ENHANCED PRODUCTION OF BIOETHANOL FROM HIGHLY EFFICIENT SALT AND ETHANOL TOLERANT YEAST ISOLATED FROM MURCHA

M. Sc. Thesis

2011

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Jeni Maharjan

Roll No. : BT 010/066

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Recommendation

This is to certify that the research work entitled **"Enhanced production of bioethanol from highly efficient salt and ethanol tolerant yeast isolated from Murcha"** has been carried out by **Ms. Jeni Maharjan** under our supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. We, hereby, recommend this thesis for final evaluation.

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Certificate of Evaluation

This is to certify that this thesis entitled **"Enhanced production of bioethanol from highly efficient salt and ethanol tolerant yeast isolated from Murcha"** presented to evaluation committee by Ms. **Jeni Maharjan** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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Jeni Maharjan

Glossary Acronyms

µg	Microgram
µl	Microlitre
µm	Micrometer
ADP	Adenosine diphosphate
ATP	Adinosine triphosphate
CBP	Consolidated bioprocessing
DNA	Deoxyribonucleic acid
DNS	Dinitro salicylic acid
g/g	gram/gram
gm	gram
L	Liter
mg	Milligram
ml	Millilitre
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
nm	Nano meter
^0C	Degree Celsius
OD_{600}	Optical Density at 600nm
RNA	Ribonucleic acid
rpm	Rotation per minute
SSEF	Simultaneous saccharification and extractive fermentation
SSF	Simultaneous saccharification and fermentation

SStF	Solid state fermentation
T_{max}	Maximum temperature
T_{min}	Minimum temperature
T_{opt}	Optimum temperature
TTC	2,3,5-Tris-phenyl tetrazolium chloride
v/v	volume per volume
v/v	volume/volume
w/v	weight/volume
w/w	weight per weight
XDH	Xylitol dehydrogenase
XR	Xylitol reductase
YEPD	Yeast extract peptone dextrose
YMA	Yeast malt agar
YMB	Yeast malt broth
YPD	Yeast peptone dextrose

Abstract

Ethanol is one of the oldest product known to human that is obtained through traditional biotechnology which has been known for long time. It can be made from large variety of natural lignocellulosic renewable materials such as agricultural crops, industrial and domestic wastes etc. Microorganisms used for ethanol production must have high tolerance for ethanol, must grow vigorously and produce large quantity of ethanol. Among many other organisms, yeast is still the primary choice for the fermentation. As yeasts are ubiquitous they can be isolated from variety of substrate from the environment. Total 19 yeasts were isolated from 8 samples of Murcha collected from Bhaktapur and Lubhu. Murcha is an amylolytic starter used for the production of alcoholic drink. The 8 isolates were selected on the basis of high ethanol production as compared to others. The yeasts were identified based on the morphological and physiological characterization. The isolated yeasts were found to be *Saccharomyces cerevisae, Zygosaccharomyces bailii, Pichia canadensis, Zygosaccharomyces rouxii, Saccharomycopsis fibuligera, S. exiguus and Pichia pijperi*. Among them two were *Saccharomyces cerevisiae* and these were selected on the basis of high ethanol production.

The viability count for salt tolerance found to be maximum at 14%. As the salt concentration was increased viability decreased gradually. However growth was seen up to 20% salt concentration. Likewise ethanol tolerance was seen up to 20% but the growth was very less.

At substrate concentration of 15% highest ethanol of 6.52 mg/ml was produced by S2Y4 and for 20% highest ethanol produced by S2Y8 was 7.7 mg/ml. The temperature of 24^{o}C and 28^{o}C was found to be optimum, maximum ethanol produced at 28^{o}C was 7.2 mg/ml by S2Y1 and at 24^{o}C maximum ethanol produced was 7.4 mg/ml by S2Y8. Similarly, pH of 4.5 to 6 was found to be effective for higher concentration of ethanol production. At pH 4.5 S2Y8 produced 7.9mg/ml, at pH 5 S1Y3 produced 8.2mg/ml, and at pH 6 S2Y4 produced 7.4 mg/ml ethanol. Among the nitrogen sources used ammonium phosphate and urea was found to be effective for ethanol production. 6.9 mg/ml ethanol was produced by S2Y8 using ammonium phosphate whereas S1Y5 produced 8.7mg/ml ethanol using urea. These conditions were considered as optimum for ethanol production. However the optimizing parameters were species specific.

CHAPTER ONE

INTRODUCTION

1.1 Background

The production of pure ethanol apparently began in the 12-14th century along with improvement in the art of distillation permitting the condensation of vapors having the lower boiling points. In fact the manufacture of ethanol is as old as human civilization. During the middle ages, alcohol was not only mainly used for the production or as a constituent of medical drugs, but also for the manufacture of painting pigments and other chemical industries. Due to economic improvements of the distilling process, it was only in the 19th century that this trade became an industry with enormous production, (Roehr, 2001). At the beginning of 20th century raw materials such as molasses, sulfite waste liquors and the possibility of hydrolyzing lignocellilosic materials were investigated in Germany and USA. And later found that alcohol might be used as fuel for combustion of engines, especially automobiles. This led to the invention of mass production of absolute ethanol. Another milestone in the large scale ethanol technology is the Brazil ethanol program and the ambition of this program was to provide 100% ethanol fuel using sugarcane as raw material. Beginning in the early 1980s, and continuing since then several countries especially Europe, have decided to initiate programs for large scale production of ethanol from indigenous materials. Now, ethanol has become an important industrial chemical with emerging potential as a biofuel to replace non-renewable fossil fuels (Alfenore *et al*., 2002).

The energy crisis in 1970 led to the development of low cost, sustainable and renewable energy sources i.e ethanol and has become a major focus in scientific research (Favela *et al.,* 1986; Ingledew, 1999; Pramanik, 2003; Pramanik, 2005). The energy crisis necessitates studying and discovering new processes involved in the production of utilizable compounds as alternative energy sources among which fermentation to ethanol represents a significant strategy. Leading to energy crisis, the production of bioethanol has been successfully used in Brazil where large numbers of cars are run on either gasohol (76% gasoline and 24% ethanol) or pure ethanol (Ogbonna *et al.,* 2001). Ethanol is being widely investigated as a renewable fuel source because in many respects it is superior to gasoline fuel (Jones and Ingledew, 1993). This situation has led many countries to develop and prepare preconditions for the use of ethanol as a fuel. Beside the use of ethanol as biofuel it has wide range of application in manufacturing of perfumes, aftershaves and cleaners as solvent, used in antiseptic, germicide, antifreezer and beverage ethanol (Moore *et al.,* 1988; Ogbonna *et al.,* 1989; Garcia *et al.,* 1994;

Araujo *et al.,* 1998; Lehlonen *et al.,* 1999; Ahro *et al.,* 2001; Almedia *et al.,* 2001; Teramoto *et al.,* 2002; Malluochas *et al.,* 2003; Torija *et al.,* 2003; Dragone *et al.,* 2004; Tsakiris *et al.,* 2004.). Additionally there is a growing demand of ethanol in industries such as pesticides, pharmaceuticals, paints and varnishes and for laboratory requirements (Krishna *et al.,* 1999). Furthermore it has an application in synthesizing products like ethylene, acetaldehyde and acetic acid etc. (Slapack *et al.,* 1987; Machado *et al.,* 2005; Zafar *et al.,* 2005).

In many industrial application microbes are being used to produce industrially useful and viable products by the method of fermentation. As the fermentation does not require the high technology it could be introduced and developed in many developing countries (Ogbanna *et al.,* 1997). Fermentation is core of biotechnology where current methodologies span across technologies based on the use of either solid or liquid substrate (Mazumdar-Shaw and Suryanarayan, 2003; Jones and Lassner, 2005). Solid substrate fermentation is an ancient microbial culture system that is being transformed for new purpose using new approaches of microbiology, biochemistry and biochemical engineering (Dominguez *et al.,* 2001; Murthey *et al.,* 1993; Tao *et al.,* 1997;). The use of simultaneous saccharification and fermentation (SSF) for the studies seems to be promising for alcohol productivity whether with mutants or wild types of microorganisms (Rajoka *et al.,* 2004).

1.2 Current studies

Despite the evolving trend of using bacteria for ethanol production, yeast is still the primary choice for fermentation (Chandra and Panchal, 2003). Yeasts are used in many fermentative productions such as ethanol, alcoholic beverages, baking products, and protein and vitamin supplement in human and animal diets as well as in the production of single cell proteins. However, efforts to characterize these yeasts are still limited. In the assessment of yeast isolates for economic and efficient ethanologenic processes, certain specific physiological properties are important and required. These include good tolerance to high concentrations of ethanol, sugars and acids as well as high osmotic pressure (Ansanay-Galeote *et al.,* 2001; Benitez *et al*., 1983; Ezeogu and Okolo, 1984a; Okolo *et al.*, 1990; Okolo *et al*., 2004 and Stewart *et al.*, 1984). Over the past two decades most bioethanol related researches in many other developing tropical countries have focused primarily on the isolation of local *Saccharomyces* yeasts and their use for industrial ethanol production (Bulawayo *et al.,* 1996; Ezeogu and Emeruwa, 1993; Ezeogu and Okolo, 1994a, b; Okafor, 1987; Sefa-Dedeh *et al.,* 1999). Yeasts have been isolated from many sources for industrial purposes. Such sources include cashew, apple juice (Osho, 2005), palm wine (Bechem *et al*., 2007; Nwachukwu *et al.,* 2006) and

fermenting cassava tubers (Oyewole and Odunfa, 1988) among others. Yeasts occur widely in nature and have been recovered from widely differing terrestrial as well as marine sources. Certain yeasts are more or less ubiquitous while others appear to be restricted to very specific habitats. Yeasts seldom occur in the absence of either molds or bacteria (Kreger-van Rij, 1984).

At present condition Nepal can produce 18,045m^3 ethanol annually without compromising the production of food products from sugarcane such as sugar, chaku and shakhar. This can reduce 14% import of gasoline and can save US 10 million annually if bioethanol is used. There is a chance of development in industrial sector for the production of higher amount of sugarcane. Furthermore, the use of ethanol in the transport sector will have a positive environmental effect reducing carbondioxide emissions and combating pollution in Kathmandu valley (Silveira and Khatiwada, 2010).

Traditionally ethanol has been produced form liquid or fluid via submerged microbial fermentation (Hang *et al.,* 1981). And to carry out the fermentation process the used microorganisms are just important as the substrate and have been the target of many researches. Among the different yeasts *S. cerevisiae* is the most widely used for the baking and brewing in most industries (Gunasekaran and Chandra, 1999; Michilka, 2007; Roehr, 2001).

The production of ethanol has been practiced for thousands of years (Slapack *et al.,* 1987) and is used for heating, cooking, motor fuel, etc. Ethanol is a liquid fuel that has a property of high energy, clean burning and totally renewable. The major way of producing ethanol is either the carbon containing gases such as ethylene, acetylene and carbonmonoxide derived mainly from various fossil fuels or from carbohydrates.

There are vast amount of renewable biomasses that can be utilized for the production of liquid fuel (South and Lynd., 1994; South *et al.,* 1995; Lynd., 1996; Lynd *et al.,* 1996; Goug *et al.,* 1999; Krishna and Choudhary., 2000). There are three main abundant renewable raw materials from which ethanol can be produced, which are saccharines, starch materials and cellulosic materials (Mathewsion, 1980; Shirai *et al.,* 1998; Underwood *et al.,* 2002; Zaldivar *et al.,* 2005). Plants containing high amount of celluloses including hemicelluloses and lignin and starch materials are the good sources for ethanol production, are the only sustainable source of organic fuels, chemicals and materials (Dumsday *et al.,* 1997; Stevenson and Weiner, 2002; Peterson *et al.,* 1998; Van Rensburg *et al.,* 1998). For the economical production of ethanol, low value agricultural residues can be easily converted to fermentable sugars (Converti *et al.,* 1989; Jesse *et al.,* 2002), agricultural by-products of starchy industry such as potato pulp (Oda *et al.,* 2002; Saito *et al.,* 2003) and raw starch hydrolysate (Kondo *et al.,* 2002; Rajoka *et al.,*

2004; Shigeehi *et al.,* 2004) can be the resources. Cellulose and other lignocellulosic biomass are economical than sugar crops but only after being hydrolysed to fermentable sugars (Tewari *et al.,* 1985; Grohmann *et al.,* 1996; Ogbonna *et al.,* 2001; Zhou and Ingram, 2001; Ballesteras *et al.,* 2002; Fujita *et al.,* 2004; Genansounon *et al.,* 2005; Shahbazi *et al.,* 2005). Although cellulose is the abundant low cost carbon source, hydrolysis of this polymer is problematic to yield the ethanol (Banat *et al.,* 1998).

The sugar crops that are suitable for industrial fermentation include sugarcane, sugar beets, fruits, sweet potato, sweet sorghum, Jerusalem artichokes and agricultural wastes (Atiyeh and Duvnjak, 2002; Hang *et al.,* 1981; Ingledew and Kunkee, 1985; Joshi *et al.,* 2001; Michilka, 2007). Molasses is a byproduct of sugar manufacturing process and most important raw material for ethanol production, these raw materials could meet the demands of liquid fuels to the country. Making use of these cheaper renewable feedstocks and waste fruits and vegetables to ethanol will add even more to the production of the value added product. There have been numerous studies concerning the effects on the fermentation kinetics of temperature, ethanol concentration, assimilable nitrogen, nutrients, oxygen and inhibitors. Importance in ethanol fermentation has been focused on taking up renewed interest in research works in several areas such as use of improved mutant strains, yeast strain development from cheaper source, use of cheaper source of raw materials, optimum reactor design, better nutrients for optimum cell growth, optimization of fermentation factors, etc. (Bisson, 1991; Converti *et al.,* 1985; Gregory *et al.,* 1984; Insa *et al.,* 1995; Jones *et al.,* 1981; Mancilha *et al.,* 1984; Mendes *et al.,* 2004.). These days' four different fermentation operations are used in industries: batch fermentation, continuous fermentation, fed-batch fermentation and semi continuous fermentation. Among these batch fermentation is the most commonly used method for ethanol production which is the classical method that has been used from hundreds of years (Green, 2002; Mendes *et al.,* 2004). In this process, cell is grown separately from the fermentation substrate and is combined with any required enzymes or nutrients.

The trend of modern life involving huge energy consumption has resulted into exhaustion of natural non-renewable resources (Escande, 1997; Alexandratos, 1999; Haines, 2001). There has been high investment in the fundamental research on the thermochemical conversion of alternative solid fuels and on its area of application. The upgrading processes is being carried out to develop easy accessible energy sources of high efficiency (Hallgren, 1996; Jimenez *et al.,* 2003; Anderson *et al.,* 2005 Shahbazi *et al.,* 2005).

Fossil fuels are the main sources of most of the energy that are used. The combustion of fossil fuels give rise to the deleterious gases that influence human health in direct and indirect ways (Haines, 2001; Kikuchi, 2001). The increase in concentration of green house gases in atmosphere causes climate change, ecological problems and loss of biodiversity. Abundant data demonstrates that global climate change has warmed during the past 150 years. There is increase in irrigation demands and the range of certain pests due to warmer climate (Loaieiga, 2003; Khan and Baig, 2003; Majdi and Ohrvic, 2004). There is a need of alternative energy sources such as ethanol, methane and hydrogen as fossil fuel is depleting slowly. The renewable source of biofuel like garbage, wood, methane and alcohol are inevitably of great importance in the years to come. There is a high demand of these resources for obtaining energy (Rotty. 1979; Escande, 1997; Davis *et al.,* 2005; Rajagopalan *et al.,* 2005).

Properties of ethanol

Ethanol is a clear, colorless liquid with a distinctive smell having a chemical formula CH_3CH_2OH, with melting temperature of -114.1^{o}C and boiling temperature of 78.5^{o}C. It has a density of 0.789g/ml at room temperature. The CO_2 emission from its oxidation is much less than any other oxygenates as it consists approximately 35% oxygen, twice as much oxygen as other commonly used oxygenates.

The advantages of bioethanol

Various studies have proved ethanol to be an ideal fuel
- sustainable and renewable resources
- reduces dependence on oil
- reduces air pollution
- possesses enormous potential for rural economic development
- will extend currently shrinking supply of petroleum

1.3 Objectives

1.3.1 General objectives

Isolation and characterization of yeasts from Murcha samples to study salt and ethanol tolerance and hence ethanol productivity.

1.3.2 Specific objectives

Isolation and characterization of different yeasts form Murcha samples.

Study on salt and ethanol tolerance of isolated yeasts.

Selection of efficient yeast for the production of ethanol.

Optimize ethanol production from salt and ethanol tolerant yeast to compare their productivity

Production of ethanol from osmostressed yeast.

1.4 Rationale and Scope

The production of fuel ethanol by fermentation requires the ability to produce high ethanol concentrations rapidly while maintaining good yields. Rapid fermentation and high alcohol levels are desirable to minimize capital costs and energy required for distillation, while good yields are necessary for process economics. High ethanol producing yeast must possess characteristics of both high alcoholic forming power as well as high alcohol resistance. These days yeast is increasingly used in genetic engineering, single cell protein and enzyme production, microbiological assays and ethanol production. Yeasts are ubiquitous in nature, they occur in soil, plants, berries, fermenting foods, etc. though they are present in vast areas only small number of yeasts are associated with the production of fermented and microbial foods. Murcha selected for the isolation of the yeasts are starter cake used as source of fermenting yeasts are indigenous to Nepal. And Murcha consists of amylolytic as well as alcohol producing yeasts. The isolation of yeasts that could utilize vast amount of substrate is important and beneficial in the fermentation and production of ethanol. The yeasts that are used in industries must sense and response to stress conditions rapidly and adapt to these adverse environmental factors by adjusting their metabolic activities to avoid substantial viability loss. And the capability of cells to tolerate various stresses is one of the important criteria to select industrial strains for efficient ethanol fermentation. Osmostress caused by high sugar have been reported in wine yeast, but there are few

reports that specifically investigate the effect induced by elevated sodium chloride. So here in this study, salt induced osmotic stress conditions were applied to understand the fermentation performance of the isolated yeast. The products synthesize by yeast during stress conditions have shown to protect cells against high temperature by stabilizing proteins and membrane integrity. The ethanol tolerance by the yeast is also one of the important factor as higher the ethanol tolerance of the yeasts it is less inhibited by the product during fermentation.

CHAPTER TWO

LITERATURE REVIEW

2.1 Murcha

Murcha is generally found in Asian countries and is known by various name such as Murcha or Marcha in the Himalayan regions of India, Nepal and Bhutan, Ragi in Indonesia, Loogpang in Thailand, Bodod in Philippines, Chinese yeast in Taiwan (Hesseltine *et al.,* 1988) and Nuruk in Korea (Park *et al.,* 1977). Murcha are not food but are mixed dough inoculum used as starter culture to ferment variety of starchy substrate that is used for the production of alcoholic beverages. It can be found as dry, round or flattened, creamy - white to dusty white, solid cake like structure ranging from 1.9 to 11.8 cm in diameter and weighing from 2.3 to 21.2 gram (Tsuyoshi *et al.,* 2004).

2.1.1 Composition of Murcha

Murcha are traditionally prepared from crushed raw rice mixed with wild herbs such as *Albizia myriophylla* and a pinch of previously prepared powdered Murcha. For the preparation of Murcha, glutinous rice is soaked and is mixed with the roots of *Plumbago zeylanica,* leaves of *Buddleja asiatica,* flowers of *Vernonia cinerea,* ginger, red chili and a pinch of powdered old Murcha. Then the mixture is made into paste by adding little water and kneaded into flat round cakes and is placed on bamboo stripes bedded with fresh fronds of ferns (*Glaphylopteriolopsis erubescens*) for 2-3 days at room temperature then sundried for further 2-3 days. Thus prepared Murcha can be stored in dry place for more than a year (Thapa, 2002). These are mildly acidic with pH 5.2 and moisture 13% w/w and ash 0.7% w/w (dry weight basis) (Tamang and Sarkaar, 1995). The process of making Murcha is practiced as a hereditary trade that passes from mothers to daughters as it is exclusively produced by women. It is a kind of trade secret as the actual composition for making Murcha is not known by the outsiders.

2.1.2 Microorganisms found in Murcha

The microflora of Murcha consists of amylolytic as well as alcohol producing yeasts, starch degrading molds and lactic acid bacteria (Tamang and Sarkar, 1987). The use of various herbs contributes to the different microflora found in the Murcha. It consists of various molds, bacteria and yeasts. The filamentous molds such as *Mucor circinelloides* and *Rhizopus chinensis*, yeasts such as *Saccharomycopsis fibuligera* and *Pichia anomala*, and bacteria such as *Pediococcus pentosaceus* have been found (Tamang and Sarkar, 1996). Lactic acid bacteria such as *P. pentosaceus*, *Lb. bifermentans*, and *Lb. brevis* are

also present in Murcha (Hesseltine and Ray, 1988; Tamang and Sarkar 1995; Tamang *et al.,* 2007). Besides these other large variety of organisms are also found (Tamang and Sarkar, 1995).

The first preliminary study of the microbiology of Murcha samples collected from Sikkim was done by Kobayashi *et al.,* 1961, who reported *Rhizopus oryzae, Mucor praini*, and *Absidia lichtheimi*. *Hansenula anomala var. schneggii* (*Pichia anomala*) in Murcha collected from Kalimpong in the Darjeeling hills was reported by Batra and Millner, 1974. Mucor and *Rhizopus* spp. from murcha samples in Nepal was isolated by Hesseltine *et al.,* 1988. In Bhutanese murcha Uchimura *et al.,* 1990 reported the dominant yeast to be *Saccharomycopsis,* and also molds *Penicillium spp.* and *Aspergillus spp*.

