Isolation and characterization of yeast for bioethanol production, using sugarcane molasses.

A DISSERTAION

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SUBMITTED BY

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To whom it may concern

This is to certify that the thesis entitled "**Isolation and characterization of yeast for bioethanol production, using sugarcane molasses"** submitted by Armanul Naser is a record of student's own work carried out by him under our joint supervision and guidance in the Microbiology Laboratory, Department of Mathematics and Natural Sciences (MNS), BRAC University. It is further certified that the research work presented here is original, has not been submitted anywhere else for a degree and suitable for the partial fulfillment of the degree of Masters of Science in Biotechnology, BRAC University, Dhaka.

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DEDICATED

TO

MY BELOVED FAMILY

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Abstract:

In view of the anticipated shortage of the traditional supplies of fossil fuels there is a great deal of interest in the production of ethanol as an alternative biofuel in recent years. The present study describes the search for potential yeast isolates from various fruit peels capable of producing ethanol. The main objective of this research work was to isolate & characterize stress tolerant, high potential ethanol producing yeast strains from various fruit peel. Two yeast isolates Pa and Or have been characterized on the basis of morphological and physico chemical characters. Based on morphological appearance of vegetative cell under microscope, colony character and physico chemical characters the isolates were identified to be Yeast. Isolates were thermotolerant, pH tolerant, ethanol tolerant as well as osmotolerant. They were resistant to Chloramphenicol (30µg/disc) and nalidixic acid (30µg/disc). The isolates showed no killer toxin activity against E. coli. Ethanol producing capability of the strains were studied using sugarcane molasses as substrate .Ethanol production percentage was estimated by Conway method. The highest bioethanol production capacity of the yeasts were found to be 7.39% and 5.02 for Pa & Or respectively at pH 5.0, 30^{o}C temperature in media with, initial reducing sugar concentration 6.5% for Pa and 5.5% for Or isolate in shaking condition. Addition of metal ions increased the rate of ethanol production highest to 10.61% by KH_2PO_4. This study revealed that indigenous yeast isolates could be used to benefit the fuel ethanol, spirit and industrial alcohol industries.

List of Tables

Page number

List Of Figures

Page Number

LIST OF ABBREVIATIONS

pH- Negative logarithm of hydrogen ion concentration

ºC- Degree Centigrade

Temp- Temperature

Conc. - Concentration

DNS- 3, 5Dinitro salicylic acid

g or gm- Gram

hrs- Hours

fig. Figure

O.D- Optical density

µl- Micro liter

et.al.- And other people

Kg- Kilogram

L- Liter

mg- Milligram

ml- Milliliter

µm- Micrometer

Ppm- Parts per million

No. /no.-Number

V/v- Volume Per volume

W/v- Weight per volume

% - Percentage

/ - Per

α- Alpha

µ mol- Micro mole

ATP - Adenosine triphosphate

NADH - Nicotinamide adenine dinucleotide

Psi - Pound per square inch

Rpm - Round per minute

YPD - Yeast extract peptone dextrose

YMM- Yeast maintenance media

$CuSO_4$- Copper sulphate

$K_2Cr_2O_7$- Potassium dichromate

$MgCl_2$- Magnesium chloride

$CaCl_2$- Calcium chloride

CHAPTER 1. INTRODUCTION

1.1 Significance of the study

The energy crisis necessitates studying and discovering new processes involved in the production of renewable compounds as alternative energy sources among which fermentation of ethanol using renewable resources represents a significant alternative. In fact ethanol is being widely investigated as a renewable fuel source because in many respects it is comparable to gasoline fuel (Jones and Ingledew, 1993). This situation has led many countries including Brazil to use ethanol as a fuel especially from food crop. In Bangladesh sugarcane resource can be used to produce a variety of commercial products that can be marketed domestically, regionally and internationally. In economic and environmental terms, the three products that have special significance are sugar, ethanol, and electricity. Bangladesh through its potential in developing large sugarcane production can develop proper strategy of use ethanol as a fuel especially from cane sources.

Yeast alcohol is the most valuable product for the biotechnology industry with respect to both value and revenue. Approximately 80% of ethanol is produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae*. Yeast alcohol technology has undergone significant improvements during the last decade though profit margins are not attractive. Contamination, limited availability of raw materials and proper design of fermentation process are the major limitations causing reduced alcohol yields and quality. In view of the importance of alcohol as an alternative for liquid fuel, several investigations in ethanol fermentations are currently reported. The price of the sugar source is an important parameter when considering the overall economy of production and it is of great interest to optimize alcohol yields to ensure an efficient utilization of carbon sources (Bai et al., 2008; Carlos et al., 2011).

Bioethanol is an eco friendly fuel that can be used in unmodified petrol engines (Hansen et al., 2005). Combustion of ethanol results in relatively low emission of volatile organic compounds, carbon monoxide and nitrogen oxides. The emission and toxicity of ethanol are lower than those of fossil fuels such as petroleum, diesel etc. (Wyman & Hinman, 1990). Molasses a byproduct of sugarcane or sugar beet processing industries is widely used as a raw

material for the production of ethanol for economic reasons, and different strains of yeast have been selected for efficient ethanol production (Takeshige and Ouchi, 1995, Beuchat, 1983, Haegerdal et al., 1982). Utilization of molasses for the production of ethanol will provide value addition to the byproduct through fermentation. Molasses is the noncrystallizable residue remaining after sucrose purification. It has some advantages: it is a relatively inexpensive raw material, readily available, it does not require starch hydrolysis and has been already used for ethanol production. Molasses obtained after sugar beet processing contains about 60% sucrose and 40% other components. The non sucrose substances include inorganic salts, raffinose, ketose, organic acids and nitrogen containing compounds. Molasses is used in the production of baker's yeast, ethanol, citric, lactic and gluconic acids, as well as glycerol, butanol and acetone production, as an ingredient of mixed feeds and in the production of amino acids (Belitz et al., 2009, Satyanarayana et al., 2009). The fermentative yeast *Saccharomyces cerevisiae* is largely used in ethanol production using such renewable biomass as sugar cane or sugar beet molasses as the main carbon source. (Echegaray et al., 2000,Sanchez and Cardona, 2008). In the present study these types of *S. cerevisiae* were selected as production microorganisms on account of their commercial availability and an extensive application in food industry.

1.2 Objectives

- To isolate ethanol producing indigenous yeasts,
- To evaluate the efficiency of yeast isolates for ethanol production in cane molasses under different conditions,
- To determine the effects of different parameters on ethanol production,
- To optimize the conditions for maximal ethanol production.
- To determine the effect of metals on ethanol production.

1.3 Research hypothesis

After detailed characterization and optimization of physico chemical parameters for ethanol production of the selected yeast isolates, these can be used as a potential strain for ethanol production industrially.1.4 Expected results

Highly efficient stress tolerant yeast strain for ethanol production would be obtained. The yeast strain thus obtained could be useful for ethanol fermentation industry.

CHAPTER 2. REVIEW OF LITERATURE

Alcoholic fermentation has been carried out using a number of sugary materials depending upon their availability and suitability in particular geographic situations. Various raw materials like sugarcane juice and molasses (Morimura et al., 1997 and Agrawal et al., 1998), sugar beet, beet molasses (EI Diwany et al., 1992 and Agrawal et al., 1998), sweet sorghum (Bulawayo et al 1996) and starchy materials like sweet potato (Sree et al 1999), corn cobs and hulls (Beall et al., 1992 and Arni et al., 1999), cellulosic materials like cocoa, pineapples and sugarcane waste (Othman et al., 1992) and milk/cheese/whey using lactose hydrolyzing fermenting strains (Silva et al., 1995, Ghaly and Ben Hassan 1995) have been reported. The crucial aspects of microbial selection and adaptation include: substrate selection and preparation, suitable microbial strain selection and adaptation optimization of fermentation conditions and improvement of fermentation technology. Of these, simple sugar bearing materials are the easiest to process, since yeast ferment these directly while other carbohydrates like starch/cellulose have to be first hydrolyzed to fermentable sugars using current commercial technologies like: physio chemical and enzymatic preparations before they can be fermented to yield ethanol. The fermentable carbohydrates in molasses are sucrose and other sugars mainly glucose and fructose. The non sugars may consist of nitrogenous substances like gums, polysaccharides, wax, sterols, pigments and salts of calcium, potassium and magnesium (Rao, 1983).

The 1st generation biofuel faced many problems, the most known is food Vs energy crisis, due to the dependence on edible crops as food and feedstocks. Thus, there was a need for a 2nd generation technology which depends on non food sugary materials as feedstocks. Several advantages are offered by renewable energy resources such as being indigenous, increasing security of supply and reducing dependency on oil import (Jegannathan et al., 2011) and can contribute to a cleaner environment (Chaudhary and Qazi., 2006)

2.1 Yeast

Yeasts are unicellular fungi that can be classified into two phylogenetic groups i.e. teleomorphic and anamorphic ascomycetous or teleomorphic and anamorphic basidiomycetous yeasts that reproduce by budding or fission and that form their sexual states (i.e. asci), which are not enclosed in a fruiting body (Boekhout & Kurtzman, 1996; Kurtzman & Fell, 1998; Querol & Belloch, 2003). During sexual reproduction ascomycetous yeasts (i.e. yeasts of interest in this study) form asci, which contain varying numbers of ascospore shapes. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores (Van der Walt, 2000).

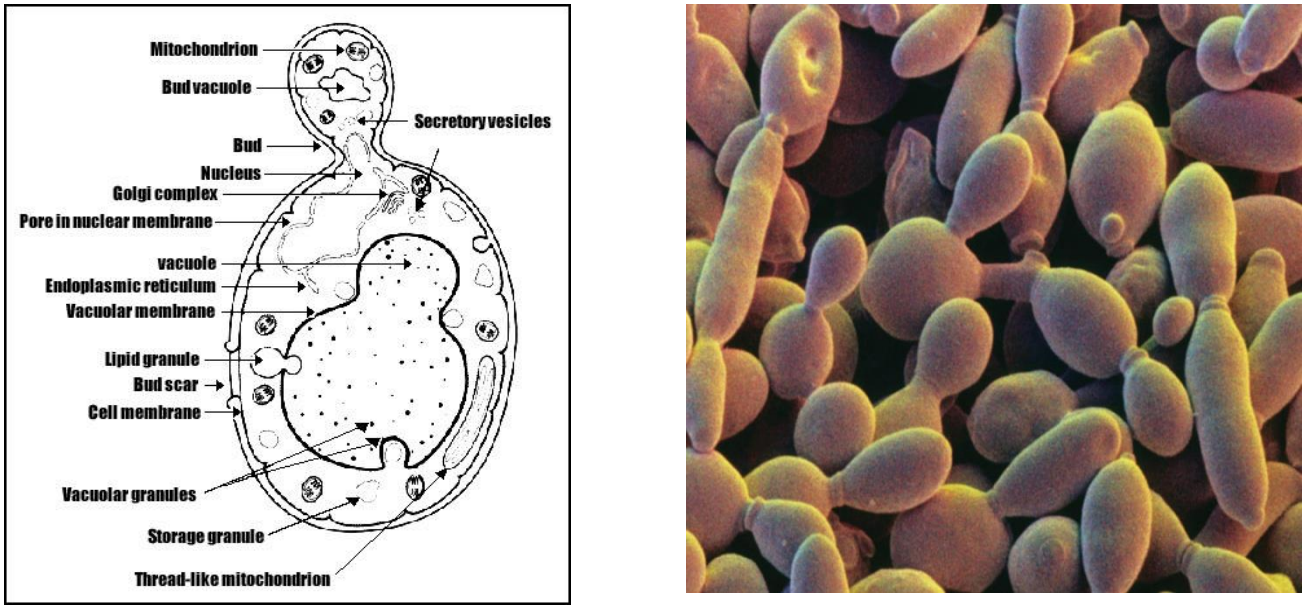


Fig 1 : Yeast cell morphology

2.1.1 Yeast and fermentation

For much of human history, alcohol (ethanol) production has been accomplished by 'wild' yeast strains i.e. the yeast population naturally resident on the fermentation feedstock or other ingredients. By the early 1900's, yeast strains were routinely selected from 'good' fermentations and used over and over again. This practice, despite the crude yeast propagation techniques being employed, led to improved consistency and quality of final products. When commercial yeast producers (driven mainly by the ever changing needs of the bread baking industry) emerged, they developed improved yeast propagation, quality control, and drying methods that further improved consistency and quality of ethanol production. Worldwide, nearly all ethanol production is accomplished using a single genus and species of yeast, namely *Saccharomyces cerevisiae* Many hundreds of yeast species have been identified in nature An unlimited number of strains are possible; literally thousands of yeast strains have been selected for specific purposes. Hundreds of specialty strains have been commercialized for ethanol or CO_2 production, including specific strains for baking, wine, beer, distilled beverages, and fuel ethanol. In practice, more than 90% of the glucose from starch is converted to ethanol and carbon dioxide by the yeast cell in fermentation mode.

