* ETP53. Like this study, Azmi *et al.* (2009) found that the ethanol (35.3 g/L)**,** which was produced by coculture of ragai tapai and *S. cerevisiae* mixed after 10 hours ragai tapai fermentation, was higher than simultaneous mixing (0 hours) of ragai tapai and the yeasts. On the other study, the 24 hours delay in inoculation of *K. marxianus* MTCC1389 to *A. niger* MTCC1349 on wheat flour gave substantially higher ethanol (23 g/L) than simultaneous and 48 hours delay (Manikandan and Viruthagiri, 2009).

6.4.6. Coculturing of yeasts with amylolytic fungus on soluble and sorghum flour

The aim of coculturing is to reduce the production cost by removing hydrolysis stage. However, if the yeast and the fungus require different growth variables, optimal ethanol production may not be successful attained. For example, the amylolytic fungi requires more oxygen where as the ethanologenic yeasts need a lower concentration of dissolved oxygen.

The ethanol produced by *S. cerevisiae* ETP23 was more than 2 times of *K marxianus* ETP87 and 3 times of *P. fermentans* ETP22 during coculturing with *A. niger* JCM22344. S. cerevisiae is crab tree positive, able to produce ethanol under higher oxygen condition (Vieira *et al.*, 2013) where as *K. marxianus* is crab tree negative (Zoppellari and Bardi, 2013); therefore, this might be the reason for significantly lower ethanol by *K. marxianus* ETP87. Furthermore, the *P. fermentans* and *K. marxianus* might be less competent to *A. niger*.

The result on ethanol production with *S cerevisiae*, in this study, was much lower than the high ethanol concentration (23g/L) after 48 hours fermentation by mixed culture of *A. niger* and *K. marxianus* on wheat bran flour (Manikandan and Viruthagiri, 2009). The time course of ethanol production by mixed culture of *A. niger* and *S. cerevisiae* on potato starch was 24 hours (Jeon *et al.*, 2008) and 48hours (Abouzied and Reddy, 1986).

6.4.7. Biomass production

The yeast dry biomass is needed for the production of various intracellular products such as intracellular enzymes, lipids and others. Producing biomass together with ethanol is economically very important since it enable to get two very important valuable products from one pot production system. Dry yeast biomass of *S. cerevisiae* ETP23, *K marxianus* ETP87, and *P. fermentans* ETP22 was produced from sorghum flour treated and fermented by different methods. Acid hydrolysates supported the production of highest (6.5-7.8 g/L) biomass for all yeast and coculturing the least (3.3-4.0 g/L). Suresh and Rao (1999) showed that 15 g/L biomass was produced by the coculture of *A. niger* and *S. cerevisiae* VSJl which were grown in damaged sorghum grain at 30°C for 5 days.

6.5. CONCLUSION AND RECOMMENDATIONS

Simultaneous saccharification and fermentation (SSF) of both sorghum and soluble starch resulted better ethanol than separate hydrolysis fermentation (SHF). This shortens ethanol production time and reduces production cost by saving energy and human power. Therefore, SSF is a promising alternative for ethanol production.

Coculturing of *S. cerevisae* ETP53, *K. marxianus* ETP87 and *P. fermentans* with *A. niger* JCM22344 is feasible for ethanol production. Coculturing eliminates the enzymatic starch hydrolysis steps as it is currently employed in factories; thereby, it improves starch to ethanol conversion.

Highest yeast biomass was obtained from yeasts cultivated on acid hydrolysates where as the lowest was attained from cocultured yeasts. Unlike ethanol production, *S. cerevisae* ETP53 has given lowest yeast dry weight than *K. marxianus* ETP87 and *P. fermentans* ETP22.

The yeasts and the fungus may need different oxygen concentration, pH, and other variable; therefore, it is highly recommended to optimize these growth variable for both fungus and starch. Further investigation is needed to know the effect of cellulase and protease enzymes on exposing the sorghum flour granules for amylase degradation. Furthermore, it is advisable to optimize the amylase enzyme activity for various parameters. Analyzing the nutrient content (specially amino acids and minerals) of acid hydrolysates of sorghum flour helps to know the deficiency of nutrients and to enhance the fermentation process.

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Chapter 7

Bioethanol Production from Whey Using Non-*Saccharomyces* Yeast, *Kluyveromyces marxianus*

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Abstract

Ethanol production from non-food substrate is strongly recommended to avoid competition with food production. Whey, which is rich in nutrients, is one of the non-food substrate for ethanol production by Kluyveromyces *spp. The purpose of this study was to optimize ethanol from different crude (non-deproteinized, non-pH adjusted and non-diluted) whey using* K. marxianus *ETP87 which was isolated from traditional yoghurt. The sterilized and non-sterilized whey were employed for* K. marxianus *ETP87 substrate to evaluate the yeast competition potential with lactic acid and other microflora in whey. The effect of pH and temperature on ethanol productivity from whey was also investigated. Peptone, yeast extract, ammonium sulfate (*$(NH_4)_2SO_4$*), and urea were supplemented to whey in order to investigate the requirement of additional nutrient for ethanol optimization. The ethanol productivity corresponded with the pH and lactose concentration. The ethanol obtained from non-sterilized whey was slightly and statistically lower than sterilized whey. The whey storage at* 4^oC *didn't guarantee the constant lactose presence at longer preservation time. Significantly high amount of ethanol was attained from whey without pH adjustment (3.9) even if it was lower than pH controlled (5.0) whey. The thermophilic yeast,* K. marxianus *ETP87, yielded high ethanol between* $30\text{-}35^oC$ *and the yeast was able to produce high ethanol until* 45^oC *and significantly lower ethanol was recorded at* 50^oC*. The ammonium sulfate and peptone enhanced ethanol productivity where as yeast extract and urea depressed the yeast ethanol fermentation capability slightly. The* K. marxianus *ETP87, the yeast isolated from traditional yoghurt, is capable to produce ethanol from non-sterilized and non-deproteinized substrates.*

7.1. INTRODUCTION

Cheese whey is the liquid remaining after the precipitation and removal of milk casein during cheese-making. It is estimated that 130 million tons of whey is produced worldwide and increasing at a rate of 3% per year (Ariyanti *et al.*, 2014). More than half (50%) of the whey produced worldwide is disposed to rivers, lakes, and other water bodies which represents a significant loss of resource and serious pollution problems (Christensen *et al.*, 2011; Ariyanti and Hadiyanto, 2013). Consequently, treatment of whey seems to be a major problem for medium and small-scale cheese-making plants.

Cheese whey has been used for production of ethanol since it contains lactose and other nutrients required for microbial growth (Kargi and Ozmıhcı, 2006). It contains about 7% solids comprising of 10–12% proteins, lactose (74%), minerals (8%) and fat (3%) (Koushki *et al.*, 2012). Thus, exploitation of microorganisms and whey for production of bio-ethanol and other valuable products offers best alternative to tackle pollution problems.