2.1.3 Yeasts in Murcha

Saccharomycopsis fibuligera is the most dominant yeast in Murcha (Tamang and Sarkar, 1995), and is typically found growing on cereal products (Hesseltine and Kurtzman, 1990). It has been assumed that two types of amyolytic yeasts are found in Murcha, mostly *Saccaromycopsis* that degrade starch and produce glucose, and alcohol-producing yeasts that grow rapidly on the resultant glucose to produce ethanol (Tamang and Sarkar, 1996; Thapa, 2002). Tsuyoshi et al., 2005 isolated several yeast strains from Murcha and main species were *Saccharomyces bayanus*, *Candida glabrata, Pichia anomala, Saccharomycopsis fibuligera, Saccharomycopsis capsularis* and *Pichia burtonii*. Among the microbes, saccharifying yeast species belonging to *Saccharomycopsis* predominate, suggesting that they are involved in amylolytic fermentation (Hesseltine and Kurtzman, 1990; Tamang and Sarkar, 1996).

2.1.4 Role of different microbes in fermentation

Among the yeasts found in Murcha the high amylolytic is shown by *Sm. fibuligera*, *Sm. capsularis* and *P. burtonii* whereas *S. bayanus, C. glabrata*, and *P. anomala* shows alcohol producing capacity (Tsuyoshi *et al.,* 2005; Tamang *et al.,* 2007). *Rhizopus spp*. and *Sm. fibuligera* shows the saccharifying activities wheras liquefying activities are shown by *Sm. fibuligera* and *S. cerevisiae* (Thapa, 2001). *Rhizopus sp*. and *Sm. fibuligera* degrade cereal starch and produce glucose, and then alcohol-producing yeasts species of *Saccharomyces* and *Pichia* rapidly grow on the resulting glucose to produce ethanol. A considerable amount of glucoamylase is produced by *Rhizopus spp*. (Ueda and Kano, 1975) and by *Sm. fibuligera* (Ueda and Saha, 1983). Among the lactic acid bacteria *Pediococci* are more dominant than lactobacilli (Tamang *et al.,* 2007). These lactic acid bacteria in Murcha play an important role in imparting flavor, antagonism, and acidification of substrate.

2.2 Yeast

Yeasts are unicellular eukaryotic micro-organisms classified in the kingdom Fungi, with about 1,500 species currently described (Kurtzman and Fell, 2006). Yeasts, which form one of the important subclasses of fungi, are rather more complex and usually larger than bacteria. Yeasts are distinguished from most fungi by their usual existence as single ovoid cells doubling every 1-3 hours in favourable media (Wayman and Parekh, 1990). The most common asexual method for the reproduction is the budding or fission but some may reproduce by mitosis. Though yeasts cells are unicellular some may become mutlicellular by the formation of pseudohyphae (Kurtzman and Fell, 2005). The size of yeast greatly vary depending upon the species, the size typically measures form 3–4 µm in diameter but some may reach 40 µm (Walker *et al.,* 2002). Unlike some yeast *Schizosaccharomyces pombe*, reproduce by fission (Balasubramanian and Glotze, 2004). All the yeast species are characterized by similar sets of features i.e. morphological and physiological. This type of description, in which physiological characters are important, distinguishes yeast taxonomy from other fungal taxonomy (Kreger-van Rij, 1984).

Saccharomyces cerevisiae is the dominant yeast among the many yeasts species. It is among the almost 800 yeast species described in taxonomy by Kurtzman and Fell, 1998. Many other different species can be interesting for the variety of reasons ranging from their use in specific technological applications. The attention should be given to those non-conventional yeasts (NYC) or non-Saccharomyces yeasts as well. In the last years there has been an attraction towards some species such as: *Kluveromyces lactis* as a possible utilize of residual whey in dairy industries, methylotrophic yeasts for the production of heterologous proteins for the study of peroxisome biogenesis and *Yarrowia lypolytica* for its ability to grow on particular substrates and its high protein excretion capacity. In spite of the importance of these yeasts there is a need of basic knowledge about enzymes of fundamental pathways, their regulation and the genes encoding.

Table 2.1: Some present and potential uses of yeasts in the food, beverage and fermentation industries.

Ale fermentation	***Saccharomyces cerevisiae***
Bread and dough leavening	*S. cerevisiae, S. exiguus, S. rosei*
D-Arabitol (sweetener)	*Candida diddensiae*
Emulsifier	*C. lipolytica*
Ethanol fermentation	*S. cerevisiae*
Fish and poultry feeds (astaxanthin)	*Phaffia rhodozyma*
Fodder and single cell protein	*C. utilis*
Lactose and milk fermentation	*C. pseudotropicalis, Kluyveromyces fragilis, K. lactis*

Ale fermentation	*Saccharomyces cerevisiae*
Lager beer fermentation	*S. carlsbergensis*
Wine fermentation	*S. cerevisiae*
Xylitol (sweetener)	*T. candida*
D-xylose fermentation	*C. shehatae, Pichia stipitis, P. segobiensis*

Modified from Jacobson and Jolly, (1989).

2.2.1 Yeast physiology

Though yeast are unicellular some may become mutlicelllular by the formation of string of connected budding cells known as pseudohyphae or false hyphae, as seen in most molds (Kurtzman and Fell, 2005). Identification of yeast genera can often be achieved by morphological tests supplemented with a few physiological tests. With regard to the later, sole carbon and nitrogen source assimilation by yeasts may be determined by auxanography which nowadays can be conveniently carried out using commercially available kits: for example, Analytical Profile Index (API) strips (BioMérieux, France) or the automated/computerized BCCM/Allev 2.00 system (Louvain-la-Neuve, Belgium). Sugar assimilation and fermentation tests are commonly accomplished using glucose, galactose, maltose, sucrose, lactose, raffinose, trehalose, xylose, rhmanose, mannitol, inositol, ribose, arabinose, cellobiose, D-glucoronate, starch, sorbose, mellibiose, erythritol and glycerol. With regard to fermentation of these sugars, Scheffers, 1987 has argued that the anaerobic liberation of CO_2 into Durham tubes is not very accurate for detecting slowly fermenting yeast species. Ethanol production assays are deemed to be more appropriate determinants of sugar fermentation by yeasts (Walker, 1998). Yeasts are used in many industrial processes, such as the production of alcoholic beverages, biomass and various metabolic products. Some of these products are produced commercially while others are potentially valuable in biotechnology (Kurtzman and Fell, 1997). Some yeast species have potential to be uses in food, beverage and fermentation industries (Jacobson and Jolly, 1989).

2.2.2 Isolation of yeast

From widely differing aquatic and terrestrial sources, yeasts can be recovered. Most of the yeasts occur widely, whereas some are confined to the restricted habitats. Yeasts seldom occur in the absence of either molds or bacteria (Kreger-van Rij, 1984). Thus, selective method has to be applied to for the recovery of yeasts, employing media which permit the yeast to grow while suppressing molds or bacteria. The composition of media is such that yeasts are capable of developing at pH levels and water activities which reduce or inhibit the growth of bacteria. Sometimes antibiotics are used to suppress bacteria. But the fungi static agents should be used with cautious as they may inhibit the growth of yeast as well. The cultures are usually incubated at the temperature range of

20-25^{o}C as most of the yeasts are mesophilic but the temperature range may be different for the psychrophiles and mesophiles. Davenport, 1980 has described several media containing antibiotics for the isolation of yeasts. And these media may contain the compound which can be either antibiotic or inhibitors or selective media with nitrogen and carbon sources, for the isolation of particular genus, species, or of yeasts with a particular property.

Scheda, 1966 stated that yeast cultures are best maintained on the medium which contains glucose as the only source of carbon as this reduces the risk of changes in growth and fermentative patterns due to the selection of mutants.

2.2.3 Classification and identification of yeasts

However, different tests such as morphological, physiological and biological tests have commonly been used for phenotypic characterization of yeast species; the methods are not so reliable due to strain variability. And this does not allow differentiation between yeasts species belonging to same species. Recek *et al.,* 2002 genetic characterization using molecular techniques provides more powerful means of identification and differentiation among strains. Ribosomes are the indispensable component for the identification based on the genomic analysis and their structures are strictly conserved (Nishimura and Mikata, 2000).

Physiological properties primarily serve to describe and identify yeast species and, to a very minor extent, genera. And the tests that are mostly used for the routine identification are fermentation and growth on carbon sources, growth on nitrogen sources, and requirement of vitamins, growth at various temperatures and on media with content of sugar or sodium chloride, hydrolysis of urea, and resistance to antibiotics. There is not standardized method as many of these tests and the results depend on the techniques employed.

The chief characteristics used to classify yeasts are microscopical appearance of the cells, their mode of sexual reproduction certain physiological (especially nutritional) activities, certain biochemical futures comparison of genomes in terms base sequences, by DNA hybridization or RNA/DNA sequence comparison (Barnett *et al*., 2000).

Table 2.2: Criteria used in yeast species classification and identification.

Morphological Characteristics	Physiological characteristics
Giant colony morphology	Fermentation of sole carbon source
Cell morphology in liquid media	Assimilation of sole carbon source
Mode of vegetative and/or sexual Reproduction	Assimilation of sole nitrogen sources
Spore characteristics	Pigment production
Pellicle formation at liquid surface	Osmophilia

2.3 Colony morphology

Individually yeast cells appear colorless, but when grown on artificial solid media they produce colonies which may be white, cream colored, or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts. Physiological characteristics are also used to a great extent in determining yeast species (Alexopoulos, 1962).

2.4 Mode of reproduction

The yeasts were classified into budding yeasts and fission yeasts by (Alexopoulos, 1962) depending on their types of asexual reproduction. And this is a type of asexual reproduction. The budding yeasts reproduce by budding; the bud enlarges until it is separated from mother cell by constriction at the base. Under some conditions bud do not separate and gives rise to pseudo mycelium. Sexual reproduction in yeasts takes place either between two somatic cells or between two ascospores which unite to form a zygote cell. But the majority of industrial yeasts reproduce by budding (Glazer and Nikaido, 1995).

2.4.1 Spore

Ascospores are confined in ascus and the number depends on the number of cell divisions that takes place. The fungal belonging to the phylum Ascomycota produces the ascospores. Ascospores are generally found in the clusters of four or eight spores within the ascus. In response to nutrient depletion the baker's yeast give rise to the stress-resistant haploid spores. The diploid cells of *S. cerevisiae* modify their growth in response to nutrient availability and in the presence of nutrients they grow in budding form. Whereas the low or poor nitrogen source such as proline will trigger the formation of pseudohyphal form (Gancedo, 2001). And the sporulation occurs when there complete absence of nitrogen and the presence of nonfermentable carbon sources such as acetate in the medium (Esposito and Klapholz, 1981).

Ascospores are of different types such as globose and ovoid are formed in *Debaromyces, Saccharomyces, Schizosaccharomyces* and *Saccharmycodes*, in *Pichia* and some species of *Hansenula*, the ascospores are hat-shaped. But in other species the germinating spores bud or form germ tubes, which results in bursting of the persistent ascus wall (Alexopolos *et al.,* 1996). Miller 1989 pointed out that ascospore are much more durable than somatic cells. Endospores are usually observed in old cultures and are vegetative cells which are formed within discrete cells and hyphae on YM agar, Potato dextrose media and corn meal agar that are kept at room temperature. So far there is not any media that has been devised to stimulate the development of endospores.

More than 15 different methods for inducing ascosporulation in yeasts have been recommended following the first report by DeSeynes in 1868 describing the formation of ascospores by yeasts on the surface of water (DeBecze, 1959). The various method of ascospore formation either based on the starvation of cells or the cultivation of cells in the minimal amount of directly assimiable carbohydrates. But certain methods are specific for particular strains or genera.

2.4.2 Pseudomycelium formation

The development of pseudohyphae was observed and described in industrial yeast strains more than 100 of years ago. Since then, the ability of yeasts to form pseudomycelim has been used as an important taxonomic criterion (Kurtzman and Fell, 1998). Wide variety of yeasts including the genera *Candida, Endomyces, Pichia, Saccharomyces*, and *Yarrowia* shows the development of pseudomycelium (Kurtzma and Fell, 1998). However, pseudohyphal growth depends not only on the yeast species, but also on the growth conditions. In general, formation of pseudomycelium seems to be favored by poor conditions. Pseudomycelium and the elongated cells have been observed in the cultures that have been grown markedly below the optimum temperatures. Another factor that stimulates the formation of pseudomycelium is the partial anaerobsis. This factor has found its application in the so-called Dalmau plate technique, where part of the surface of an agar streak is covered with a coverslip and where the portion of the culture beneath the glass produces pseudomycelium more readily than the other part of the culture (Kurtzman and Fell, 1998; Ernst, 2000). The standard procedure for observing pseudohyphal growth on solid medium is still used for taxonomical classification of yeasts (Kurtzman and Fell, 1998).

2.5 Fermentation of carbon sources

Carbohydrates such as mono-, di-, tri-, and polysaccharides are generally used for physiological features to distinguish different yeasts that the given organism can use the

carbohydrate as the source of carbon and energy under semi-anaerobic and aerobic condition. And they have the ability to grow in the presence of glucose or sodium chloride (as a measure of osmotolerance) and the relative ability to hydrolyze and utilize lipids. These various properties help investigators to determine which yeast strains are suitable for the investigation to be used for particular application (Glazer and Nikaido, 1995).

Yeasts can also be categorized in several groups according to their modes of energy production, utilizing respiration or fermentation. And these processes are mainly regulated by environmental factors on the availability of glucose and oxygen. Thus yeasts can adopt to varying growth environments, and within a single species, the prevailing pathways will depend on the actual growth conditions. As for example *S. cerevisiae* can utilize glucose in several different ways depending on the presence of oxygen and other carbon sources.

Table 2.3: Principal modes of respiration in yeasts (biochemie.web.med).

Types	**Examples**	**Respiration**	**Fermentation**	**Anaerobic growth**
Obligate repires	*Rhodotorula spp.* *Cryptococcus spp.*	Yes	No	No
Anaerobic respires	*Candida spp.* *Kluyveromyces spp.* *Pichia spp.*	Yes	Anaerobic in pregrown cells	No
Aerobic fermenters	*S. pombe*	Limited	Aerobic and anaerobic	No
Facultative aerobic fermenters	*S. cerevisiae*	Limited	Aerobic and anaerobic	Facultative
Obligate fermenters	*Torulopsis*	No	Anaerobic	Yes

2.5.1 Assimilation of carbon sources

Though there are many yeast which employ sugars as the main carbon and energy but there are particular yeasts which can utilize non-conventional carbon sources such as nitrogen, phosphorous and sulfur. And most of them are capable of assimilating simple nitrogenous sources to synthesize amino acids and proteins. Aspects of the metabolism of inorganic compounds such as biopolymers, pentoses, alcohols, polyols, hydrocarbons, fatty acids and organic acids have been studied in detail, predominantly only in *Saccharomyces cerevisiae*. There is a need in other species as well.

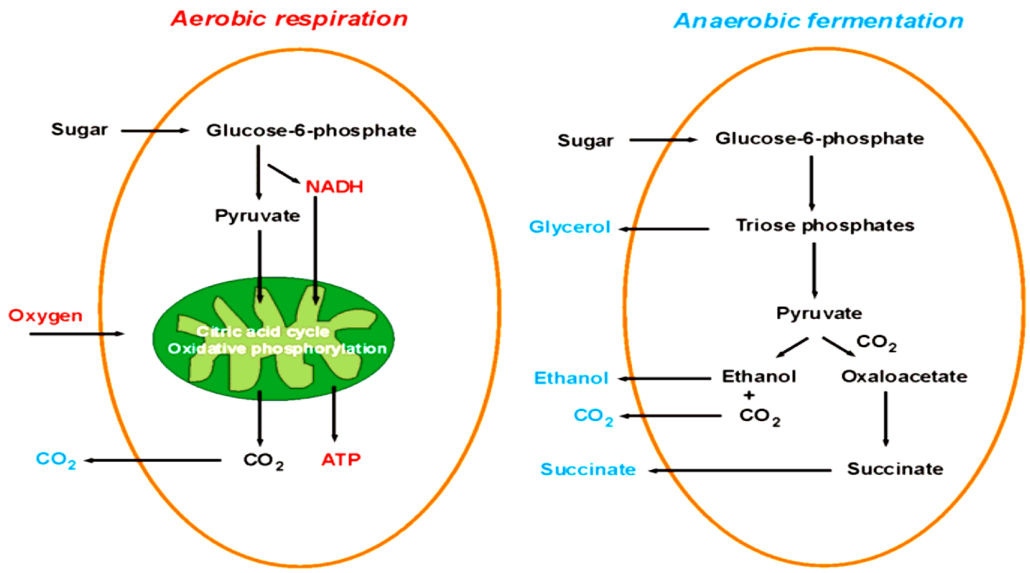

Fig 2.1: Metabolism in yeast under aerobic and anaerobic conditions

Sugars are utilized by almost all known yeasts and the common process in their metabolism is the conversion of glucose 6-phosphate or fructose6-phosphate to pyruvate through the common glycolytic pathway and the metabolic product of the pyruvate is different depending on the yeast species and the cultivation conditions. Some specialized yeasts can directly metabolize starch, inulin, cellulose, hemicelluloses, or pectin but for utilization first the carbon sources have to be hydrolyzed by non yeast enzymes.

The ability to ferment sugars is measured by the production of carbon dioxide. There appear to be no exceptions to the rule that when a yeast strain ferments a carbohydrate it is also able to grow on it. However, the reverse does not hold true: many yeasts grow aerobically on sugars they cannot ferment. Yeasts belonging to the genera *Kluveromyces, Saccharomyces* and *Zygosaccharomyces*, ferment glucose vigorously whereas others such as *Rhodosporidium* and *Stweigmatomyces*, do not noticeably ferment any sugars. Several tests have been devised to detect the production of carbon dioxide from carbohydrates (van der Walt, 1970).

Only few yeast species can ferment pentose sugars to ethanol, although many yeasts can grow aerobically on pentoses. The inability of *S. cerevisiae* to ferment could be circumvented by introducing genes for xylose reductase (XR) and xylitol dehydrogenase (XDH) form xylose fermenting species like *Pichia* by recombinant DNA technology. Though this process has been applied the efficiency of xylose fermentation remains low.

Disaccharides (maltose, sucrose, melibiose, lactose or cellobiose) can be easily used as nutrients by the action of corresponding hydrolases that breaks down these

disaccharides into their respective monosaccharides. Thus the hydrolysis is coupled to transport either disaccharide or the resulting monosaccharides.

Table 2.4: Disaccharides as substrates in yeasts.

Disaccharide	**Extracellular hydrolysis**	**Intracellular hydrolysis**	**Products**	**Organism**
Maltose		Maltase	2Glucose	*S. cerevisiae*
Sucrose	Invertase		Glucose+ Fructose	*S. cerevisiae*
Melibiose	α- galastopyranosidase		Glucose+ Galactose	*S.carlsbergensis*
Lactose		β-galactosidase	Glucose+ Glactose	*Kluveromyces*
Cellobiose	β-glucosidase		2 Glucose	*Brettanomyces*

Table 2.5: Assimilation of nitrogen sources

Nitrogen source	Metabolites	Examples
Urea	Ammonium (urea ammonium hydrolase)	Many yeasts
Nitrate	>Nitrite > Ammonium	*Candida spp, Hansenula spp*.

Table 2.6: Use of unusual nutrients in yeasts

Carbon source	**Metabolites**	**Examples**
Starch	Glucose	*Candida spp, Pichia spp*
Cellulose	Glucose	
Hemicelluloses	Glucose, Xylose	*Candida spp, Pichia spp*
Pectin	Galactoronic acid	*Candida spp, Kluveromyces*
Inulin	Fructose	*Candida spp, Kluveromyces*
Xylose	Pyruvate > Ethanol	*Candida spp, Pichia Kluveromyces*
Organic acids	Acetyl-CoA	*Many yeasts*
Protein	Amino acids	*Candida spp, Kluveromyces, S. cerevisiae*
Lipids	Fatty acids + Glycerol	*Candida spp, Pichia spp, Yarrowia lypolytica*
Alkanes	Fatty acids	*Candida, Pichia, Yarrowia lipolytica*
Methanol	GAP + DAP	*Hansenula, Pichia pastoris, Candida*

2.6 Osmotolerance in yeast

The studies on the inhibitory effects of high levels of inorganic salts on sugar fermentation was motivated by the high concentrations of electrolytes in fermented foods and in raw materials such as molasses-enriched sugar cane stalks. And this

inhibitory action was first reported by Tajima *et al*. 1966 and Umemoto *et al*. 1967. The salt tolerance of the yeast strains could be the desirable characteristics used for fermentation of molasses (Tajima *et al*. 1966). Due to these studies, researchers have replaced the heterogeneous mixture of inorganic salts found in molasses with pure sodium chloride for more convenient experimental methodology. To date, however, few kinetic studies have been reported on the effects of sodium chloride on entrapped yeasts growing in semisolid media (Tanner *et al.,* 1981), mainly because it is difficult to track cells attached to solids.