Table 1: Ethanol production by different *S. cerevisiae* strains

Strain	% Ethanol produced
S.cerevisiae	5.8-11.16
Zygosaccharomyces sp.	4.2
S.ellipsoids	9.7
Schizo.pombe	8.7
Schizo.mallaeri	7.8

(ref. Recycling, residues of agriculture and industry, pp202, M.S.Kalra)

2.1.2 Yeast fermentation conditions

The yeast dose rate also has effect on performance. A higher dose rate will result in a faster start of fermentation, which helps to control contamination. Dose rates must be optimized for cost effective performance. Nutrition is another important parameter to take into consideration. For

optimal fermentation, yeast requires building block substances (C, N, P, S, O) in optimal ratios, minerals (e.g., K, Na, Mg, Ca, Zn, Fe, Mn, Cu, Co) and vitamins (B1, B5, B6, Biotin, etc.). Oxygen is normally present at very low levels in commercial scale ethanol fermentations. In practice, the process cannot be completely anaerobic because oxygen is required for production of unsaturated fatty acids that are essential for yeast growth and ethanol production. Most substrates for commercial ethanol production have been found by to be nitrogen limited. It is therefore recommended to add to grain-based ethanol fermentations yeast nutrients containing a nitrogen source usable by yeast. High ethanol concentration also stresses yeast. Avoidable yeast stress factors, such as high temperatures, high osmotic pressure, high sodium (and other ions) concentration, and high concentrations of organic acids

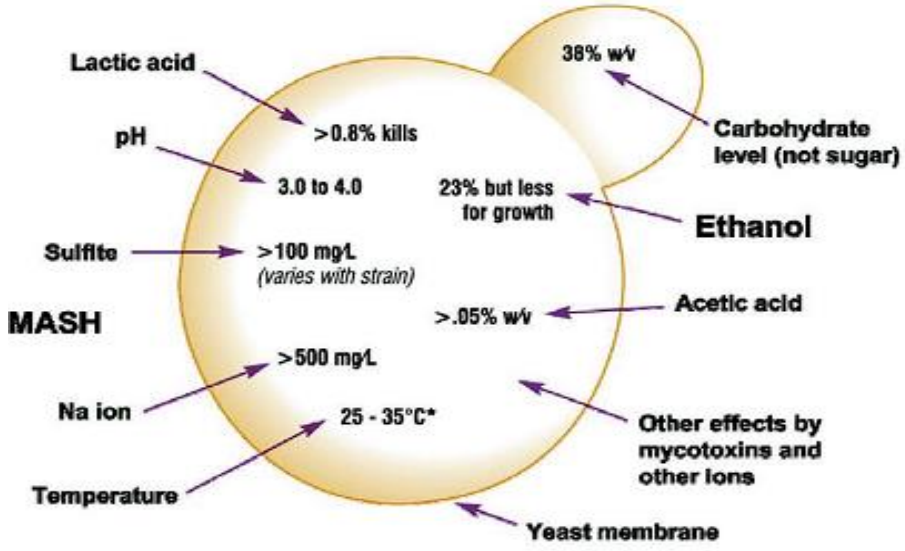


Fig 2 : Stress factors that effect yeast metabolism

2.1.3 Yeast strain selections

Some widely used, high alcohol productivity strains are *Saccharomyces cerevisiae*, *S.uvarum* (formerly *S.carlsbergensis*), and candidautilis. *Saccharomyces anamensis* and *Schizosaccharomyces pombe* are also used in some instances. Kluyveromyces species, which ferment lactose, are good producers of ethanol from whey. Ethanol production by yeast is characterized by high selectivity, low accumulation of byproducts, high ethanol yield, high

fermentation rate, good tolerance toward both increased ethanol and substrate concentrations, and lower pH value. Viability and genetic stability of yeast cells under process conditions and at high temperature are also desirable. Finding a strain that has all these characteristics is difficult. The most important ones used for making ethanol are members of the *Saccharomyces* genus, bred to give uniform, rapid fermentation and high ethanol yields, and be tolerant to wide ranges of, temperature, pH levels, and high ethanol concentrations. Yeasts are facultative organisms which mean that they can live with or without oxygen. In a normal fermentation cycle, they use oxygen at the start, and then continue to thrive once it has all been used up. It is only during the anaerobic (without oxygen) period that they produce ethanol (Basappa, 1989).

2.1.4. Sugar degradation pathways of yeasts

There are three pathways yeast (usually *Saccharomyces cerevisiae*) can obtain energy through the oxidation of glucose

a) Alcoholic fermentation under anaerobic conditions

The pyruvate resulting from glycolysis is decarboxylated to acetaldehyde (ethanal) which is reduced to ethanol. This pathway yields only two more molecules of ATP per molecule of glucose over the two resulting from glycolysis and of course is the major pathway in wine making.

b) Glyceropyruvic fermentation

During winemaking 8% of glucose follows this pathway and it is important at the beginning of the alcoholic fermentation of grape when the concentration of alcohol dehydrogenase (required to convert ethanal to ethanol) is low

c) Respiration under aerobic conditions

Glycolysis of glucose yields pyruvate and two molecules of ATP per molecule of glucose. Pyruvate is then oxidized to carbon dioxide and water via the citric acid cycle and oxidative phosphorylation. This pathway yields a further 36-38 molecules of ATP per molecule of glucose and obviously the yeast would prefer this route

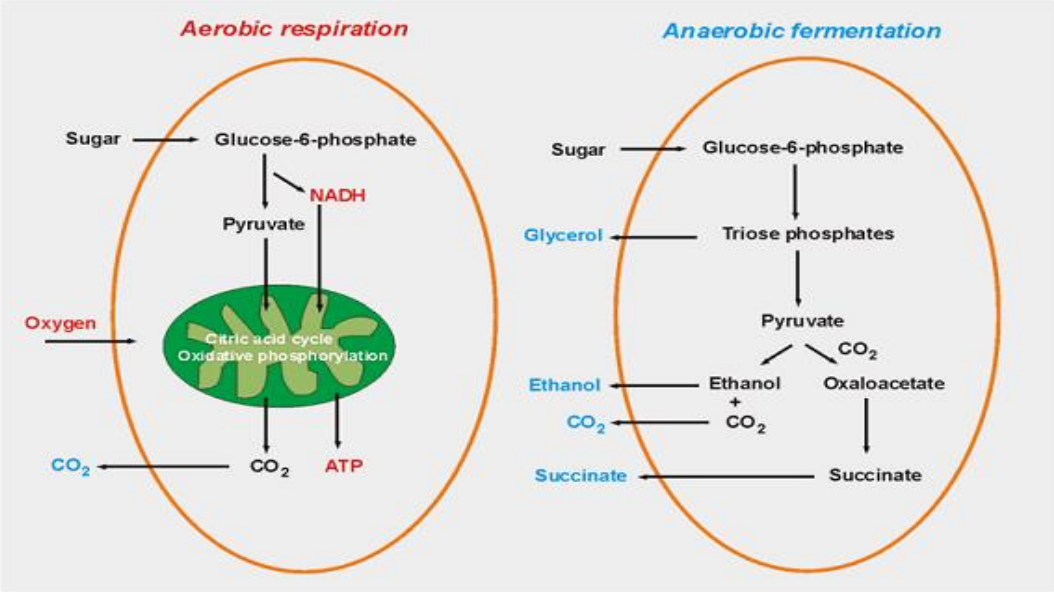


Fig 3: Metabolism of yeast under aerobic and anaerobic conditions

2.2 Bioethanol

Bioethanol from biomass sources is the principal fuel used as a petrol substitute for road transport vehicles. The high price of crude oil makes biofuels attractive (Bryner and Scott, 2006). Also, because biomass fuels are renewable, they help reduce greenhouse gas emissions from fossil fuels (Ibeto and Okpara, 2010). Bioethanol can be obtained from a variety of feedstocks including cellulosic, starchy and sugar sources. These feedstocks include corn, sugar cane, bagasse, sugar beet, sorghum, switch grass, barley, hemp, potatoes, sunflower, wheat, wood, paper, straw, cotton and other biomass materials. Currently the largest producers in the global biofuel are the United States and Brazil, where millions of tons of sugar are processed (Mobile Emissions Today, 2006). Although, at the moment bioethanol is mainly used in blends with gasoline as E10 and E20 (10 and 20% of ethanol mixed with 90 and 80% gasoline respectively), the demand has soared. For instance, consumption of bioethanol in most countries of the European Union is far greater than the quantity produced in those countries (Wikipedia, 2009a). According to a study by Hart's Global Biofuels Center (a division of Hart Energy Publishing LP, one of the world's largest energy industry publishers), the Global biofuel use may double by 2015 (Johnson, 2009).

2.3 Raw materials for bioethanol production

- Sugars
- Starch
- lignocellulosic biomass

2.3.1 Ethanol from sugars

Main feedstock for ethanol production is sugar cane in the form of either cane juice or molasses (by product of sugar mills). About 79% of ethanol in Brazil is produced from fresh sugar cane juice and the remaining percentage from cane molasses (Wilkie et al., 2000). Sugar cane molasses is the main feedstock for ethanol production in India; cane juice is not presently used for this purpose (Ghosh and Ghosh, 2003). Beet molasses are other source of fermentable sugars for ethanol fermentation. The most employed microorganism is *Saccharomyces cerevisiae* due to its capability to hydrolyze cane sucrose into glucose and fructose, two easily assimilable hexoses. Aeration is an important factor for growth and ethanol production by *S. cerevisiae.* Although this microorganism has the ability to grow under anaerobic conditions, small amounts of oxygen are needed for the synthesis of substances like fatty acids and sterols. The oxygen may be supplied through the addition to the medium of some chemicals like urea hydrogen peroxide (carbamide peroxide), which also contributes to the reduction of bacterial contaminants as claimed in the patent of (Narendranath et al., 2000). Other yeasts, such as *Schizosaccharomyces pombe* , present the additional advantage of tolerating high osmotic pressures (high amounts of salts) and high solids content (Bullock., 2002; Goyes and Bolan os, 2005). In fact, a fermentation process using a wild strain of this yeast has been patented (Carrascosa., 2006).

2.3.2 Bioethanol from molasses

Production of ethanol from molasses constitutes part of the sugar refining process. The overall process consists of the following steps.

- **Crushing:** Sugar cane is chopped at a sugar mill to facilitate handling and processing
- **Sugar cane extraction:** This is effected in a counter current flowing warm water. The solids after extraction (bagasse) containing less than 0.5% sugar are squeezed dried to remove maximum sugar solution (Liquor).

- **Raw sugar production:** Sugar-containing liquor is concentrated in evaporators. Crystalline sugar is separated in centrifuges. This process is repeated several times yielding raw sugar.
- **Fermentation from molasses:** Liquid residue from sugar production (molasses) containing approximately 50% sugar and 50% mineral matter is mixed with yeast and minerals and is used as fertilizer.
- **Distillation:** The fermented mash, now called "beer" contains about 10% alcohol as well as all the non fermentable solids from the feedstock and the yeast cells. The mash is pumped to a continuous flow, multicolumn distillation system where the alcohol is removed from the solids and the water. The alcohol leaves the top of the final column at about 96% strength and the residue mash called stillage is transferred from the base of the column to the co product processing area.
- **Denaturing:** Ethanol that will be used for fuel is denatured at the time of the transport with a small amount (0-5%) of some product such as gasoline to make it unfit for consumption (Beer *et al*., 2006).

Table 2: Some fermentation process for ethanol production from sugarcane molasses using *S. cerevisiae*

Some fermentation processes for ethanol production from sugar cane molasses using *S. cerevisiae*

Regime	Configuration	Ethanol conc. in broth, g/L	Productivity, g/(L h)	Yield, % of theor. max.	References
Batch	Reuse of yeast from previous batches; yeast separation by centrifugation	80–100	1–3	85–90	Claassen et al. (1999)
Fed-batch	Stirred tank with variable feeding rate (exponent. depend. with time)	53.7–98.1	9–31	73.2–89	Echegaray et al. (2000)
Repeated batch	Stirred tank; flocculating yeast; up to 47 stable batches	89.3–92	2.7–5.25	79.5–81.7	Morimura et al. (1997)
Continuous	CSTR; cell recycling using a settler; flocculating yeast; aeration 0.05 vvm	70–80	7–8		Hojo et al. (1999)
	Biostill; residence time 3–6 h; cell recycling by centrifugation; recycled stream from distillation column to fermentor	30–70	5–20	94.5	Kosaric and Velikonja (1995)
Continuous removal of EtOH	Removal by vacuum; cell recycling	50	23–26.7		Costa et al. (2001); da Silva et al. (1999)

2.3.3 Ethanol from starch

Starch is a high yield feedstock for ethanol production, but it needs to be hydrolysed to simple sugar for use in ethanol fermentation. Starch was traditionally hydrolyzed by acids, but the specificity of the enzymes, their inherent mild reaction conditions and the absence of secondary reactions due to their specificity have made the amylases to be the catalysts of choice generally to be used for this process.

Ethanol is produced almost exclusively from corn in the USA. Corn is milled for extracting starch, which is enzymatically treated for obtaining glucose syrup. Then, this syrup is fermented into ethanol. Fermentation is performed using *S. cerevisiae* and is carried out at 30–32 °C with the addition of ammonium sulfate or urea as nitrogen sources. Proteases can be added to the mash to provide an additional nitrogen source for the yeast resulting from the hydrolysis of corn proteins (Bothast and Schlicher., 2005).

Although in France ethanol is mostly produced from beet molasses, it is also produced from wheat by a process similar to that of corn. Some efforts have been made for optimizing fermentation conditions. For example, Wang et al., 1999, have determined the optimal fermentation temperature and specific gravity of the wheat mash.

Cassava represents an important alternative source of starch not only for ethanol production, but also for production of glucose syrups. Cassava starch has a lower gelatinization temperature and offers a higher solubility for amylases in comparison to corn starch. The hydrolysis of cassava flour has been proposed for the production of glucose in an enzymatic hollow fiber reactor with 97.3% conversion (Lo´ pez Ulibarri and Hall., 1997). Cassava flour production is more simple and economic than cassava starch production.

2.3.4 Ethanol from lignocellulosic biomass

The importance of lignocellulosic biomass as a feedstock for ethanol production is evident. Lignocellulosic complex is the most abundant biopolymer in the Earth. It is considered that lignocellulosic biomass comprises about 50% of world biomass and its annual production was estimated in 10–50 billion ton (Claassen et al., 1999). In general, prospective lignocellulosic materials for fuel ethanol production can be divided into six main groups: crop residues (cane bagasse, corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose wastes (newsprint, waste office paper, recycled paper sludge); and herbaceous biomass (alfalfa hay, switchgrass, reed canary grass, coastal Bermudagrass, thimothy grass),

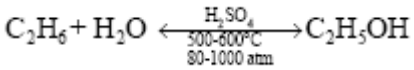
Table 3: Different substrates for ethanol production and their comparative production potential

Crop	Ethanol production potential (L/ton)
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other cellulose biomass	280

Source: Nigam and Agarwal, 2004.

2.4 Bioethanol production processes:

Bioethanol fuel is mainly produced by the sugar fermentation process, although it can also be manufactured by the chemical process of reacting ethylene with steam.



So far, industries have adopted saccharification and fermentation process for producing bioethanol from woody biomass, where woody biomass is first dissolved into sugars and then the sugars are fermented by yeast and changed into ethanol that is finally refined by distillation.

Glucose (a simple sugar) is created in the plant by photosynthesis.

$6\ CO_2 + 6\ H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6\ O_2$

During ethanol fermentation, glucose is decomposed into ethanol and carbon dioxide.

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 + \text{heat}$

Fermentation is the slow decomposition by micro-organisms of large organic molecules (such as starch) into smaller molecules such as ethanol. Ethanol fermentation can be described as the biochemical process by which sugar such as glucose, fructose and sucrose are converted into cellular energy thereby producing ethanol and carbondioxide as metabolic waste products. Yeasts carry out ethanol fermentation on sugar in the absence of oxygen. Because the process does not require oxygen, fermentation is classified as anaerobic.