The fermentation of lactose in crude whey is ineffective from an economic view point due to high dilution and low concentrations of ethanol (25 g/L) achieved, requiring high energy inputs for distillation (Oda *et al.*, 2010; Gabardo *et al.*, 2014; Athanasiadis *et al.*, 2002). The production cost of ethanol would be economical when the lactose concentration raised to 100g/L (Oda *et al.*, 2010). However, this process by itself, is energy demanding process. The simple method to elevate the low sugar concentration is supplementation of cheap carbon sources (Oda *et al.*, 2010).

Ethanol production from whey is mediated mainly by yeasts that are capable of fermenting lactose in whey. Most of *Saccharomyces* species can't ferment lactose because they lack the β-galactosidase enzyme system that degrade lactose into glucose and galactose. Nevertheless, most of the *Kluyveromyces* species are able to ferment lactose to ethanol of which. *K. marxianus* and *K. lactis* are extensively studied for this purpose (Aktaş *et al.*, 2006).

K. marxianus has useful physiological features for ethanol production. It is a thermotolerant yeast with high growth rate (Lukjanenko *et al.*, 2014). It has the capability to ferment different substrates and many strains have already been recognized as GRAS (Generally Recognized As Safe) (Zoppellari and Bardi, 2013). However, it has a Crabtree-negative character and the fermentative metabolism is linked to the limitation of oxygen and can't grow under anaerobic conditions**.** This study was performed to evaluate ethanol production by *K. marxianus* ETP87 from non-sterilized or non-pasteurized, non-deproteinized, and non-diluted crude whey at different conditions.

In Ethiopia, there are more than 32 dairy industries with a projected capacity of 20,000 tones of whey (Abebe, 2016; SNV Netherlands Development Organisation, 2008). The potential of using

the ever-increasing whey waste to produce bio-ethanol and biomass from yeast and other microbes is all the more important for a dual function of commodity production and environmental protection. The aim of this study was to evaluate ethanol production capability of *K. marxianus* ETP87 from crude whey.

7.2. MATERIALS AND METHODS

7.2.1. Whey

The crude whey was obtained from Shola Dairy Farm (Addis Ababa), Tigist Mini Dairy Processing Unit (Debre Berhan), Amanual Dairy (Debre Berhan) and different rural households near Debre Berhan. The pH of the whey was measured within 2 hours of sampling.

The ethanol content of the whey was measured according to Patricia (1995) before it was used for ethanol production. The lactose content of whey was determined as reducing sugar using lactose as a standard according to Ariyanti *et al.* (2014). A standard curve of lactose was prepared by using 2.3, 2.7, 3, 3.5 and 4 mg/L.

7.2.2. Yeast

The bioconversion of lactose present in crude whey into ethanol was performed by the yeast, *K. marxianus* ETP87, isolated from traditional yoghurt, maintained in culture collection at Addis Ababa University. This yeast was selected since it fermented lactose as efficient as galactose and glucose. The strain was maintained on agar slant composed of lactose 20 g/L, yeast extract 10 g/L, peptone 20 g/L and agar 20 g/L.

7.2.3. Experimental conditions

The whey was directly used for ethanol production without deproteinizing and defatting and it was compared with deproteinized by simple heating (Ariyanti *et al.*, 2014). However; clear filtrate was obtained by filtering boiled crude whey though muslin cloth and Whatman paper № 1; it was autoclaved at 121°C for 15 minutes. Ethanol production from autoclaved one was compared with un-autoclaved whey to assess the competition of the yeast with lactic acid bacteria.

7.2.4. Supplementation of whey with nitrogen sources

In order to analyze effects of nitrogen supplementation, the whey substrate was also supplemented with yeast extract (0.55%), peptone (1%), and ammonium sulfate (0.33%). inoculated with 5.6×10^6 *K. marxianus* ETP87 cells and incubated on a bath shaker (Clifton, England) at 28°C and 100 strokes per minutes for 36 hours**.** The ethanol released was determined (Patricia, 1995) and the biomass was quantified by viable count methods that correspond to specific dry weight from YPD control medium containing yeast extract 1%, and peptone 2%, lactose 4%).

7.2.5. Effect of pH and temperature

The effect of whey pH (pH 3.9) on ethanol production was evaluated against the control where the medium pH was adjusted to 5.0 with sodium hydroxide (Christensen *et al.*, 2011) and it was incubated at 30°C for 36 hours. Likewise, the effect of temperature on the same was evaluated on the medium (adjusted to pH 5 and fortified with peptone) by incubating at 25, 30, 35, 40, 45, and 50°C for 36 hours. The *K. marxianus* ETP87 inoculum size was 5.6×10^6 yeast cells. The number of yeasts was counted by viable count.

7.2.6. Ethanolic fermentation

Whey fermentation was conducted in 250 ml flask containing 100 ml crude whey in which its pH was 3.9. The media in the flasks were inoculated with 5.6×10^6 *K. marxianus* ETP87 seed cultures which was aerobically grown in broth media (lactose, 20 g/L; peptone, 20g/L; and yeast extract, 10 g/L) for 24 hours at 30°C. The flasks were covered by cotton-rolled with aluminum foil. The fermentation was carried at 30°C on water bath shaker at 100 rpm and samples were taken for ethanol, reducing sugar, and biomass determinations at different time interval.

7.2.7. Biomass determination

Yeast biomass was determined by counting CFU (colony forming unit) and it was converted into dry weight using standard curve. The direct dry weight measurement was not employed since the precipitated protein contributed in weight increment**.**

7.2.8. Lactose determination

Lactose in whey was determined as a reducing sugar (Ariyanti *et al.*, 2014). The whey was boiled to precipitate proteins and filtered with Whatman №1 using vacuum suction in order to prevent precipitate formation during whey-DNS mixture boiling. Lactose instead of glucose was used to formulate the standard curve.

7.3. RESULTS

7.3.1. Ethanol production from different whey sources

In this study, attempt was made to optimize ethanol production from whey using a local non-*Saccharomyces* yeast, *K. marxianus* ETP87 (Table 7.1). Effect of keeping whey in refrigerator on ethanol production was also evaluated.

After keeping the whey in refrigerator for 2 weeks, the lactose content of the whey samples was reduced by 6% in whey from Shola dairy and by 21-25% in whey from other whey samples might be due to the consumption of lactose and production of ethanol by lactic acid microflora and natural ethanologenic yeasts. The lower reduction of lactose content was attained from whey under industrial scale cheese making process in Shola Dairy that was free from direct human hand contact where as all others involved house hold cottage-scale processing at Amanual dairy, and Tigist dairy.

