UNIVERSITY OF BORÅS

SCHOOL OF ENGINEERING

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Master thesis

Subject Category: Industrial Biotechnology

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ABSTRACT

Environmental and economic issues have drawn the world's attention to produce and utilize energy from renewable sources for sustainable development. One of the attempts includes the production of ethanol from various substrates. Many researchers have focused on utilizing lignocelluloses biomass as substrate for the production of ethanol, which mainly contains cellulose and is a cheap and abundantly available material in the world. One of the major problems faced by researchers during production of ethanol from the lignocellulosic biomass is the stress tolerance of yeast cells, due to the nature of the hydrolysed substrate (lignocellulosic material treated with Nitro methyl cellulose (NMC)). One of the solutions for this problem is to encapsulate the yeast cells. Encapsulation is an attractive method, which can enhance the stress tolerance of the yeast cells in the reactor, and also aid in maintaining a high yeast concentration inside the bioreactor and thereby increase the volumetric productivity of ethanol. This report includes a major study on the sodium chloride and ethanol stress tolerance of alginate chitosan alginate (ACA), alginate chitosan (AC) and APTES treated ACA encapsulated yeast biomass in medium containing different concentrations of glucose under anaerobic conditions. AC capsules shows significant results towards osmotic stress and ethanol stress compared with that of freely suspended cells in stress conditions.AC capsule encapsulated yeast tolerated osmotic stress better than ACA capsules in 2M of NaCl where as freely suspended yeast cells unable to tolerate 2M of NaCl . At 100^{th} hour in AC capsules glucose consumption was 12 g/l where as in ACA capsules glucose consumption at same 100^{th} hour was 2 g/l. At 10% ethanol concentration yeast inside ACA capsules showed 5 g/l of glucose consumption but in freely suspended yeast cells there is no glucose consumption as they cannot tolerate higher stress levels.

CONTENTS

INTRODUCTION

Developed and developing countries in the world are suffering from economic and environmental issues associated with energy usage in daily lives. There is a large dependency between a country's economy and its energy sector, where most of the energy is derived from petroleum, more than half of which is imported. Many potentially important resources including petroleum are found in unstable regions of the world and large numbers of countries therefore tend to import these resources from where they are abundantly available. This resulted in severe price shocks that led to substantial economic disorders during the 1970s. As a result, many countries in different parts of the world were willing to start using alternative energy sources that could reduce oil imports and build up their economic strength. [1]

Additionally, the environment has been continuously affected by emissions created by the transportation sector. About two-thirds of the pollution in major urban cities originated from vehicular traffic during the late 1990s, which had a great impact in causing environmental disorders. It was therefore necessary to employ different strategies to reduce greenhouse gas emissions to prevent the depletion of ozone. [1]

In the 1880s Henry Ford designed a car model 'Ts' that ran on farm ethanol made from corn. However, petroleum derived fuels (fossil fuels) quickly came to dominate the market as low prices persisted for several decades until the oil crisis in the 1970s. The situation then demanded an alternative energy source, giving rise to the birth of organizations such as National Alcohol Programme in Brazil and the "gasohol" programme in USA. Later again the oil prices started declining over the following decades which did not encourage bioethanol production. However during the late 1990s, the world realized that excess consumption of fossil fuels may lead to their depletion and also that high amounts of pollutions were generated. [2]

Ethanol is an oxygenated fuel and like that of petroleum fuels, ethanol has a high-octane value. Blending ethanol to petroleum-based fuels can decrease usage of fossil fuels and reduce greenhouse gas emissions. As a strategy to reduce global warming and to improve energy security globally, large production and utilization of ethanol as an alternative fuel or additive to the current fossil fuel has been a notion worldwide. However, this can only be achieved by utilizing the inedible parts of food crop as feedstock for bioethanol production in order to maintain balance between fuel and food production. [3]

Most of the ethanol producing industries utilizes sugars or starch obtained from corn and grains due to the ease of substrate handling and processing. Recent focus of researchers has been to develop methods to utilize lignocellulosic materials such as straw and wood etc. as a source for bioethanol production, as they are cheap, renewable and available in large amounts. However, the production process becomes more challenging if lignocellulosic materials are to be used. [3]

In addition, high levels of pollution have been a problem especially in urban areas where large amounts of fossil fuels have been consumed in the recent decades. Ethanol produced from

lignocellulosic biomass resources (bioethanol) has been regarded as the fuel, which can potentially match the features of petroleum at a low price. Ethanol blended with gasoline, when used as fuel, decreases the amount of carbon monoxide produced and unburned hydrocarbons that lead to smog formation. This in turn can reduce air pollution to a great extent and also create a demand for bioethanol. [2] Ethanol can be produced from various feedstock available abundantly in different land conditions. As a matter of fact, over 90% of the world's bioethanol production is from crops, but this leads to negative impact on decreases food availability to mankind. On the other hand, ethanol production using lignocelluloses as substrate is more challenging, mainly because of its recalcitrant structure, which makes it hard to degrade to fermentable sugars. Lignocellulosic material containing cellulose, hemicelluloses and lignin, which contain highly branched heteropolymers and aromatic polymers. [2]