2.6.1 Effect of salt on growth of the cells

Parameters such as elevated levels of inorganic electrolytes i.e. salt in the liquid growth medium have been found to influence the yeast activity. (i) Cell growth and multiplication: (a) the number of viable yeast cells per unit volume of liquid growth medium decreases as salt content increases, (b) the biomass of the culture (i.e., the total weight of yeast cells per unit volume of liquid growth medium) decreases as salt content increases, and (c) the length of the lag phase (i.e., the incubation period between inoculation of the culture and detectable initiation of cell growth) lengthens as salt concentration increases. (ii) Utilization of the primary carbon and energy source is reduced. (iii) Change in concentration of metabolic products: (a) there is a decrease in the production of ethanol as salt content increases and (b) there is an increase in the concentration of other fermentation products (such as glycerol, acetaldehyde, etc.) as salt content increases.

2.6.2 Adjustment of cells in osmostressed condition

Saccharomyces cerevisiae is unicellular eukaryote is an ideal model for studying cellular and molecular mechanisms of salt tolerance. Its small genome, which has been sequenced (Wodicka *et al*., 1997; Dujon 1996; Goffeau *et al*., 1996), adds to several other advantages. Yeast cells tend to accumulate compatible solutes like glycerol and trehalose to counteract high external osmolarity during salt stress (Brown, 1990; Gadd *et al*., 1987; Mackenzie *et al*., 1988; Albertyn, 1994).

The numbers of plant membrane proteins, such as the potassium, amino acid, and sugar transporters, have been isolated by functional complementation of yeast mutants (Anderson JA et al. 1992) as many ion flux mechanisms are highly conserved in yeasts. For salt tolerance the accumulation of glycerol is essential (Albertyn *et al*., 1994) and glycerol-deficient mutants are available for evaluating the functions of other sugar polyols in stress tolerance. Yeast provides an unsurpassed system for genetic analysis, transformation and functional characterization of cell-specific functions. When the cells

are exposed to NaCl, cells experience both osmotic stress and ion toxicity. In response to low external osmotic potential, the accumulating glycerol compensates for the difference between the extra and intra cellular water potential (Brown *et al*., 1990). For reducing sodium toxicity, yeast cells have to maintain low cytosolic Na^+ concentrations and this is achieved by several mechanisms: by restricting Na^+ influx, rapidly extruding Na^+ and/or efficiently compartmentalizing sodium into vacuoles. The genetic evidence indicates both mechanisms are essential for yeast salt tolerance (Yagi *et al*., 1988; Ushio *et al*., 1992).

During the last decade, the understanding of molecular physiology of osmotic response in yeasts has undergone considerable improvements. Genetic analysis of mutants and expression analysis by proteomics has helped in understanding the metabolic complexity. Moreover, the advent of DNA microarray technology and its use in large-scale transcript analysis during saline stress conditions enlarged greatly the knowledge on stress response. There has been much progress in the recent years concerning our understanding of molecular biology of osmotic and ionic stress responses in yeast cells (Varela and Mager, 1996). As external osmotic pressure changes it induces changes in the expression of certain genes in yeast which are involved in controlling the levels of compatible solutes like glycerol. With regard to exponentially growing yeasts, Singh and Norton 1991 have shown that when active *S. cerevisiae* cells are transferred to a medium with 8% NaCl, cellular trehalose accumulated immediately and rose to 10 fold a few hours after transfer. Van Dijck *et al*., 1995 have noted that any correlation between cellular trehalose and yeast stress resistance appears to hold only for non-fermenting cells. Although some authors have questioned the evidence implicating trehalose in the osmotic protection of yeast cells (Serrano, 1996), trehalose is now widely recognized as general stress metabolite in yeast. Wiemken, 1990 has proposed that the primary role of trehalose in yeast is as a stress protectant molecule. This is because trehalose has been shown to act in yeast not only as an osmoprotectant, but also as an antidesicant (Gadd *et al*., 1987), cryoprotectant (Hino *et al*., 1990), thermoprotectant (De Virgillo *et al*., 1994) and chemical detoxicant (Attfield, 1997).

2.7 Ethanol fermentation

The biological process in which sugars are converted into cellular energy to produce ethanol and carbon dioxide as metabolic products is referred as ethanol fermentation. During ethanol fermentation one mole of glucose is converted into two moles of ethanol and two moles of carbon dioxide. The equation below summarizes the fermentation of glucose:

$$C_{12}H_{22}O_{11} + H_2O + \text{invertase} \rightarrow 2\ C_6H_{12}O_6$$

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

On the weight basis each gram of glucose can theoretically give rise to 51% alcohol. However the yield attained in practical fermentations does not exceed 90-95% of theoretical value. This occurs due to the requirement for some nutrients to be utilized in the synthesis of new biomass and other cell maintenance related reactions.

One glucose molecule is broken down into two pyuruvate molecules before the fermentation takes place which is known as glycolysis. Glycolysis is summarized by the chemical equation:

$$C_6H_{12}O_6 + 2\ ADP + 2\ P_i + 2\ NAD^+ \rightarrow 2\ CH_3COCOO^- + 2\ ATP + 2\ NADH + 2\ H_2O + 2H^+$$

The ethanol fermentation is greatly affected by the nature of the substrate used. So, the raw materials used for the ethanol fermentation has great importance in the fermentation process (Prescott & Dunn, 1987; Baptista *et al*., 2006).

2.8 Ethanol fermenting yeasts

The genera of yeasts that are most frequently involved in ethanol production are *Saccharomyces*, *Brettanomycespecies*, *Cadida, Kluyveromyces, Debaryomyces, Trolopesis and Clavispora.*

Table 2.7: Yeasts capable of producing ethanol from different carbohydrates

Glucose and sucrose	**Lactose**	**Starch**	**Insulin**	**Xylose**	**Cellobiose**	**Cellodextrin**
S.cerevisiae	*C.pseudotropicalis*	*S.diastaticus*	*C.pseudotropicalis*	*K.marxianus*	*K.cellobiovorus*	*C.wickerhamii*
S.ellipsoideus	*T.cremoris*	*k. marxianus*	*K.frangilis*	*C. tropicalis*	*B.anomalus*	
S.carlsbergensis	*K.frangilis*	*C. tropicalis*	*k. marxianus*	*C. shehatae*	*C. lustanae*	
S. ovifofmis	*Brettanomyces spp*	*C. shehatae*	*Torulopsis spp.*	*k. cellobiovorus*	*C.molischiana*	
S .pombe	*Torulopsis spp.*	*S. alluvius*	*S.occidentalis*	*clavispora spp*	*C. versalis*	
	Debaryomyces spp	*S. castelli*	*S. castelli*	*P.tannophius*	*C.wickerhamii*	
		S.occidentalis	*C. macedoniensis*	*P. stipitis*		
			C. kefyr	*B.anomalus*		
			C.memebranaefaciens	*B.clussenii*		
			K.thermotolerans			
			S. fermentati			
			S. cheresiensis			
			S. kluyveri			
			S. moldevorans			
			D.castellii			
			T. delbrecki			
			T. prectoriensis			
			T.globosa			

Source : Modified from Spencer and Spencer(1997).

2.9 Yeast isolates selection for ethanol production

Generally, to obtain high quality and yield of ethanol in ethanol industry, selection of fermentative yeast is very essential. In selecting yeasts for the efficient production of ethanol fuel (as opposed to potable ethanol) microbiologist have set out certain requirements of yeasts (Panchal *et al.,* 1981), the following are the most important one. An ideal yeast for fuel ethanol production should be ethanol tolerant and osmo tolerant (Spencer and Spencer,1997), acid tolerant, thermo tolerant, genetically stable, rapid and efficient fermenter, easy to propagate, able to utilize wide range of substrates, generate minimum heat during fermentation, possess flocculating or non flocculating characteristics depending upon the process requirements, possess killer activity, de-repressed for di- or polysaccharide uptake in the presence glucose, resistant to certain toxic wastes, absence of metabolites other than ethanol . It is safe to assume that there is no single yeast strain used in the industry today posses the entire above characteristic and hence the research activity in this have continued in this area (Panchal *et al.,* 1981).

There are several factors that affect the yeast fermentation performance that have been investigated. The factors may be the yeast strain employed, the mode of substrate feeding, nutrient supplementation, fermentation temperature, osmotic pressure, oxygen, intracellular ethanol accumulation and yeast ethanol tolerance.

2.9.1 Ethanol tolerant Yeasts

Yeasts are involved in the conversion of carbohydrate to ethanol and the cells should be tolerant to high concentration of both substrates and products as well as should be able to produce ethanol for the optimal conversion (Walker, 1998). Among the most eukaryotic organisms, *Saccharomyces* are the most ethanol tolerant that can produce over 20% ethanol (Casey and Ingledew, 1986). However, there are different ways of improving ethanol production: increasing the range of substrate used as feed stock, improving the efficiency of substrate conversion to ethanol, raising fermentation temperature, or improving tolerance to ethanol and osmotic pressure. For these reason, attention has been given to yeasts other than *Saccharomyces* capable of fermenting substrates not accessible to the former such as insulin, starch, lactose, cellobiose, hemicelluloses, or xylose (Ingledew, 1993 and Walker, 1998). One of the important factors for the ethanol tolerance is the lipid composition of plasma membrane that is consistent with the structural changes observed in the cell membrane of microorganisms tolerant to high concentration of ethanol (Ingram and Buttke, 1984). Environment and the nutrition conditions also affect the ethanol tolerance (Ingledew, 1993). However under fixed conditions non-isogenic strains differs in their ability to tolerate ethanol, and tolerance is a reproducible characteristics implying that it is

genetically controlled (Jime'nez and Beni'tez 1987, 1988). Genetic analysis confirmed that the polygenic character, and that the genes responsible for ethanol tolerance are different in different strains (Jime'nez and Beni'tez, 1986). For this reason hybridization has generated yeasts more tolerant to ethanol than their parental. Different yeast strains have different alcohol tolerances (Aldiguier *et al.,* 2004; Priest *et al.,* 2004) but the potential to reach ethanol yields as high as 23 % (v/v) is feasible (Thomas *et al.,* 1993).

2.10 Factors affecting fermentation

The ethanol production also depends on various stress factors such as increased temperature, low pH and weak organic acids and during fermentation yeasts may encounter lactic acid as well as acetic acid (Graves *et al*.,). There may be the loss of yeast cell viability, reduced yeast growth, increased fermentation times, decreased fermentation rates etc. (D'Amore *et al.,* Narendranath *et al.,* Thomas).

The economic loss due to reduction in ethanol yield can be prevented by selecting the desirable yeasts that are capable of tolerating various forms of stress and are capable of synthesizing large quantities in the presence of such stress. The speed and efficiency of the fermentation process can be improved by employing the strains having the ability to respond and adapt to stressful conditions and maintaining high resistance during fermentation.

2.10.1 Inoculum size

One of the important factor affecting fermentation process and productivity is the volume of microorganism (Sree *et al.,* 2000). Beside the inoculums size, other conditions can modify the inoculums effects. Increase in the inoculums size of *S. cerevisiae* in co-culture from 4 to 12 % gave the remarkable increase in the rate of ethanol production and within 2 days of fermentation more than 96% of theoretical maximum yield was obtained (Abouzied and Reddy, 1986). The increase in inoculums size of *S. cerevisiae* above $3.6*10^5$ cells/100ml decreased the ethanol yield (EI-Diwany *et al.,* 1992).

2.10.2 Effect of ethanol on yeast fermentation

Growth is one of the most sensitive cellular activities to inhibition by ethanol, followed by survival or loss of reproductive ability. The adverse effects of ethanol on growth, viability and metabolism are caused primarily by ethanol induced leakage of plasma membrane i.e. increased membrane fluidity at higher ethanol concentrations (Ingram, 1990; Lioyd *et al.,* 1993). Swiecilo *et al.,* 2000, reported that excess amount of ethanol can cause mitochondrial DNA damage and degrades bio membranes in yeast cells. The

cytoplasmic rigidity as well as the fatty acid constituents of the cell membrane can be dissolved by the ethanol (Osho, 2005). There are many reports that show the relationship between the fatty acid compositions of lipid membranes and ethanol stress tolerance (You *et al.,* 2003). The measurement of ethanol tolerance involves determination of ethanol effects on cell growth, ability on the fermentation and batch culture performance (Ekunsanmi and Odunfa, 1990). The ethanol tolerance of yeasts may be affected by medium composition, and many other surrounding environmental factors and number of genes (Chi *et al.,* 1991). In fact there are many strains which relatively tolerate high ethanol (Nichols *et al.,* 2003). The process having high specific rates and yields through the microorganism having increased ethanol tolerance has been developed (Skotnicki *et al.,* 1983). The strains tolerating the high ethanol are able to extend the fermentation process for longer period of time as well as produce distinct products in the presence of ethanol. The strains that are resistance to ethanol are also resistant to other stresses like osmotic pressure and oxidative and heat (Swiecilo *et al.,* 2000). Achievement of thermal and high ethanol tolerant strains led greater ethanol yield in fermentation process than the control strains. So it is possible to convert pre-treated sugar material more efficiently into ethanol at industrial scale.

The ethanol production varies from strain to strain in cultural condition and the types of process adapted. And a number of reports have been published on the production of ethanol submerged fermentation techniques using different strains of yeast (Mariam *et al.,* 2009). *S. cerevisiae* among all the yeast was proved to be more successful for ethanol production as compared to other species (Ergun & Ferda, 2000). This is due to the fact that some species adopt different metabolic pathways by having special genes or special enzymes such as invertase genes and invertase enzymes respectively for the conversion of sugars to ethanol or other metabolites (Fregonesi *et al.,* 2007).

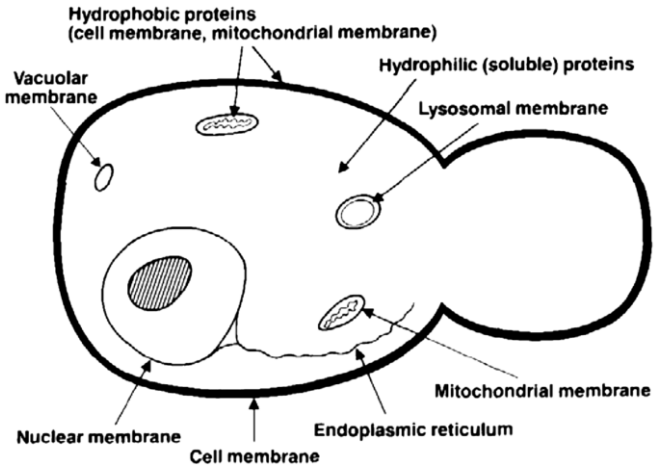

Fig 2.2: Possible target sites for ethanol inhibition in yeast cells (D'Amore and Stewart, 1987 cited by Zhao and Bai, 2008)

Ethanol inhibits the alcoholic fermentation as well as limits the concentration of ethanol which can be produced by a given strain of yeast. The maximum concentration of ethanol which can be produced by yeast strain varies up to 20% by volume. Wayman and Parekh, 1990 stated that ethanol produced during fermentation is much more inhibitory to cell growth than that from exogenous source. The toxic effect of ethanol has been attributed to damaging the cell membrane or changing its properties. The extent of ethanol tolerance of certain yeasts is highly strain dependent and appears to be related to the unsaturated fatty acid and the fatty acyl composition of the plasma membrane (Wayman and parekh, 1990).

In fact ethanol is quiet an effective antizymotic agent. Increasing concentration of ethanol will be initially inhibitory and laterally lethal to the yeast. The mechanism of ethanol toxicity have been extensively studied in yeast, mainly due to the fact that fermentation impairment by product inhibition is of distinct commercial significance for alcohol producers. Ethanol induced toxicity and ethanol tolerance in yeast has been reviewed by (Ingram and Bukkte, 1984; Casey and Ingledew, 1986; Oliver, 1987; Jones, (1987, 1990), D'Amore *et al.,* 1990 and Mishra, 1993). With regard to growth, ethanol acts as a non-competitive inhibitor of yeast growth rate at relatively low concentrations. Although intracellular ethanol increases as fermentation progresses (D'Amore *et al.,* 1990), it is generally accepted that because ethanol diffuses very rapidly across the cell membrane it does not accumulate in yeast cells (Guijarra and Lagunas, 1984). Nevertheless, the fact is that external ethanol is much less toxic than fermentatively derived ethanol remains an anomaly (Jones, 1988).

Watson and Cavicchioli, 1983, suggest that alcohol and heat both affect membrane lipid which play key roles in the stress physiology on yeast cells. The inhibitory effects of ethanol are enhanced synergistically not with high temperature but also with nutrient limitation especially of Mg^{2+} and other metabolic by products such as other alcohols, aldehydes, esters, organic acids, fatty acids, carbonyl and phenolic compounds.

2.10.3 Measurement of ethanol concentration

There are varieties of techniques that can be applied for the determination of ethanol concentration in aqueous solution (Dubowski, 1980). It can also be determined with good precision by oxidation with acid dichromate solution (Kiransree *et al.,* 2000). The ethanol in the known masses of the solution is oxidized to acetic acid using known mass standard potassium dichromate in the presence of sulfuric acid. Among many other techniques, gas chromatography is the most common method for clinical samples as well as for alcoholic beverages. Methods such as enzymatic oxidation with alcohol dehydrogenase and chemical oxidation with acid dichromate are also used. To

determine ethanol concentration in wine industry, measurement of specific gravity of distillate is used (Hyun- Beom Seo, 2009). Due to the time consuming and laborious process of distillation (Lazarova *et al.,* 1987 and Varma *et al.,* 1984), alternative methods such as solvent extraction and refractrometry is used for ethanol measurement (Lazarova *et al.,* 1987). In addition to these methods, dichromate oxidation method has been used as substitute for measuring specific gravity in alcoholic beverages (Fletcher and van Standen, 2003 and Pilone, 1985). It has been reported that dichromate oxidation method can be used directly to determine ethanol concentration in clinical laboratories (Bennett, 1971) as well as in culture broths of yeast *Candida shehatae* (Isaran-Kura-Na-Ayudhya *et al.,* 2007).

No doubt that gas chromatography technique is rapid, simpler and more convenient; there is a need of such technique that is more economic. Also there is a need of higher throughput determination of ethanol concentration in many industries and research fields such as selection of strain having high productivity, development of bioethanol production process, process monitoring and control in alcoholic beverage production (Hyun-Beon-Seo, 2009).

2.10.4 Effect of Oxygen

Though oxygen is an important factor for survival and growth for aerobic microorganisms, it has negative effects on anaerobic and microaerophilic microbes. The poisonous property of oxygen is due to reactivity of oxygen species that is formed with many cellular components. The molecular state of oxygen is not very reactive one but partially reduced forms of oxygen known as active oxygen species are very reactive that attack proteins, lipids and nucleic acid (Fridovich, 1998). When employing pulse of dissolved oxygen (1 to 10 mg/L) during the batch fermentation, *S. cerevisiae* can stimulate alcoholic fermentation (Rosenfeld *et al.,* 2003). Alfenore *et al.,* 2004, while studying fermentation parameters and kinetics verified that average productivity of 2.6 g/L/H were obtained in cultures without oxygen limitation. Under respiratory condition, fermentation occurs during the late exponential phase (Dukan and Nystrom, 1999). When there is greater amount of oxygen in the culture there occur an altered redox state and the NADH oxidase activity is greater at that time. As a consequence of this sugar fermentation is shifted towards mixed fermentation and acetic acid, formic acid is produced (Lopez de Felipe *et al.,* 1997).

2.10.5 Effect of nutrients

Yeasts require very simple media for their growth but it has complex nutritional requirement to undergo optimal fermentation. Any media containing fermentable

carbohydrates that supplies energy and carbon skeleton for biosynthesis, adequate nitrogen for protein synthesis, mineral salts, etc. is enough for the growth. Carbon sources may be monosaccharides, disaccharides and trisaccharides (Priest and Campbell, 1996). Though yeasts are not capable of digesting many essential vitamins but are required for enzymatic reactions. Among the vitamins biotin is important vitamin that is used by yeast during fermentation.

Helena da Cruz *et al.,* 2003 concluded that nitrogen and carbon are the main nutrients for the fermentation and this implies that the mutual interaction of these nutrients may play important role in metabolism of yeasts. The growth medium containing maltose or glucose supplemented with more complex structural nitrogen source such as peptone induced higher biomass accumulation and ethanol production. In *S. diastaticus* Amore *et al.,* 2002 reported by doubling the nutrient components in the medium resulting in the production of 9.1% (w/v) ethanol. Most yeast grows well on variety of amino acids, purines and pyrimidines as sole source of carbon.

2.10.6 Effect of temperature

One of the most important physical parameter is the temperature which influences the yeast growth. There is different temperature range which can be tolerated by different organisms which provides the enormous scope for a range of fermentations. And the variation in temperature may affect the concentration of ethanol production (Slininger *et al.,* 1987). And the fact is that lower temperatures imply lower costs and more manageable conditions (Sanchez *et al.,* 2004). Most laboratory and industrial yeasts generally grow best between 20-30^{o}C. Like all microorganisms, yeasts exhibit characteristics, or cardinal, minimum, optimum and maximum growth temperatures (T_{min}, T_{opt} and T_{max}, respectively). The maximum temperature for the growth is relatively constant within a species (van Uden, 1984). For *S. cerevisiae* strains, T_{max} values range from 35-43^{o}C whereas strains of *S. bayanus* and *S. pastorianus* fail to grow above 35^{o}C. The lowest T_{max} values for yeasts are around 20^{o}C, while the highest are around 50^{o}C (Slapack *et al.,* 1987), although some thermoduric strains of *Kluveromyces maxianus* can grow at 52^{o}C (Banat *et al.,* 1992). Actual values of T_{max} are not only species-dependent, but also growth condition-dependent. For example, the influences of carbon sources (Gross and Waston, 1996), media water potential (Bloomberg and Adler, 1992) and the presence of ethanol (van Uden, 1984) and growth factors (Phaff *et al.,* 1978) have been shown to play role in dictating T_{max} values.