The highest ethanol production of 12.49 g/L with 88.09% efficiency was obtained from whey provided by Shola dairy. The ethanol produced by *K. marxianus* ETP87 from the three dairies was almost twice as much ethanol as from whey collected from households. In rural areas, the milk is stored for at least 3 days (data not shown) to make cheese and hence this would promote the growth of lactic acid bacteria and competent yeasts which lower the pH and produced ethanol (up to 2.3 g/L) in the whey, respectively (Table 7.1). The Amanual whey also contained small amount of ethanol (0.6 g/L). No ethanol was detected in Tigist mini-dairy and Shola dairy within 2 hours after sampling.

Table 7.1 shows that the yeast dry weight obtained from Tigist (7.67 g/L) and Shola (7.12 g/L) dairies upon36 hours incubation was significantly higher than the yeast biomass in whey collected from house hold (5.48 g/L) showing 18% difference in ethanol production between the industrially processed whey and the house hold whey.

Table 7.1 Ethanol and biomass produced by *K. marxianus* ETP87 from different whey incubated at 30°C for 36 hours

Whey samples	pH	Lactose (g/L) Sampling	2 week later	Ethanol (g/L) by microflora on time of sampling	microflora 2 weeks in refrigerator	Ethanol by *K. marxianus* ETP87	Efficiency (%)	Biomass (g/L)
Tigist dairy	4.5	34.4±1.3	27.3±1.1	0	1.2±0.09	11.62±0.7	66.23	7.67±0.5
Amanual dairy	3.8	28.6±1.2	21.5±0.9	0.6±0.08	1.7±0.08	10.54±0.8	72.26	6.20±0.4
Household	3.1	18.7±0.8	14.4±0.7	2.3±0.1	1.4±0.07	6.43±0.5	67.42	5.48±0.4
Shola dairy	4.2	27.8±1.2	26.1±1.1	0	0.8±0.08	12.49±0.9	88.09	7.12±0.7

7.3.2. Ethanol production from non-sterilized and sterilized whey

The effect of sterilization of whey for ethanol production is shown in Table 7.2. Accordingly, the ethanol concentration reduced after the whey was preserved for 4 days and longer in refrigerator under both sterilized and non-sterilized conditions. However, the sterilized whey resulted in higher ethanol yield than non sterilized whey. The ethanol reduction percentage was very significant, especially in non-sterilized whey when the whey was preserved for 4 days and longer in refrigerator at 4°C.

Table 7.2 Ethanol produced from non-sterilized and sterilized whey

Refrigerator days	Non Sterilized whey Ethanol produced	% of reduction	Sterilized whey Ethanol produced	% of reduction
0	11.71	0	11.68	0
1	11.46	2.13	11.69	+0.09
2	10.34	11.7	11.48	1.71
3	10.97	6.3	10.77	7.79
4	10.01	14.51	11.12	4.79
5	9.22	21.26	10.56	9.59
6	8.61	26.47	10.21	12.59
7	8.18	30.15	9.63	17.55
8	7.54	35.61	9.44	19.18
14	5.67	51.58	8.37	28.34
Average	9.11		10.36	

At the 14th day preservation, 51.58% and 28.34% of ethanol produced from non-sterilized and sterilized whey was reduced due to preservation and contamination showing an almost 50% reduction between sterilized and non-sterilized whey.

7.3.3. Effect of whey pH

The effect of untreated whey (pH 3.8-4.5) and pH controlled (pH 5.0) whey was investigated to evaluate the capability of the yeast to ethanol production from crude whey. Figure 7-1 illustrates that the pH controlled whey (pH 5) yielded higher ethanol than whey without pH control. Accordingly, the increase in ethanol production due to pH adjustment in Tigist, Amanual and Shola whey was 13, 16 and 15%, respectively, which was significantly different from untreated acidic crude whey.

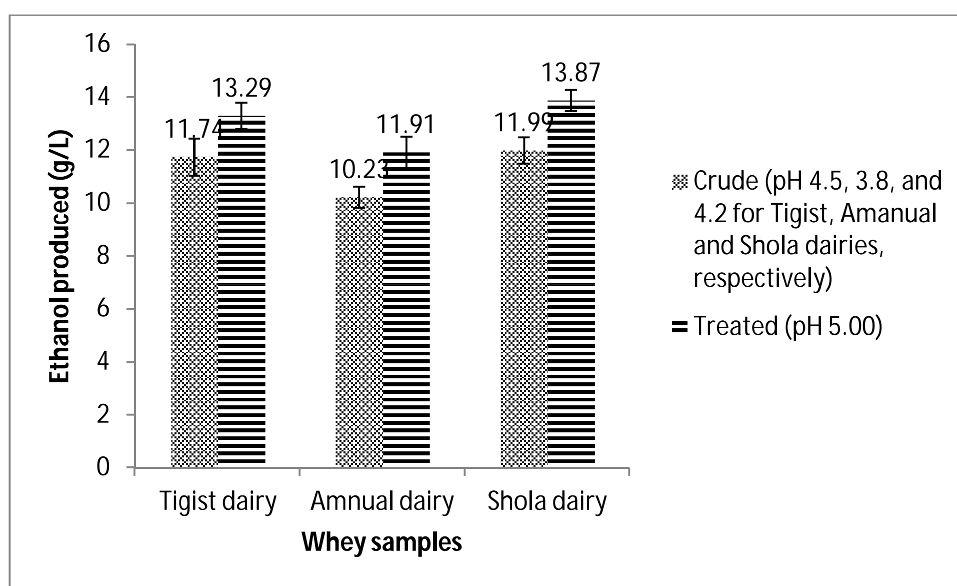

Figure 7-1 Ethanol produced from pH controlled and crude whey incubated at 30°C for 36 hrs

7.3.4. Effects of external nutrient additions to whey

Similarly, the effect of nitrogen additives (peptone, yeast extract, urea, and ammonium sulfate) on ethanol yield from crude whey was assessed and the results are shown in Figure 7-3. The highest ethanol content (15.9 g/L) was recorded from supplementation of peptone followed by 15.4 g/L ethanol produced from (yeast extract and peptone) and 13.6 g/L ethanol produced from whey supplemented with ammonium phosphate. On the contrary yeast extract (9.4 g/L) and urea

(10.2) supplement showed the lowest ethanol yield that did not show any difference from the control treatment. This indicates that *K. marxianus* ETP87 showed best ethanol yield when whey was fortified with peptone, followed by ammonium sulfate. Generally, irregularities of ethanol concentration were observed when whey were supplemented with different nutrients.