Lignocellulosic biomass is the most abundant source for cellulose on earth. Its properties make it suitable as a feedstock for ethanol production. About 10-50 billion tons of lignocellulosic biomass is produced annually and is accounted to be 50% of the world's biomass produced. [4]

Lack of an appropriate microorganism to efficiently ferment all the sugars released during hydrolysis of lignocellulosic substrates is one of the major factors affecting bioethanol production at a large scale, thus preventing the usage of lignocelluloses. There are ongoing efforts to construct an organism with a combination of different traits from various organisms that have performed well in pilot scale and are capable of producing ethanol from lignocellulosic sugars. [2]

In this thesis project the performance of encapsulated yeast cells was compared to suspended yeast at various stress levels. This was performed in order to see if encapsulated cells showed any benefits in producing bioethanol from lignocellulosic waste at different stress conditions. Experiments were performed in defined medium at different sodium chloride concentration and ethanol to investigate the encapsulated and suspended yeasts tolerance against osmotic and ethanol stress.

SOCIAL AND ETHICAL ISSUES

Bio-ethanol production plays a major role in social and ethical issues. According to green facts research paper, bio-fuels are produced directly or indirectly from organic matter such as plant materials and animal waste. Unprocessed biomass, like animal dung, charcoal and fuelwood, has been used for energy sources in the past. Besides the conventional usage, advancement in technology has facilitated to extract bio-fuels from waste materials, such as wood waste and crops. In addition to that, bio-fuels may also be derived from the by-product waste of other industries such as food, agriculture and forestry.

Bio-fuels are classified based on raw materials as follows -:

Primary bio-fuels, such as fuel-wood, wood chips and pellets, are used in heating, cooking or electricity production.

Secondary bio-fuels are obtained from from processing of biomass and which include liquid bio-fuels such as biodiesel and ethanol that can be used for transport and industrial processes. [5]

As said by Buyx, there is variety of alternatives for transport fuel such as hydrogen but it is not put into production and there are moderately serious ethical concerns on deforestation and biodiversity loss, risks to food security, some human rights breaches.
One of the ethical issues is corn ethanol production in the United States which showed effect on food prices and food security, from past decade food prices are increased as U.S corn crop was focused on bio-fuels, which in turn led to sharp rises in the price of staples. Even though bio-fuels alone didn't contribute to rise in food prices but continuous production, in long term, may affect food prices worsely. The land usage for biofuels and its effect on greenhouse gas emissions are some ethical issues raised along with biofuel production worldwide. Although bio-fuels can reduce greenhouse gas emissions the effects of land use cannot promises benefits of greenhouse gas emissions.
Few case studies revealed on Malaysia that it focuses mainly on palm oil diesel production. Brazil is produces ethanol from sugarcane which causes deforestation is a major concern; there have been reports of conditions on the sugarcane mills that amount to slave labour as well as reports of very unhealthy working conditions and of informal child labour in the mills. [6]

In the production of agro-fuel, USA, next to Brazil produces more ethanol from maize. Rapeseeds and sunflower oil are also being used in the production of biodiesel. Deforestation occurred due to forest land is being used for palm oil plantations , overall it causes to biodiversity , economies disruption for land grabs by government and private sector and rising of food prices.

Some ethical principles need to put into practical policies such as Bio-fuels progress should not be at the cost of people's essential human rights, together with health, water and food. Bio-fuels should be environmentally sustainable, has to help in reduction of total greenhouse gas emissions; the costs and benefits of bio-fuels should be distributed in an equitable way so that poor countries should not effect. [7]

In this project, there is a production of ethanol from encapsulated yeast cells in order to overcome stress conditions during fermentation with the substrate mixture of ligno-cellulosic waste. However, at first performed on using defined media to observe performance of yeast cells, so production of ethanol concern ethical and social issues.

BACKGROUND

One of the most widely accepted and used organism for bioethanol production is *Saccharomyces cerevisiae*, commonly known as baker's yeast. This organism is capable of hydrolysing sucrose present in cane sugar into the hexoses, glucose and fructose. Although this yeast is capable of producing ethanol under anaerobic conditions, it requires some

amounts of oxygen for the production of fatty acids and sterols. [8] *S. cerevisiae* produces ethanol upon fermentation of substrate.

Pretreatment and hydrolysis of lignocellulosic materials

The pretreatment of lignocellulosic substrates is considered to be one of the most important steps in the bioethanol production process it is in fact accounted as the second most expensive unit cost in the process representing 33% of the total process cost. This clearly states that it is very essential to build up more economical and efficient pre-treatment technologies, to finally reduce the ethanol production cost. In addition, the pre-treatment also affects various operational steps by determining fermentation toxicity and other process variables. [1]

The pretreatment of lignocellulosic material is very challenging, which complicates the fermentation step. The lignocellulosic matrix majorly consists of three polymers, cellulose and lignin, which are bound together by hemicelluloses. Lignocellulosic materials consist of cellulose (32-54%), hemicelluloses (11-37%), lignin (17-32%) other extractives (1-5%) and ashes (0-2%) as shown in Fig. 1. [10] The prime objective of the pretreatment step is to break down the matrix to increase the amorphous content of cellulose by decreasing the crystallinity. Amorphous cellulose is more accessible for enzymatic degradation. Various chemical, physical and biological methods have been proposed for the pretreatment process. [9]