During combustion, ethanol reacts with oxygen to produce carbon dioxide, water and heat:

$C_2H_5OH + 3\ O_2 \rightarrow 2\ CO_2 + 3\ H_2O + \text{heat}$

Bioethanol can be manufactured from numerous sources. They can be produced from raw materials containing fermentable sugars such as sucrose rich feedstock namely juices, sugarcane

and beet etc. They can also be produced from some polysaccharides that can be hydrolyzed for obtaining sugars that can be converted to ethanol (Cardona and Sanchez, 2007). Starch contained in grains is the major polymer used for ethanol production. Lignocellulosic biomass (a complex polysaccharide) is the most promising feedstock considering its great availability and low cost. However large scale commercial production from cellulosic materials is still a major challenge.

2.5 Benefits of bioethanol

Domestic production and use of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits (Charles *et al*., 1992), create jobs in rural areas, reduce air pollution, global climate change and carbon dioxide build up (Ofoefule *et al*., 2009) bioethanol, unlike gasoline, is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NOx emissions from combustion (Lang *et al*., 2001).

Ethanol provides energy that is renewable and less carbon intensive than oil. Bioethanol reduces air pollution due to its cleaner emissions and also contributes to mitigate climate change by reducing greenhouse gas emissions (GHG). In comparison to gasoline utilization, reduction of greenhouse gas emissions occurs, since much carbon dioxide is taken up by the growing plants as is produced when the bioethanol is burnt, with a zero theoretical net contribution. Several studies have shown that sugarcane-based ethanol reduces greenhouse gases by 86 to 90% if there is no significant land use change (Isaias *et al*., 2004; Goettemoeller and Goettemoeller, 2007) and ethanol from sugarcane is regarded as the most efficient biofuel currently under commercial production in terms of GHG emission reduction (Rajagopal and Zilberman, 2007).

Since, bioethanol is of plant origin, the carbon dioxide (CO_2) emitted when it is blended with gasoline and burned is not counted as greenhouse gas emissions, it is recaptured as a nutrient to the crops that are used in its production. Bioethanol is appropriate for the mixed fuel in the gasoline engine because of its high octane number. Its low cetane number and high heat of vaporization impede self ignition in the diesel engine. Since, it is an octane enhancing additive and removes free water which can plug fuel lines in cold climates; ignition improver, glow plug, surface ignition and pilot injection are applied to promote self ignition by using diesel bioethanol blended fuel (Lang *et al*., 2001). Some countries have already been blending gasoline with

bioethanol produced from such materials as sugar cane. In Japan, a blended fuel containing three percent bioethanol was introduced in fiscal 2004 (Anonymous, 2005).

2.6 Disadvantages of bioethanol

Disadvantages of bioethanol include its lower energy density than gasoline, its corrosiveness, low flame luminosity, lower vapor pressure (making cold starts difficult), miscibility with water and toxicity to ecosystems (Mustafa *et al*., 2008). For ethanol to be currently economically viable, requires massive Government Federal subsidies and price supports. In the USA, even the biggest of proposed ethanol supports an increase in mandated ethanol consumption from 7.5 billion gallons a year to 15 billion gallons a year, as called for in their energy bill by congress would barely dent America's oil consumption, which is approximately 150 billion gallons annually (Wikipedia, 2007).

Scientists report that ethanol may end up contributing to global warming more than fossil oil, where rainforests are destroyed to produce it. This is also applicable to other biofuels such as biodiesel (Sanchez, 2007). Another concern about ethanol as a gasoline substitute is the fact that only about 5 million automobiles out of 135 million currently in America are Flexible Fuel Vehicles (FFV) cars that are equipped to run on a blend of 85% ethanol and 15% gasoline (known as E85).

2.7 Factors affecting fermentation of ethanol

2.7.1 Effect of temperature on ethanol fermentation

Ethanol production by thermotolerant yeasts has been extensively studied, because thermotolerant yeasts are capable of growth and fermentation during the summer months in non-tropical countries as well as under tropical climates (Ueno et al ., 2001). Cooling costs during the process of ethanol production are expensive; hence, by using thermotolerant yeasts cooling and distillation costs can be reduced (Sree et al., 1999; Ueno et al., 2002). Considerable difficulties are associated with fermentation in tropical areas owing to the lack of heat tolerance in conventional industrial yeast strains. These difficulties include high ambient temperature, especially in the summer, coupled with an exothermic fermentation reaction, the compound effect of which leads to inhibition of yeast fermentation ability (Anderson et al., 1986). As the

temperature increases, productivity decreases sharply because of greater ethanol inhibition (Sree et al., 1999). Moreover, Anderson et al., (1986) and Ueno et al., (2003) reported that thermotolerant yeast can produce > 6% ethanol within 24 hours at 40 °C

Stress due to temperature has been the most studied abiotic factor, where both heat and cold induce the synthesis and storing of a group of proteins that increase stress resistence (Hiraishi et al., 2006; Yacoob and Filion 1987). Most of the time yeast performances are researched to valorize biomass in this way it is necessary to find thermo tolerant yeast. For an efficient conversion of fibrous biomass to ethanol another desirable microorganism characteristics is the capacity to ferment other sugars beside glucose, such as d-xylose and cellobiose obtained from hydrolysis of the material (Banat et al., 1988; Miller 1969).

2.7.2 Effect of metal ions on ethanol fermentation

The role of biogenic microelements in the metabolic state of microorganisms and higher organisms (human and animal) has become an especially interesting field for research (Huges et al., 1991). Microelements play an important role in the cellular metabolism, primarily due to their requirements as cofactors for a large number of enzymes (Gadd et al., 1992 and Berg et al., 2002). Apparently, metal ions are vital for all organisms, and therefore ion transporters play a crucial role in maintaining their homeostasis. The number of studies of the processes involved in the uptake of trace metals by the yeast *Saccharomyces cerevisiae* has increased considerably in recent years. This yeast has become a model microorganism for studying metal transporters and their accumulation in the cells (Nelson 1999 and Cohen et al., 2000). However, excess amounts of the same metal ions are toxic and can cause damage to the function that they serve. Yeasts are known for their ability to accumulate metal ions from aqueous solutions by different physicochemical interactions, e.g. by adsorption and absorption, or by a metabolism dependent mechanism (Gadd 1990 and Brady et al., 1994). Sorption processes are dependent on disposable functional groups on the cell surface and on the nature of metal ions. Thus, the concentration of free ions, ligand electronegativity, metal cation, ligand charge and the cavity size have a great influence on the selectivity of metal uptake (Frausto da silva et al., 1976 and Williams 1981). Furthermore, the composition of nutritive medium to which the microorganism is exposed affects the amount of metal uptake (Grupa et al., 1992), because of the cell wall structure and the metabolic state of the cell (Gadd 1990 and Norris et al., 1977). Therefore, it may be taken in

consideration that the growth in different media can influence the capacity and the selectivity of metal uptake by creating other binding sites or diverse enzymatic systems within the cells.

2.7.3 Effect of ethanol concentration on fermentation

Ethanol is an inhibitor of yeast growth at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol concentrations reduce cell vitality and increase cell death (Birch and Walker 2000). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of petite mutations, altering metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu et al., 2007). The main sites for ethanol effects in yeast are cellular membranes, hydrophobic and hydrophilic proteins and the endoplasmic reticulum (Walker, 1998). For both ethanol stress and heat shock, vacuole morphology is altered from segregated structures to a single, large organelle (Meaden et al., 1999). Membrane structure and function appear to be a predominant target of ethanol. Exposure of yeast to ethanol results in increased membrane fluidity and consequential decrease in membrane integrity (Mishra and Prasad, 1989). A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzymes and these proteins may be denatured (Hallsworth et al., 1998).

A recent study used high resolution quantitative analysis to examine the growth behavior of a *S. cerevisiae* SGKO collection in the presence of 8% (v⁄v) ethanol (Yoshikawa et al., 2009). Liquid cultures were used to determine the specific growth rates of each deletion strain in the absence or presence of ethanol stress, facilitating more sensitive analyses of growth performance compared to previous gene deletion screens using ethanol supplemented agar plates (Kubota et al., 2004; Fujita et al., 2006; van Voorst et al., 2006). After excluding results for strains that had growth defects in the absence of ethanol, 446 deletion strains were observed to be ethanol sensitive, and 2 deletion strains were ethanol tolerant (Yoshikawa et al., 2009).

Jimenez and Benitez (1986) and Du Preez et al., (1987) pointed out that ethanol tolerance is particularly important since ethanol tolerance can hardly be avoided during fermentation although substrate inhibition can be avoided through stepwise addition of substrate.

2.7.4 Effect of salt concentration on ethanol fermentation

Microorganisms such as the yeast, *Saccharomyces cerevisiae,* develop systems to counteract the effect of osmotic stress such as salt stress (NaCl) (Andreishcheva et al., 1999). Salt induced stress results in two different phenomena: ion toxicity and osmotic stress (Mager et al., 2001). Defense responses to salt stress are based on osmotic adjustments by osmolyte synthesis and cation transport systems for sodium exclusion (Yancey, 2005). Exposing yeast cells in a hyper osmotic environment leads to a rapid cell dehydration and arrest cell growth (Yale, 2001). Under these conditions, cellular reprogramming or "adaptation" represents major defenses including accumulation of compatible solutes to balance the intracellular osmotic pressure with the external environment (Gacto et al., 2003). Under stress conditions yeast cells enhance intracellular accumulation of osmolytes, and polyols in particular glycerol and compatible ions such as, amino acids and fatty acids in cell membranes (Butinar et al., 2005). Other products synthesized by yeast during stress conditions are trehalose and glycogen that may collectively represent 25% of the dry cell mass depending on the environmental conditions (Herdeiro et al., 2006). The disaccharide trehalose accumulates has been shown to protect cells against high temperature by stabilizing proteins and maintaining membrane integrity (Cipak et al., 2006). Elevated osmotic pressure of the medium also activates the plasma membrane H+-ATPase (Hamilton et al ., 2002) and the Na+/H+ antiporter which use the proton electrochemical gradient on the plasma membrane as a driving force to eject excessive intracellular Na+ (Kogej et al ., 2007). In some yeasts adaptation to higher salinity is accompanied by an increased viscosity of the membranes due to an increased level of unsaturated fatty acids or an increased length of fatty acyl residues (Gostincar et al ., 2008).

2.7.5 Effect of pH on ethanol fermentation

Control of pH during ethanol fermentation is important for two reasons:

1) The growth of harmful bacteria is retarded by acidic solution.

2) Yeast grows well in acidic conditions (Mathewson, 1980). With increase in pH yeast produces acid rather than alcohol. Molasses has naturally alkaline pH and must be acidified prior to fermentation (Hodge and Hildebrandt, 1954).

The rate of ethanol production by yeast cells is highly affected by the pH of the fermentation medium. More acidic and basic conditions, both retard the yeast metabolic pathways and hence the growth of cells (Willaert & Viktor, 2006). Productivity may decrease by increase and decrease in pH due to the lower metabolic rate of the yeast cells. It may also be due to the growth of other microbes with the increase in pH, as the fermentation carried out without sterilization (Amutha & Paramasamy, 2001; Kourkoutas et al., 2004). In addition, pH of the surrounding medium change the configuration and permeability of the cell membrane thus reduced the rate of sugar fermented enzymes There are some evidences that the rates of yeast growth and fermentation are decreased as the initial pH is decreased towards 3.0 (Heard et al.,1988 and Ough 1966) however the yeast strains examined in another study (Charoenchai et al., 1998) exhibited similar growth behaviour at either pH 3.0, 3.5 or 4.0, and there was no firm evidence that initial pH over the range 3.0 to 4.0, would provide a selective growth advantage to any one particular species.

2.7.6 Effect of sugar concentration on ethanol fermentation

High substrate concentrations are inhibitory to fermentation (Jones et al 1981) due to osmotic stress. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. Hexose sugar is the primary reactant in yeast metabolism. Under fermentative condition, the rate of ethanol production is related to the available sugar concentration (Park and Sato, 1982; Atiyeh and Duvnjak, 2001). At very low substrate concentration, the yeast starved and productivity decreases (Levenspiel, 1980). An important secondary effect of higher sugar content is catabolite repression of the oxidative pathways (Moss et al., 1971). Higher concentration of sugars in case of sugarcane juice media causes metabolic repression of yeast which results reduced yield of ethanol. Amount of sugar utilization and ethanol production varied in the presence of different sugars like mono & disaccharides. This is because monosaccharides such as glucose is transported across cell membrane by a simple facilitated transport mechanism whereas disaccharides like sucrose is converted to glucose & fructose at the cell surface using invertase which is present in yeast cell wall (Johannes et al., 1994). In this way variation in ethanol content shown by yeast cells as a result of variable sugars used as a carbon source is due to their different pathways of metabolism.

It is observed that higher utilization of sugars corresponds to higher protein utilization by yeast cells, and ultimately higher ethanol yield.

2.7.7 Effect of inhibitory effect on ethanol fermentation

Monomeric sugars are not the only products of dilute acid hydrolysis, since liberated sugars undergo secondary reactions and are converted to other chemical compounds which are mostly toxic for fermenting microorganisms (Klinke et al., 2004; Larsson et al., 1999). Monosaccharides, especially those produced in the early stage of dilute acid hydrolysis (mostly from hemicellulose), are decomposed to furan derivatives, namely furfural and HMF. The formation of furfural and HMF from lignocellulosic materials is a first order reaction (Rodriguez Chong et al., 2004; Saeman, 1945). The furan derivatives can be further converted to formic acid and levulinic acid. Acetyl group in hemicelluloses through the hydrolysis can result in acetic acid in the resultant hydrolyzate. These weak acids with relatively high pKa at the higher concentrations have negative impact on the cell viability, even though their toxicity is pH dependent. Undissociated forms of these acids can penetrate through the plasma membrane and dissociate in the cytoplasm and disturb neutral intracellular pH. Phenolic compound mostly originate from lignin decomposition and to a minor extent from the aromatic wood extractives (Taherzadeh and Karimi, 2007). Inhibition mechanism in phenolic compound and *S cerevisia* and other eukaryotic microorganisms have not yet been completely elucidated, largely due to the heterogeneity of the group and the lack of accurate qualitative and quantitative analyses. Phenolic compounds may partitione into biological membranes causing loss of integrity thereby affecting their ability to serve as selective barriers and enzyme matrices (Almeida et al., 2007; Palmqvist and Hahn Hägerdal, 2000). Low molecular weight (MW) phenolic compounds are more inhibitory to *S. cerevisiae* than high MW compounds. Treatment of hydrolyzate with laccase, a lignin oxidizing enzyme, resulted in less inhibition of fermentation (Jönsson et al., 1998).