Yeasts capable of utilizing a variety of substrate effectively yield ethanol at temperature of 28-35^{o}C (Kosaric and Vardar-Sukan, 2001). Though the initial rate of ethanol production is higher at increased temperatures, the overall productivity of fermentation

is decreased due to ethanol inhibition (Jones *et al.,* 1983). Fermentation process is always accompanied with evolution of heat that raises the temperature of the fermenter and the microorganisms employed there may be the need of cooling the system. So to circumvent such problems different systems with temperature and moist controls have been designed and developed (Aidoo *et al.,* 1984; Abdel-Fattah *et al.,* 2000). As the initial wort gravity is increased, there is decreasing the rate and extent of fermentation as well as ethanol production (D'Amore, 1992). The fermentation process carried out by the thermophilic organism has been the interest to industrial alcoholic fermentation as at high temperature both pentose and hexose fractions of biomass can directly be fermented (Morgan *et al.,* 1985; Patel *et al.,* 1988; Atala *et al.,* 2001; Ryabova *et al.,* 2003). The necessity to cool the fermenter becomes the major operation and the cost factor in the production of ethanol. Fermentation in industries is usually carried out at ambient temperature of 25-35^{o}C but temperature exceeds 40^{o}C during fermentation which decreases the cell viability and productivity. Maintenance of high cell viability is a major characteristic of fermentation to get high ethanol yield.

The lower growth rate, oxygen solubility and the change in cellular composition of the yeasts can be observed if the temperature is above the optimum. Under oxygen limited conditions, yeasts require nutritional supplements for growth (Slapack *et al.,* 1987 and Thomas *et al.,* 2002). Slapack *et al.,* 1987 says that an increase in temperature does not inhibit substrate uptake nor does it significantly alter enzyme levels.

2.10.7 Effect of pH

Every microorganism varies in their optimal pH requirements for growth and other activities. The varied nature of pH requirement of different groups of microorganisms is exploited where successions of microorganisms take over from each other as pH changes during different phases of fermentation process. There is significant influence of hydrogen ion concentration on industrial fermentation as it acts in controlling bacterial contamination, fermentation rates and by-product formation. The best ethanol yields are generally obtained at pH 4.5-5.7. At higher pH, more glycerol and organic acids are formed at the expenses of ethanol (Wyaman and Parekh, 1990). The increased conversion of glucose is independent of the presence of nutrient supplements in the medium (Thomas *et al.,* 2002). Acetic acid is produced from acetaldehyde if the pH is adjusted to 7 or above, due to the increased activity of aldehyde dehydrogenase due to glycerol production which inhibits ethanol fermentation (Wang *et al.,* 2001).

The acidity of the medium may add a stress to the microbial growth at high temperature (Brock, 1978). Fermentation product may be indifferent to pH values. Almeida *et al.,* 2001 found that pH value had no significant influence on the fermentation parameters

and productivity while studying high gravity wort fermentation by *Saccharomyces cerevisiae*. The ethanol production rate optimum in pH range of 5.5 to 6.0 and an ethanol yield of 0.41 to 0.46 g/g has been reported in wood hydrolysate to ethanol fermentation by yeasts.

For acidifying media either hydrochloric acid or the phosphoric acid is preferred. The use of organic acids is not recommended for general isolation as such acids are only slightly dissociated at pH 3.5-4.0 and high concentrations of undissociated acids may have inhibitory effect on yeasts. Although many yeast can be isolated from the acidic media i.e. at pH 3.7, some species, notably those of genus *Schizosaccharomyces*, are inhibited by very acid media and are best isolated at moderately acidic media with the pH range of 4.5-5.0. And the exceptions are *Zygosaccharomyces bailii*, *Z. bisporus, Schizosaccharomyces pombe* and some strains of *Pichia membranifaciens* and similar species.

2.11 Adaptive physiological responses of yeasts to ethanol

Decrease in membrane saturated fatty acids e.g palmitic acid

Increase in membrane unsaturated fatty acids e.g oleic acid

Acceleration of squalene and ergosterol biosynthesis

Increase in phospholipid protein ratio

Enhanced mitochondrial superoxide dismutase activity

Elevated levels of cellular trehalose

Stimulation of stress protein biosynthesis and acquisition of thermotolerance

Increased synthesis of cytochrome P450 and increased ethanol metabolism

Although there is no unified definition of ethanol tolerance in yeasts, it may generally be described as the ability of particular strain of yeast to withstand higher levels of ethanol without any deleterious effects on its viability and metabolic activities.

Table 2.8: Role of Mg^{2+} ions in ethanol stress protection in yeasts

Experimental observations	**References**
Mg^{2+} partially prevents the increase in proton and anion permeability caused by ethanol	Petrov and Okorokov, 1990
Mg^{2+} supplementations reduce the decline in yeast fermentative activity	Dombek and Ingram, 1986
Mg^{2+} increases ethanol production during fermentation of high sugar media	D'Amore *et al*., 1988 Walker *et al*., 1996

Experimental observations	References
Mg^{2+} maintains huge cell viability and ethanol production in rapid fermentations	Dasari *et al*., 1990
Mg^{2+} is responsible for the difference in toxicity between produced and added ethanol	Dasari *et al*., 1990
Mg^{2+} protects cells from ethanol toxicity and prevents ethanol-stress protein synthesis	Birch and Walker, 1996 Ciesarova *et al.,* 1996

Jones *et al*., 1981; Casey *et al*., 1984; D' Amore *et al*., 1991 and Ingledew, 1993 stated that ethanol tolerance is known to decrease with increased temperatures. When the carbohydrate concentration in the fermentation mash is increased these problems are more observed (Panchal and stewart, 1980; D'Amore *et al.,* 1991; Laluce *et al.,* 1991; Thomas *et al.,* 1993). The relationships between fermentation conditions and ethanol production rate have not received sufficient attention in the fuel alcohol industry. In the comprehensive study reported by Jones and Ingledew, 1994, wheat mashes with initial specific gravities of 14.0-36.5g/100ml, were fermented at temperatures of 17-33^{o}C. They also found that the time required to complete fermentation of mash of a given specific gravity decreased as the fermentation temperature was increased.

2.12 Fermentation

For the production of ethanol as well as for the valuable compounds several fermentations methods are being carried. The methods are batch fermentation, fed batch fermentation and continuous fermentation.

2.12.1 Fed batch fermentation

This type of fermentation is the production technique in between batch and continuous fermentation. Here the substrate is fed into the system without diluting the culture and once the fermentation reaches certain stage for example when aerobic conditions cannot be maintained anymore, culture is removed and the biomass is diluted to the original volume with the sterile medium containing the feed substrate. It has advantages over both continuous and batch fermentations. The production of product can never be depleted and the nutritional environment can be maintained constant during the fermentation process when there are controlled conditions. And there is no need of special piece of equipments as that in batch fermentation. In addition to this, here the levels of inhibitory compounds formed from pretreatment of cellulosic biomass and ethanol inhibition can also be held low enabling high ethanol productivity (Longobarch, 1994; Gapes *et al.,* 2000; Cannizaro *et al.,* 2004; Ezeji *et al.,* 2004; Rudolf *et al.,* 2004,

2005). This also reduces the cost of enzymes in cellulose hydrolysis (Attfield *et al.,* 1997; Valentinotti *et al.,* 2003).

In the fed batch fermentation yeast fermenting starch molecules into ethanol are used than in batch fermentations (Nakamura *et al.,* 1997). In batch system *Sacchararomyces* and *Kluveromyces* have been reported to produce ethanol in a range of 4 to 8% (w/v) (Brady *et al.,* 1994; Bannat Marchant, 1995; Bannat *et al.,* 1996; Uneo *et al.,* 2002). In the comparative studies employing fed batch cultures, ethanol production has been reported from 0.26 to 15% (Xu *et al.,* 1996; Nilsson *et al.,* 2002; Alfenore *et al.,* 2004). Due to some difficulties related to the continuous variation of the reaction volume, no remodeling is done for this process in the past though it has crucial importance in industries.

2.12.2 Continuous fermentation

Though batch cultures are capable of high yield, continuous fermentation is used for large scale ethanol production (Dumsday, 1997; Gyamerah and Glover, 1996). And the main advantage of continuous fermentation is the ability to use high concentration of yeast/bacteria which results in rapid fermentation, the process can also be carried out in small fermenter. Scgugerl 2002, has reviewed integrated processing of biotechnology products and explained that continuous removal of ethanol from the fermentation broth following its production has an important advantage in improving yield and conversion as compared to conventional process. The relative ethanol yield between 70 to 92% of the theoretical value of sugar cane black strap molasses has been documented during oscillatory phase of undisturbed continuous ethanol fermentation (Borzani, 2001).

Continuous cascade fermenters are used to ferment saccharified corn mash to ethanol using yeast. There has been a report of 100% conversion of glucose and the fermentation yield of ethanol is 95% of the theoretical value (Krishna *et al.,* 2000). The use of thermotolerant/thermophilic microorganisms in the continuous fermentation is expected to overcome the problem of product inhibition due to the continuous removal of alcohol from a fermenter (Scopes, 1997; Atala *et al.,* 2001; Lopez *et al.,* 2005).

Novel approaches such as simultaneous saccharification and fermentation (SSF), simultaneous saccharification and extractive fermentation (SSEF), solid state fermentation (SStF) and consolidated bioprocessing (CBP) have been worked out for fermentation, particularly ethanol fermentation.

SSF process is a viable option for direct bioconversion of cellulosic materials into fuels and chemicals as it reduces the cost of process and is economically feasible. It involves

the enzymatic saccharification and simultaneous yeast/bacterial fermentation of sugars to ethanol in same vessel. Moreover, it has been successfully applied for the production of ethanol from pretreated biomass (Krishna *et al.,* 19980.

CBP is a one step process which involves direct conversion of cellulose by pure cultures to fuel ethanol. Cellulolytic enzymes, hydrolysis of biomass and fermentation of the resulting sugars to desire products i.e. ethanol occurs in one step (Lynd *et al.,* 2002). It is applicable in the production of any fermentation product from cellulosic biomass. And CBP strategy has greatest potential of any process improvement ever analyzed to lower the cost of ethanol production from cellulosic biomass. Although no natural microorganism exhibits all the features desired for CBP, a number of microorganisms, both bacteria and fungi, possess some of the desirable properties.

Cost of the traditional raw materials is the limiting factor in the production of bioethanol. There are large amounts of low value agricultural residues as well as agro-industrial wastes can be converted to sugars and fermented to valuable products such as ethanol. Katzen and Fowler, 1994 have shown that majority of sucrose lost with bagasse fibers may be recovered and converted to ethanol. For the production of α- amylase banana waste is used as a substrate (Krishna and Chandrase- Karan, 1996) and also used for lactic acid production (Chan- Blanco *et al.,* 2003). Mixtures of waste vegetables and fruits are good source for the production of alcohol and other products (Traverso *et al.,* 2000).

2.13 Bacteria and yeast in fermentation technology

Research over the past years has indicated the potential use of Gram negative bacterium *Zymomonas mobilis* for the cost effective production of ethanol from various carbohydrate sources. It has an advantage over the traditionally used yeasts such as *S. cerevisiae* and *S. uvarum*, which include lower biomass production, no requirement of oxygen control, higher specific rates of ethanol production and high ethanol tolerance up to 13% v/v. These microbes are competitive with other recombinant bacterium and yeasts which are capable of converting xylose to ethanol (VIgants *et al.,* 1996; Rogers *et al.,* 1997; Lawford and Rousseau, 1999; Zikmanis *et al.,* 2001). Due to reduced substrate instability to cell mass production, higher ethanol yield 98% of theoretical is expected by *Z. mobilis* than by yeast 95% of theoretical value (Krishna *et al.,* 2000).

Apart from prokaryotes, yeasts have long been employed in ethanol fermentation. Among yeasts *S. cerevisiae* is used for many diverse purposes and is one of the economically most important micro-organism that is used in large scale biotechnological processes. Under the oxygen limited conditions yeasts exhibit alcoholic fermentation.

But the naturally occurring *Saccharomyces* cannot metabolize xylose though they are used for ethanol production form starches and cane sugar (Sedlak and Ho, 2004). They are generally resistant to many stresses such as nutritional starvation, osmotic pressure, oxidation temperature and in some cases dehydration/rehydration or freezing or thawing (Attfield *et al.,* 1997; Canizzaro *et al.,* 2001) and high sugar stress (Devanner *et al.,* 2005; Malacrino *et al.,* 2005). However brewing yeasts age as they are subjected to characteristics genetic, metabolic and morphological changes but the young cells exhibit extended generation time prior to division and are less tolerant to a variety of stress factors (Sampermans *et al.,* 2005).

2.13.1 Biotechnological and bioengineering aspects

Biotechnology relies on mainly on inexpensive substances for biosynthesis and processes that function at low temperatures and consume less energy. The field of biotechnology covers food production, provision of chemical feedstock to replace petrochemical sources, waste recycling, pollution control, agriculture and new products to aid in the advancement of medicine, veterinary sciences and pharmaceuticals. The technology has covered wide fields of knowledge ranging from economic through understanding of chemistry and life science to technology (Bruchhart and Ingram, 1992; Nguyen *et al.,* 1999; de Vrije *et al.,* 2001; Jones and Lassner, 2005; Rajagopalan *et al.,* 2005). Besides fermentation which is core of the biotechnology, enzyme engineering embraces production, isolation, purification use in soluble form and for immobilization of enzymes. There is a need of environmentally compatible highly efficient enzymes that are free from product and substrate inhibition for the conversion of various pretreated biomass to various sugars and then for alcohol production (Bothast *et al.,* 1996).

Bioengineering is the close cooperation between technologists and geneticists. And to improving the microorganisms ability for converting useless materials into valuable ones, metabolic engineering has been employed (Bothast, 1994; Ostergaard *et al.,* 2000). By using the techniques such as mutations, selection and adaptation high ethanol producing strains have been obtained. Improvements in genetics have been made either to enlarge the range of substrate utilization or to channel metabolic intermediates specifically toward ethanol (Dien *et al.,* 2001; Nichols *et al.,* 2003; Narayanan *et al.,* 2004). Genetic manipulation has created recombinant yeast and bacterial strains including enteric bacteria for ethanol production (Ingram *et al.,* 1998; Marullo *et al.,* 2004). Though some wild type microorganisms utilize sugars present in cellulose and hemicelluloses but results in low ethanol production. Therefore, there is a need of superior organism that can efficiently utilize lignocellulosic biomass to ethanol which can be achieved through genetic manipulation (Dien *et al.,* 2002; Nichols *et al.,* 2003).

The advantage of engineered microbes is that they can efficiently ferment sugar mixtures containing hexoses and pentoses effectively into selective products (Dien *et al.,* 2002). Traditional microbes such as *S. cerevisiae* and *Z. mobilis* used for ethanol production do not metabolize pentose sugars (Sanchez *et al.,* 2004). Both yeasts such as *Saccharomyces* and *Pichia spp* and bacteria such as *E. coli, Klebsiella* and *Zymomonas* have been genetically engineered to ferment glucose, xylose and arabinose sugars (Mielenz, 2001). *Z. mobilis* has been metabolically engineered hence, genes of this bacterium has been encoded in many strains. A lot of experiments have been done in order to obtain fermentation process that yield high product or reduce the number of steps involved. High concentration of ethanol is obtained from cooked corn starch by *Saccharomyces spp* at different temperature ranges and ammonium sulfate addition (Chi *et al.,* 1995).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Collection of Murcha samples

Murcha samples were collected from different places of Bhaktapur and Lubhu area. Collected samples were then stored in refrigerator until processed.

3.2 Isolation of yeast from Murcha sample

0.1 gm of each samples were added to 10 ml of sterile distilled water in a test tube, vortex for a minute and serially diluted up to 10^{-6}. These diluted samples were pour plated on the labeled PDA plates with the help of a loop. The plates were then incubated at 28^{o}C for 48 hrs. Pure yeast colony was obtained by repeated sub-culturing on the PDA plates.

3.3 Identification of isolated yeasts

3.3.1 Morphological characterization

Cells were grown on solid and liquid media. Yeast cells were observed microscopically to examine their size and shape, budding pattern, filament types and form, structure and mode of formation of ascospores.

3.3.2 Growth on solid media

The morphology of cells and their appearance on the solid medium i.e. PDA (Appendix IA) was examined after incubating at 28^{o}C for 48hrs. From the culture, texture, color and the surface of the colonies were recorded.

3.3.3 Growth on liquid media

The cells were cultured in 10ml of YPD liquid medium (Appendix IB). A loopful of actively growing cells was inoculated in the tubes containing the medium which was incubated at 28^{o}C for 48 hrs. The culture was examined for the visible growth on the medium and the cells were observed under the microscope.

3.3.4 Pellicle formation (Middelhoven, 2002)

To observe the pellicle formation GYEP broth (Appendix IC) was used. A loopful of yeast culture from YPD broth was inoculated to the wall of the tube at liquid surface. After incubation for 3 days at 25^{o}C without shaking the culture was examined for the formation of sediment and a pellicle.

3.3.5 Ascospore formation (Kurtzman *et al.*, 2005)

The isolates were examined for ascospore formation. For this two types of media were used: sporulation and presporulation media (Appendix IIA). First a loopful of young culture of 48hrs old was inoculated in presporulation media and incubated at 25^{o}C for 3 days in shaker to induce the ascospore formation.

Then the sporulation media was inoculated with a drop of culture from presporulation media. The inoculated media was further incubated at 25^{o}C to induce ascospore formation. The culture was examined microscopically for ascospore production at weakly intervals for 3 weeks.

The smeared slide was heat fixed and then carbol-fuschin (Appendix IIB) was added to it and steamed for 5 minutes (Kreger-van Rij, 1984). Then the slide was decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. The slide was rinsed in water and counter stained with 1% methylene blue. The mature ascospores were stained red whereas the vegetative cells were stained blue.

3.3.6 Pseudomycelium formation

There are various methods of stimulating the pseudomycelium formation in the yeasts. The classical method for stimulating pseudomycelia is the growth of organism in media that are relatively poor sources of nutrients and include potato water or cornmeal agar (Kurtzman and Fell, 1998).

For the formation of pseudomycelium, sterilized petri dish containing U shaped glass rod supporting a slide was taken. Another petri dish containing melted PDA was taken aside. The glass slide was dipped in the molten agar and replaced on the glass rod. After the surface of the agar has been solidified, yeast is lightly inoculated into it and sterile cover slip is placed over it. Little water was poured into that petri dish to prevent the agar from drying out. Then the slide was incubated at 25^{0}C for 48hrs. After incubation slide was examined microscopically for the formation of filaments (Middlehoven, 2002 and Kreger-van Rij, 1984).

In the diploid strains of *Saccharomyces cerevisiae*, pseudohyphal can be stimulated by starvation for nitrogen. Apart from natural conditions or routine laboratory practice, several specific substances have been described that stimulate the development of pseodomycelium that include camphor, penicillin, auxin and certain salts of cobalt or boron.

3.3.7 Differentiating wine yeast and wild yeast

2,3,5-Tris-phenyl tetrazolium chloride (TTC) assay was also performed to differentiate Wild yeast and Wine yeast. For this assay, two types of medium were used Upper medium and the Lower medium. The pure yeast culture was first inoculated in YMB at 30^{o}C for 4 hrs. From this culture, 0.1 ml was spread on Lower medium (Appendix **II**C) and allowed to dry then Upper medium (Appendix **II**C) was poured over that. The plates were then incubated at 30^{0}C for 48hrs. Color change was observed after the incubation.

3.3.8 Utilization of various carbohydrates (Middelhoven, 2002)

The identification is based on the utilization of different sugars as a sole source of carbon. From this method the pattern of carbon compound utilization can be studied which in many cases is species-specific. For the utilization of various carbohydrates the media containing carbon sources, peptone and yeast extract was used (Appendix **I**B). The various carbon sources include: D-Glucose, D-Galactose, D-Xylose, Rhamnose, Sucrose, Maltose, Lactose, Mannitol, Inositol, Ribose, Arabinose, Cellobiose, D-Glucoronate, Starch, Trehalose, Melibiose, Sorbose, Erythritol and Glycerol. In the tube containing media, Durhams tubes were also inserted to collect any gas that may be produced that indicates the fermentation took place.

3.3.9 Acetic acid tolerance on yeasts

This test is carried out by streaking young culture on agar plates containing glucose, tryptone, yeast extract and agar(appendix **I**E) which after sterilization is cooled down to approx. 45-50^{o}C. Then 1% glacial acetic acid is added quickly and mixed and is poured in the petri dishes. This test is used to discriminate *Zygosaccharamyces spp.*

3.4 Physiological characterization

The fermentation test was also carried out for all the 19 isolates only 8 were able to produce ethanol in significant manner. And those were morphologically distinct as well and were selected for further experiment.

3.4.1 Studying ethanol tolerance of isolated yeast

YEPD medium was used for detecting yeasts for ethanol tolerance (Osho, 2005). To that medium ethanol with varying percentages i.e. from 5% to 26% v/v differing by 3% v/v was added. The media was then inoculated with 0.5ml of young yeasts culture. The initial optical density of each tube was read at 615nm against the medium blank. The increase in optical density was recorded as evidence of growth.