One of the major factors affecting the pretreatment step is the difference in the ratio of lignin and hemicellulose content in lignocelluloses, which varies from one feedstock to another. This makes it difficult to choose the appropriate method for pretreatment. [7]

Lignocellulose biomass needs to be hydrolysed to simple sugars before fermentation, for the fermentative microorganisms to be able to utilize and convert them to ethanol. Hydrolysis can be performed by enzymes (enzymatic hydrolysis), which breaks down celluloses and hemicelluloses into simple sugars. However, the composition of lignocellulosic biomass makes it difficult to hydrolyse by enzymes. It is therefore necessary to perform pretreatment of lignocellulosic biomass prior to enzymatic hydrolysis. [1]

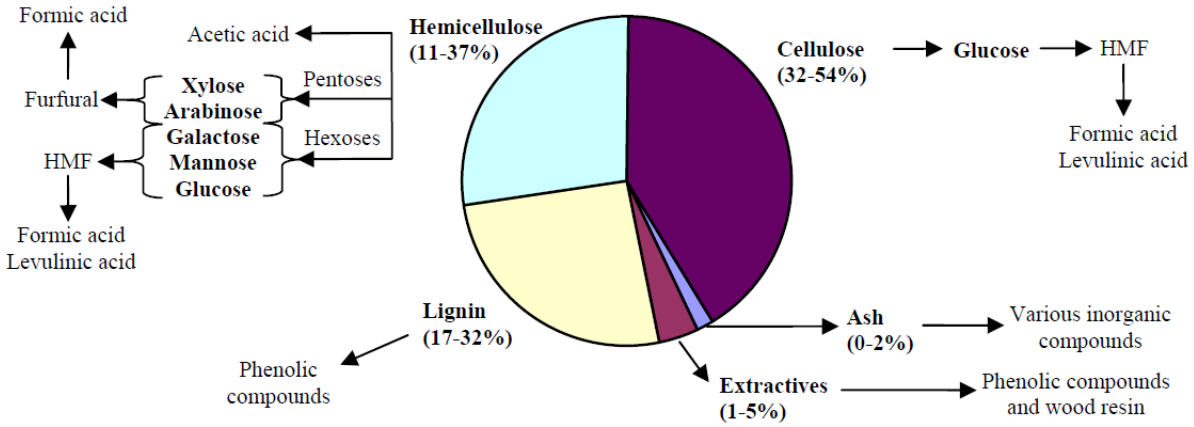

Figure.1. Composition of lignocellulosic materials and their potential hydrolysis products. [10]

In addition there are also certain methods for hydrolysis of lignocelluloses where there is no need to apply chemicals or enzymes. For example, lignocelluloses can be hydrolysed by using electron beam irradiation or microwave irradiation or can be hydrolysed by gamma-rays, but these processes are not commercially feasible and hence not applied. [10]

Minimization of inhibitory compounds formation during the pretreatment and hydrolysis is very essential before the fermentation process as the inhibitors can inhibit the microorganism during fermentation, thus resulting in reduced ethanol yield and productivity. [2]

Metabolically engineered microorganisms

Attempts have been made to construct and metabolically engineer an organism, which possess essential and desired traits obtained from other organisms i.e.; modifications or introduction of new biochemical reactions using recombinant DNA technology to improve the product formation. [2]

Genetically modified bacteria such as Z*ymomonas mobilis* have shown higher yield and productivity during ethanol production when compared to the wild type. However, using genetically modified stains in fermentation technology especially for the production of bio-ethanol has not yet been cost-effective. [14] When compared to *Z. mobilis,* the yeast *Saccharomyces cerevisiae* showed to be a better organism in tolerating the concentrations of the end product and other compounds which are present in the hydrolysate and thus is the preferred organism in the existing fermentation industry so far. [11]

During fermentation of hydrolysates prepared from lignocellulosic materials *Saccharomyces cerevisiae,* face various difficulties, such as different stress conditions. During osmotic stress, yeast releases water from the cell to the environment to even out the osmotic difference. For the yeast cells this leads to water scarcity and ion toxicity. It causes the cells to shrink and ultimately death due to lack of water, so in order to prevent this; the cells accumulate glycerol, which is produced in the cytosol of the cells. The cells also accumulate xylitol, mannitol, arabitol and meso-erythritol to adjust its osmolarity to the outer environment conditions. These solutes will even out the osmolarity difference between the outside and inside of the cells, increasing the osmotic tolerance of the cells [12].

Glycerol plays an important role in the cells physiological conditions and helps during harsh situations like osmotic stress, managing cytosolic phosphate levels and maintaining the NAD^+/NADH redox balance. [12] Glycerol, which is a by-product produced during

fermentation of sugars to ethanol, and acts as a carbon source for yeast in the deficiency of glucose.