2.7.8 Effect of oxygen on ethanol fermentation

The microorganisms involved in ethanol fermentation are facultative microbes since they are able to grow with or without the utilization of oxygen. Thus, two of different pathways of pyruvate metabolism are available (Abbott, 2005). In the presence of oxygen, more cell biomass is produced from initial substrate and the growth rate is increased (Alfenore, 2005). However

for ethanol production, oxygen must be restricted from entering the fermenter. But, small concentration of oxygen must be proved to the fermenting yeast as it is a necessary component for the biosynthesis of polyunsaturated fats and lipids (Cysewaski and Wilke, 1977 and Sa'nchez and Cardona, 2008). According to Ksaric and Vardar sukan(2001), typical amount of oxygen maintained in the broth is 0.05-0.10 mmHg. Any value higher than this will promote cell growth at the expense of ethanol productivity. The oxygen concentration which triggers aerobic or anaerobic growth processes, is however, varies from culture to culture depending on substrate concentration and cell density (Munnecke, 1981).

2.8 Bioethanol production and utilization status in different parts of the world:

The global annual potential bioethanol production in the world as a whole are from the major crops such as corn, barley, oat, rice, wheat, sorghum and sugar cane. Lignocellulosic biomass such as crop residues and sugar cane bagasse are included in feedstock for producing bioethanol as well. Asia is the largest potential producer of bioethanol from crop residues and wasted crops and could produce up to 291 GL year of bioethanol. Rice straw, wheat straw and corn stover are the most favorable bioethanol feedstocks in Asia. The next highest potential region is Europe (69.2 GL of bioethanol), where most bioethanol comes from wheat straw**.** Corn stover is the main feedstock in North America, from which about 38.4 GL $year^{-1}$ of bioethanol can potentially be produced. Globally rice straw can produce 205 GL of bioethanol, which is the largest amount from single biomass feedstock. The next highest potential feedstock is wheat straw**,** which can produce 104 GL of bioethanol (Kim and Dale, 2004).

In 1993 only 60 million litres of bioethanol was produced in Europe compared to a little over 900 million litres the previous year. From 2004 to 2005 there was an increase of almost 70%. This increase can be explained by: (1) higher production in France due to new government support, (2) crisis in distillation measures in the wine sector resulting to a much bigger volume of wine alcohol in the market and (3) growth in Germany because of new production capacity coming on-stream (Miguel, 2006).

EU production in 2005 was around 900 million litres. However, total consumption was close to 1,200 million litres. Sweden, Germany and the UK consumed much more than they produced and are therefore strong import markets while Spain is a strong exporter. Production in Sweden

is about 60 million litres home-grown production while the remaining is about 100 million litres of wine alcohol converted to fuel grade ethanol. Traditionally, it was Spain buying the highest volumes, but in 2005 Sweden took over. In comparison with the USA and Brazil, EU ethanol for fuel production is still very modest. The two giants are now in competition for the title of world biggest producer. The European Council had already called for 8% increase in production by 2015. However, it is believed that this target can be surpassed. Minimum targets of 10% by 2015, 15% by 2020 and 25% by 2030 have also been proposed (Miguel, 2006).

In Europe mainly grain (barley, wheat and rye) and some sugar beet have been used for bioethanol production (Enwald, 2007) and in USA, corn (Tavares, 2007). France is a frontrunner in the EU, producing over 800 million liters ethanol from sugar beet and wheat in 2004 (Murray, 2005). Cooperatives have a significant role in sugar-to ethanol plants in the USA (Jacobs, 2006). In the years 2004-2007, local farmers in Häme, in Southern part of Finland, also started to source alternative use for sugar beet due to expected drastic reduction of domestic sugar production in the near future.

Japan, is developing a technology to produce bioethanol from waste paper and other materials by using cellulolytic yeast which is genetically engineered from cellulose.

Production of bioethanol from sugar cane and corn is currently costly; it requires large facilities. In contrast, the technology currently under development uses waste paper that cannot otherwise be recycled, which makes it possible to reduce costs and to use resources effectively. The existing bioethanol manufacturing methods require much time and involve high costs for the processes of fermenting and distilling starch. By using a special kind of bacteria, the new technology can drastically shorten the processes.

Shingoshu was developing the technology, aiming to enter the market in 2007 and is expecting an annual production of 36,500 L of bioethanol by 2012, which would mean an annual reduction of CO_2 emissions by about 51,700 t (Anonymous, 2005).

Asia Pacific ethanol production is expected to grow tremendously in the coming years and could represent as much as 20% of global ethanol production by 2015 (Johnson, 2009). If India's own projections are realized, it could outpace Brazil in ethanol production and exporting by 2015.

Nonetheless, despite India's ethanol production expansion, Hart projects that Brazil will remain the leading global biofuels exporter (Johnson, 2009).

In China, cassava is considered as the alternative starch-based feedstock to replace corn in ethanol production. For instance, The China nation Cereals, Oils and Foodstuffs Corp, (COFCO) has built Cassava ethanol plant in Beihai, Guangx. This plant operated as Bioenergy Co Ltd is the country's first fuel ethanol plant based on a non grain feedstock (Austin, 2008). There are also proposals to build several ethanol plants using cassava in other countries such as Nigeria and Thailand. Production of bioethanol at a competitive price with fossil fuel by saccharification and fermentation of lignocellulosic biomass has been achieved through investigation of biomass resources in Asian countries through experimental study for hydrothermal pretreatment, following saccharification and its evaluation. These led to process design for optimum ethanol production. The rationalization of fermentation and pretreatment processes resulted in a new fermentation process based on high speed fermentation method by a high bacteria concentration. The rationalization process enabled the cost reduction by 30% compared to that of the new National Renewable Energy Laboratory (NREL) process (29.8/kg-ethanol) (Yamaji *et al*., 2006).

Brazil is the world's second largest producer of ethanol fuel and the world's largest exporter. Brazil in 2007 exported 933.4 million gallons (3,532.7 million liters), representing almost 20% of its production and accounting for almost 50% of the global exports. Since 2004, Brazilian exporters have as their main customers the United States, Netherlands, Japan, Sweden, Jamaica, El Salvador, Costa Rica, Trinidad and Tobago, Nigeria, Mexico, India and South Korea (Sanchez, 2007). Together, Brazil and the United States lead the industrial production of ethanol fuel, accounting together for 89% of the world's production in 2008. In 2008, Brazil produced 24.5 billion litres (6.47 billion US liquid gallons), which represents 37.3% of the world's total ethanol used as fuel (The World Bank, 2008; Renewable Fuels Association, 2009).

In Nigeria, ethanol production for transportation is still at the research and developmental stage. They are yet to be commercially produced in the country though serious efforts are in progress to get the technology commercialized. Some private concerns have also indicated plans to commence cultivation of some energy crops for the production of bioethanol in some parts of the country (Sambo, 2007). Some of the research and developmental works in bioethanol are also

already being focused on using feedstock that are either not edible, are wastes or are not widely consumed to reduce competition with food. This is expected to reduce concerns on food security and they include; Ethanol production from a three leaved yam (*Dioscorea demetorium*) an alternative source of fuel or fuel extender (Garba *et al*., 1997). Evaluation of ethanol production from enzymatically hydrolyzed S*accharin officinarum* bagasse (Udotong, 1997). The pilot plant designed to produce 100 litres per day of fuel grade ethanol from sugar cane was carried out, test run and eventually commissioned by the Kaduna State Government of Nigeria (Ofoefule *et al*., 2008). These are also in addition to other research works going on in the area of bioethanol using other local sources such as cassava. Nigeria is actually the world's largest cassava producer (Drapoch, 2008).

2.9 Prospects of bioethanol production from various biomass materials

The main sources of sugar required to produce ethanol are derived from fuel or energy crops. These crops are grown specifically for energy use and include corn, maize and wheat crops, waste straw, willow and poplar trees, sawdust, reed canary grass, cord grasses, Jerusalem artichoke, miscanthus and sorghum plants, wheat grains and/or straw (Coppola *et al*., 2009). To avoid conflicts between human food use and industrial use of crops, only the wasted crop, which is defined as crop lost in distribution, is considered as efficient feedstock. Bioethanol feedstocks are plants that have high sugar or starch content like sugarcane or corn. It can only use the sugars and starches from the fruit and not from the entire plant. In Europe, the feedstock used for bioethanol is predominantly wheat, sugar beet, corn and wastes from the wine industry. Wheat has proven to be a very good raw material for the bioethanol production and is considered as a primary commodity for the bioethanol production also in Australia (Mojovic *et al*., 2009).

2.10 Prospect of bioethanol in perspective of Bangladesh

South Asia with a geographical area of 5.1 million hectares is home to 1.5 billion people and is witnessing a sustained and rapid economic growth. As a result primary en'ergy demand in this part of the globe has increased by 64 per cent since 1991 reaching 584 mton (million tons of oil equivalent) in 2003-04. All countries in this region are net importers of petroleum fuels. In this context, as an indigenous and renewable energy source, the use of biofuels can play a vital role in reducing the dependence on petroleum import and catalysing the rural economic development.

In the last few years, interest in these green fuels has grown dramatically followed by the equivalent market responses. In Bangladesh, at present LPG is making inroads and biofuels are yet to make an impact. Private companies are making plans to manufacture fuel alcohol in the country. An investment of $4.5 million have made for a 12000 litres/day ethanol plant, which uses molasses as a feedstock (Bangladesh Observer, 2005).

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

- Molasses (Local market Pabna, Bangladesh)
- Fruit peel (Pineapple, Orange)
- DinitroSalicylic acid (DNS), Sulphuric acid, sodium-potassium tartarate ($KNaC_4H_4O_6.H_2O$), Sodium hydroxide, Indicators (methylene blue), YPD (yeast extract, peptone, dextrose) medium, Sodium-Metabisulfite, Phenol, Potassium Sodium-Tartarate, NAOH, Potassium Iodide, Potassium dichromate, Solubole starch, sodium acetate, carbol fuchsine etc.
- Spectrophotometer
- Incubator
- Autoclave
- Orbital Shaker
- Glasswares: Laboratory distillation apparatus, Neubauer counting chamber (Cell counter), Petri dishes, slants, micro-pipettes, Bunsen burner, hot plate, Desiccators, Balance micro burette, etc.

3.2 Isolation and screening of stress tolerant yeasts

Fruit samples (pine apple and orange) were collected from local market. 1 gm amount of sample was soaked in 250 ml YMM broth at 30 °C for 3 days. After 3 days incubation each 100 µl of suspension was spread into a plate containing YMM media, which consisted of 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 15 g agar, in 1 liter water, initial pH 5.5 (Kreger-van Rij, 1984), and was incubated aerobically at 30 °C for 3 days. Single colony formed was picked and the cells were observed under microscope.

3.3 Maintenance of culture

The culture of yeast was maintained by subculturing on slants using YMM (Yeast maintenance Media) media, incubating for 48 hrs at 30ºC and thereafter storing in a refrigerator at 4ºC for future use.

3.4 Characterization of the selected yeast isolate (Pa and Or)

3.4.1 Morphological characterization

According to the method of (Kreger-van Rij 1984) and (Kurtzman and Fell 1997), the morphology of the vegetative cells of yeast was grown in liquid and on solid media.

3.4.2 Growth on solid medium

In the present study, morphology of cells of the selected isolates and their appearance on YEPD agar media was examined. The medium was autoclaved at 121°C and 15 psi and poured on petridish and cooled. After cooling, the plates were inoculated by 48 hrs old yeast strain and incubated at 30°C for 48 hrs. The following features of the appearance of cultures were recorded; texture, color and surface of colonies.

3.4.3 Growth in liquid medium

Selected isolates Pa and Or were cultured in YPD liquid medium. The medium was autoclaved at 121°C and 15 psi and cooled. 15 ml portion of the medium was distributed into McCartney tubes and after then inoculated with half loopful of 48 hrs old selected yeast strain and incubated at 30°C for 3 days. The culture was examined for the growth visually on the surface of YPD liquid medium and the shape of cells by compound microscope (Olympus, Japan).

According to Lorenz *et al*., (2000), the morphological changes in the culture yeast (*Saccharomyces cerevisiae*) leads to filament formation under unfavorable fermentation conditions. This causes scum formation and foaming. The budding yeast *S. cerevisiae*, starved for nitrogen, differentiates into a filamentous growth form.

3.5 Physicochemical characterization

3.5.1Carbohydrate fermentation test:

Tryptone broth was used as a basal medium for fermentation tests. The ability of the Yeast to use sugar is an important factor for their growth & alcohol production. There are various types of sugar available but Yeasts are generally capable of using limited number of sugar. Yeast

fermentation broth media was used for identification the ability of the yeasts to ferment specific carbohydrates. 0.01% phenol red was used as indicator. Fermentation tubes with 9 ml of basal medium provided with indicator were made. Then the medium was sterilized in autoclave at 121°C and 15 lb/inch^2 for 20 min. 1 ml of filtered sterile 1% sugar was taken in each tube. One durham tube was introduced in each of the fermentation tube before sterilization of basal medium. The tubes were then inoculated in duplicate with fresh culture of the yeast isolate and allowed to incubate at 30°C for 48 hrs. Ability to ferment seven different carbohydrates was examined anaerobically. Capability of fermentation was assessed by looking for the formation of gas (CO_2) in Durham tube and colour change of the fermentation media Deep pink to yellow due to the formation of acids and gas (Warren and shadomy, 1991). In this study the fermentation tests of the following carbohydrates and sugar alcohol were made:

Monosaccharides: Glucose, Fructose, Xylose.

Disaccharides: Sucrose, Maltose, Lactose, Trehalose.

3.5.2 Detection of thermo tolerance

YPD liquid medium was used for detecting thermotolerance and growth in liquid media of selected yeast isolates. The medium was autoclaved at 121 °C and 15 psi and cooled. 10 ml portion of the medium was distributed into McCartney tubes and then inoculated with half loopful of 48 hrs old selected yeast isolates. The initial optical density of each tubes was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 32°C, 37°C, 40°C and 44°C for 3 days for observing thermo tolerance of yeast strain. The increase in optical density in a tube was recorded as evidence of growth. Without it, growth on YEPD agar media at 25°C, 30°C, 32°C, 37°C, 40°C and 44°C was also observed to ensure thermotolerence of the strain.