3.4.2 Studying salt tolerance of isolated yeasts (Logothetis *et al.*, 2007)

For this process the isolated pure colony were pre grown in YMB for 24-48hrs. To the YMB medium various concentration of salt (NaCl) ranging from 5- 25 % were added in different tubes (at difference of 5%). 0.5 ml of the pre grown cells were then inoculated in YMB medium containing salt. The tubes were then incubated at 28^{o}C. The optical density was taken at the interval of 24 hrs against the medium blank. Increase in OD is taken as evidence of the growth.

3.5 Laboratory ethanol production and optimization

3.5.1 Inoculum preparation

The inoculum for fermentation was prepared in YEPD broth. And the broth was incubated at 28^{o}C for 24 to 48 hrs.

3.5.2 Optimization and fermentation of ethanol from isolated yeast

The fermentation process carried out by yeast is known to vary with respect to substrate concentration, temperature, nitrogen sources, hydrogen ion concentration inoculums size etc.

3.5.2.1 Effect of temperature

For evaluating the effect of temperature on the fermentation process, fermentation media (Appendix IF) was inoculated with 0.5 ml of young cultures. The media was then incubated at different temperature i.e. 20^{o}C, 24^{o}C, 28^{o}C, 32^{o}C for 72hrs. The temperature at which the maximum ethanol is produced is taken for further incubation. Ethanol detection was done by acid dichromate method.

3.5.2.2 Effect of pH

The pH of the medium was adjusted in the range between 3.0 to 8.0 i.e. 3, 3.5, 4, 4.5, 5 5.5, 6, 7 and 8. The pH was adjusted with 1 N HCl or 1N NaOH according to Thomas *et al.,* 2000. Here also 0.5 ml of the young culture was inoculated and incubated at optimum temperature for 72 hrs. The optimum conditions for ethanol production were applied for further inoculation.

3.5.2.3 Effect of glucose concentration

The effect of glucose concentration on growth and ethanol production was tested by incorporating different glucose concentrations in the media varied from 5-30%, differing by 5%. i.e. 5, 10, 15, 20, 25 and 30 percent glucose in the media. The media was adjusted with optimum pH and inoculated with 0.5 ml of culture. Then media was incubated at optimum temperature for 72 hrs. At 24 hr the sample was collected for the determination of ethanol content in the media. The ethanol content was determined by potassium dichromate method and the sugar depletion was determined by DNS method (Miller, 1959).

3.5.2.4 Effect of various nitrogen sources

The nitrogen sources used were ammonium nitrate, ammonium phosphate, ammonium chloride, yeast extract and urea. 1% of each nitrogen source was added to YPD medium and taken in different tubes. The inoculum was also prepared on YPD medium. 0.5 ml of inoculum was inoculated in media containing different nitrogen sources. The initial optical density of each tube was read off on Spectrophotometer at 660 nm. YPD medium is used as blank. The cultures were then incubated at 28^{o}C for 72 hrs. Growth of yeasts was measured by Spectrophotometry at 660 nm (Norrell and Messley, 1997; Ueno *et al.,* 2001). Ethanol production reading was taken every 24 hrs for 3 days. The best source of nitrogen to produce high amount of ethanol was selected.

3.5.3 Fermentation of ethanol from osmostressed yeast

The cells were grown in YEPD medium containing salt from 2% to 14%. And each of these preconditioned cells were used for the fermentation. For the fermentation 100ml of the fermentation broth was taken in a 100ml conical flask and 1 ml of the preconditioned cells were added to the respective conical flask. The flasks were then incubated at 28^{o}C for 3 days. The ethanol productions was measured and compared.

3.5.3 Quantitative estimation of ethanol by potassium dichromate method

3.5.3.1 Ethanol estimation by acid dichromate

Each sample was taken and centrifuged at 10000 rpm for 5 minutes in microfuge tube. Then one ml of sample was transferred to test tube and 200µl of acid dichromate (Appendix **III**A) was added to the sample. The solution was then boiled for 10 minutes along with the standard ethanol. During boiling, the color of the solution changes from orange to green. The solutions were allowed to cool then the OD was read at 600nm.

3.5.3.2 Standard graph for ethanol

Stock solution of 10mg/ml was prepared by dissolving ethanol to distilled water. Different concentrations of ethanol were prepared from stock solution (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mg/ml). Standard graph was plotted with the values obtained from OD. Then concentrations of unknown samples, corresponding to the obtained OD values were noted.

3.5.4 Determination of glucose depletion by DNS method (Miller, 1959)

For estimation of glucose, 3, 5- Dinitrosalicylic acid method (DNS method) was employed during the study. The sample was taken and diluted to appropriate dilution. Then 1 ml of the sample was taken in a test tube and 3ml of DNS reagent (Appendix **III**C) was added and was boiled for 5 min. The solution was cooled and absorbance read at 547nm. Along with the sample, substrate blank as well as glucose standard was also prepared.

3.5.4.1 Preparation of stock solution of glucose

1 gm of glucose was dissolved in distilled water in a 100ml of volumetric flask and final volume was made up to 100ml so that the concentration of solution becomes 10,000µg/ml. Working solution was made from stock solution by adding 1ml of solution to 9 ml of distilled water in a test tube.

3.5.4.2 Standard curve of glucose

Different concentrations of glucose were prepared form working solution (50, 100, 200, 300, 400, 500 and 600 µg/ml) in distilled water. 1ml of each concentration was added to test tube along with 3ml DNS solution. The test tubes were kept in boiling water for 5 minutes and cooled. A blank was also prepared (1 ml distilled water and 3 ml DNS solution). Then absorbance was read at 547nm and graph was drawn by plotting absorbance against concentration.

CHAPTER FOUR

RESULTS

The experiment was carried out to different types of yeasts isolated from Murcha sample that could be useful for the bioethanol production. The aim was to isolate such strain that could utilize hexose sugars as well as pentose sugars producing more ethanol. During the experiment, best yeast cell was preconditioned with salt stress and fermentation was carried out if the stressed cells could produce ethanol efficiently as compared to wild cells. The eight selected yeasts were able to tolerate salt and ethanol up to 20% though there was minimum growth. The various morphological and physiological tests were carried out to differentiate the yeasts strains. The effects of parameters such as temperature, inoculum size, pH, substrate concentration and different nutrients were included in this experiment in order to screen for significant factors for bioethanol production.

4.1 Isolation of yeasts form Murcha samples

Total of 8 Murcha samples were collected from different places from Lubhu and Bhaktapur. The Murcha samples were designated as S1 and S2, S1 for Lubhu and S2 for Bhaktapur. From S1 total 10 isolates were isolated and from S2 total 9 isolates were isolated. 19 pure colonies were isolated by repeated subculturing. Most of the colonies were creamy white with convex. The isolates were preserved in agar slants for further use. Colony morphology, microscopic features and carbohydrate utilization tests were performed for the identification. And the charts are shown below.

4.1.1 Colony morphology

Table 4.1: Colony characteristics of isolated yeasts.

S.N.	Colony characteristics				
	Size(mm)	Color	Configuration	Margin	Elevation
(S1Y1)	1.5	White		smooth	Flat
(S1Y2)	1	Creamy white	Round	Smooth	Raised
(S1Y3)	0.5-1	White	Irregular	smooth	Flat
(S1Y4)	1.5-2	White	Round	Smooth	Flat
(S1Y5)	1.5	Creamy white	Round	smooth	Flat
(S1Y6)	2.5	White(rhizoid)	Round	smooth	Raised
(S1Y7)		Dry white	Irregular	Irregular	Flat
(S1Y8)	2-2.5	White	Round	Smooth	Flat
(S1Y9)	1.5-2	Creamy white	Round	Smooth	raised
(S1Y10)	0.5	Dry	Round	Smooth	Flat

S.N.	Colony characteristics				
	Size(mm)	Color	Configuration	Margin	Elevation
		white(rhizoid)			
(S2Y1)	2	White	Round	smooth	Raised
(S2Y2)	2-2.5	White	Round	Smooth	Raised
(S2Y3)	1-1.5	Dry White	Round	Smooth	Flat
(S2Y4)	3	Creamy white(dry)	Round spreading	Smooth	Flat
(S2Y5)		Dry white	Irregular	Irregular	Flat
(S2Y6)		Dry white	Round	Smooth	Flat
(S2Y7)	2.5	White	Round	Smooth	Raised
(S2Y8)	<0.5	Creamy white	Round	Smooth	Raised
(S2Y9)	2	Creamy white	Round	Smooth	Raised

Most of the colonies were between size 1-2mm and form round, smooth with flat colonies.

4.1.2 Microscopic structure

Cell morphology was observed under microscope as shown in Appendix IV. When observed under the microscope budding cells as well as non-budding cells were observed by using methylene blue as a dye. The cells were of different size when observed under 40× optical zoom.

Seven isolates form oval budding cells, six isolates form ellipsoidal cells, one form circular cells, one form filamentous cells whereas one form small circular cells. From the morphology of cells it can be said that those that form different type of cell belong to different species of yeast.

4.1.3 Pellicle formation

Among the nineteen isolates six of them produce pellicle when grown in liquid medium (Appendix V1). The pellicles were observed as white layers on the surface of the walls. Not all the yeasts form pellicles.

4.1.4 Ascospore formation

Not all the isolated yeasts form the ascospore, some were able to form (Appendix V3a). The formation of ascospore plays a pivotal role in yeast identification. For the indication of ascomycetous yeasts the method of Kreger-van Rij 1984 and Krutzman *et al*., 1997

was used. After incubation for 3 weeks in sporulation medium at 25^{o}C the ascospores were observed. 1-2 spheroidal ascospores were observed (Appendix v3b).

4.1.5 Wild and wine yeast

TTC was used to differentiate wine yeast and wild yeast among the isolates. Only one yeast was found to be wine yeast which appeared pink colored upon incubation for 48 hrs whereas rest of them were pale in color (Appendix v4a and 4b).

4.1.6 Acetic acid tolerance

Among the isolates only two isolates were able to tolerate 1% acetic acid within 48 hrs of incubation. None of the other isolates were able to grow though incubated for 96 hrs. There is possibility that these isolates may belong to *Zygosaccharomyces spp*. (Barnet *et al*., 1983) (Appendix v2).

4.2 Fermentation of carbohydrates

The preculture was prepared by inoculating one loopful of colony in YPD broth by incubating at 28^{o}C after 24 hrs (Arthe *et al*., 2008). In this study isolates showed variation in utilization of twenty different sugars. Most of the isolates utilized Glucose, Galactose, Sucrose and maltose. Only few utilized inositol, cellobiose, starch, mannitol, etc. Among the isolates some vigorously used these carbohydrates.

Table 4.2: Utilization of various carbohydrates by isolates

Yeast / Carbohydrates	S2y1	S2y6	S1y6	S2y8	S1y5	S2y3	S1y9	S1y7	S1y3	S1y4	S2y4	S1y1	S1y2	S2y7	S2y5	S2y2	S1y10	S1y8	S2y9
Budding	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pellicle	-	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-	-	+	-
Filaments	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	D	-	-	+	-	+	-	-	+	-	+	+	+	-	+	+	D	-
Sucrose	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+
Maltose	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	-	-	-
Inositol	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Cellobiose	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	+	-	-	+	D	-	-	-	D	-	-	+	-	-	-	+	-	-	-
D-Xylose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

D-Ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucoronate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DL- lactate																			
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose																			
Melezitose																			
D-glucosamine																			
α-Methylglucoside																			
Glucitol																			
D-gluconate																			
Ethylamine																			
Acety-D-glucosamine																			
Glucitol																			
Urease																			

*+= positive, -= negative, D= delayed positive (more than 3 days)

Table 4.3: Comparative chart of isolated yeast with standard yeast

	S. cerevisiae	**S2y1**	**S1y1**	***Z. bailii***	**S1y5**	***P. canadensis***	**S1y3**
Budding	+	+	+	+	+	+	+
Filaments	-	-	-	-	-	+	-
Fragments	-	-	-	-	-	-	-
Pellicle	-	-	-	-	-	W	+
D-Glucose	+	+	+	+	+	+	+
D-Galactose	V	+	+	V	+	-	-
D-Sorbose	-	-	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	+	+
L-Arabinose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	W	-
Sucrose	+	+	+	D	+	+	+
Maltose	+	+	+	V	+	+	-
Trehalose	+	-	-	+w	-	D	-
Cellobiose	-	-	-	-	-	+	-
Melibiose	V	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-
Raffinose	+	-	-	-	-	-	-
Melezitose	V	-	-	-	-	+	-
D-Glucoronate	-	-	-	-	-	-	-

	S. cerevisiae	**S2y1**	**S1y1**	***Z. bailii***	**S1y5**	***P. canadensis***	**S1y3**
Starch	-	+	+	-	D	-	D
Glycerol	-	-	-	+w	-	+	-
Mannitol	-	-	-	+	-	D	+
Inositol	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-
Fermentation of glucose	+	+	-	+	+	+	+
Urease	-	-	-	-	-	-	-

*+=positive, -=negative, W=weak response, D=delayed positive,V=variable,

	Z.rouxii	**S1y4**	***Saccharomycopsis fubuligera***	**S2y4**	***S.exiguus***	**S2y7**	***P.pijperi***	**S2y8**
Budding	+	+	+	+	+	+	+	+
Filaments	-w	-	+	-	-	-	W	-
Fragmenting	-	-	-	-	-	-	-	-
Pellicle	-	-	V	+	-	-	+	-
Glucose	+	+	+	+	+	+	+	+
Galactose	+	+	-	-	+	+	-	-
D-Sorbose	-	-	-	-	-	-	+	-
D-Ribose	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	+	-
L-Arabinose	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	-	+
Maltose	-	-	+	+	-	-	-	+
Trehalose	-	-	V	-	+	-	-	-
Cellobiose	-	-	+	-	-	-	+	-
Melibiose	+	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Raffinose	+	-	V	-	+	-	-	-
Melezitose	-	-	V	-	-	-	-	-
Starch	-	-	+	+	-	-	-	+
Glycerol	D	-	+	-	-	-	+	-
Mannitol	+	-	V	-	-	-	+	-
Inositol	-	-	W	-	-	-	-	-
Erythritol	-	-	V	-	-	-	-	-
Fermentation of glucose	+	+	+D	+	+	+	+	-
Urease	-	-	-	-	-	-	-	-

Eight yeasts isolates were selected on the basis of high ethanol production as compared to other isolates among the 19 isolates. The comparative study of those 8 yeasts was done with the standard yeast. From the comparative study S1Y1 and S2Y1 was found to

be similar to *Saccharomyces spp*, S2Y7 was found to be similar to *Saccharomyces spp*, S1Y3 and S2Y8 was found to be similar to *Pichia spp*, S1Y5 was found to be similar to *Zygosaccharomyces spp*, S1Y4 was found to be similar to *Zygosaccharomyces* whereas S2Y4 was found to be similar to *Saccharomycopsis spp.*

When theses isolates were compared with the species it was found that S1Y1 was 75.6% similar to that of *Saccharomyces cerevisiae,* S2Y1 was 78.3% similar to that of *Saccharomyces cerevisiae,* S1Y5 was 86.48% similar to *Zygosaccharomyces bailii,* S1Y3 was 64.86% similar *to Pichia canadensis*, S1Y4 was 82.05% similar to *Zygosaccharomyces rouxii*, S2Y4 was 67.56% similar *Saccharomycopsis fibuligera*, S2Y7 was 89.18 % similar to *S. exiguus* and S2Y8 was 64.86% similar to that of *Pichia pijperi*.

4.3 Study of salt tolerance in yeast

The isolated yeasts were subjected to the medium containing various concentration of salt. The viable cell count was done to monitor the effect of increasing concentration of salt on growth of yeasts cells. As the incubation temperature increases viability decreases gradually. This may be due to the depletion of nutrients. The fermentation capability of those yeasts were also conducted and only those yeasts were selected which produce high ethanol. For every salt concentration maximum growth was seen during 3 to 4 days of incubation and beyond that growth slowly decreased. Several studies have been done by Oda and Tonomura, 1993 and Bagum *et al.,* 1998 which shows an increase in fermentation performance by yeast previously exposed or grown on hyperosmotic media i.e. with high NaCl concentration.

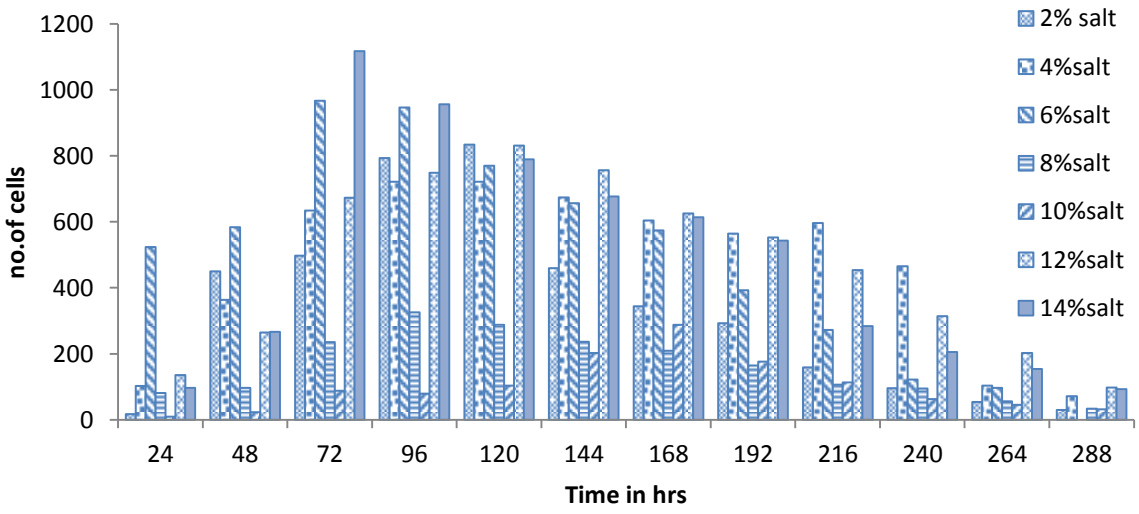

Fig 4.1: Viability cell count of yeast at different salt concentration

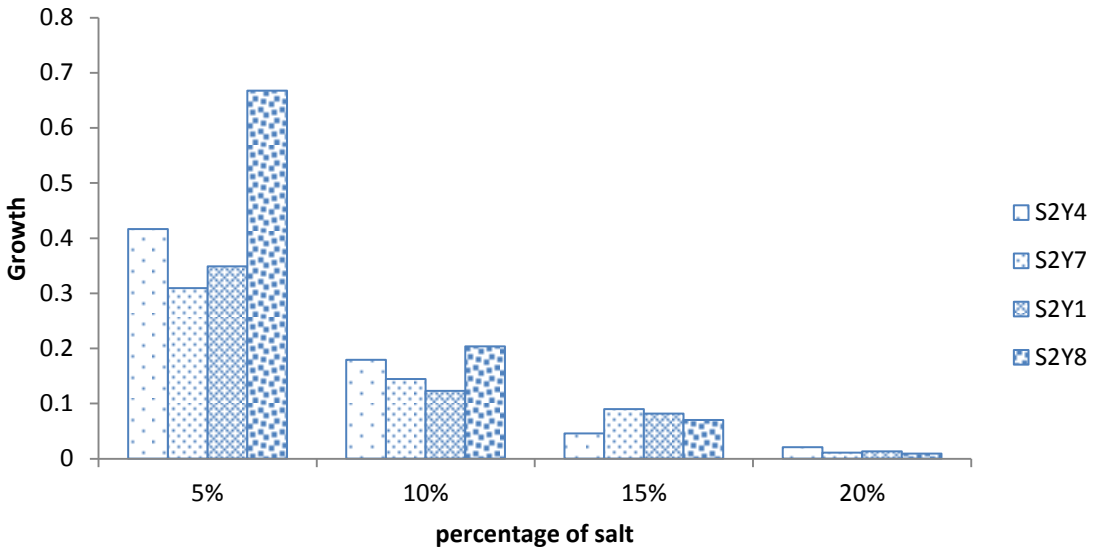

Fig 4.2: Amount of salt tolerated by yeast isolated from Bhaktapur

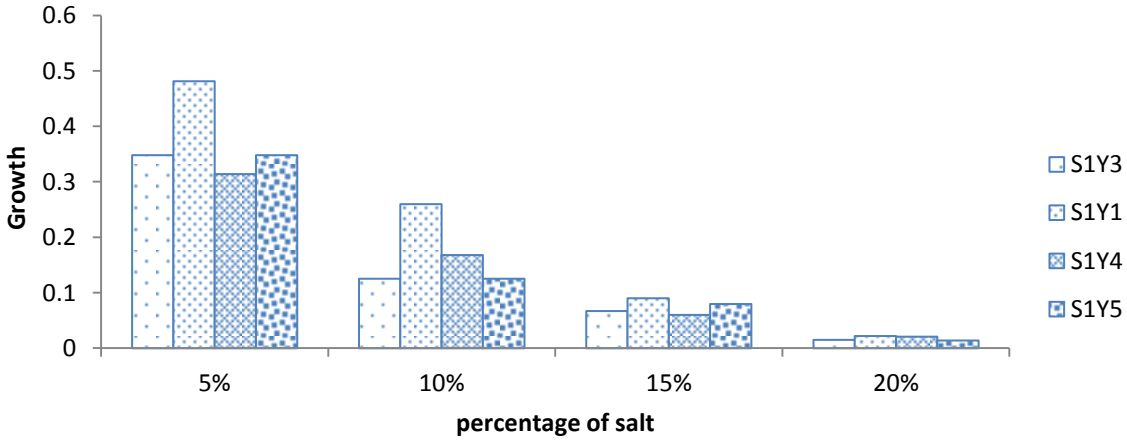

Fig 4.3: Amount of salt tolerated by yeast isolated from Lubhu

Among the isolates only eight yeasts were able to tolerate maximum salt concentration as shown in above figure. 20% salt is tolerated by the isolates S2Y4, S2Y9, S2Y1, S2Y8, S1Y1, S1Y3, S1Y4 and S1Y5.