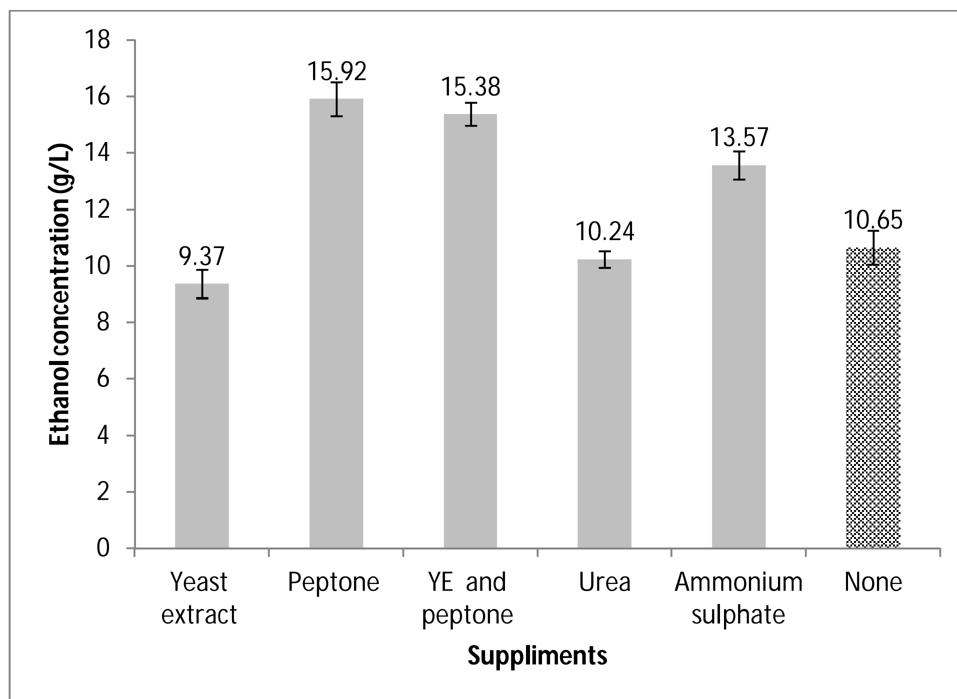

Figure 7-3 Nitrogen source supplimenattion on ethanol productivity incubated at 30°C for 36 hours

7.3.5. Effect of Temperature

The effect of temperature (25-50°C) on ethanol and biomass production was evaluated and the result is shown in Figure 7-4. The maximum ethanol (11.86 g/L) and biomass production (8.16 g/L) were obtained under incubation temperatures of (30-35°C). The ethanol production decreased by 35.9% from 35 to 45°C and followed by a sharp decline by 81.9% at 50°C but biomass productivity reduced slowly (by 4.7, 18.2, and 36.3%) when the temperature raised from 35 to 50°C (Figure 7-4). The ethanol declined faster than biomass after optimum temperature and hence ethanol production is more heat sensitive than biomass production.

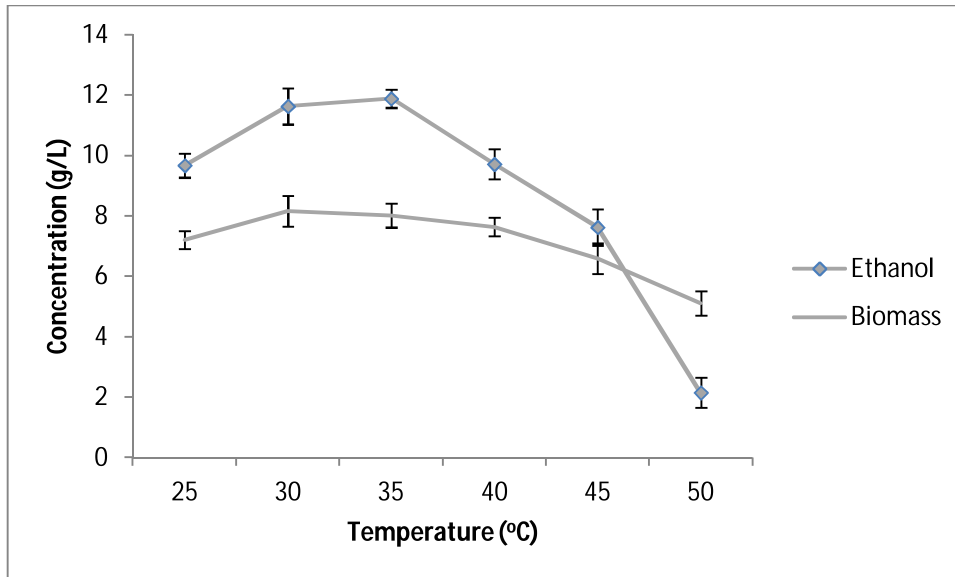

Figure 7-4 Ethanol and biomass production from crude whey fortified with peptone incubated at different temperature for 36 hour

7.4. DISCUSSION

7.4.1. Ethanol production from different whey sources

The pH of whey was variable from 3.1 to 4.5 which was very acidic; however, the acidity of whey in other studies was in the range of pH 4.3 and pH 5.0 depending upon the composition of milk, the variety of cheese made, the storage and handling mechanism of the milk, and the cheese-making process employed (Ariyanti and Hadiyanto, 2013; Koushki *et al.*, 2012). The low pH might reduce the ethanol concentration unless the yeast is low pH tolerant.

The lactose content of the whey was between 18.7 and 34.4 g/L (Table 7.1) which was lower than other reports (34.5 to 48.0 g/L) (Zafar and Owais, 2006; Ariyanti *et al.*, 2014). Relatively high lactose continent in whey (50-60 g/L) was also reported by Ozmihci and Kargi (2007). However, according to Christensen *et al.* (2011) lactose content can be as low as (19.3 g/L) depending upon storage room temperature and the load of lactose consuming lactic acid bacteria in whey.

The dry biomass of *K. marxianus* ETP87 that grew in different whey ranged from 5.48 to 7.67 g/L. This was slightly lower than the 8.9g/L biomass obtained from whey by *K. marxianus* MTCC 1288 after 22 hours of incubation (Zafar and Owais, 2006). In other studies, the application of fed batch fermentation process using *K. marxianus* at 30°C resulted in 13.4 g/L dry weight (Ariyanti *et al.*, 2014) and 4.43 g/L biomass (Ariyanti and Hadiyanto, 2013) indicating process conditions affect ethanol production from whey. Like ethanol, the lower biomass might be due to low lactose content of the whey.

7.4.2. Ethanol production from non-sterilized and sterilized whey

The ethanol concentration decreased as the whey was kept in refrigerator longer. However, the pattern of reduction was abrupt for the first 4-5 days in the non-sterilized whey compared to the steady decrease from the sterilized whey. Zoppellari and Bardi (2013) recommended that whey has to be stored at 4°C for no more than 3 days.

In general, storage condition potentially mattered a lot to control the lactic acid bacteria. As a result, cold storage condition helps to keep the lactose concentration maximum (greater than 4.5 g/L). Therefore, it is advantageous to properly store whey without pasteurization or sterilization before ethanol fermentation in order to reduce the production cost.

The highest ethanol production of ethanol (11.7g/L) was attained on both crude non sterilized and non refrigerated whey by the yeast. *K. marxianus* was able to ferment the lactose present in untreated crude whey with ethanol concentration of 7.9626 g/L in agitated culture (Ariyanti *et al.*, 2014). On the other study, 8.64 g/L ethanol was attained by *K. marxianus* from non sterilized crude whey within 16 hours of fermentation in agitated culture (Ariyanti and Hadiyanto, 2013). Therefore, it could be possible to produce ethanol from crude and non-sterilized whey using *K. marxianus* and hence the production cost could be reduced.