3.5.3 Detection of ethanol tolerance

The medium for the detection of ethanol tolerance of thermotolerant yeast was modified. YPD liquid medium was used for detecting yeasts for ethanol tolerance. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. One ml of various concentrations of absolute ethanol was varied from 5 to 20% (v/v), and then added to different flask of the same medium to constitute varying percentages of ethanol differing by 1-3% (v/v) from one flask to the others. Forty ml portion of the medium was distributed into 125 ml flask, and then inoculated with

selected yeasts. The initial optical density of each flask was read off on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 40 °C for 5 days. The increase in optical density in a flask was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts.

In the present study, YPD broth was prepared containing 5%, 6%, 9%, 10%, 12%, 15%, 18% and 20% of absolute ethanol. Each McCartney contained 15 ml of YPD liquid media with appropriate concentration of ethanol and blank media was used as a control. Then each was inoculated by half loopful of yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm.

3.5.4 Growth at different pH in Liquid Media:

YEPD liquid medium was used for detecting the ability to grow at different pH. The medium was autoclaved at 121 °C and 15 psi and cooled. YEPD broth was prepared at pH 2-10. Each McCartney contained 15 ml of YEPD media with different pH and blank media was used as a control. Then each was inoculated by half loopful of yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm for growth.

3.5.5 Osmotolerence observation :

YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18% and 20% of NaCl. Each McCartney contained 15 ml of YEPD liquid media with appropriate concentration of salt and blank media was used as a control. Then each was inoculated by half loopful of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm.

3.5.6 Chloramphenicol,and Nalidixic acid resistance test:

Sensitivity to Chloramphenicol and nalidixic acid were evaluated by growing isolates in MEA in the presence of 30 µg/ml discs, Sample collection using the method of Kirby et al. (1966). In this study YPD agar medium was used for detecting yeasts for Chloramphenicol and nalidixic acid resistance. Chloramphenicol and Nalidixic acid disc (30 µ/L) was placed into the center of the

already inoculated petridish. Then the plate kept at 30°C for growing. The zone of inhibition by the disc was recorded as an evidence of Chloramphenicol and nalidixic acid sensitivity.

3.5.7 Determination of Killer toxin production capacity of yeasts:

Yeast killer toxins are protein compounds, which are active against members of the same species or closely related species, and the activities of these toxins are analogous to the activities of bacteriocins in bacterial species (Lowes et al., 2000). The capability to produce killer toxin can confer an advantage over more sensitive competitive strains growing in a fermentative process (Soares and Sato, 2000). Investigations revealed that the occurrence of the killer phenotype in yeast is widespread in alcohol fermentations for beverage production such as in breweries, wine, plants and more recently in sugarcane producing plants (Soares and Sato, 2000). The concentration of sensitive cells influences the sensitivity of the bioassay; In addition, the composition of the medium and buffer solution may contribute to the sensitivity of the assay (Panchal et al., 1985). As a rule, nutritionally rich organic media are more suitable than synthetic media. In most case, glucose yeast extract-peptone agar or malt agar with sodium citrate-phosphate buffer are used. It is apparent that both the level and expression of killer toxin activity depends on a number of variables. In particular, the assay condition can be crucial for detecting killer with low activity of those organisms that are weakly sensitive (Kurtzman and Fell, 1997). Ribéreau Gayon et al., (2000) described the action of a killer strain on a sensitive strain is easy to demonstrate in the laboratory on an agar culture medium at pH 4.2-4.7 at 20° C. The sensitive strain is inoculated into the mass of agar before it solidifies; then the strain to be tested is inoculated in streaks on the solidified medium. If it is a killer strain, a clear zone in which the sensitive strain cannot grow encircles the inoculums streaks. In this study the following was followed to observe killer toxin production by Yeast:

- First the target bacteria was inoculated in Nutrient broth for 24 hours
- 10 ml Molten Agar (3%) were added to already inoculate Nutrient broth.
- Then poured on plate & let to solidify
- Streak Yeast on plate in 2 to 3 rows
- Incubated at 25° C for 24 hours
- Observed clear zone of Inhibition

3.6 Pretreatment of Molasses for Ethanol Production:

Sulfuric acid is used to convert calcium salts in molasses to calcium sulfate salts. Calcium acts as an inhibiting agent during fermentation of molasses by yeast. Sulfuric acid decreases the fermentation medium pH that controls bacterial contamination. Sulfuric acid in diluted molasses can precipitate calcium and reduce the scaling. Blackstrap molasses contains many nutrients for fermentation. In addition the organism requires some other nutrients. Urea is used as a nitrogen source in molasses fermentations for ethanol production, but in alcoholic beverage it may produce carcinogenic ethylcarbamate, which is unacceptable. Diammonium phosphate may be added in Phosphorus deficient molasses. In our present study 0.30 ml concentrated Sulfuric acid and 0.10 gm urea was used in 250 gm molasses to treat and diluted it with tap water.

3.7 Fermentation media preparation:

Sugarcane molasses was used as a Fermentation media for the study. Molasses was collected from local market of Bangladesh at the district of Pabna. It contains 20%-25%% of reducing sugar. In all experiments, reducing sugar concentration was estimated only.

Composition of Fermentation Media:

Molasses 250 gm

Urea 0 .10 gm

Conc. (H_2SO_4) 0 .30 ml

Tap water was added up to 1000 ml and the media was boiled. After boiling the media was autoclaved at 121° C and 15 psi. The reducing sugar concentration was maintained by boiling the media.

3.8 Preparation of yeast cell suspension:

A 48 hrs old slant culture of yeast cell was added aseptically to autoclaved molasses fermentation media (10 ml) and the tube was shaken gently to form a homogeneous suspension.

3.9 Fermentation of molasses:

Fermentation was carried out in Erlenmeyer conical flasks. 250 ml fermentation media was taken into 500 ml Erlenmeyer flasks and added the homogenous suspension of yeast was inoculated into the media in an aseptic condition. The flask was cotton plugged and incubated at different temperatures in an incubator under non shaking and shaking condition.

3.10 Estimation of reducing sugars:

The reducing substance (sugar) obtained due to the enzymatic reaction was determined by DNS method (Miller *et al*., 1959).

3.11. Procedure

The estimation of total reducing sugar was based on the dinitrosalycylic acid (DNS) method. A double beam UV scanning spectrophotometer was used for measuring absorbance. Reducing sugar contents before fermentation and after fermentation were determined by taking 1.0 ml diluted solution (1 ml sample in 100 ml distilled water) with 3.0 ml of DNS reagent in a test tube. Blank containing 1.0 ml of distilled water and 3.0 ml of DNS was run parallel. The tubes were heated in a boiling water bath for 15 min. After cooling the tubes at room temperature, 8 ml of distilled water was added to each and absorbance was noted at 540 nm using spectrophotometer. Reducing sugar concentration was determined from the standard curve of glucose and multiplies by dilution factor. A standard curve of glucose was prepared that shown

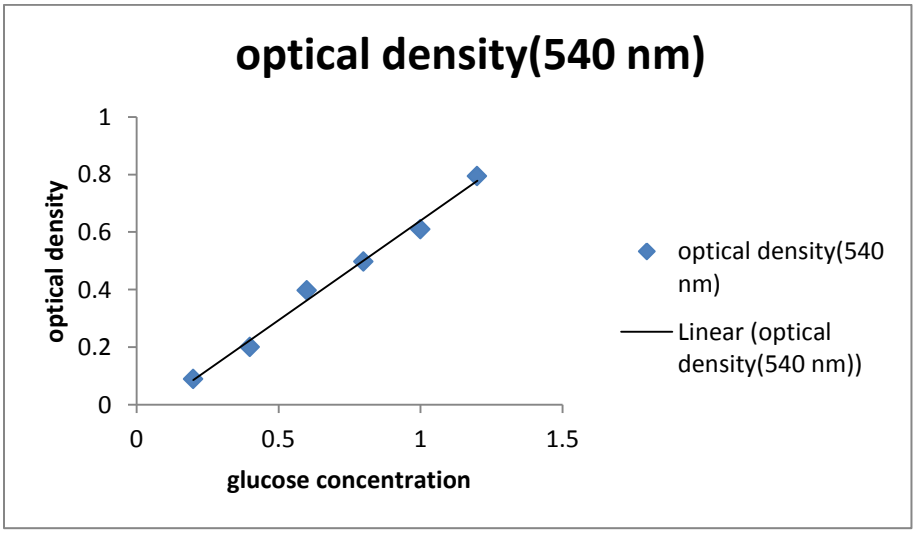


Fig-4: Glucose standard carve for reducing sugar estimation.

3.12 Estimation of Ethanol: Conway Method:

Ethanol was determined by Redox titration. In this method ethanol is oxidized to ethanoic acid when ethanol react with excess of potassium dichromate solution (0.05 N) and unreacted dichromate is then determined by adding potassium iodide (50% KI) solution which is oxidized by the potassium dichromate. Potassium iodide reacts with potassium dichromate and creates iodine. Then the iodine is titrated with a standard solution of sodium thiosulfate (0.1N). The titration reading is used to calculate the ethanol content after fermentation. One ml fermented solution was diluted 250, 500 and 1000 times with distilled water and each one ml diluted solution was taken as a sample. A Conway unit was used for ethanol detection by this procedure. One ml potassium dichromate was placed into the Conway unit center and sample was placed around the center. The Conway unit was covered by a glass plate for 24 hrs for reaction. The water and ethanol slowly evaporate, come in contact with Potassium dichromate and then oxidized. More ethanol evaporates until eventually all the ethanol from the fermented dilute solution has left the sample and reacted with the dichromate. One Conway unit was used as a blank and in that unit 1 ml distilled was used as a sample.

3.12.1 Alcohol Estimation (calculation)

Conway unit center (1 ml 0.05 N potassium dichromate solution)

↓

Round (1 ml sample)

↓

Reaction complete within 24 hours

↓

Conway unit center (50% KI solution 0.5 ml + 1-2 drop soluble starch)

↓

Microburatte (0.1 N sodium thiosulfate)

↓

Titration

↓

Colourless

$$\text{Percentage of Ethanol (\%)} = \frac{(\text{TR of blank sample- TR of FS}) \times 11.6 \times 0.1 \times \text{DF} \times 100}{.793 \times 1000}$$

= Alcohol percentage gm/100 ml

Where,

Density of Ethanol 0.793 g/ml, DF-Dilution Factor, FS-Fermented solution ,TR-Titration Reading, **Volume of sodium thiosulfate used: 11.60 cm^3**

3.13

Optimization of fermentation process:

Fermentation process carried out by yeast is known to vary with respect to substrate concentration, temperature, pH, N-source and inoculums size. It is therefore imperative to optimize the fermentation conditions for yeast cells so that the production efficiency increases. Various factors were investigated affecting ethanol production from molasses.

3.13.1 Effect of sugar concentration:

To study the effect of sugar concentration on ethanol production by *S. cerevisiae*, the production media was prepared by diluting molasses to reducing sugar concentration 4.5%, 5.50%, 6.0%, 6.50%, 7%, and 7.5%, and fermentation was carried out in a volume of 250 ml media in a 500 ml conical flask. A forty eight hrs old inoculum of yeast was added to the medium. Samples were withdrawn at different time and estimated for residual sugars (Miller, 1959) as well as ethanol content in the media.

3.13.2 Effect of pH

To study the effect of pH on ethanol production by *S. cerevisiae*, fermentation media with different reducing sugar concentration was used for the production of Ethanol. Fermentation was carried out at pH 5.0 in a volume of 250 ml media in a 500 ml conical flask. A forty eight hour old inoculum of yeast was added to the medium. Samples were withdrawn at different time and estimated for residual sugars (Miller, 1959) as well as ethanol content in the media.

3.13.3 Effect of Agitation

To study the effect of agitation on ethanol production by *S. cerevisiae*, fermentation media with different reducing sugar concentration was used. Fermentation was carried out at pH 5.0 and temperature 25°C, 30°C, 35°C in a volume of 250 ml media in a 500 ml conical flask. A forty eight hour old inoculum of yeast was added to the medium and then flasks containing the same sugar concentration kept both in shaking (120 rpm) and non shaking condition and thereby ethanol content was measured after 48 hours.

CHAPTER 4. RESULTS

4.1 Morphological characterization

According to the method of Kreger van Rij (1984) and Kurtzman and Fell (1997), the morphology of the vegetative cells of yeast was observed grown in liquid and on solid media.

4.1.1. Growth on solid medium

Yeast isolates formed butyrous and smooth white raised colonies on YEPDA medium. The budding stage of the yeast isolates was observed under (40X) microscope and confirmed them to be yeast.

Fig-5: Colonies on YEPD agar medium after incubation for 48 hours at 30°C.

4.1.2 Growth in liquid medium

After 3 days of incubation at 30°C, heavy, dry climbing pellicles were formed on the surface of YEPD broth medium.

Fig-6: Growth in liquid medium and surface film formation

4.1.3 Microscopic observation:

The cell morphology of the ethanol tolerant Pa and Or strain observed under compound microscope showed an ovoidal to elongate having single, pairs, or triple budding cells. The strain reproduces vegetatively by budding.

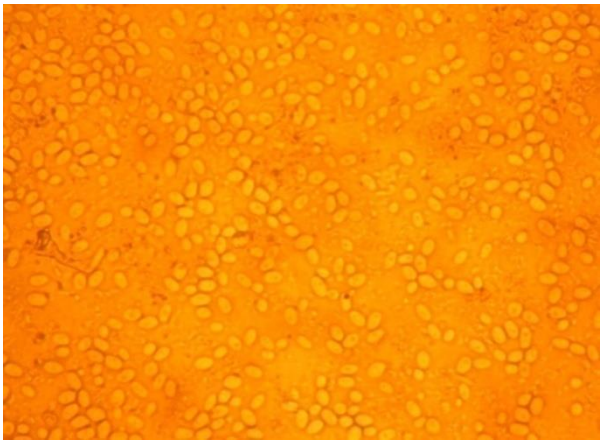


Fig-7: The cell morphology of Pa isolate under compound microscope.