The Fps1p channel is responsible for glycerol movement in and out of the cell; this channel is located in the plasma membrane and helps in the transportation of glycerol. The Fps1p channel closes during osmotic shock, which leads to accumulation of glycerol inside the cell. In normal conditions the Fps1p channel is open; in such a situation the glycerol escapes very quickly and does not stay inside the cells. [13] There are two homologous genes *GPD1* and *GPD2* in yeast *Saccharomyces cerevisiae*, which synthesize glycerol-3-phosphate dehydrogenase. *GPD1* has the major role during osmotic stress; osmotic stress induces *GPD1* expression, whereas *GPD2* expression is not affected by stress, but is active during anaerobic conditions. [14]. Yeast can come across high concentrations of ethanol, high concentrations of sugars, osmotic stress, heat shock, oxidative stress and acidic or alkaline conditions during fermentation. If the yeast cannot tolerate the above conditions, the cells can die or stop their fermentation which leads to an abolished ethanol production. To overcome some of these difficulties, immobilization of the cells has been shown to be beneficial [28].

Immobilisation:

Immobilisation is the technique where cells or enzymes or medicine are trapped in a solid matrix to protect themselves from inhibitors or toxins. Among the various methods (bead entrapment, coacervation, emulsion or interfacial polymerization) of cell immobilization, encapsulation of cells is very attractive and has several advantages over the conventional entrapment method. In encapsulation the cells are enclosed within a semipermeable membrane. The semipermeable membrane of the capsule allows the passage of essential nutrients for the cell metabolism and can also in some cases protect the cells from toxic compounds or inhibitors present in the medium, see Fig 2. [15] Improved cell stability and inhibitor tolerance in lignocellulosic substrate have been found to be achieved by encapsulation. [16] When compared to gel core beads, high cell concentration can be achieved inside the capsules. The capsules are also mechanically robust for prolonged usage.

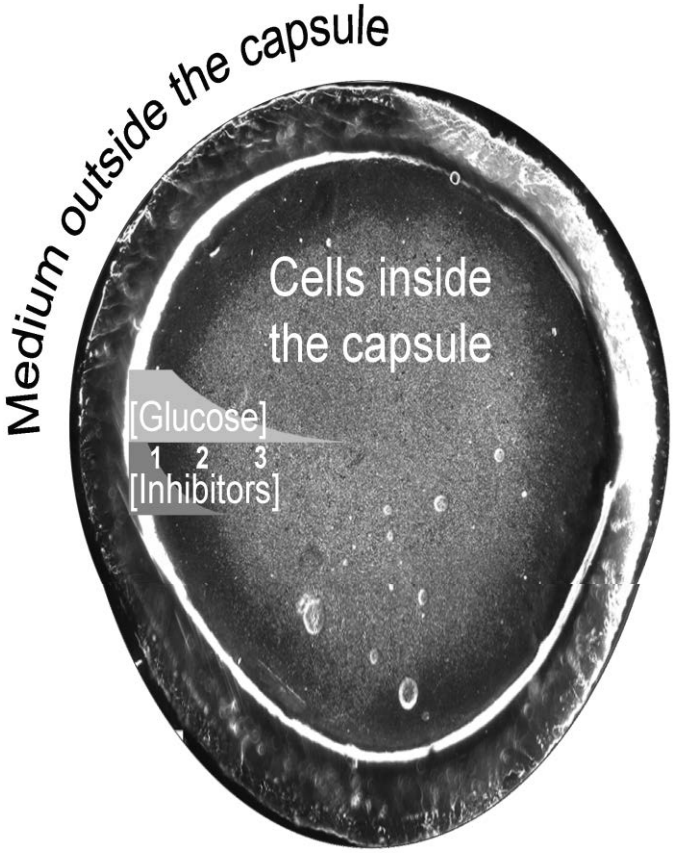

Figure 2. Schematic representation of the hypothesised concentration profiles in the cell pellet of the capsules.[26]

The immobilisation of cells with the help of a polymer membrane microcapsule was first performed by Chang (1964). His idea was to trap the cells in a semipermeable membrane where the cells are surrounded by a biocompatible matrix which protects the cells from destruction by foreign invaders but allows the entry of essential nutrients, which the cells demand for their growth, and exit of toxic compounds. Thus, the idea of encapsulation of cells gave rise to many new innovations and applications in biotechnology as well as in medicine. The type of membrane used for encapsulation depends on its application in various fields while the membrane is usually made up of either natural or synthetic polymer since polymer has the capability to exist in multiple phases such as solids, liquid and gels, which allows them to endure and satisfy different conditions. [16]

Microbial cells, which are encapsulated, can for example, produce therapeutic molecules or biofuels such as ethanol. The use of calcium cross-linked alginate gel beads for encapsulation of yeast cells has been intensively investigated as they enable the mass production of cells in the gel beads. One of the major disadvantages of cell encapsulation using alginate gel beads is the loss of their structural integrity. Therefore, to improve the stability of alginate gel beads,

various strategies have been proposed to prepare mechanically robust gel beads, one of which was to chemically conjugate alginate with methacrylic groups, termed methralic alginate (MA). The MA beads were exposed to UV to induce cross linking networks to develop interpenetrating networks (IPN) forming IPN-MA, through a cross linking reaction. The elastic module of the IPN-MA gel was 1.6 times higher than that of alginate gel and also showed high strength and ultimate strain when compared to alginate gel beads. [18]

It has been reported that, when the polymer beads are modified by coating them with oppositely charged polymers better properties are achieved, such as when chitosan is used to change the permeability of alginate membranes, where chitosan is a polycationic and alginate is a polyanionic polymer. [19]

Microencapsulation

Compounds like alginate, chitosan, carboxymethyl cellulose (CMC) and calcium plays very important roles in the capsule formation; each with specific properties which are important in the capsule formation and will be explained in more detail in the coming sections.