7.4.3. Effect of whey pH

pH affects strongly the nutrient (specially ions) transport across the membrane. Whey is acidic (less than 5) and hence it is mandatory to evaluate the effect of pH on ethanol production from whey by *K. marxianus* ETP87. A lower ethanol concentration was attained from whey in which its pH is not adjusted than pH adjusted. The lower ethanol production from untreated whey is due to the effect of lower pH on sugar utilization rate by yeasts (Kargi and Ozmıhcı, 2006).

However, the result suggests that *K. marxianus* ETP87 is capable of producing ethanol from crude whey without controlling its pH.

In this study, ethanol production by *K. marxianus* (10-12 g/L from untreated whey) was significantly higher than the amount produced (7.96 g/L ethanol) from non-pH adjusted acidic whey using fed batch mechanisms (Ariyanti *et al.*, 2014), and 8.64 g/L of ethanol produced from fermentation of crude whey without pH control (Ariyanti and Hadiyanto, 2013) under 20 hr incubation. The higher concentration in this study may be due to the longer incubation time (36 hours) that could enable the yeast to adapt to the acidity in whey. Likewise, Mohd Azhar *et al.* (2017) concluded that longer incubation period is required to achieve sufficient amount of ethanol production when the pH is lower (particularly, less than 4.0).

7.4.4. Effects of external nutrient additions to whey

Although crude whey is rich in protein, vitamins, and minerals, the content of directly usable form of nitrogen containing organic compounds such as proteosepeptone, peptides and low molecular weight products formed by the enzymatic degradation of the caseins is lower (Gabardo *et al.*, 2014). Therefore, the effect of nitrogen containing organic and inorganic compounds like peptone, yeast extract, urea, and ammonium sulfate on ethanol yield from crude whey were assessed. High ethanol concentration was attained from whey supplemented with peptone and ammonium sulphate; however, supplementation of yeast extracts and urea to whey reduced the ethanol concentration compared to unsupplemented control.

Some studies showed conflicting results regarding the addition of growth supplements to whey. For example, the fortification of growth supplements such as ammonium hydroxide (NH_4OH, 0.07%) and mono ammonium phosphate ($NH_4H_2PO_4$, 0.25%) to crude whey resulted in increased ethanol concentrations (by 13%) by *K. marxianus* (Koushki *et al.*, 2012). On the contrary, Kargi and Ozmıhcı (2006) indicated that highest ethanol yield was obtained in whey without any external N and P sources indicating that the whey contained sufficient nutrients for bio-ethanol production by the yeasts.

7.4.5. Effect of Temperature

Temperature affects membrane fluidity, nucleic acid stability, nutrient transport, and enzyme structure and function and hence it needs to know the optimum temperature for ethanol production. *K. marxianus* ETP87 which was inoculated to whey were incubated in the temperature range of 25 to 50°C in order to investigate optimum ethanol production. Optimum ethanol (11.8g/L) was obtained at 35°C.This was much lower than biomass yield (17 g/L), and similar to ethanol yield (8.1 g/L) under 30°C by *K. marxianus* (Ariyanti *et al.*, 2014).

Kourkoutas *et al.* (2002) found optimal ethanol (6.5 g/L) at 45°C using immobilized *K. marxianus*, but with a significantly lower (1.8 g/L) ethanol concentration at 50°C that might be related to ethanol loss due to evaporation. However, the boiling point of ethanol is near 78°C which is higher than the incubation temperature (50°C). On the other study, ethanol production sharply declined at 50°C due to greater ethanol inhibitory effect of ethanol at higher temperature (Banat *et al.*, 1996).

Generally, the ability of *K. marxianus* ETP87 to ferment lactose at higher temperature (40°C and higher) makes it to be good candidate for industrial application. Among the known yeast species used in fermentation processes, *K. marxianus* is thought to have the best performance for ethanol production at higher temperature (Mohd Azhar *et al.*, 2017).

7.5. CONCLUSIONS AND RECOMMENDATIONS

Keeping the non-sterilized whey in refrigerator at 4°C does not guarantee lactose existence without reduction and hence the whey has to be employed for ethanol production as soon as the whey is produced. Ethanol can be produced by *K. marxianus* ETP87 from non-sterilized, non-deproteinized, and non-pH adjusted whey.

The ability of *K. marxianus* ETP87 to produce ethanol from whey at 45°C confirms that the yeast is promising to apply for ethanol production from warm whey before it is dominated by lactic acid bacteria.

It is good to know the lactic acid bacteria and other microbe loads in the whey before ethanol is going to be produced from non-sterilized whey. Investigating the quantification of different

minerals (cations and anions), amino acids, other form of nitrogen, and vitamins present in whey is important to fully realize the production of ethanol from this substrate.