4.1.4 Fermentation of carbohydrates:

In this study, *Saccharomyces cerevisiae* showed variation in terms of utilization of seven different sugars (Table-4). The strain utilized Glucose, sucrose maltose, dextrose and fructose, xylose and trehalose but failed to grow on lactose . The following results After 48 hours

Table-4: Fermentation of different carbohydrates by Pa isolate

Carbohydrate	**Before Fermentation Color of the medium**	**After Fermentation**	
		Color of the medium	Gas production
Glucose	Pink colour	(yellow)	yes
Sucrose	Pink colour	(yellow)	yes
Maltose	Pink colour	(yellow)	yes
Lactose	Pink colour	(no colour change)	no
Fructose	Pink colour	(yellow)	yes
Xylose	Pink colour	Yellow	yes
Trehalose	Pink colour	Yellow	yes

Table-5: Fermentation examination of different carbohydrates by Or strain

Carbohydrate	**Before Fermentation Color of the medium**	**After Fermentation**	
		Color of the medium	Gas production
Glucose	Pink colour	(yellow)	yes
Sucrose	Pink colour	(yellow)	yes
Maltose	Pink colour	(yellow)	no
Lactose	Pink colour	(no colour change)	no
Fructose	Pink colour	(yellow)	no
Xylose	Pink colour	(no colour change)	no
Trehalose	Pink colour	(yellow)	yes

Fig-8(a): Media colour before fermentation

Fig-8(b) Media colour after fermentation of carbohydrate.

4.2 Tolerence of selected yeast strains to different environmental conditions

4.2.1(a) Effect of temperature on growth of yeast isolate Pa

Six YPD Agar containing plates were strick yeast cell and incubated for 48 hours at 25°C, 30°C, 35°C, 37°C, 40°C and 45°C. The yeast isolate Pa was able to grow at 25°C-44°C. To confirm the results obtained from solid media, thermotolerance was repeated in liquid media. Growth in liquid media is shown in table(6,7)

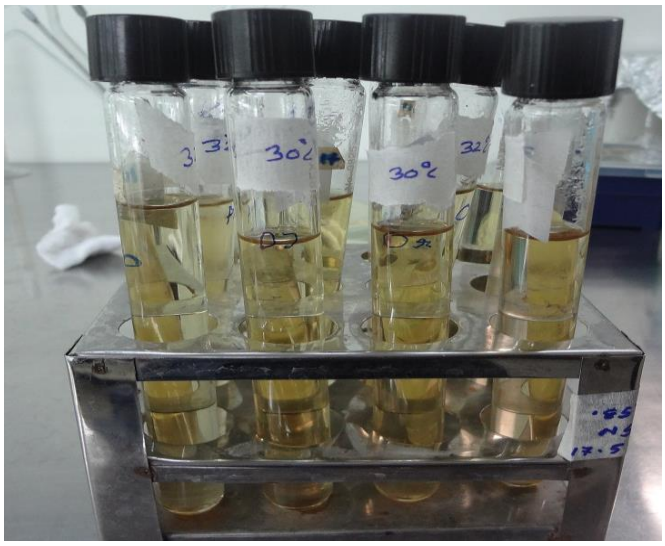


Fig-9(a): Growth before inoculation

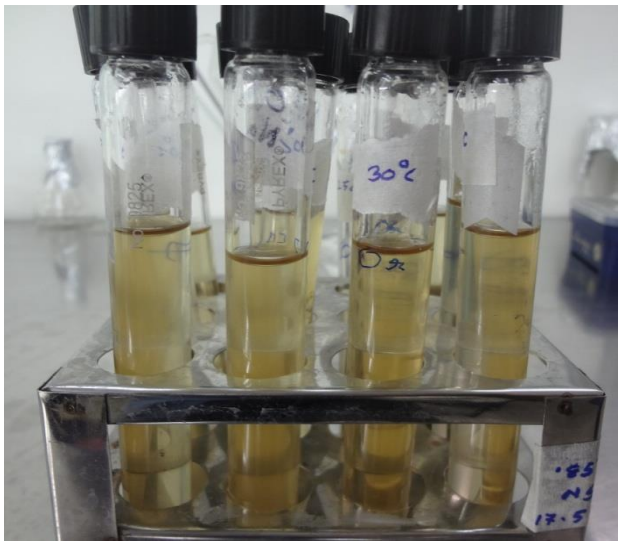


Fig-9(b): Growth (after inoculation) after 48 hours incubation at various temperature

Table6- Growth of Pa isolate in liquid media at different temperatures

Temperature	**Initial optical density (O.D)**	**O.D(after 48 hours of growth)**
25°C	.269	1.435
30°C	.346	1.537
32°C	.322	1.730
37°C	.441	2.236
40°C	.461	2.079
44°C	.482	.848

From the table-6, it is evident that the Pa yeast isolate is highly thermotolerant as it is able to grow up to 44 °C.

4.2.1(b) Effect of temperature on growth of yeast isolate Or

Six YPD Agar containing plates were stricked with yeast cell and incubated for 48 hours at 25°C, 30°C, 35°C, 37°C, 40°C and 45°C. The Yeast isolate Or was able to grow at 25°C-44°C. To confirm the results obtained from solid media, thermotolerance was repeated in liquid media.

Table7- Growth of Or isolate in liquid media with different pH

Temperature	**Initial optical density (O.D)**	**O.D (after 48 hours of growth)**
25°C	.356	1.112
30°C	.211	.717
32°C	.255	1.488
37°C	.559	2.420
40°C	.519	2.171
44°C	.515	.501

From the table-7, it is evident that the Or yeast isolate is highly thermotolerant as it is able to grow up to 40 °C.

4.2.2(a) Effect of ethanol concentration in the media on growth of yeast isolate,Pa

The isolate was selected for screening of yeasts tolerant to ethanol and the results are shown in table-8. It can be observed from Table 8 that the isolate Pa can grow up to 15% ethanol containing liquid YEPD media. Maximum growth was seen in 5% ethanol containing media. Growth were recorded at 5%, 8%, 10%, 12%, 15%, 18% and 20% of ethanol containing liquid media and the Table shows the change in O.D at 48 hours with ethanol concentration in the media.

Table 8- Growth of Pa isolate in liquid media with different ethanol concentration

Ethanol percentage	Initial optical density (O.D)	O.D (after 48 hours of growth)
5%	.328	1.851
6%	.187	1.542
9%	.242	1.406
10%	.336	1.314
12%	.184	.736
15%	.317	.339
18%	.287	.282
20%	.220	.210

4.2. 2(b)) Effect of ethanol concentration in the media on growth of yeast isolate, Or

The isolate was selected for screening of yeasts tolerant to ethanol (table-9) .It was observed that the isolate can grow up to 12% ethanol containing liquid YEPD media. Maximum growth was seen in 5% ethanol containing media. Growth were recorded at 5%, 8%, 10%, 12%, 15%, 18% and 20% of ethanol containing liquid media and the Table shows the change in O.D at 48 hours with ethanol concentration in the media.

Table 9- Growth of Or isolate in liquid media with different ethanol concentration

Ethanol percentage	Initial optical density (O.D)	O.D (after 48 hours of growth)
5%	.254	.687
6%	.236	.557
9%	.298	.530
10%	.250	.456
12%	.229	.236
15%	.223	.163
18%	.174	.151
20%	.259	.153

4.2.3 (a) Effect of pH on growth of yeast isolate Pa

The ethanol producing Pa isolate is able to grow lower to higher pH. The isolate can grow up to pH 10. Maximum growth was seen at pH 4. After 48 h cell density was recorded at 600 nm and given gradually for evidence of growth:

Table10- Growth of pa isolate in liquid media with different pH

pH	Initial optical density (O.D)	O.D (after 48 hours of growth)
2	.349	.868
3	.430	1.813
4	.390	1.893
6	.405	1.789
7	.199	1.442
8	.200	1.460
9	.279	1.210
10	.418	1.425

4.2.3(b) Effect of media pH on growth of yeast isolate, Or

The ethanol producing Or isolate is able to grow lower to higher pH. The isolate can grow up to pH 10. Maximum growth was seen at pH 6. After 48 h cell density was recorded at 600 nm and given gradually for evidence of growth:

Table11- Growth of Or isolate in liquid media with different pH

pH	Initial optical density (O.D)	O.D (after 48 hours of growth)
2	.359	.863
3	.382	1.487
4	.274	1.37
6	.304	1.489
7	.197	1.320
8	.229	1.265
9	.217	1.215
10	.425	1.207

4.2.4 (a) Effect of salt concentration in the media on growth of yeast isolate Pa

The isolate Pa can successfully tolerate up to 9% sodium chloride salt in the media and this is an index of osmotolerance. However, at higher concentration growth was reduced.

Table12- Growth of Pa isolate in liquid media with different salt concentration

NaCl percentage	Initial optical density (O.D)	O.D (after 48 hours of growth
6%	.232	1.372
9%	.219	.648
12%	.267	.443
15%	.202	.229
18%	.269	.263
20%	.254	.237

4.2.4(b) Osmotolerence of Yeast isolate, Or

The isolate Or can successfully tolerate up to 12% sodium chloride salt concentration but at higher concentration growth reduced.

Table13- Growth of Or isolate in liquid media with different salt concentration

NaCl percentage	Initial optical density (O.D)	O.D(after 48 hours of growth)
6%	.204	.877
9%	.237	.246
12%	.242	.248
15%	.197	.154
18%	.290	.184
20%	.257	.197

4.3 Killer toxin test

Killer toxin test was carried out for the both Pa and Or strains against E. coli, showed negative result ie, (no clear zone of inhibition by Yeast was observed)

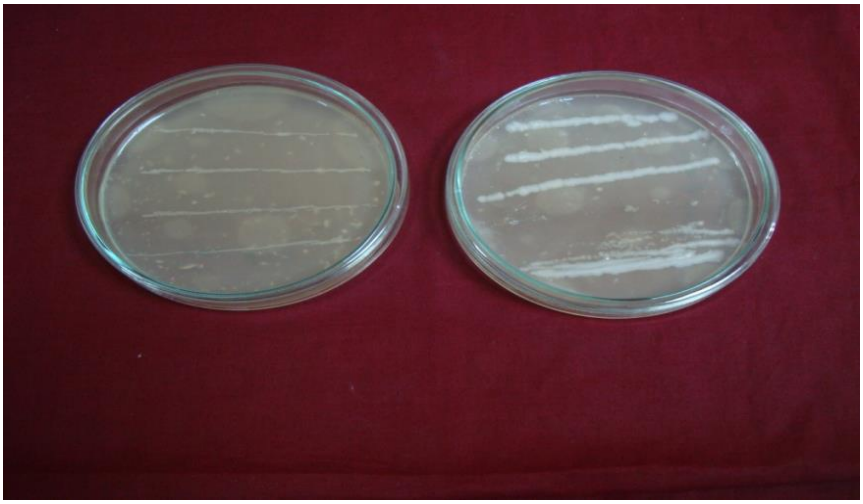


Fig 10 : killer toxin test by Pa and Or strain against E. Coli

4.4 Antibiotic resistance test by chloramphenicol and nalidixic acid

Antibiotic resistence test by chloramphenicol and nalidixic acid showed that both the strains Pa and Or were resistant to the antibiotics mentioned above.

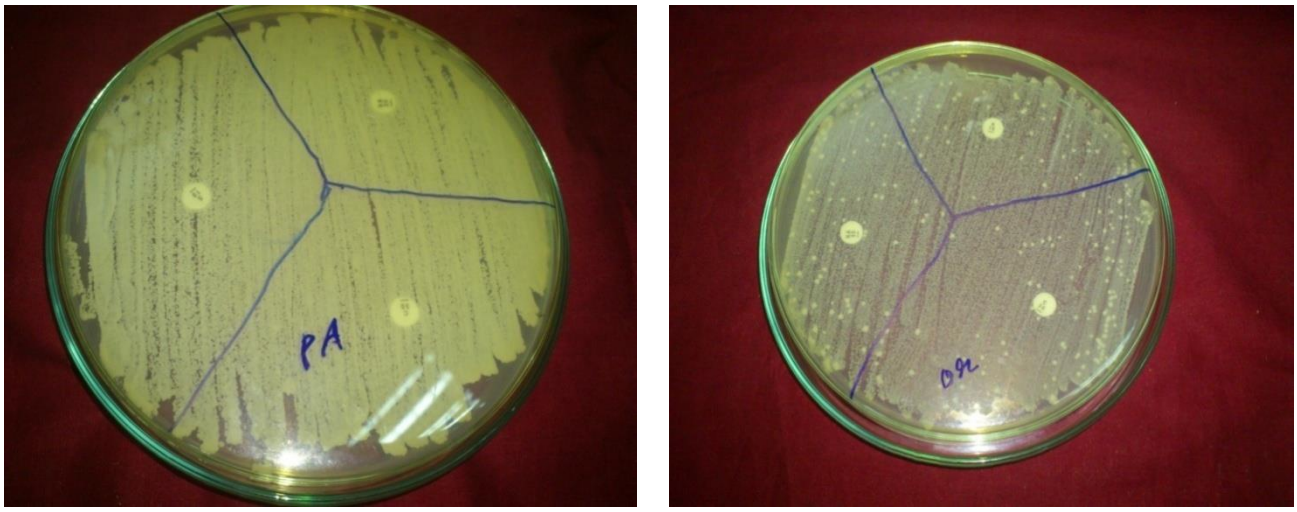


Fig-11: Antibiotic resistance test by chloramphenicol and nalidixic acid

4.5 Ethanol production

4.5.1 Ethanol Production by Pa Yeast strains at 24, 48, 96 hours fermentation under shaking condition at 120 rpm at 6.5% glucose and pH 6

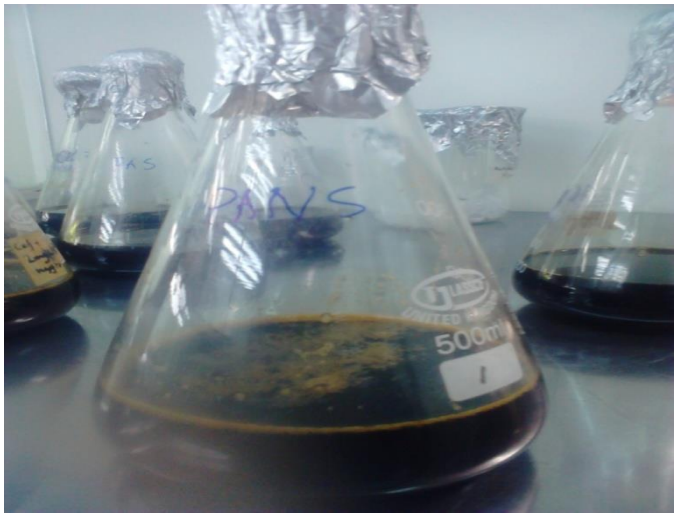


Fig 12(a) : Fermentation in shaking condition

Fig-12(b) Fermentation in non shaking condition

Under shaking condition (120 rpm), at 30°C temperature using Initial reducing sugar concentration of 6.5% of the fermentation media and pH 5.0. Maximum ethanol production was 4.83% at 48 hrs