Alginate is extracted from seaweed; it is an anionic polysaccharide, non-toxic and biodegradable. It can be extracted from marine brown algae and even from a few species of bacteria. Sodium alginate is a sodium salt of alginic acid, it is a linear polymer consisting of 1, 4 linked β-D mannuronic acid (M) and α-D guluronic acid (G). Alginates rich in G-residues make stronger alginate membranes. Sodium alginate is water soluble and can be ionically cross-linked with divalent cations like calcium or polyvalent cations to form an insoluble gel network. Due to the fact that it is eco-friendly, calcium-alginate gels can be used for encapsulation of cells, enzymes, proteins and vaccine. It also has reswelling ability, helping in tolerating acidic conditions. [20] Calcium chloride is mixed with carboxymethyl cellulose (CMC), a chemically modified cellulose product obtained through a carboxymethylation process. CMC is mixed with calcium chloride in order to increase the viscosity that helps in obtaining spherical capsules. In the encapsulation process, chitosan strengthens the capsule; there is a strong electrostatic interaction between the amine group of the chitosan and the carboxyl groups of the alginate which leads to a chitosan-alginate complex, which strengthen the membrane. Chitosan or N-deacetylated chitin is a cationic polysaccharide consisting of ß (1-4) linkages 2-acetamide-2-deoxy- ß-D-glucopyranose (GlcNAc) and amino-2-deoxy-ß–D glucopyranose units used in formation of polyelectrolyte complex products with polyanions like alginate, xanthan etcetera. These complexes are widely used in enzyme immobilization, drug delivery etcetera. [21]

In recent years, lots of research activities along with technical modifications have been in progress to develop mature technologies for bioethanol production based on lignocellulosic biomass. Various biotechnology-based companies, which are currently operating in Canada, USA and Sweden, are working on the optimization of this technology and setting up pilot plants. [11]

It is evident that when the yeast cells are exposed to a small dose of any stress, they become resistant to similar doses of other stresses as the yeast cells develop cross protection against different stresses. This phenomenon explains that the yeast cells use a general mechanism of cellular protection that comes into action when the cells are exposed to different stresses. [22]

AIM OF THE PROJECT

During this experimental period, three different types of capsules; Alginate-Chitosan (AC) capsules, Alginate-Chitosan-Alginate (ACA) capsules and Alginate-Chitosan-Alginate treated with APTES (3- Aminopropyl triethoxysilane) capsules with yeast inside were prepared and compared to each other, with free cell cultivations as reference, to observe their response and tolerance levels to different stress conditions such as osmotic and ethanol stress when cultivated anaerobically.

Overview of the experimental process:

As was discussed earlier about the global fuel crisis and the rising demand for bioethanol as an alternative energy source, many countries in the world are starting to produce ethanol. However, there are still many drawbacks in using lignocelluloses as substrate due to its high pretreatment cost and production of inhibitors during pretreatment and hydrolysis, which finally affects the ethanol yield. Recent developments and ongoing research activities should make it possible to overcome these difficulties.

Encapsulation of ethanol producing organisms is one of the developments, which are undergoing research as it to some extent can protect the organisms from being affected by the inhibitors.

A laboratory *S. cerevisiae* strain, CBS 8066, was used to carry out the experiments throughout the project. Previous research has dealt with the formation of various inhibitors during the pretreatment of lignocellulosic substrate and their effect on yeast and ethanol production. The current research deals with the encapsulation of yeast to investigate how tolerant they are when cultured at different stress conditions, mainly when cultivated in medium with high osmolarity and high ethanol concentration at anaerobic conditions.

After the initial encapsulation of the yeast, the capsules undergo various treatment steps to make them stronger and more rigid, and thereby able to withhold the yeast throughout the cultivation. Four hours from the start of the anaerobic fermentations, the viability of the yeast was assayed and two hours from this time the intracellular glycerol content was determined following a defined protocol by *Ylitervo et al* [25].

METHODOLGY

Pre-cultivation

The laboratory strain *S. cerevisiae* CBS 8066 was preserved on Petri-plates containing 2% nutrient agar medium and stored at 4˚C. Inoculation of culture medium was done by picking

up a colony of CBS 8066 and placing it into 100 ml DGM (defined glucose medium) containing 50 g/l glucose, magnesium sulphate 500mg/l , potassium dihydrogen phosphate 2000 mg/l ammonium sulphate 5000mg/l, trace metal 6.7 g/l and vitamins 1ml/l and incubating it in a shaking water bath at a temperature of 30˚C, at 130 rpm (26).