7.6. REFERENCES

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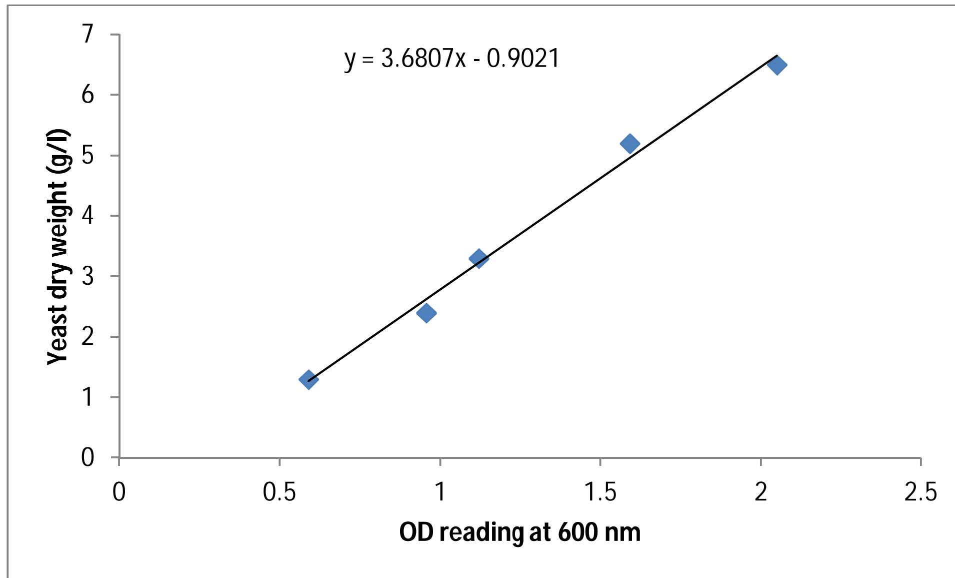
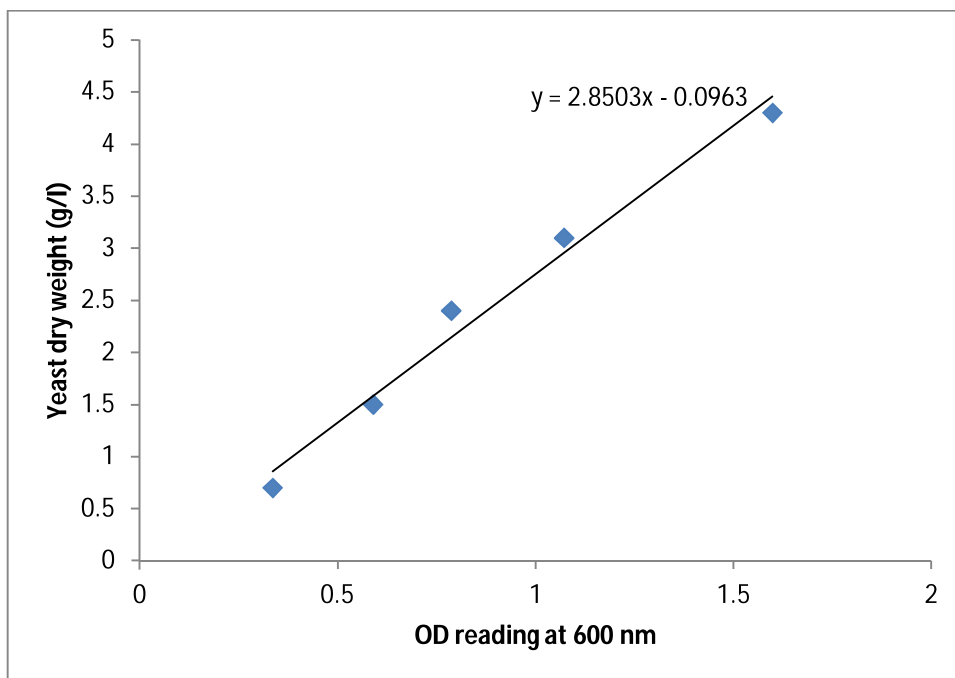
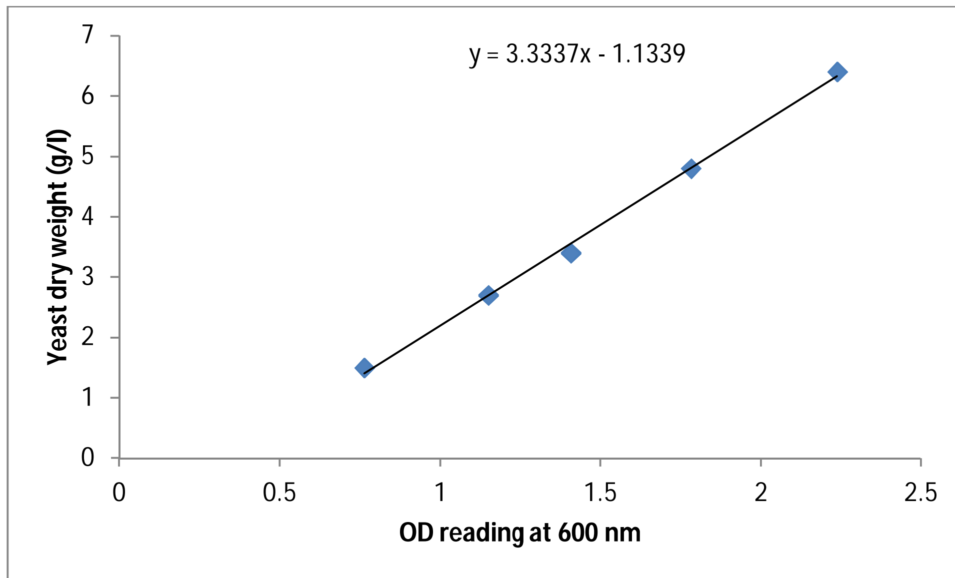
Appendix



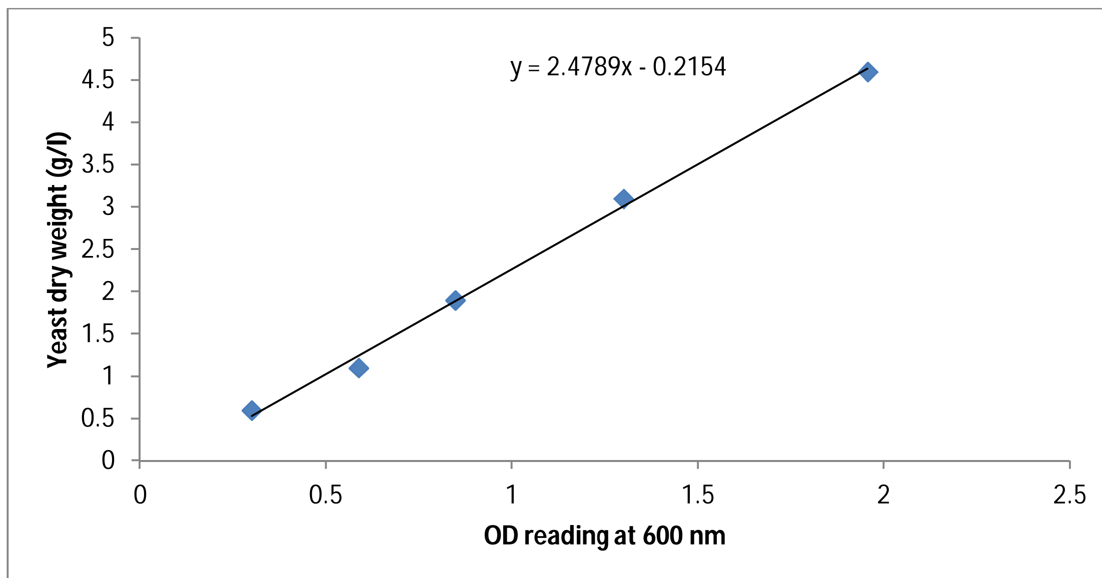
Appendix 1 Fig. 1 Standard curve of *Pichia fermentans* ETP22 for dry weight determination.