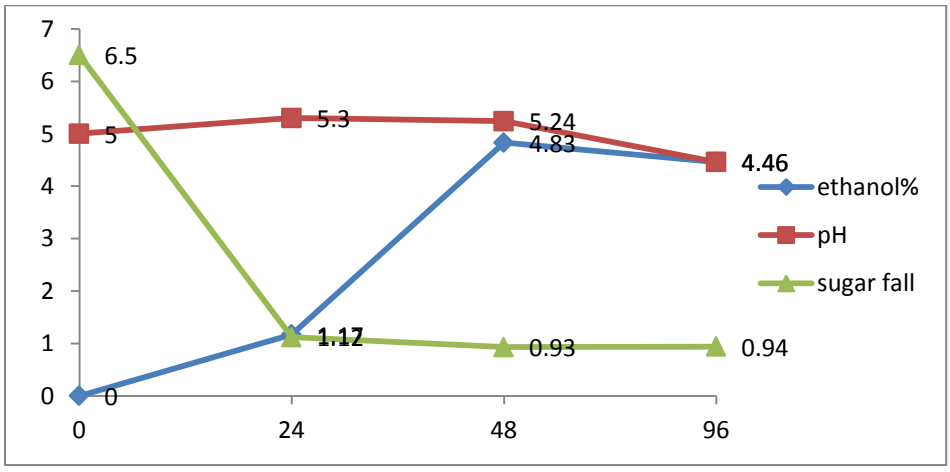


Fig 13: Production of ethanol, pH and sugar depletion at initial 6.5% sugar concentration by Pa isolate

4.5.2 Ethanol Production by yeast isolate, Pa at 24, 48, 96 hours fermentation at 7.5% glucose and pH 6 under shaking condition at 130 rpm

Under shaking condition (130 rpm), at 30°C temperature using initial reducing sugar concentration of the fermentation media 7.5% and pH 6.0 maximum ethanol production was 7.02% at 96 hrs.

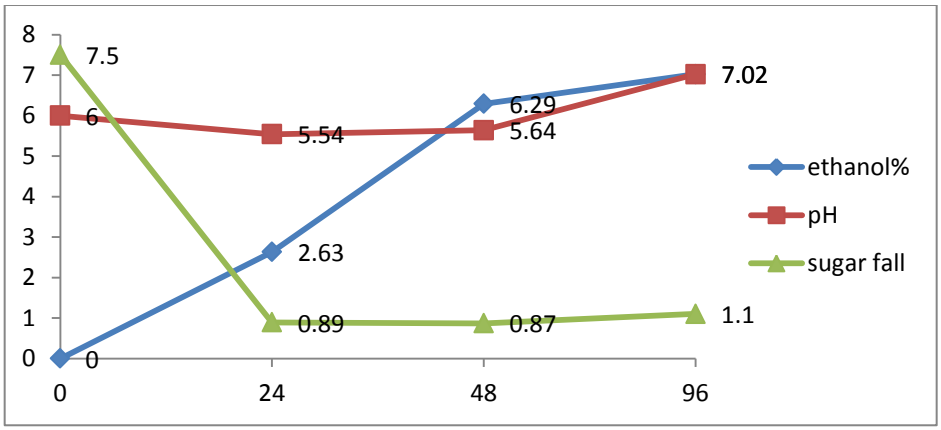


Fig 14 : Production of ethanol, pH and sugar depletion at initial 7.5% sugar concentration by isolate, Pa

4.5.3 Ethanol Production by yeast isolate, Pa at 24, 48, 96 hours fermentation (All Shaking at 140 rpm) at 8.5% glucose and pH 5.0 under shaking condition at 130 rpm

Under shaking condition (140 rpm), at 30°C temperature using initial reducing sugar concentration of the fermentation media 8.5% and pH 5.0 maximum ethanol production was 5.93% at 48 hrs.

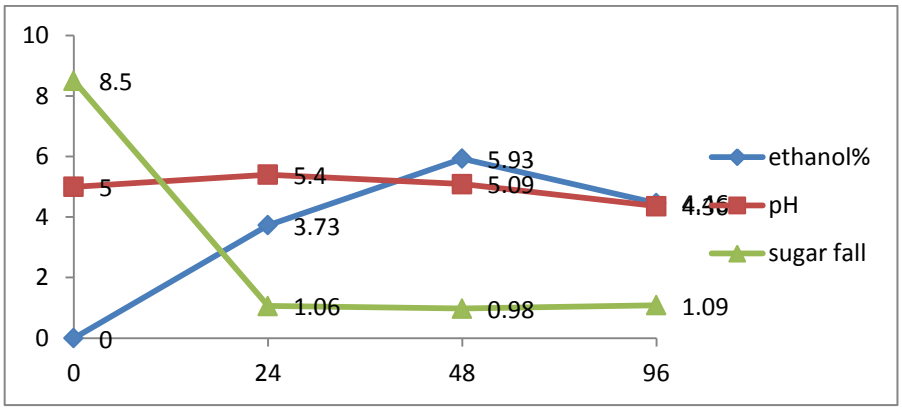


Fig 15 : Production of ethanol, pH and sugar depletion at initial 8.5% sugar concentration by Pa isolate

4.5.4 Ethanol Production by Pa yeast isolate at 24, 48, 96 hours fermentation at 9.5% glucose and pH 5.0 under shaking condition at 130 rpm

Under shaking condition (130 rpm), at 30°C temperature using initial reducing sugar concentration of the fermentation media 8.5% and pH 6.0 maximum ethanol production was 6.66% at 48 hrs

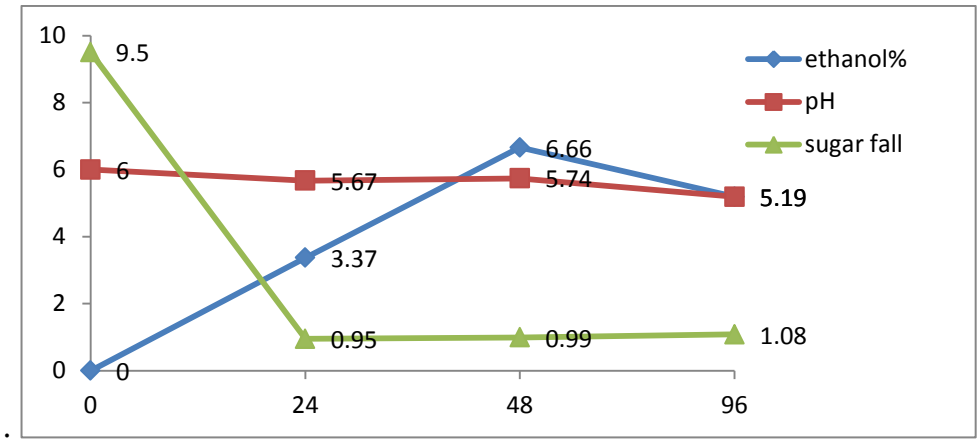


Fig 16: Production of ethanol, pH fall and sugar depletion at initial 9.5% sugar concentration by isolate, Pa

4.5.5 Effect of metals on ethanol production

To determine the effect of metals on ethanol production $MnCl_2$, $CuSO_4$ KH_2PO_4 $ZnSO_4$, were added at different concentrations in 250 ml fermentation media and the fermentation was carried out in different 500 ml conical flasks in the presence of different metals. In every conical flask 0.10g/250ml, metals were added into the fermentation media. Ethanol production was observed at 30°C; pH5.0 and using the initial reducing sugar concentration of 7.5% in shaking condition.

Table 14-Production of ethanol at 30°C temp in presence of metals (shaking condition 120 rpm).Pa & Or strain

Metals	Ethanol production % (48 h)pa	Ethanol production% (48h)or
$MnCl_2$	9.50	8.04
$CuSO_4$	7.58	5.85
KH_2PO_4	10.61	7.98
$ZnSO_4$	9.48	7.52

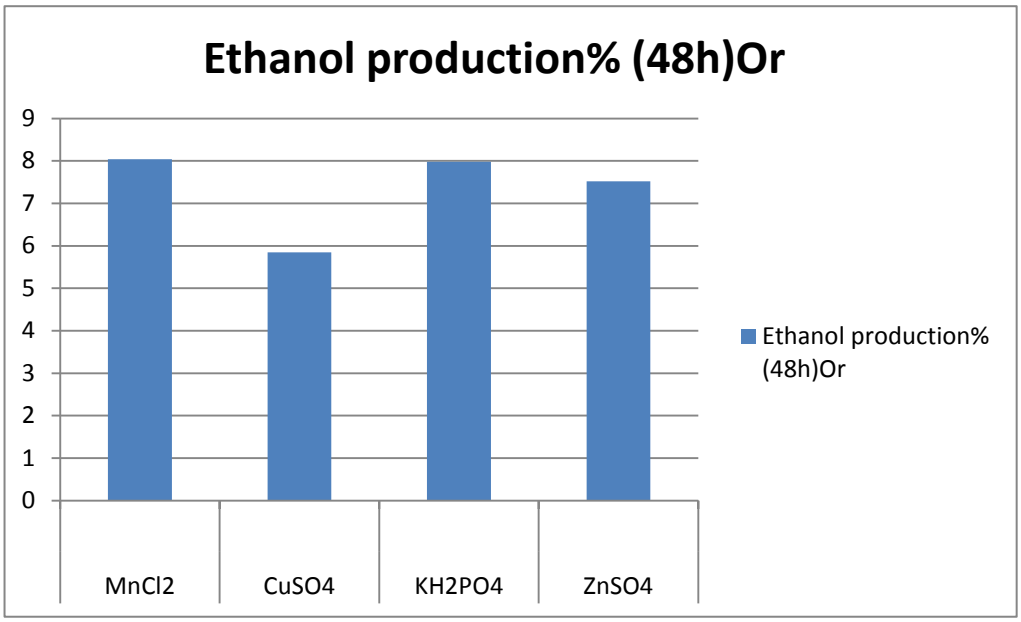


Fig 17: Production of ethanol at 30°C temperature in presence of metals (shaking condition 120 rpm) by Or strain at 48 hours

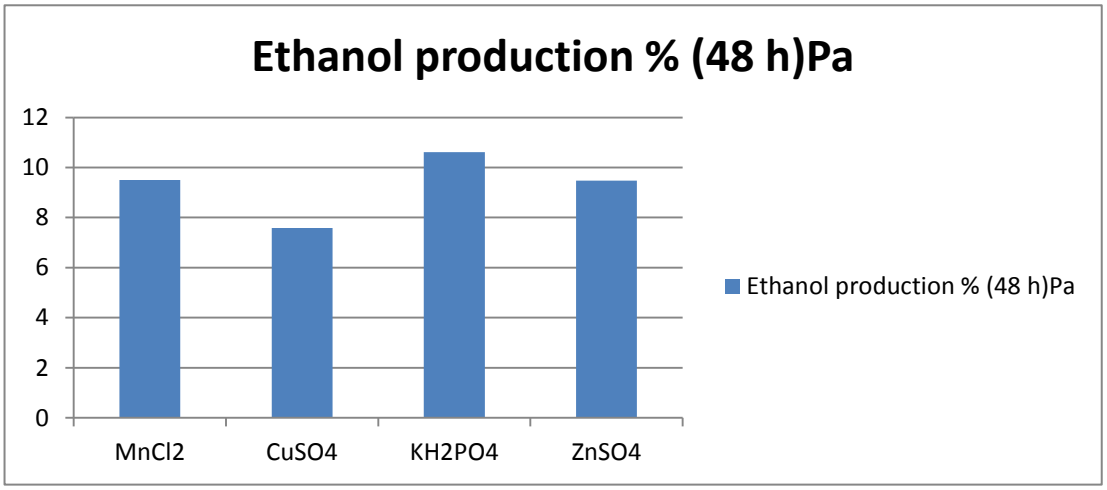


Fig 18: Production of ethanol at 30°C temperature in presence of metals (shaking condition 120 rpm) by Pa strain at 48 hours

4.6. Ethanol production under shaking and non shaking condition by Or isolates

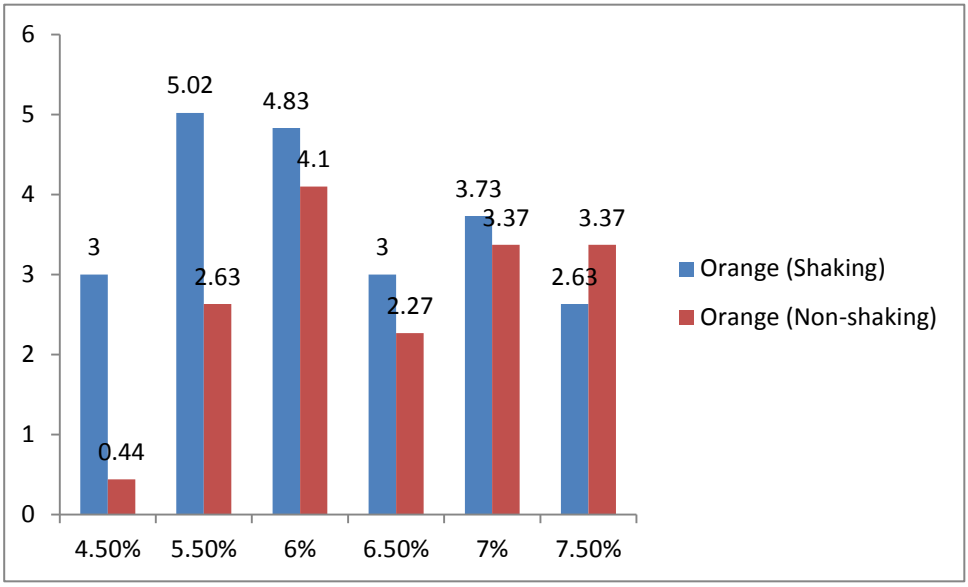


Fig 19: Production of ethanol under shaking and non shaking condition by Or strain at different sugar concentrations and initial pH 5.