Encapsulation procedure

Various encapsulation techniques are available; the *liquid droplet-forming method* was used for carrying out the encapsulation process in the current work. The method was as follows:

1. Pre-cultivated yeast from 100 ml medium was harvested by centrifugation at 3400 g for 5 minutes and the pellet was re-suspended in 100 ml 1.3% (w/v) sterile $CaCl_2$ solution containing 1.3% (w/v) carboxymethylcellulose (CMC).
2. A 1-litre sterile glass bottle containing sterile 250 ml of 0.6% (w/v) sodium alginate and 0.1% (v/v) Tween 20 was used for capsule formation.
3. Capsules were formed by dripping the CMC-yeast-$CaCl_2$ solution into the stirred sodium alginate solution through syringe needles (as shown in Fig. 3) for 5 minutes.
4. The capsules were then allowed to gel for another 5 minutes, and were thereafter washed with ultra-pure water (autoclaved milli-Q water) for 10 minutes and thereafter hardened in 1.3% (w/v) $CaCl_2$ solution for 20 minutes.
5. Thereafter, the capsules were treated in a 0.2% (w/v) low molecular weight chitosan solution at a volume ratio of 1:5 in 1 l Erlenmeyer flasks in a water bath at 30˚C at 140 rpm.

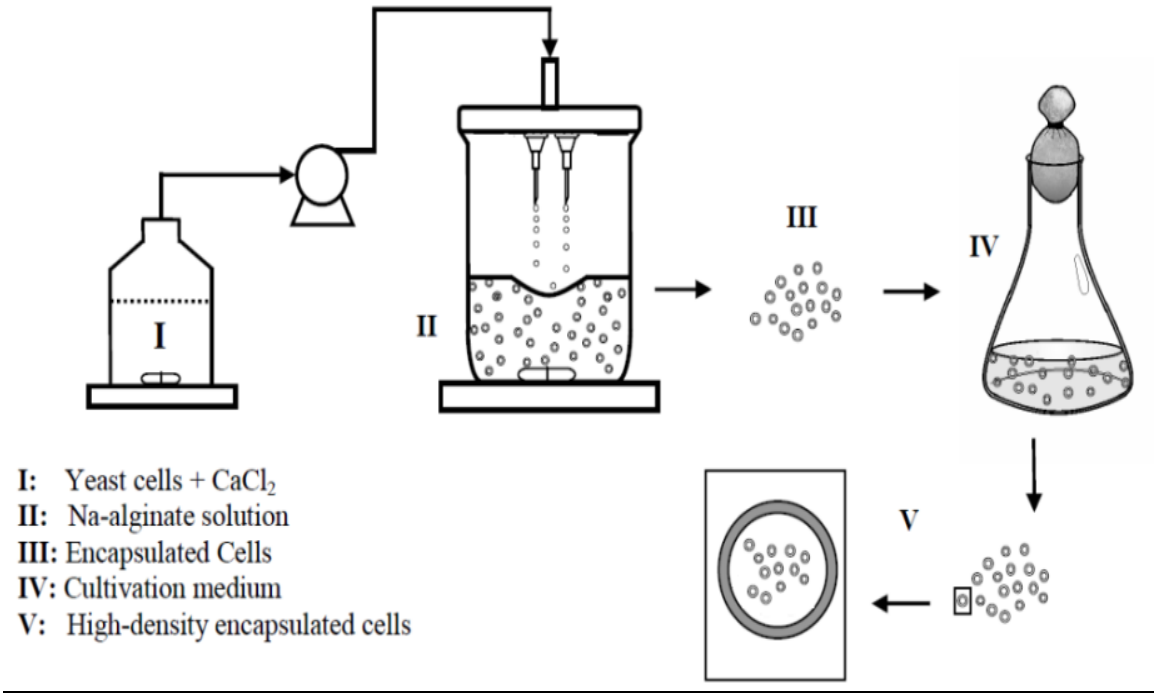

Figure 3: Schematic description of cell encapsulation.[16]

Different types of capsules preparation (following the above mentioned procedure):

ACA capsules:

1. The chitosan treatment for ACA capsules lasted for 10 hours in a water bath at 30˚C at 130 rpm.
2. The capsules were then equilibrated by washing with a large amount of 0.9% NaCl (approximately 1:5 ratios) for at least 15 minutes.
3. Later, the capsules were coated with 0.05% (w/v) alginate solution (to form ACA capsules) by suspending the capsules in the alginate solution (at 1:5 ratio) in a 1 l Erlenmeyer flask at 30˚C for 12 hours at 140 rpm.
4. The capsules were thereafter transferred to 500 ml of DGM, 40 g/l glucose for 24 hours in a 1 l Erlenmeyer flask in a water bath at 30˚C at 130 rpm.
5. After 24 hours, the medium for the capsules was changed with another 500 ml medium containing 40 g/l glucose as the yeast in the capsules consumed most of the nutrients present in the media.

6. After 48 hours, the capsules were transferred to anaerobic fermentation flasks to investigate their tolerance levels under different stress conditions.

AC capsules:

1. The yeast capsules preparation was followed by treating the capsules with chitosan for 24 hours in a 1 l Erlenmeyer flask in a water bath at 30˚C at 140 rpm.
2. After 24 hours of chitosan treatment, the capsules were equilibrated in 0.9% (w/v) NaCl (with a large amount, approximately 1:5 ratio) for at least 15 minutes in order to lower the Ca^{2+} concentration inside the capsules.
3. The cell-seeded capsules were later transferred into DGM containing 40 g/l glucose (100 ml DGM for 15 ml of capsules) and cultivated for 24 hours in a shaker bath at 30˚C at 130 rpm.
4. The capsules were thereafter rinsed with sterile 0.9% NaCl and transferred to fresh medium for another 7 hours.
5. After the encapsulated yeast was grown in the medium for 31 hours, the yeast capsules were subjected to different stress conditions at anaerobic conditions.