All yeasts were able to grow well in 5% salt, there was increase in growth up to 3 days and it can be said that low amount of salt enhanced the growth of the cells. Whereas there was decrease in growth in cells when the percentage of salt was increased, though there was decrease in growth the cells tolerated salt up to 20% but the growth was very minimum. But these yeasts cells can be said to be halotolerant as they were able to grow at 20% salt. Among these yeasts growth was maximum in S2Y8 and S1Y1 as compared to others.

4.4 Study of ethanol tolerance in yeast

Eight isolates having high fermentative capacity were also studied for ethanol tolerance. The maximum ethanol tolerance was up to 20% (v/v) by the isolates S2Y7, S2Y1, S1Y1, S1Y4 and S1Y5 however the growth was minimal at this percentage. Whereas others tolerated salt up to 17%. Among the isolates S2Y8 tolerate only 8% of salt and S1Y3 tolerated 11% of salt. Those which could tolerate high amount of alcohol were also relatively rapid fermenters than others.

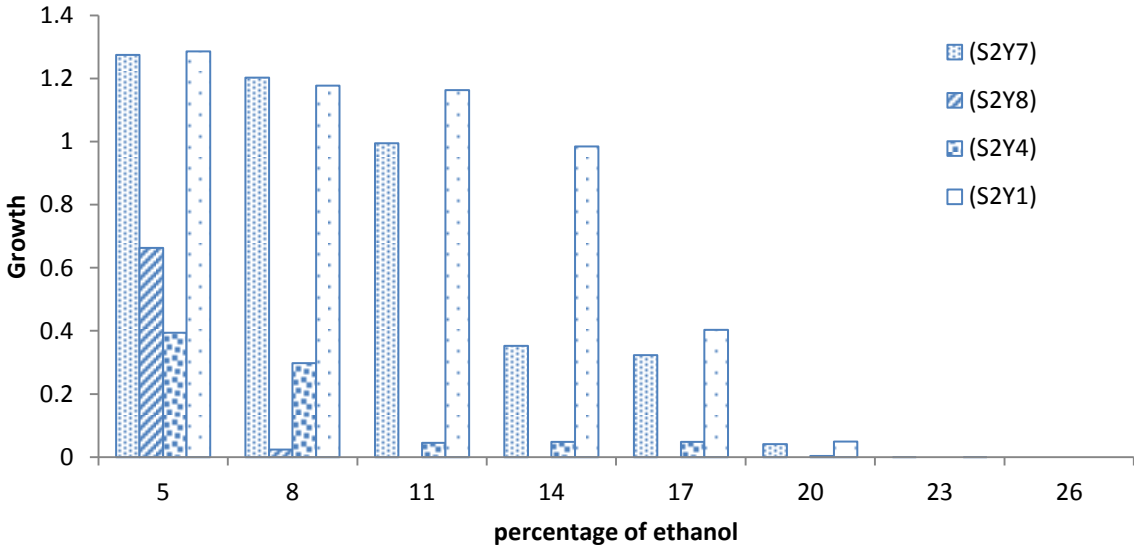

Fig 4.4a: Amount of ethanol tolerated by yeasts isolated from Bhaktapur

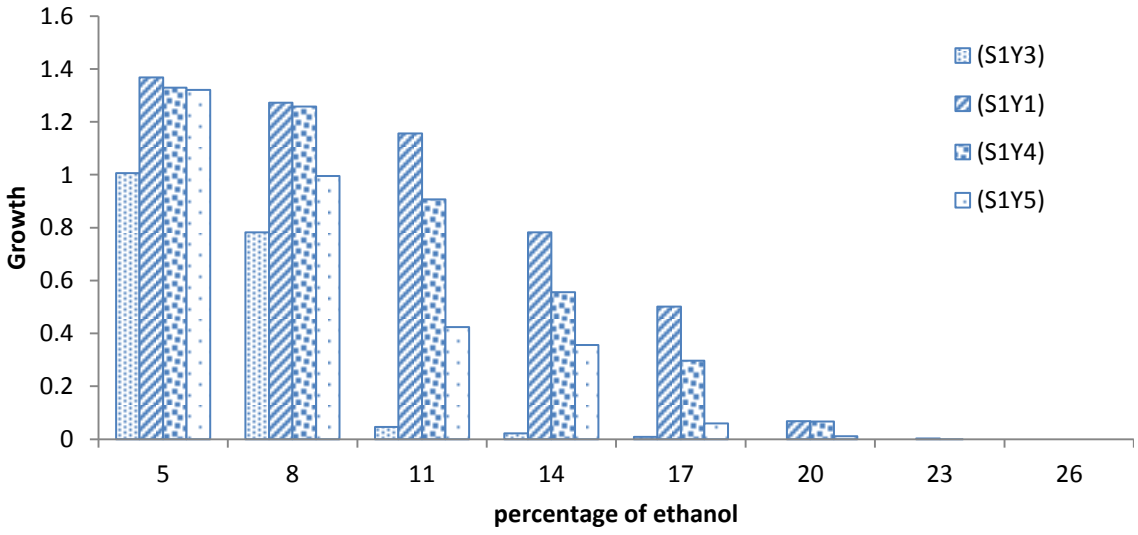

Fig 4.4b: Amount of ethanol tolerated by yeasts isolated from Lubhu

The growth was decrease when the percentage of ethanol was increased. There was linear decrease in ethanol tolerance by the yeasts. As the growth was seen at the 20% of ethanol, this means that they were able to tolerate maximum ethanol of 20%. Among all the isolates maximum growth was seen in S1Y1.

4.5 Ethanol production and optimization of growth conditions

For optimizing the growth parameters, all readings (OD), were recorded after 24 hours of incubation. The sole source of carbon and energy for cell growth in these fermentations was glucose.

4.5.1 Effect of sugar concentration on ethanol production

Sugar concentration up to 30% was applied during the experiment. Samples were taken every 24 hours for the study of growth. The ethanol concentration was measured at 600nm using spectrophotometer. The increase in the concentration showed an increase in optical density which confirmed the growth. But the maximum ethanol production was found at 15% and 20%. As the concentration of sugar increases the optical density decreased gradually. The optical density was highest at 15% and 20% and lowest at 5% and 30%.

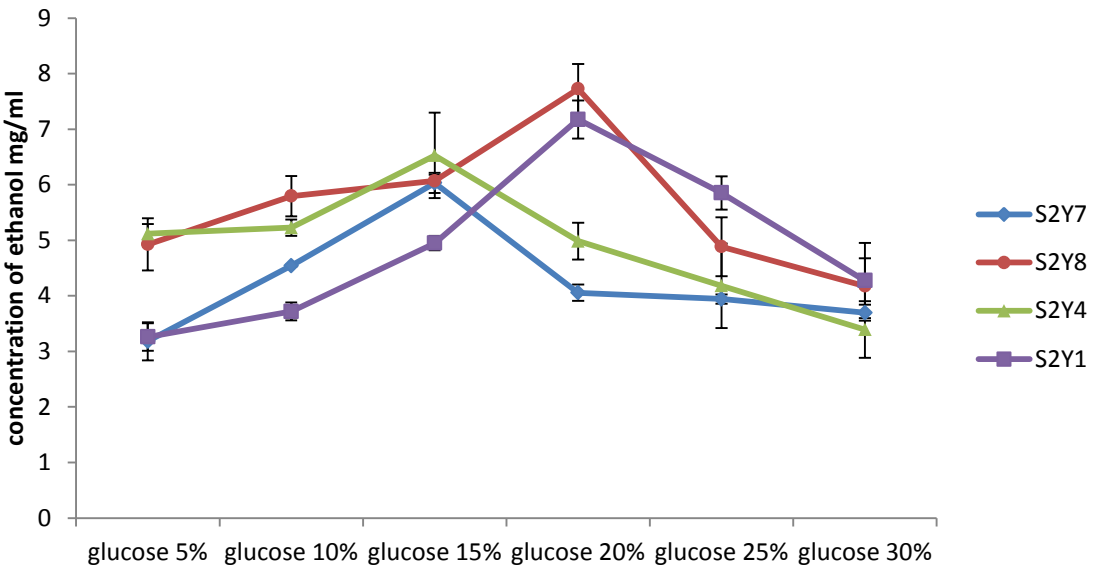

Fig 4.5a: Ethanol production by yeasts at different concentration of glucose (Samples from Bhaktapur)

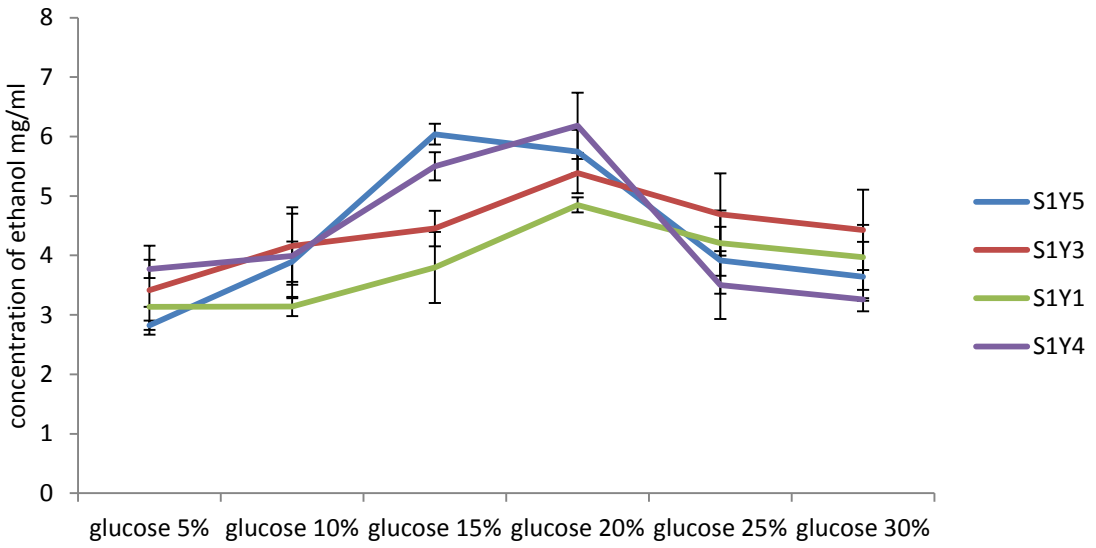

Fig 4.5b: Ethanol production by yeasts at different concentration of glucose (Samples from Lubhu)

As different species have different optimum value, the production of ethanol also depends on optimum substrate utilization. Among the 8 isolates 3 produced better ethanol at 15 % substrate concentration. For these isolates 15 % was considered as optimum substrate concentration. Whereas 5 isolates produced better ethanol at 20% substrate concentration which was considered optimum for those isolates. The highest ethanol production was 7.7 mg/ml by S2Y8 at 20% substrate whereas 6.5 mg/ml ethanol was produced by S2Y4 at 15% substrate. The production of ethanol by others were 7.1 mg/ml by S2Y1 at 20%, more than 6 mg/ml by S1Y5 at 15% ,5.3 mg/ml by S1Y3 at 20%, more than 7mg/ml by S2Y1 at 20%, 4.8mg/ml by S1Y1 at 20% and more than 6mg/ml by S1Y4 at 20%. Form this experiment we can say that substrate concentration of 15% and 20% can be used for better production of ethanol for those yeast.

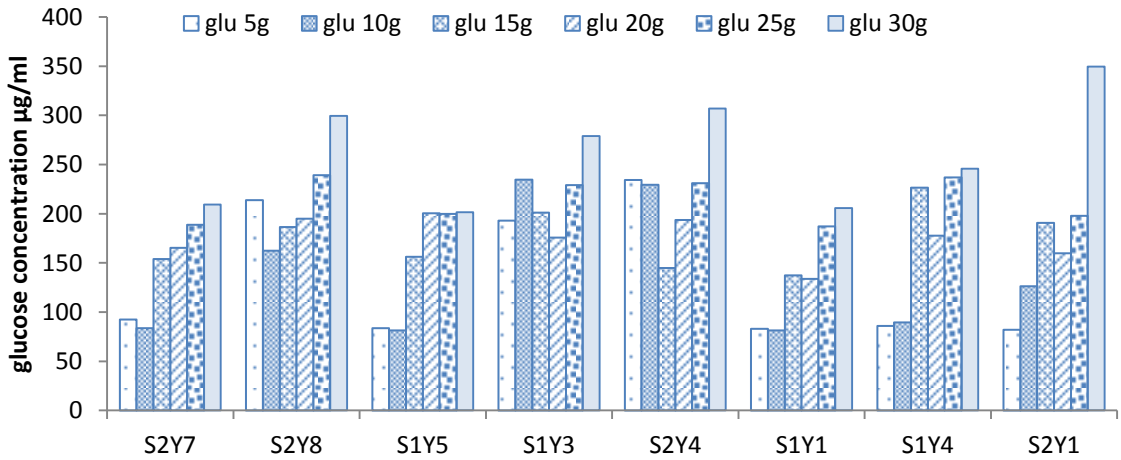

Fig 4.5c: Glucose depletion during ethanol fermentation where the initial concentrations of glucose were 5gm, 10gm, 15gm, 20gm, 25gm and 30gm

Here, the depletion of sugar was also estimated by DNS method. Sugar depletion was found proportional with the ethanol production.

4.5.2 Effect of temperature on ethanol yield

The major constraints that determine the ethanol production is the temperature. For the determination of optimum temperature for fermentation, temperature varying from 20 to 36^{o}C was used.

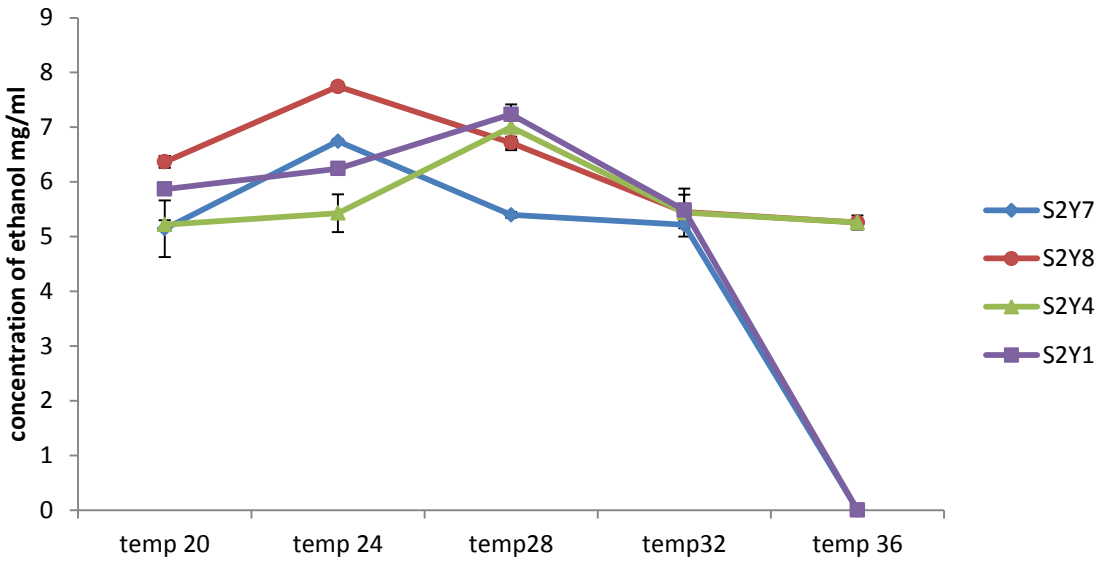

Fig 4.6a: Ethanol production at different temperature ranging from 20 to 36^{o}C (Samples from Bhaktapur)

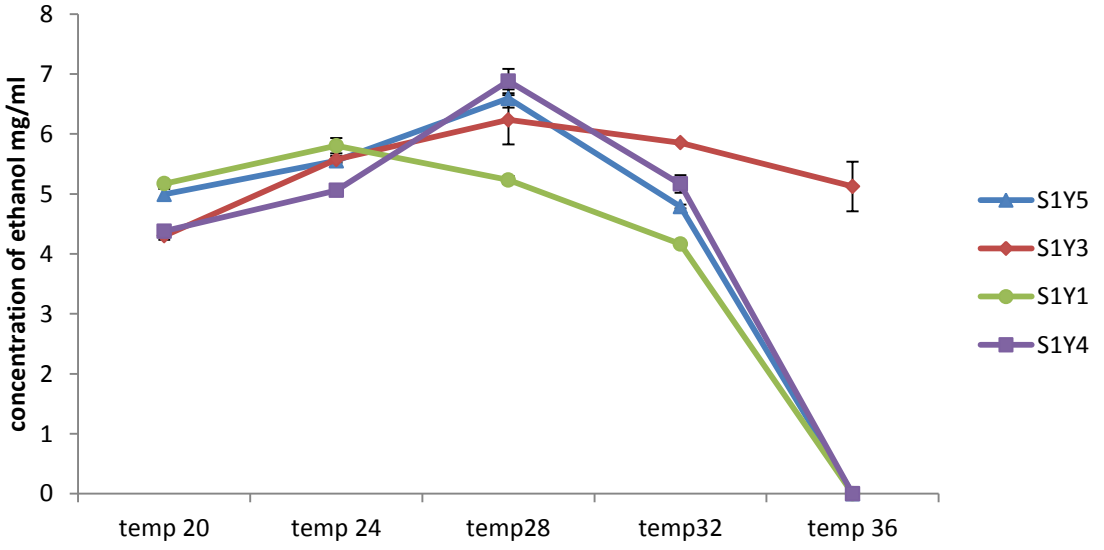

Fig 4.6b: Ethanol production at different temperature ranging from 20 to 36^{o}C (Samples form Lubhu)

The fermentation was carried out for 72 hours. No growth was observed at 36°C. However increasing the temperature beyond 28°C the growth as well as concentration of ethanol decreased. So 28°C and 24°C was selected as optimum for ethanol production, as 5 isolates produce maximum ethanol at 28°C and 3 isolates produce maximum ethanol at 24°C as shown in figure. The maximum ethanol produced at 28°C was 7.2 mg/ml by S1Y1 and at 24°C maximum ethanol produced was 7.7mg/ml by S2Y8. At 28°C production of ethanol was 6.9mg/ml by S2Y4, 7.2 mg/ml by S2Y1, 6.5 mg/ml by S1Y5, 6.03 mg/ml by S1Y3 and 6.8 mg/ml by S1Y4. Whereas at 24°C, S2Y7 produced 6.7 mg/ml and S1Y1 produced 5.8 mg/ml ethanol. This indicates that optimum temperature is species specific.

4.5.3 Effect of pH on ethanol yield

Initial sugar concentration of 15% and 20% and optimum temperature of 24 and 28°C was selected for further studies and subjected to pH treatments of 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7. The fermentation took place in almost every pH but maximum results were obtained at pH 4.5, 5 and 6.