4.7 Ethanol Production under shaking and non shaking condition by Pa isolate

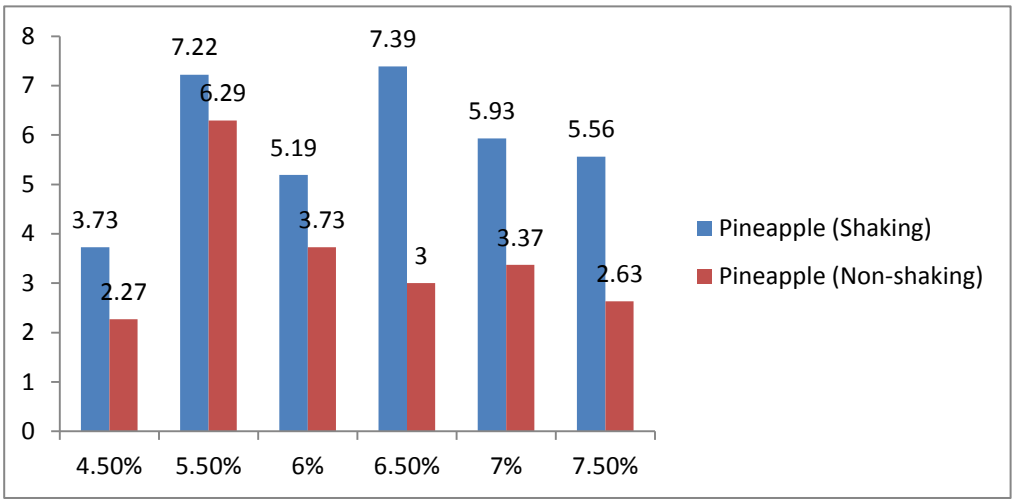


Fig 20 : Ethanol production under shaking and non shaking condition by Pa strain at different sugar concentrations and initial pH 5.

4.8 Comparison of ethanol production by different yeast isolated from different fruits at different glucose concentration

Table 15: Alcohol production comparison at different sugar concentrations in shaking and non shaking condition

Glucose conc.	Orange (Shaking)	Orange (Non-shaking)	Pineapple (Shaking)	Pineapple (Non-shaking)
4.5%	2.03	0.44	3.73	2.27
5.5%	5.02	2.63	7.22	6.29
6 %	4.83	4.1	5.19	3.73
6.5%	3	2.27	7.39	3
7%	3.73	3.37	5.93	3.37
7.5%	2.63	3.37	5.56	2.63

4.9 Comparison of glucose depletion by various yeast isolated from different fruits at different glucose concentration

Table 16: Glucose depletion comparison at different sugar concentration in shaking and non shaking condition

Glucose conc.	Orange (Shaking)	Orange (Non-shaking)	Pineapple (Shaking)	Pineapple (Non-shaking)
4.5%	1.56→0.56	1.73→1.37	0.33→0.25	1.47→1.01
5.5%	3.19→1.73	4.05→3.99	0.63→0.62	3.60→1.35
6 %	0.82→0.72	4.89→3.27	0.85→0.75	3.84→1.72
6.5%	4.28→1.80	5.55→5.41	0.81→0.60	4.77→3.42
7%	4.29→3.44	6.35→5.85	1.09→0.92	5.21→3.97
7.5%	6.32→4.48	6.68→4.82	2.66→1.02	5.54→3.84

4.10 Comparison of pH change by various yeast isolated from different fruits at different glucose concentration

Table 17: pH fall comparison at different sugar concentration in shaking and non shaking condition

Glucose conc.	Orange (Shaking)	Orange (Non-shaking)	Pineapple (Shaking)	Pineapple (Non-shaking)
4.5%	4.61→4.73	4.69→4.65	4.8→4.83	4.72→4.73
5.5%	4.56→4.5	4.73→4.77	5.18→5.27	4.84→4.98
6 %	4.86→5.07	4.87→4.85	5.24→5.18	4.9→4.95
6.5%	4.75→4.57	4.88→4.85	5.2→5.24	4.93→4.97
7%	4.87→4.61	4.93→4.89	5.09→5.37	5→5.05
7.5%	4.64→4.62	4.75→4.76	4.87→5.15	4.81→4.91

CHAPTER 5: DISCUSSION

In this study, two yeasts were isolated from Pineapple and Orange peel. Based on their colony characteristics (white and creamy texture), ovoid microscopic shape, the presence of budding pattern (multipolar), all isolates were found belongs to *sacharomyces* type unicellular ascomycete according to Lodder (1971) and (Boekhout and Kurtzman, 1996). The isolates were tested for fermentation of carbohydrates, isolate Pa was able to utilize 6 sugars out of the seven tested sugars and Or isolate was able to utilize 5 sugars indicating that they were diverse in sugar utilization (table 4 & 5).

In general, yeasts are mesophilic with upper limit growth temperature between 28°C and 38°C. This is the reason why the operating temperature must be maintained between 30 and 35 °C in typical yeast fermentation reactors (Ueno et al., 2001). However, there are only a limited number of reports on the successful selection and isolation of yeasts capable of growth or fermentation at or above 40 °C (Pellegrino et al., 1999; Sree et al., 1999). As the temperature increases, productivity decreases sharply because of greater ethanol inhibition (Sree et al., 1999). Moreover, Anderson et al., (1986) and Ueno et al., (2003) reported that thermotolerant yeast can produce > 6% ethanol within 24 hours at 40 °C. In the present study it has been revealing that the isolate Pa can survive upto 44°C temperature and isolate Or can survive almost at 40°C temperature. As such these isolates can be regarded as mild thermotolerant.

The ethanol concentrations are the major influencing factors during the fermentation process. Extremely high ethanol concentrations in the fermentation cultures have been shown to inhibit or depress the fermentation process.

A growth inhibition at ethanol levels of less than 3% v/v (Canganella et al., 1993) has been reported for *C. thermocellum* . Like the palm wine yeast, TBY 1 and TGY 2 isolates exhibited remarkably high ethanol tolerance comparable to industrial yeasts such as sake and distiller's yeasts associated with high level of ethanol tolerance (Casey and Ingledew 1986 and Hayashida et al., 1974). The level of ethanol tolerance of 16 % (v/v) by the yeast strain TGY 2 is in agreement with Nwachukwu et al .(2006) on *Saccharomyces cevisiae* isolated from raffia palm wine. It is interesting to note that isolates TBY 1 and TGY 2 showed resistance to 15.5% and

16 % (v/v) ethanol concentration. Almost similar tolerance of at16.5 % (v/v) ethanol have been observed for Saccharomyces cerevisiae (Teramoto et al., 2005). In our present study we have seen the isolate Pa can tolerate 15% ethanol and isolate Or can tolerate upto 12% ethanol which is much more consistent with the previous results that the isolates are highly ethanol tolerant(Table 8 & 9).

(Roukas., 1994) found that the optimum pH range for ethanol production from carob pod extract by Ca alginate immobilized *S. cerevisiae* was 3.5-5.5 and reported that this was due to the good yeast growth over the pH range of 3.5-6.5. In our study Pa and Or isolate can grow in a wide pH range from 2 to 10, but pH 4.0 for Pa isolate and pH 6 for Or isolate showed optimum result (Table-10, 11).

The Pa yeast isolate showed better tolerance to osmotic pressure than the Or yeast isolate up to 15% sodium chloride equivalent of osmotic pressure (Table 12& 13). Some workers (D' Amore et al., 1988; Dombek and Ingram, 1986) have reported changes in the growth dynamics of yeasts upon exposure to various osmotic stress conditions. The decrease in logarithmic growth rate constants of the test yeasts in relation to increasing osmotic pressure is therefore consistent with the views expressed by these workers (Osho, 2005). Increase in medium sugar level is believed to affect the relative proportion of total medium sugar converted to alcohol (D' Amore et al., 1988; Jimenez and Benitez, 1986; Nagodawithana and Steinkraus, 1976). The decline in yeast ethanol productivity at high medium glucose levels as observed in this study is in close agreement with the finding of several other researchers of the *Saccharomyces* genus in medium of high osmotic pressures (D'Amore et al, 1988; Dombek and Ingram, 1986).

After the optimization of substrate concentration, pH and temperature additional nutrient supplements were also tested further increase the ethanol yield. Consequently, effect of metals ($MnCl_2$, $CuSO_4$ KH_2PO_4 $ZnSO_4$) on ethanol production, were analyzed. All the isolates produced the high ethanol after supplementation with the metals. Highest ethanol production of

10.61% was observed by Pa yeast isolate with KH_2PO_4 supplementation in shaking condition. Or isolate showed highest ethanol production of 8.04% by $MnCl_2$ supplementation in shaking condition (Fig: 17, 18).

Based on the results of tolerance and ethanol production at different sugar concentration at 30°C temperature and pH 5 in shaking and non shaking condition Pa isolate showed better result than Or isolate. So a series of experiments had been conducted at different physico chemical conditions to optimize ethanol production by the Pa isolate. To know the optimum pH for ethanol fermentation, the solutions were kept at pH 5 and 6 with different initial sugar concentration. The molasses were diluted and fermentation was carried out in 500ml flasks. In shaking condition (130 rpm), at 30°C temperature using Initial reducing sugar concentration of the fermentation media at 7.5% and pH 6.0 maximum ethanol productions was 7.02% at 96 hrs (Fig-14). In shaking condition, a low ethanol yield of 4.83% was observed at ambient temperature in 48 hours and maximum ethanol produced was 7.02% at 96 hours at ambient temperature using initial reducing sugar concentration of 6.5% and 7.5 % (Shown in Fig13, 15). To know the optimum shaking condition experiments were carried out at different rpm(120,130,140) and showed the optimum result 7.02% ethanol production at 130 rpm.

CHAPTER 5. CONCLUSION

The experimental results reported in the present study revealed that both the Pa and Or isolates are highly ethanol tolerant, thermotolerant as well as moderate osmotolerant and can survive at various pH ranges. The ability to produce ethanol from sugarcane molasses was tested for both isolates. Under all circumstances Pa isolate showed better result than the Or isolate in terms of productivity. The best results (the highest ethanol concentration) were obtained with the Pa isolate, the fermentation results that evaluated the arrangements for the alcohol produced under variation in initial sugar concentrates and inoculums reveals that, for Pa isolate the use of 7.5% initial sugar concentration with pH 6.0 and 130 rpm shaking condition gives the maximum ethanol production. These isolates could be used at industrial level for fermentation of various raw materials in order to obtain an increased production of bioethanol.

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APPENDIX

Appendix-A

Microbiological Media

Media used were prepared methods using appropriate compositions. Components used high grade. All media were sterilized by autoclaving for 20 minutes. The compositions used for different media have been shown below:

1) Composition of YMM media

Molasses	2%
Agar	2%
Peptone	0.50%
Yeast extract	1%
Malt extract	1%

2) Composition YEPD agar media

Yeast extracts	3.0g
Peptone	10.0g
Dextrose	20.0g
Distilled water	1.0L
Agar	15.0g

3) Composition YEPD liquid media

Yeast extracts	3.0 g
Peptone	10.0 g
Dextrose	20.0 g
Distilled water	1.0 L

4) Composition YPD liquid medium

Glucose	20 g
Peptone	20g
Yeast extracts	10g
Water	1 L

Reagents & Chemicals:

Reagents which were used in different methods together with their sources are mentioned below:

1) DNS (3, 5-Dinitro Salicylic acid) reagent:

Mix: 1 .Distill water: 141.6 ml

2. NaOH (analytical grade):2.0gm

3. DNS: 1.06gm

Dissolved above then added: 4 K-Na-Tartarate: 30.6gm

5. Phenol: 760µl

6. Na-metabisulphite: 0.83gm

The solution was filtered and stored in room temperature in dark bottle.

2) 0.1 N Sodium thiosulfate solution:

Add 25 g Sodium thiosulfate and make the volume up to 1000 ml with Distilled water.

3) 0.05 N Potassium Dichromate solution:

9.808 g dry Potassium Dichromate

↓

Pour in 500 ml 10 N sulfuric acid (No. 1-solution)

↓

62.5ml no.-1 solution dilute again in 500ml 10 N sulfuric acid

↓

0.05 N Potassium Dichromate (prepared)

4) 10 N sulfuric acid :

140 ml sulfuric acid and make the volume up to 500 ml with Distilled water.

5) 50% Potassium Iodide:

50 g KI and make the up to 100 ml with Distilled water.

APPENDIX-B

Instruments name	Model no.	Country
Autoclave(18 L)	CL-32L,APL Co.Ltd,Japan	Japan
Autoclave	Model:WAC-47, Daihan Scientific, Korea	Korea
Balance(Core series)	Adam, UK	UK
Balance(pw series)	Adam, UK	UK
Distilation plant(merit water still)	Mo-W4000 ,EURO	EURO
Freeze (-20°C)	Siemens	Japan
Haemocytometer		
Incubator	Model:DSI300D,Taiwan	Taiwan
Laminer airflowcabinet	SAARC	
Microscope	Model-CX-21,Olympus	
Microscope	Model-XSZ-107 BN	
Microscope	Model-H.903,Optima,Taiwan	Taiwan
Oven(Universal drying oven)	Model:LDO-060E,Labtech,Singapore	Singapore
pH Meter	Model-E 201-C,Shanghai RuosuaaTechnology company,China	China
pH Meter (Pocked-sized)	pHep,HI-98107,Hanna instruments	
Refrigerator	Model-0636,Samsung	
Refrigerator	Toshiba	
Spectrophotometer	Model-TGOU, UK	UK
Shaking Incubator	Model-WIS-20R, Daihan Scientific, Korea	Korea
Vortex Mixture	VWR International	
Water bath		Korea

APPENDIX-C

SERIAL NO.	NAME OF THE GLASSS WARE AND OTHERS
1	Beaker
2	Conical flasks
3	Disposable micropipette
4	250 ml Erlenmeyer flask
5	Inoculating loop
6	Knife and forceps
7	Micro pipette 100µl
8	Micro pipette 1000µl
9	Micro pipette 5000µl
10	Measuring cylinder
11	Mackartny bottle
12	Pipette 10ml
13	Petri dishes , disposable
14	Screw cap bottle
15	Spreader
16	Slide
17	Screw cap bottle
18	Screw cap test tube
19	Cotton plug
20	Spatula
21	Scotted bottle