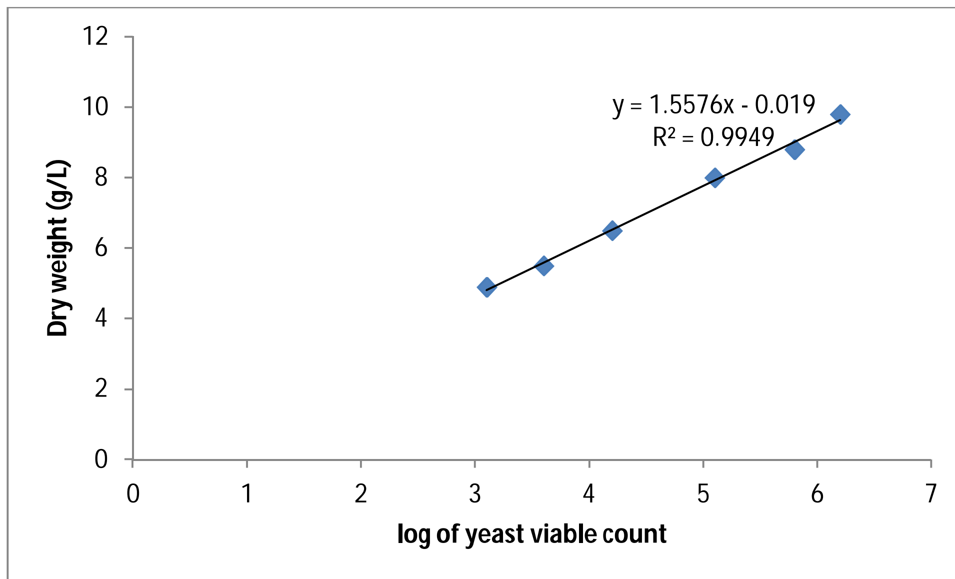
Appendix 1 Fig. 2 Standard curve of *Saccharomyces cerevisae* ETP53 for dry weight determination



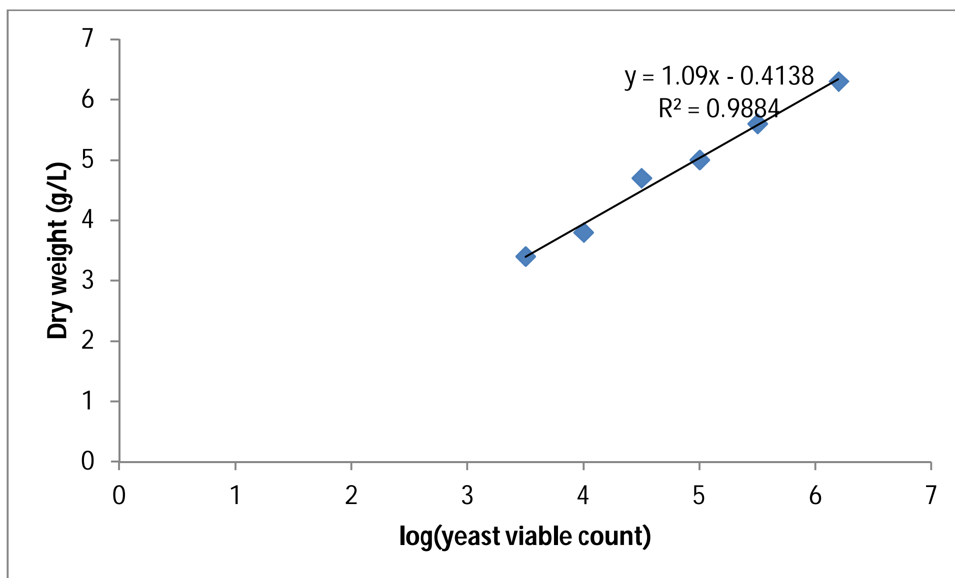
Appendix 1 Fig. 3 Standard curve of *Kluveromyces marxianus* ETP87 for dry weight determination.



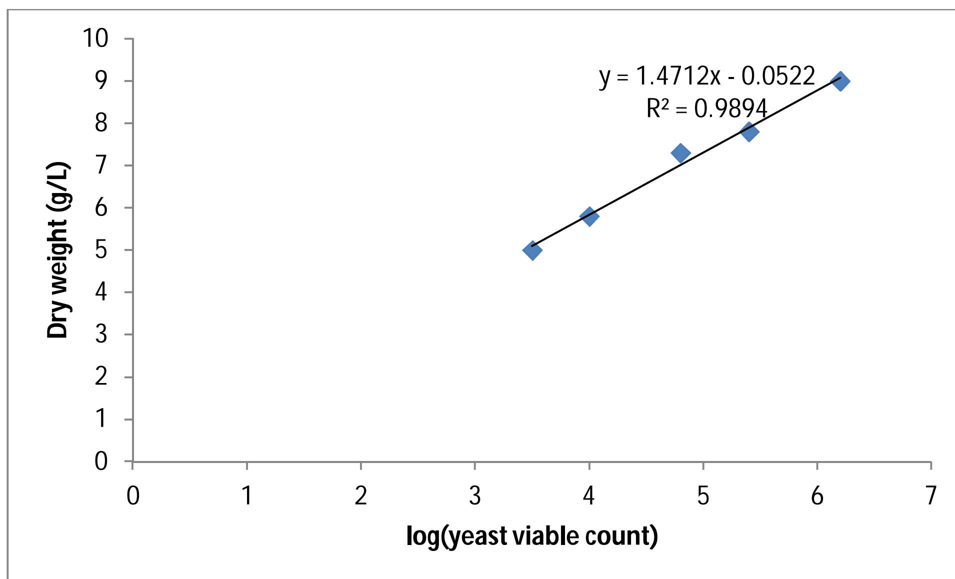
Appendix 1 Fig. 4 Standard curve of *Candida humilis* ETP122 for dry weight determination.



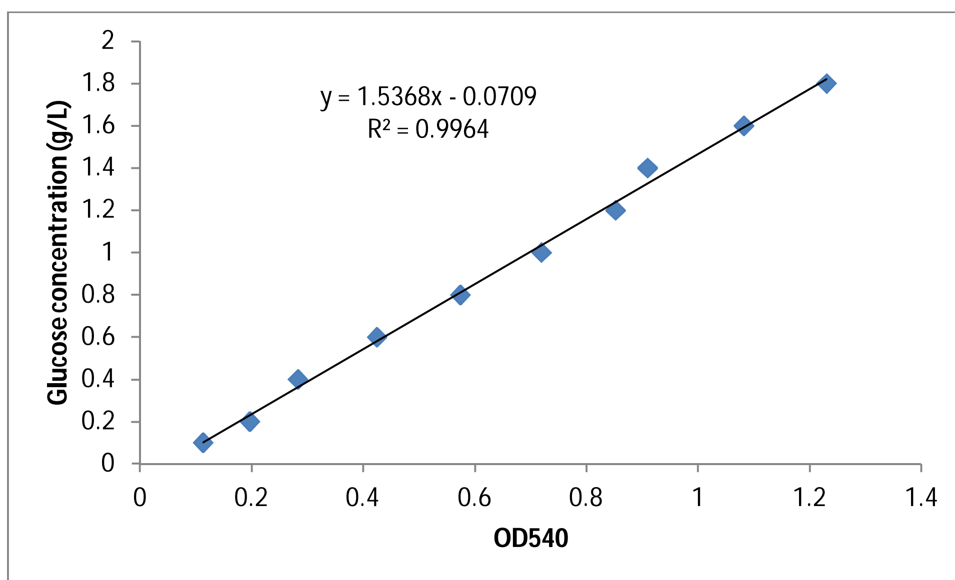
Appendix 1 Fig. 5 Dry weight Standard curve of *P. fermentans* ETP22 based on common log of number of yeast