APTES treated ACA capsules:

1. The ACA capsules were washed with sterile ultra-pure water.
2. Thereafter, the capsules were further treated with 1.5% hAPTES (hydrolysed APTES) solution (100 ml solution for 50 ml capsules) at pH 5, in a 1 l Erlenmeyer flask for 90 minutes in a water bath at 30˚C at 130 rpm.

   hAPTES preparation:

   The APTES solution was first hydrolysed in water; 1 ml APTES + 9 ml sterile water in a 50 ml sterile E-flask covered with aluminium foil in a water bath at 30˚C overnight. Thereafter the pH of the solution was carefully set to 5 and thereafter diluted to 1.5% by adding sterile water.
3. Later, the capsules were washed thoroughly with 0.9% (w/v) NaCl.
4. The capsules were then transferred to 500 ml medium, containing 40 g/l glucose, and cultivated for 24 hours in a 1 l Erlenmeyer flask in a water bath at 30˚C at 130 rpm.

5. After 24 hours, the medium for the capsules was changed as the yeast in the capsules consumed most of the nutrients present in the media.
6. After 48 hours, the capsules were transferred to anaerobic fermentation flasks to investigate their tolerance levels under different stress conditions.

Free cell cultivation:

1. Firstly, as described earlier for pre-cultivations, inoculation of the culture medium was done by picking up a colony of *S. cerevisiae* CBS 8066 and placing it into 100 ml of a DGM containing 50 g/l glucose and incubating it in a shaking water bath at a temperature of 30˚C, at 130 rpm.
2. After 24 hours, 1 ml from the previous culture was transferred into 100 ml of fresh DGM making a 1:100 dilution.
3. Later, after 23.5 hours, a defined amount of yeast cells were harvested by centrifugation at 3500g for 4 minutes and the cells were re-suspended in 100 ml fresh DGM containing 40 g/l glucose for anaerobic cultivations. The cells were grown anaerobically and samples were taken at regular intervals to analyse the amount of glucose consumed and ethanol produced to compare with the values obtained for the encapsulated yeast.

Anaerobic cultivations:

After 48 hours for ACA and APT capsules and after 31 hours for AC capsules, 50 capsules were subsequently transferred to 100 ml DGM containing 50/g/l glucose with different osmolarity and ethanol concentrations. The cultivations were performed in 250 ml Erlenmeyer flasks at anaerobic conditions. The reason for the different aerobic cultivation time was because the glucose concentration after 30 hours for AC capsules was 8.6-8.9 g/l while the glucose concentration after 48 hours for APT capsules was 5.3-5.4 g/l. The concentration of glucose at the end of the aerobic cultivations was hence with these pre-cultivations more or less similar for all types of capsules.

The conditions tested included high osmolarity media with NaCl concentrations of 1 M, 2 M and 3 M and high ethanol containing media with ethanol concentrations of 5% w/v, 10% w/v, and 15% w/v respectively. These media were used to investigate the tolerance levels of the encapsulated yeast and to observe the effect of these stresses on yeast survival and ethanol production. Samples were taken every day with the help of a syringe from the anaerobic flasks containing the yeast capsules. In the case of free cell cultivation anaerobically, samples were taken every 4 hours as the rate of fermentation was faster for cells in suspension when

compared to encapsulated yeast which may take more than a week for the fermentation to stop.

ANALYTICAL METH ODS

The medium samples were centrifuged to remove any particles and cells present. The samples were thereafter analysed by HPLC to determine the amount of ethanol and by-products produced and glucose consumed during the fermentation. Actually glucose was analyzed by ion exchange Aminex HPX-87P column (Bio-Rad Hercules, CA, USA) at 85°c. The eluent was ultrapure water at flow rate 0.6 ml/min and to quantify the sugars an evaporative light scattering detector (waters 2465, MA, USA) was used. Ethanol and glycerol concentrations was determined by Aminex HPX-87H column (Bio-Rad) at 60°c with 5mM H2SO4 as eluent at flow rate 0.6 ml/min.

VIABILITY TEST

Viability test is done to check the viability of the yeast during culturing at the different stress conditions. There are many different available methods for measuring yeast viability based on cell replication, staining, measurement of cellular components etc, (Figure 4).