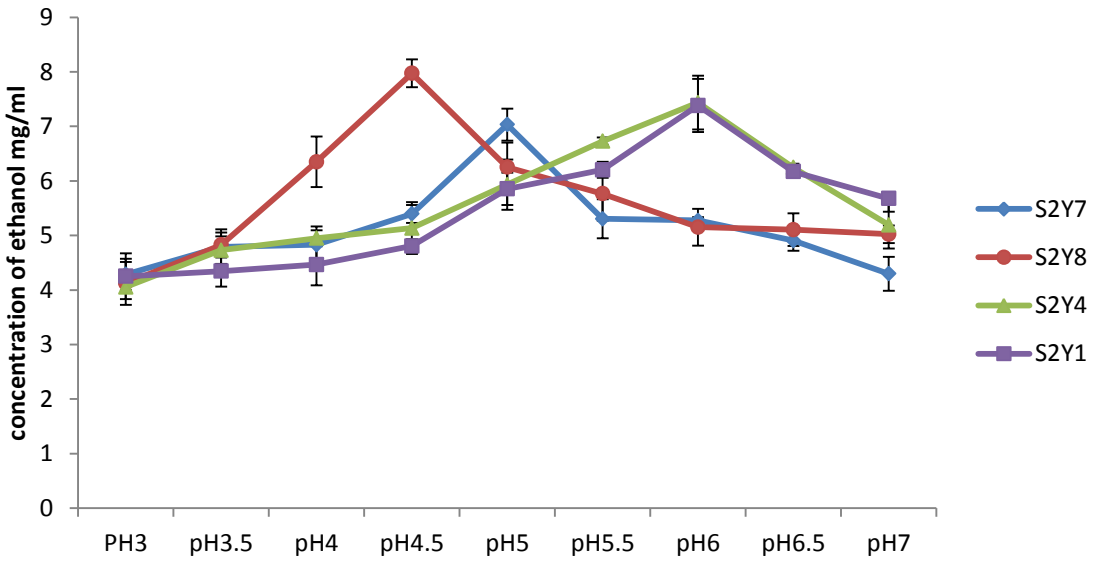

Fig 4.7a: Ethanol production at different pH ranging from pH3 to pH7 (Samples form Bhaktapur)

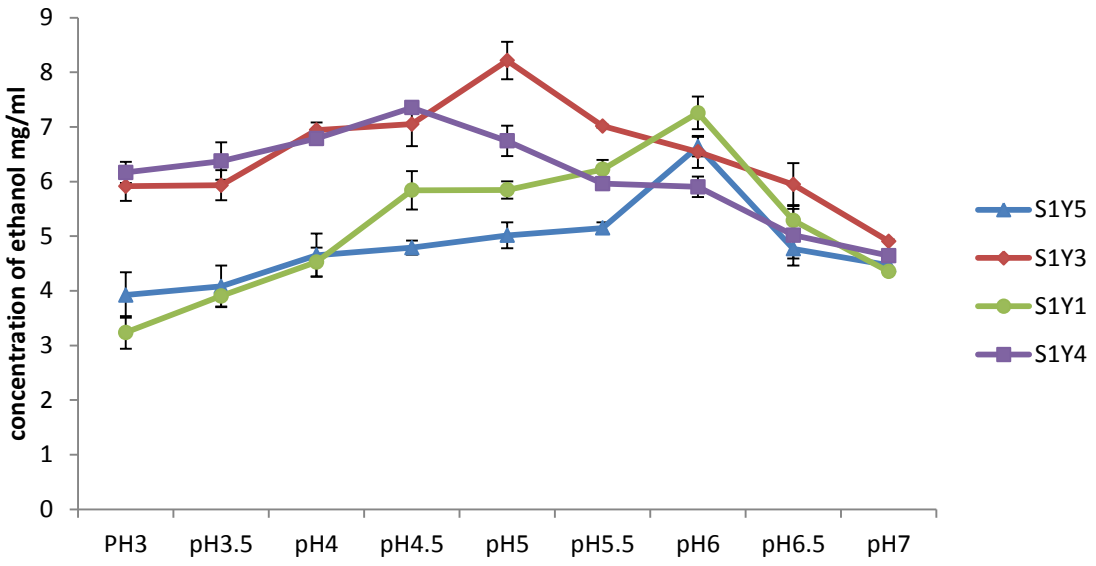

Fig 4.7b: Ethanol production at different pH ranging from pH3 to pH7 (Samples form Lubhu)

The optimum pH may vary among the yeasts isolated. 2 isolates produce maximum ethanol at pH 4.5, 2 isolates produce at pH 5 whereas 4 isolates produce at pH 6. This shows that different yeasts have different optimum pH. The highest ethanol was produced by S1Y3 8.2 mg/ml at pH5, at pH 4.5 highest ethanol produce was 7.9 mg/ml by S2Y8 and at pH 6 highest ethanol produce was 7.4 mg/ml by S2Y4. Similarly, at pH 4.5 S1Y1 produced 7.3 mg/ml ethanol and at pH 5 7.03 mg/ml ethanol was produced by S2Y7. And at pH 6 production of ethanol was 7.3 mg/ml by S2Y1, 6.6 mg/ml by S1Y5 and 7.2 mg/ml by S1Y1.

4.5.4 Effects of supplements (nitrogen sources) on ethanol yield

Among the nitrogen sources used ammonium phosphate was found to be efficient for ethanol production as compared to other.

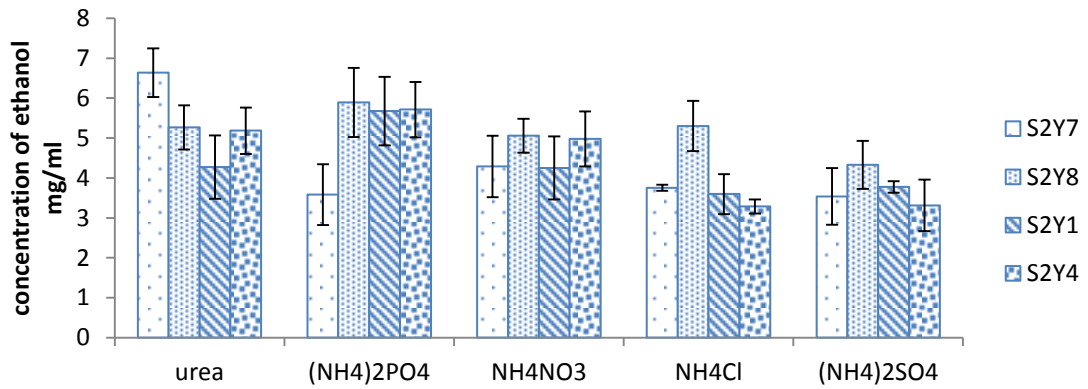

Fig 4.8a: Ethanol production from different nutrients (Samples from Bhaktapur)

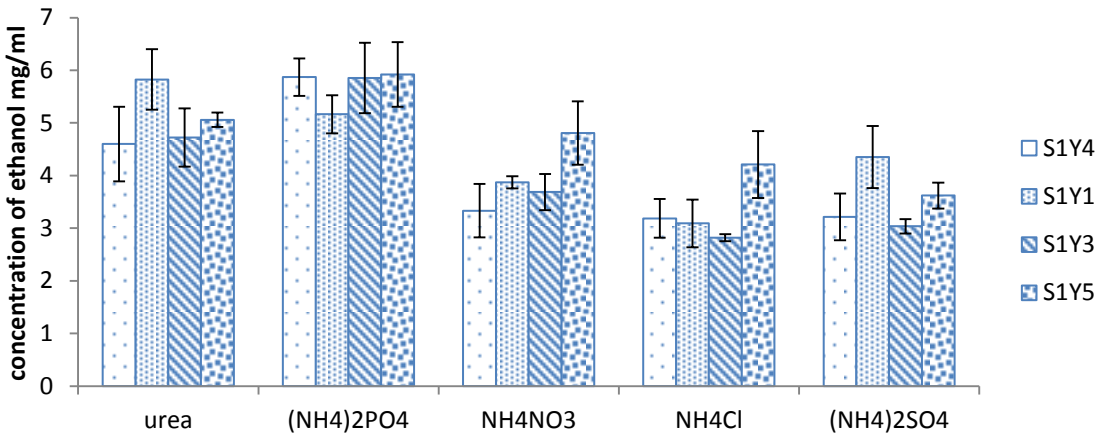

Fig 4.8b: Ethanol production from different nutrients (Samples from Lubhu)

Up to 7mg/ml ethanol was produced by S2Y8 when ammonium phosphate was used as nitrogen source. Whereas S1Y5 produced 8.7mg/ml ethanol, using urea as nitrogen source. Beside this, ammonium chloride, ammonium sulphate and ammonium nitrate were also effective in producing ethanol.

4.5.5 Fermentaion of ethanol from osmostressed yeast

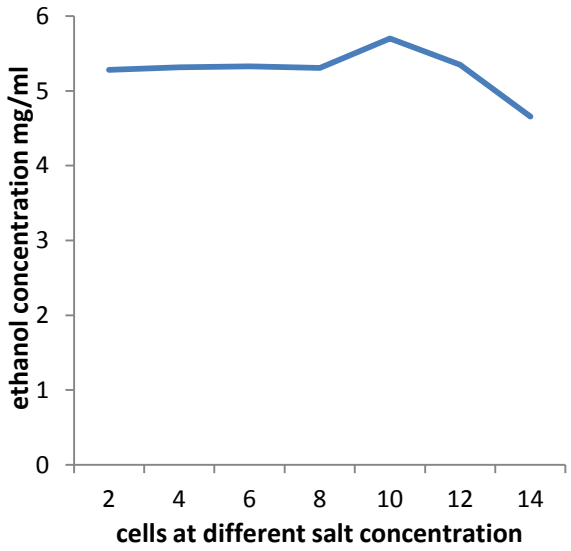

Fig4.9: Ethanol production by yeast treated with salt.

The cells were treated at different salt concentration i.e. from 2 to 14% were subjected to fermentation. The cells treated with 10% salt concentration produced high ethanol of about 5.5 mg/ml as compared to other. Cells treated with low amount of salt, seems to enhance ethanol production. Whereas in cells treated with salt beyond 10%, ethanol production decreased.

CHAPTER FIVE

DISCUSSION

The supply of non-renewable energy sources from fossil fuel such as petroleum is depleting day by day which has made challenges for society to find an alternative ways to overcome this problem (Háhn-Hägerdal *et al.,* 2006). Biofuels provide an alternative to fossil fuel dependency and emit fewer pollutants. The most important is the alternative energy supply must meet with a substantial reduction of gas emissions as it has negative impact on the environment. Its clean burning fuel can reduce air pollution since it can reduce air toxics that come from the transportation sector. By consuming bioethanol instead of petrol, we can also protect our land and water as it is less toxic, water soluble and easily biodegradable (Wayman, 1996).

Various processes have been developed for ethanol production but the worldwide demand of ethanol is generally satisfied by biotechnological fermentation process. A number of organisms including fungi, yeast and bacteria have been screened for ethanol fermentation. Among these organisms yeast cells have been extensively used to carry out the fermentation process (Bajaj *et al.,* 2001). The organisms of primary interest include *S. cerevisiae, S uvarum, S. pombe, S. vini, Kluveromyces spp* (Tao *et al*., 2005: Haq and LI, 2007).

Most of the industrially suitable microorganisms lack the mechanism for converting biomass into fuel ethanol, and this creates a major technical roadblock to developing a bioethnaol industries. Many microorganisms have been engineered in the last two decades to selectively produce ethanol. The ethanologenic bacteria such as *Escherichia coli, Klebsiella oxytoca* and *Zymomonas mobilis* are currently exploited for the ethanol production (Dein *et al*., 2003). For obtaining high ethanol yield, the strains used must produce efficient ethanol with few side products and metabolize all major sugars. Typically the sugars include glucose, xylose, arabinose, galactose and mannose (Wiselogel *et al.,* 1996).

In this research yeasts were isolated from Murcha, which is the mixed dough inocula used to prepare sweet sour alcoholic drinks. 19 yeasts were isolated among which 8 were selected for further analysis on the basis of high ethanol production. The selected yeast were identified as S2Y1 as 78.3% similar to *S. cerevisiae*, S1Y1 as 75.6% similar to *S. cerevisiae*, S1Y5 as 86.48% similar to *Z. bailii*, S1Y3 as 64.86% similar to *P. canadensis*, S1Y4 as 82.05% similar to *Z. rouxii*, S2Y4 as 67.56% similar to *Saccharomycopsis fibuligera*, S2Y7as 89.18 % similar to *S. exiguus* and S2Y8 as 64.86% similar to *P. pijper*. Several groups have analyzed and reported microorganisms in this traditional starter

culture and fermented foods. According to Tamang and Sarkar, 1995; Thapa 2001 and Tsuyoshi *et al*., 2005 commonly found yeast in Murcha are *Saccharomycopsis fibuligera, Saccharomycopsis capsularis, Pichia anomala, P. burtonii, Saccharomysec cerevisiae, Saccharomyces bayanus* and *Candida glabrata*. Many reports have shown that *S. fibuligera* is one of the predominant yeast strain found in Murcha from several regions and said to have amylolytic activity (Mura *et al.,* 1996).

The yeasts were identified based on the morphological and physiological characterization. The yeast identification was done according to the protocols given by (Middlehoven, 2002), the tests were performed with all the available sugars present in the laboratory, but as per the protocols not all the sugars were present so the identification was done in the percentage basis with the available sugars. Yeasts have a key role in the production of diverse range of products that are commercially very significant. It is important in industries to understand the ways by which yeasts respond to changes in their physiochemical environment. Yeasts are confronted with several environmental stresses caused by changes in temperature, pH, osmotic pressure, ethanol concentration and nutrient availability. These factors can cause deleterious effect on yeast physiology. And the change in yeast physiology may result in lowered yeast growth yield and diminished or weaken the fermentation performance (Walker and Dijck, 2006).

Pellicle formation is an important physiological test for the yeast identification. Pellicle is the formation of surface film which can become thick after several days of incubation. And this indicates that yeast are using aerobic metabolism. As many fermentative yeasts form thin surface film after several weeks it is necessary to incubate for several days for the qualification. Here in this experiment out of 19 yeasts, 6 isolates form pellicle, among which S1Y3 and S2Y4 were identified as *Pichia canadensis* and *Saccharomycopsis fibuligera* repectively.

Some species of *Kluveromyces* and *Pichia* form pellicles but formation of pellicles may vary within the species. Sporogenous genera forming pellicles are *Pichia, Zygopichia, Hansenula, Zygohansenula* and *Debaromyces* (Nickerson, 1944b). There is widespread occurrence and considerable economic importance of pellicle or film forming yeasts.

Ascospores were seen in S1Y3, S1Y1, S2Y4 and S2Y7. As different species form different types of spores it is important to note number as well as shape of the spores, we found mostly spheroidal spores. This may be confirmatory test for the identification of yeast (Boulton *et al.,* 1998). Those yeast forming ascospore are placed in the class Ascomycetes whereas those that cannot form ascospore are placed in Deuteromycetes (formerly the Fungi Imperfectii). The failure of not forming the ascospore does not mean

that they belong to Deuteromycetes. The unable to form ascospore may be due to some chemicals or physical conditions such as temperature, pH of the culture medium etc (Fugelsang 1980).

Media supplemented with 1% acetic acid is used for the presumptive identification of yeast that is suspected to be *Zygosaccharomyces* as *Saccharomyces* will not grow under these conditions. Among the *Zygosaccharomyces* species *Z. bailii* and *Z. bisporous* grow whereas *Z. rouxii* do not grow on acetic acid (Boulton *et al*., 1995). Only two of the isolated yeast S1Y5 and S2Y5 tolerated 1% acetic acid and S1y5 was identified as *Z. bailii.* The properties of *Zygosaccharomyces spp* are high sugar tolerance (50-60%), high ethanol tolerance (up to 18%), high acetic acid tolerance (2.0-2.5%), very high sorbic and benzoic acid tolerance (up to 800-1000mg/L), very high molecular SO_2 tolerance (greater than 3 mg/L) and high xerotolerance (Fugelsang 1980). Study by Merico *et al.,* 2003 have shown that *Z. bailii* can produce ethanol in aerobic condition as well and in their research ethanol produced was 0.11 g/g using glucose as the carbon source.

For better production of bioethanol there is a need of organism that can convert all types of sugars in the raw material to ethanol in high yield and with a high rate. Organisms that can ferment pentose sugars like xylose and arabinose, in addition to glucose, are essential for an economical process. Lignocellulose is one of the raw materials that are exploited for the ethanol production which contains pentose sugars as well. And only certain yeasts are able to convert pentose sugars to ethanol. *Sheffersomyces stipis* is one of the yeasts that have been isolated to produce ethanol from xylose. It has been demonstrated to be a yeast strain with potential for use in xylose conversion to ethanol with yield of 0.019 g/g, containing initial xylose concentration of 30 g/l supplemented with 5 g/l yeast extract (Ferreira *et al.,* 2011).

For industrial ethanol production from lignocellulosic biomass the organism should have an effective ability for xylose fermentation as xylose accounts for up to 30% total lignocelluloses sugars (Jejries and Shi, 1999). The natural xylose fermenting yeasts are *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus.*

Among the cellobiose fermenting yeast *Brettanomyces custersii* is a promising cellobiose fermenting yeast for simultaneous saccharification and fermentation (SSF) of cellulose for ethanol production. It has been reported that it produced 32 g/l of ethanol from 75 g/l of cellulose in 3 days. And this yield represents an increase of more than 16% over the yields of other fermentative yeasts and the time to achieve it is less than that of other organisms and it has greater ethanol tolerance than other cellobiose fermenting yeasts (Spindler *et al.,* 1992).

The studies have been carried out for various aspects of salt tolerance. And this has been carried out for restricted group of yeasts such as *Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Debaromyces hansenii* and *Pichia sorbitophilia* (Lucas *et al.,* 1990). Here in this experiment some isolated yeast here could tolerate up to 20% salt though the viability was low. Research done by Attfield 1997 and Posas *et al.,* 2000 concluded that hyperosmolarity exerted by high NaCl concentration or other osmolytes induces both specific and general stress responses, and this has been extensively studied at physiological, genetic as well as in transcriptional level.

The isolates exhibited remarkably high ethanol tolerance comparable to other industrial yeasts associated with high level of ethanol tolerance (Nwachukwu *et al*., 2006 and Hayashida *et al.,* 1974). Almost all yeasts tolerated ethanol up to 14%, above this value there was sharp decrease in the growth which was similar to the ethanol tolerance by *S. cerevisiae* i.e. 14.5 % (v/v) (Teramoto *et al.,* 2005). Bai *et al.*, 2008 has reported that ethanol inhibition is favored by the presence of fermentation by-products such as acetaldehyde and acetate and other stresses such as high temperature.

Ethanol tolerance is an advantage when yeast is being used for the industrial production of ethanol (Ekunsanmi and Odunfa, 1990). During fermentation it is hard to avoid the ethanol tolerance whereas substrate inhibition can be avoided by the stepwise addition of substrate, when analyzing these factors ethanol tolerance seems to be important (Jimenez and Benitez, 1986 and Du Preez *et al.,* 1987).

Temperature also affects the ethanol tolerance of some yeasts species (Casey and Ingledew, 1986; D'Amore and Stewart, 1987; Gao and Fleet, 1988). Here for the ethanol tolerance yeast isolates were subjected up to 30% v/v ethanol differing by 3%. However yeasts could not tolerate the higher amount of ethanol. Some of them tolerated up to 20% ethanol but the growth was very less. It is obvious that yeast can tolerate ethanol up to certain amount. As the amount of ethanol increases the growth decreases linearly. High amount of ethanol may inhibit the growth of cells as it may be toxic to them. Natural habitat and the origination of isolation area may be the factor for the resistance of strain to specific temperature and ethanol tolerance (Torija *et al.,* 2003).

Gonzalez *et al.,* 2003 verified that the changes in the expression of several genes concerned with the synthesis of cell envelop components may contribute to increased ethanol tolerance of yeasts. The ethanol tolerance may be contributed by the physiological factors such as medium composition and the mode of substrate feeding (Yamamura *et al.,* 1988; Dombek and Ingram 1986 a and b), intracellular ethanol accumulation (D'Amore *et al.,* 1988), temperature and osmotic pressure (Jones *et al.,* 1981; Ohta and Hyashida 1983; Vienne and Stockar 1985; D'Amore and Stewart 1987).

Most ethanol tolerant yeasts tend to be sugar tolerant as well. And the combination of sugar tolerance and alcohol tolerance is an advantage when producing ethanol industrially (Ekunsanmi and Odunfa, 1990). So this study was important. Every organism has its optimum substrate concentration at which they grow best and produce ethanol efficiently. Here, substrate concentration of 15% and 20% was found to be efficient for the yeasts as at this percentage maximum ethanol was produced. The highest ethanol concentration of 7.7 mg/ml was produced by S2Y8 at 20% substrate whereas lowest for 20% substrate was produced by S1Y1 i.e. 4.8 mg/ml. Similarly, for 15% substrate 6.5 mg/ml ethanol was produced by S2Y4 whereas 6 mg/ml by S1Y5. However ethanol production was species specific.

Glucose inhibition is related to osmotic effects (Bajpai and Margaritis, 1987). It has been reported that when there is high concentration of glucose in the medium it has negative effect on growth of cells as well as production of ethanol and this inhibitory effect is attributed to high osmotic pressure (Ghosh and Tyagi, 1979). Due to these factors inhibitory effect is one of the major problems for ethanol production. In our study also it is found that as the concentration of glucose increases growth as well as ethanol production was decreased.

It is generally estimated that 50% of sugar in fermentation is consumed by cells during the stationary phase. But the percentage may vary depending upon inoculums size, fermentation temperature and nutrient availability (Kunkee and Bisson 1993). The rate of formation of ethanol is directly related to the utilization of sugar and conversion yield.

Temperature is also known to affect yeast metabolism which results in the formation of secondary metabolites such as glycerol, acetic acid, succinic acid, etc. (Lafon-Lafourcade, 1983). Here in the experiment some strains were able to grow and produce ethanol at low temperature i.e. 24^{o}C and some grow at temperature of 28^{o}C. This result agree with that of Fleet and Heard (1993) who reported that temperature affect the development of indigenous yeast strains and suggested that different strains are better or less suited for ethanol production at different temperatures ranging from 10 to 30^{o}C.The non-*Saccharomyces* grow well in low temperatures than that of *Saccharomyces* when grown in the same medium (Sharf and Margalith, 1983; Heard and Fleet, 1988). There is not much study done about the fermentation temperature affecting the dynamics of Saccharomyces so for this, alcoholic fermentation can be carried out at different temperatures which helps in testing the natural endurance of indigenous strains. And this makes easier for selecting one of the several strains as well as studying resistance to temperature in laboratory conditions (Torija *et al.,* 2002).

It is important to maintain a constant intracellular pH during growth. During growth and metabolism of cells many enzymes are functioning at a time. And each enzymes work best at its optimal pH and most of them are acidic in nature as yeast itself is acidophilic. When there is deviation of extracellular pH from the optimal pH, energy is needed to pump in or pump out hydrogen ions in order to maintain the optimal intracellular pH (Narendranath *et al.,* 2001 and Thomas *et al.,* 2002). If the extracellular pH deviates too much from the optimal pH range, it may become difficult for the cells to maintain constant intracellular pH and the enzymes may not function normally. When the enzyme cannot function normally it can be deactivated due to which yeast cell will not able to grow and make ethanol efficiently (Narendranath and power, 2005). The results obtained here were consistent with Misono *et al.,* 1990 who reported the optimum pH for ethanol production at pH 5.0 to 6.0, optimum pH of 4.5 was also reported by Bhandari, 1999.There is variation on the optimal pH range for yeast growth and the pH varies from 4.0 to 6.0 depending on temperature, presence of oxygen and the yeast strain. And the optimum pH value is needed for the activity of plasma membrane-bound proteins including enzymes and transport proteins (Narendranath and Power, 2005). And these explanations, supports the experiment where the maximum ethanol was produced at pH 4.5, 5 and 6. There was lower production of ethanol below 4.5 and above 6.