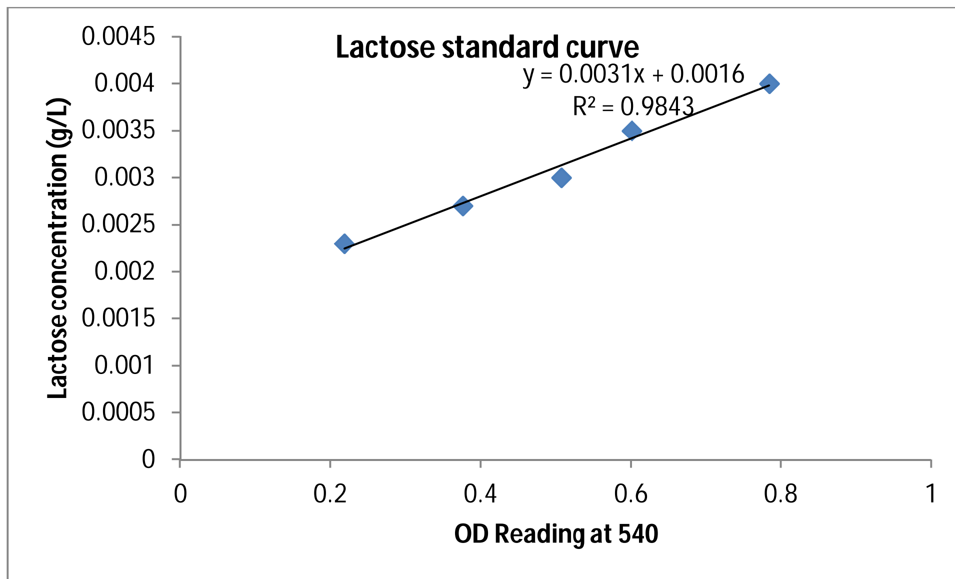
Appendix 1 Fig. 6 Dry weight Standard curve of *S. cerevisiae* ETP53 based on common log of number of yeast



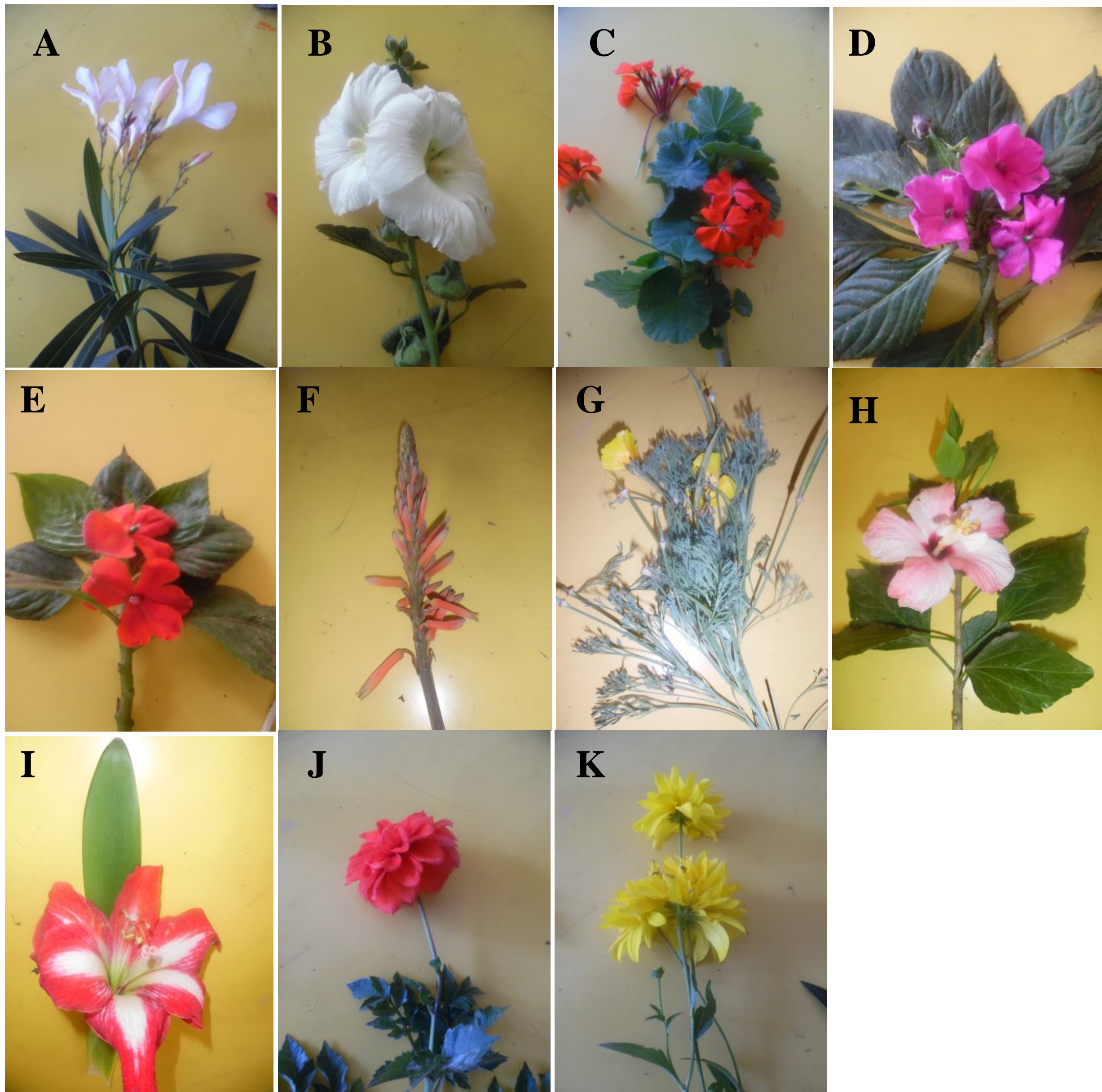
Appendix 1 Fig. 7 Dry weight Standard curve of *K. marxianus* ETP87 based on common log of number of yeast



Appendix 2 Fig. 1.Standard curve for reducing sugars using glucose as a standard



Appendix 2 Fig. 2. Standard curve for lactose determination



Appendix 3 Fig 1 Different flowers used for yeast isolation A) *Nerium oleander*, B) *Alta rosea*, C) *Pelargonium zonale*, D) and E) *Impatiens* New Gunea Hybrid, F) *Aloe excelsa*, G) *Eschscholzia californica*, H) *Hibiscus rosa-sinensis*, I) *Gladiolus* Hybrid Cultivar Grandiflorus, J) *Dahlia pinnata*, and K) *Chrysanthemum morifolium*