Effect of nitrogen additives in fermenting media was studied. Nitrogen is necessary for the growth and multiplication of yeasts and it also influences the ethanol productivity. Addition of nutrients such as ammonium phosphate, ammonium sulphate, urea, yeast extract and peptone have been reported to play vital role in boosting ethanol production and its rate (Fundora *et al.,* 2000). The additives used were urea, ammonium phosphate, ammonium nitrate, ammonium chloride and ammonium sulphate. These nitrogen sources were utilized by most of the yeasts.

Though the pattern of ethanol formation was same on ammonium phosphate and ammonium nitrate, higher amount was produced on ammonium phosphate. However some produce high yield in media containing urea as well. Same result is that of Walker 1998, according to him ammonium phosphate is commonly used as nitrogen source in the medium for the growth of yeasts as it provides source of assimilable phosphate. Some yeast can utilize low, sub toxic concentration of nitrate as a source of nitrogen. At the pH value below 6.0 in the media, nitrous acid is formed which is known to be toxic to the yeast (Rose, 1987). The low production of ethanol in the media containing ammonium nitrate is similar to Isono and Hoshino, 2000. Ammonium salts such as sulphate, phosphate and nitrate are utilized by the cells.

To understand the fermentation performance of osmostressed yeast the yeast cell was first pregrown in different salt concentration. Then those pregrown cells were used for the fermentation. It has been hypothesized that osmostress condition energized specific genes to enable yeast cells to survive under stressful conditions (Logothetis S *et al.,* 2007). When the fermentation was carried out using those osmostressed yeast cell that was preconditioned at 10% salt produced high ethanol of 5.5 mg/ml as compared to other. This may be due to the osmotic stress caused by NaCl and it has been hypothesized that mild osmotic stress would improve wine yeast fermentation due to accumulation of cellular protesting molecules.

CHAPTER SIX

CONCLUSION

Among the oldest technology ethanol production is one and is commercially produced by fermentation. Ethanol can be made synthetically as well as by microbial conversion of biomass materials through fermentation. The fermentation is carried out by the principle biological agent belonging to the genus *Saccharomyces.* These days ethanol fermentation industry is using heterogenous raw materials rather than pure glucose, so there is a need of such organism which can utilize sugar other than glucose. And the ideal yeast strain for ethanol production should be such that it would be able to tolerate various stress factors.

7 different types of yeasts were isolated from Murcha which is microbial inocula made from starchy cereals. Those yeasts were identified as *Saccharomyces cerevisiae, Zygosaccharomyces bailii, Pichia canadensis, Zygosaccharomyces rouxii*, *Saccharomycopsis fibuligera*, *S. exiguus* and *Pichia pijperi* based on morphological and physiological characteristics. The ability to ferment different carbohydrates is also one of the important parameters for identification. The available carbohydrates in lab were used for identification. Thus the percentage calculated here includes only available carbohydrates in lab. According to previous reports yeast identified in Murcha are similar to one isolated here in this research. The salt and ethanol tolerance of those identified yeast were also carried out as the tolerance to various stress is an important parameter for increasing efficiency at industrial ethanol production. As the hyperosmolarity exerted by high salt concentration has been studied at physiological, genetic and transcriptional level. In this experiment, when cells pre-grown in salt were subjected to fermentation, cells pre- grown in 10% salt produce more ethanol that at other concentrations. Thus it can be concluded that little amount of salt is beneficial for enhanced ethanol production. It has been reported that there is an increase in fermentation performance by yeast previously exposed or grown on hyperosmotic media.

The parameters such as temperature, pH, substrate concentration, supplementation of nutrients, etc are crucial for efficient ethanol production. These parameters were optimized and at optimum condition significant amount of ethanol was produced. The optimum condition for each yeast was different. Many successes have been achieved by engineering of Gram negative organism; likewise the isolated high salt and ethanol tolerant yeast can be modified. Thus the modified organism can be utilized to obtain high ethanol yield with few side products and metabolize all major sugars, typically xylose, arabinose, mannose, etc. As lignocellulosic biomass contains these complex carbohydrates there is a need of utilizing those organism that is capable of fermenting these sugars which can be achieved by recombinant DNA technology by introducing genes that are capable of using those complex carbohydrates.

Future aspects

Further research is needed to characterize salt and ethanol tolerant yeast for efficient ethanol production. As heterologous raw materials are being used for ethanol fermentation, there is a need of identification of xylose fermenting yeast and optimize the fermentation condition. For commercialization the achieved ethanol yields are too low and the organisms are not robust enough for industrial applications, so engineered microorganisms could help to produce sustainable fuels in future. The salt and ethanol tolerant yeast could be modified for better ethanol production form lignocellulosic biomass. Development of highly productive recombinant strains or good ethanol producers can be beneficial for commercial ethanol production. As there has not been much study done on the fermentation performance on yeasts that are induced by elevated levels of salt, there is a need of detail study on the fermentation performance in yeasts exposed to salt induced osmotic stress condition.

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APPENDICES

Appendix I

(A) PDA (gm/l)

Potato infusion	200.0
Dextrose	20.0
Agar	15.0

Final pH (at 25^0C) 5.5

(B) YPD liquid medium

Yeast extract	1%
Peptone	2%
Dextrose	2%

(C) GYEP broth

Glucose	2%
Yeast extract	0.5%
Peptone	1%

(D) YMB (100ml) (gm/l)

Yeast extract	0.3
Malt extract	0.3
Peptone	0.5
Glucose	1.0

pH 4.5-5

(E) Acetic acid media

Glucose	10%
Tryptone	1%
Yeast extract	2%
Agar	2%
Glacial acetic acid	1ml/100ml

(F) Fermentation medium (gm/l)

Glucose	200.0
K_2HPO_4	1.0
$K_2H_2PO_4$	1.0
$ZnSO_4$	0.2

$MgSO_4$	0.2
NH_4SO_4	2.0
Yeast extract	2.0

Appendix II

(A) Ascospore formation media (Kurtzman, 2005)

Presporulation media (g/l)

Glucose	20.0
Ammonium sulfate	2.0
Potassium dihydrogen phosphate	2.0
Yeast extract	5.0

Sporulation media (g/l)

Glucose	1.0
Potassium acetate	8.2
Yeast extract	2.5
Magnesium sulfate	1.86

(B) Preparation of 1% carbol fuschin (Kreger-van Rij, 1984)

10 gm of basic fuschin is dissolved in 100ml of methanol and to that solution 50 ml of melted phenol in flask maintained at 60^{o}C is added. The final volume is made to 1000ml.

Decolorizer

In 18ml of ethanol 0.2 ml of concentrated hydrochloric acid was added.

(C) Media for wine yeast selection

Upper medium (100ml) (g/ml)

Glucose	0.5
2,3,5-Tris-phenyl tetrazolium chloride	0.05
Agar	1.5

Lower medium (100ml) (gm/l)

Glucose	2.0
Peptone	0.2
Yeast extract	1.0
Potassium dihydrogen phosphate	1.0
Magnesium sulphate	0.4
Agar	3.0

pH 5.5-5.7

Appendix III

(A) Preparation of acid dichromate reagent

5 gm of K2Cr2O7 was dissolved in a 100 ml of 5 M Sulphuric acid on a standard flask and volume was made up to the mark.

(B) Preparation of 5M sulphuric acid

Strength of Hydrochloric acid (S_1) =18.4 M

Initial Volume (v_1) = X

To make final volume (v_2) = 100 ml

Final strength (x_2) = 5M

We have,

From Normality equation

$V_1S_1 = V_2S_2$

X x 18.4 = 100 x 5

$$X = \frac{500}{18.4}$$

X=27.17ml

27.17 ml sulphuric acid was added in 72.83 ml distilled water to make it 100ml.

(C) Preparation of DNS (Dinitrosalicyclic Acid) solution

Different ingredients used for the preparation of DNS solution are as follows:

1) Distilled water 1416ml
2) 3,5-dinitosalicylic acid 10.6 g
3) NaOH 19.8g

The above ingredients were dissolved gently in water bath at 80^{o}C until a clear solution was obtained. Then the following chemicals were added.

4) Rochelle salt (sodium potassium tartarate) 306g
5) Phenol (melted at 50^{o}C) 7.6ml
6) Sodium metabisulfite 8.3g

After dissolving the above ingredients, the solution was kept in a bottle and the bottle was wrapped to avoid photo oxidation.

Appendix IV

Fig 1: Pellicle formation

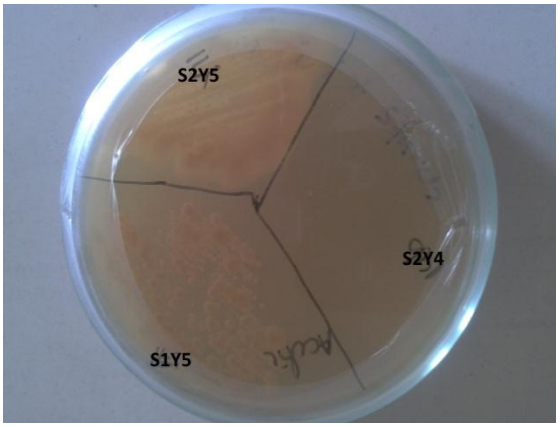

Fig 2: Acetic acid formation

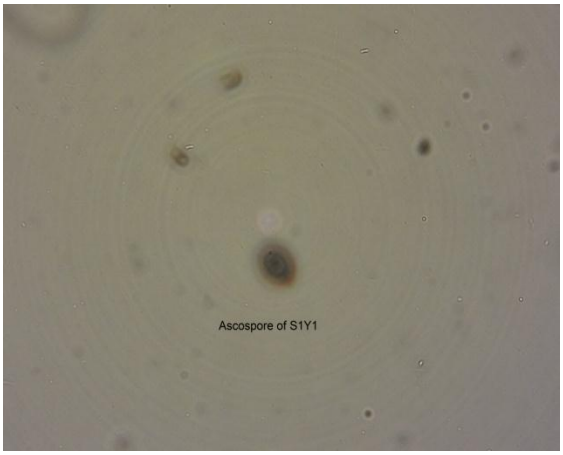

Fig 3a: Ascospore

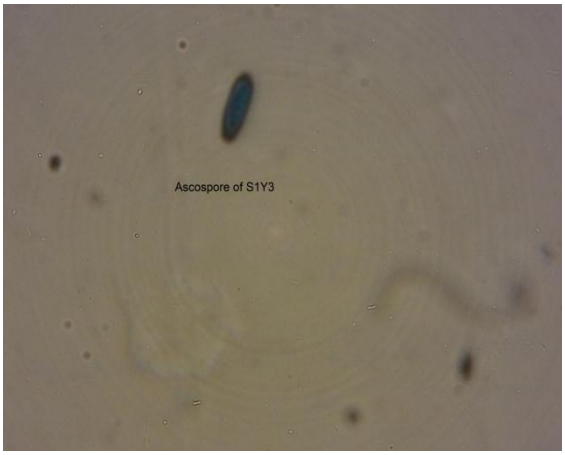

Fig 3b: Ascospore

Fig 4a: Wine yeast

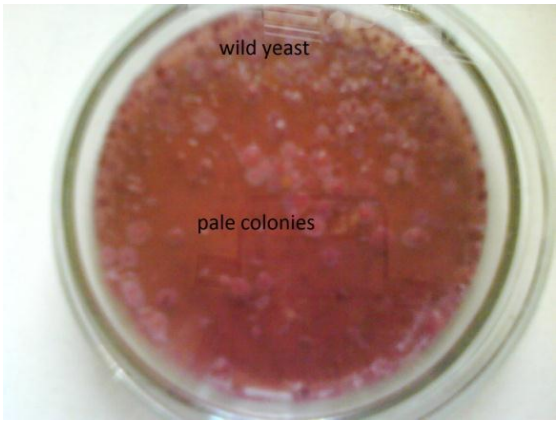

Fig 4b: Wild yeast

Appendix v

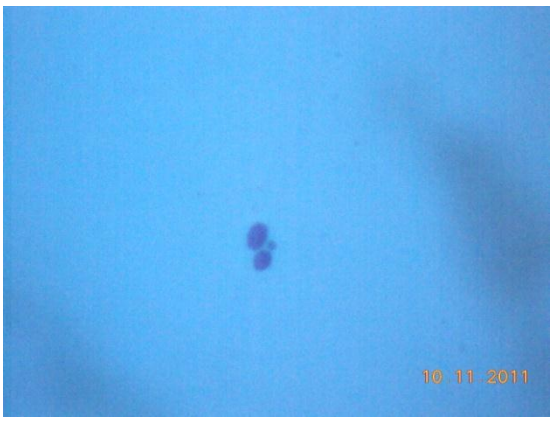

Fig 1: Cells of S2Y1 observed at 40x

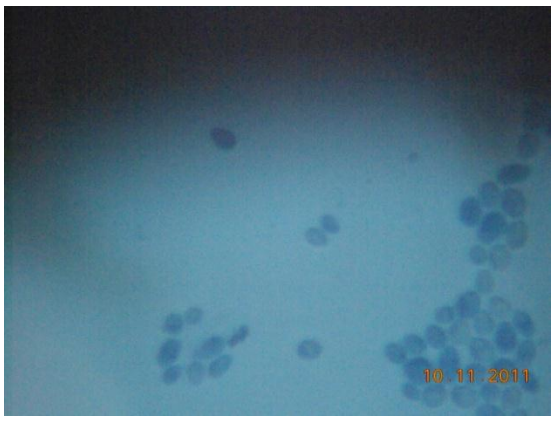

Fig 2: Cells of S1Y5 observed at 40x

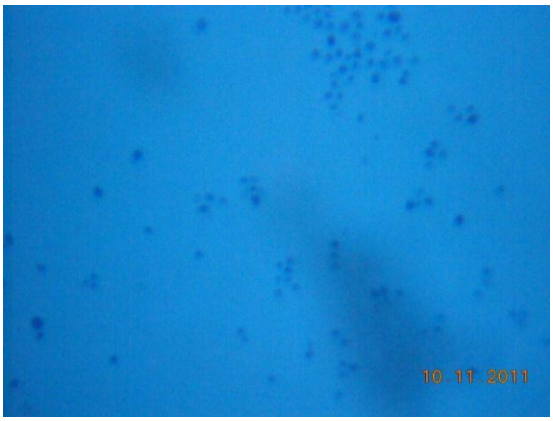

Fig 3: Cells of S1Y3 observed at 40x

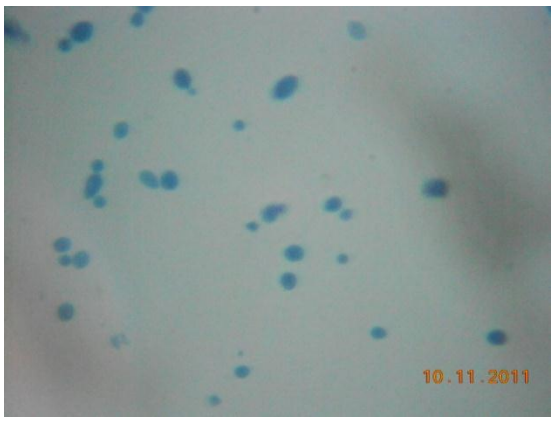

Fig 4: Cells of S2Y7 observed at 40x

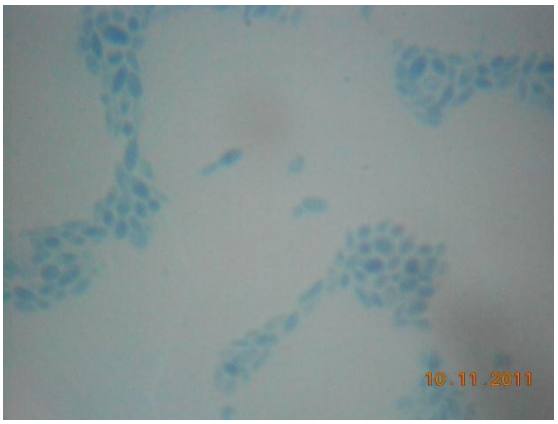

Fig 5: Cells of S2Y4 observed at 40x

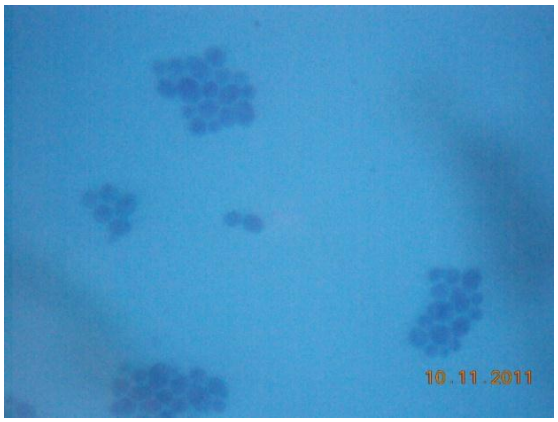

Fig 6: Cells of S2Y1 observed at 40x

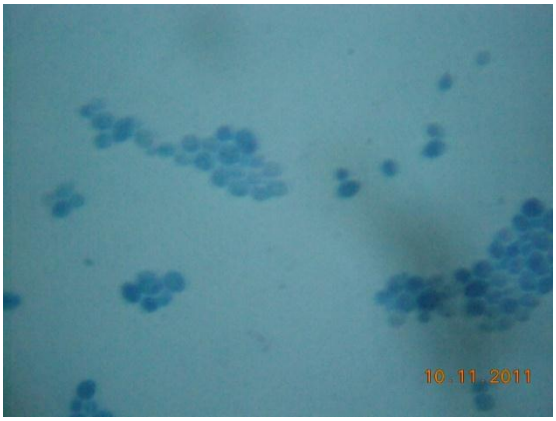

Fig 7: Cells of S1Y1 observed at 40x

Fig 8: Cells of S2Y8 observed at 40x

Appendix VI

Absorbance	Concentration
0.285	100
0.726	200
1.495	300
2.371	400
2.982	500

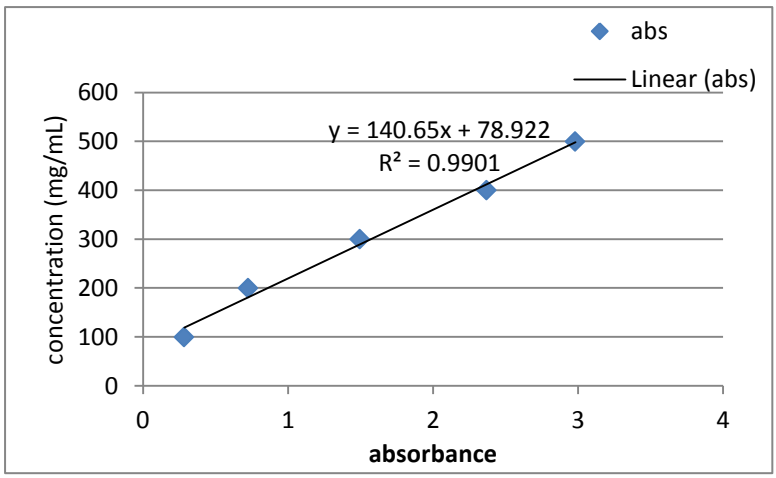

Fig 9: Calibration curve for glucose estimation

Absorbance	Concentration
0.035	0.25
0.051	0.5
0.142	1
0.434	3
0.838	6
1.042	9

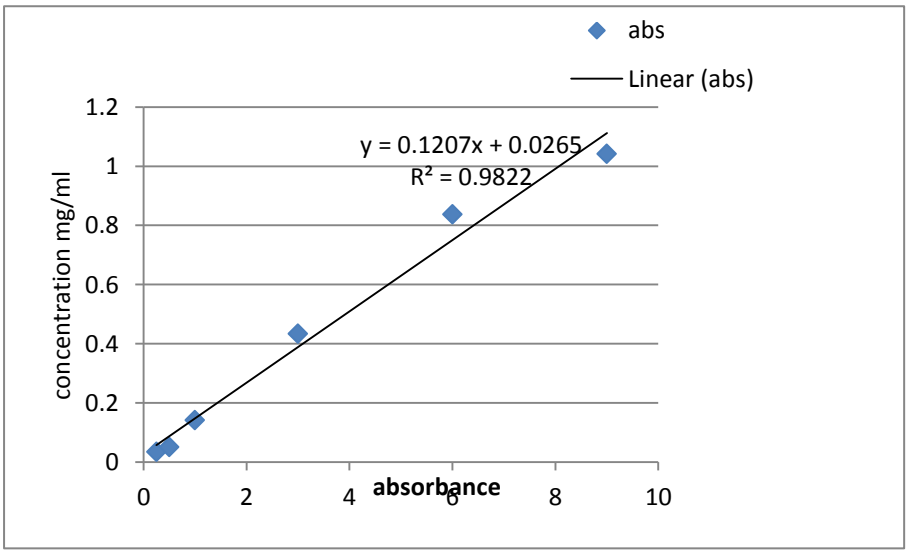

Fig 10: Calibration curve for ethanol estimation