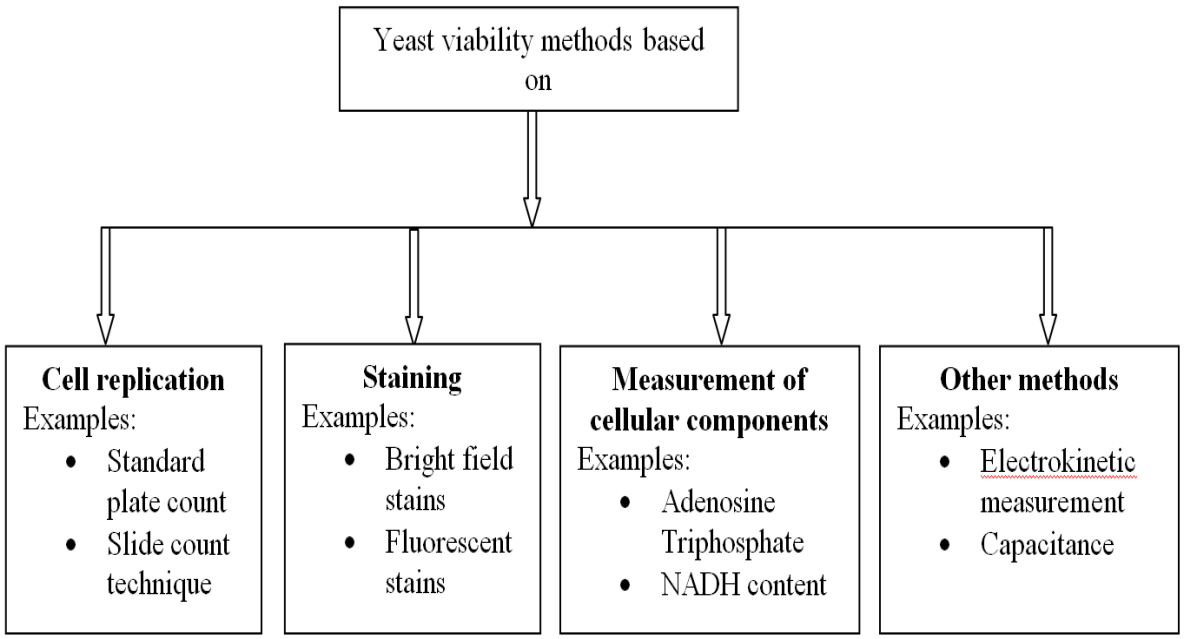

Figure 4: Summary of different methods used to measure yeast viability. [23]

Over the past few years, bright field stains have been utilized to measure yeast cell viability. Some of these stains include rhodamine, eosin, methylene blue etc. The most widely used and recommended method for viability staining is with methyleneblue. This stain has been in use since the 1990s. It is a generally and accepted fact that methylene blue is an autoxidizable dye, which upon entry into the living cells cytoplasm gets oxidized to its colourless leuco-form while the dead cells turn blue [23].

Procedure:

Methylene blue crystals were diluted in sodium citrate (0.01 % methylene blue w/v in 2% sodium citrate dihydrate solution). Two capsules from all the different stress conditions (or 0.1 ml in the case of the free cell suspension) were sampled after fourth hour from when the anaerobic cultivation was started. The capsules were crushed with the help of forceps under sterile conditions, by adding some amounts of NaCl until a clear solution was obtained, indicating that there was no more yeast inside the capsules. The NaCl containing yeast suspension was collected in a tube with the help of a pipette from which, 100 µl of sample was diluted with 200 µl of methylene blue (1:2 dilution). From this dilution, 10 µl of solution was injected into the Bürkers chamber where the cells can be differentiated by colour when observed under the light microscope. The living cells appear to be white while the dead ones appear to be blue.

DETERMINATION OF INTRACELLULAR GLYCEROL IN *S. CEREVISIAE*

Micro-organisms have the ability to adapt to different variations in the environmental conditions. Various analysis of different organisms including bacteria, fungi and some yeasts, have proved that there are a wide number of genes and proteins involved during the survival of the organism at different environmental conditions.

In this case when yeast is subjected to osmotic stress, i.e.; sudden change in the water activity of the growth medium, compatible solutes like glycerol or other polyols are synthesized as a response to counteract the higher osmolarity of the surrounding medium.

Investigations show that when cells were grown in medium with a high osmolarity, by adding large amounts of NaCl to the growth medium, the cells undergo osmotic shock and immediately shrink. The cells stop to grow during this phase. Within a couple of hours after the shock, the intracellular glycerol content increased from 3 g/l to about 35 g/l and then remains constant. [24]

Procedure:

Intracellular glycerol determination was performed 6 hours after the anaerobic cultivation was started with encapsulated yeast or free suspension under different osmotic conditions. To determine the glycerol content in the cells, firstly the cells were collected by crushing two capsules from all cultivations; 1 M, 2 M and 3 M NaCl and defined medium, in the case of encapsulated yeast while the cells were directly collected in the case of freely suspended yeast. The cells were then washed rapidly with an isotonic solution at 4˚C and placed in 2 ml of boiling 0.5 M Tris buffer pH 7.0 for 10 minutes, which maintains the pH of the cell. The combination of heat shock and the effect of Tris buffer lead to lysis of the cells, thus releasing glycerol from the cells into the buffer. The cell debris was separated by centrifugation at 15,000 g for 10 minutes at 4˚C. The glycerol content was determined by high-performance liquid chromatography.

Figure 5 Alginate chitosan capsules with filled yeast biomass

Figure 6 Encapsulated yeast cells

RESULTS AND DISCUSSIONS

It is important to investigate the yeast response at different stress conditions and to find ways to make the yeast overcome stress conditions. Because, during industrial fermentation yeast cells encounter various stresses which may damage the fermentation process. Therefore, in this project two different stress conditions: high osmolarity and ethanol stresses were studied both with encapsulated yeast and with free cells. The procedure of making the capsules were based on the protocol by Ylitervo et al [25].

FREE CELLS CULTIVATED AT DIFFERENT OSMOTIC STRESS

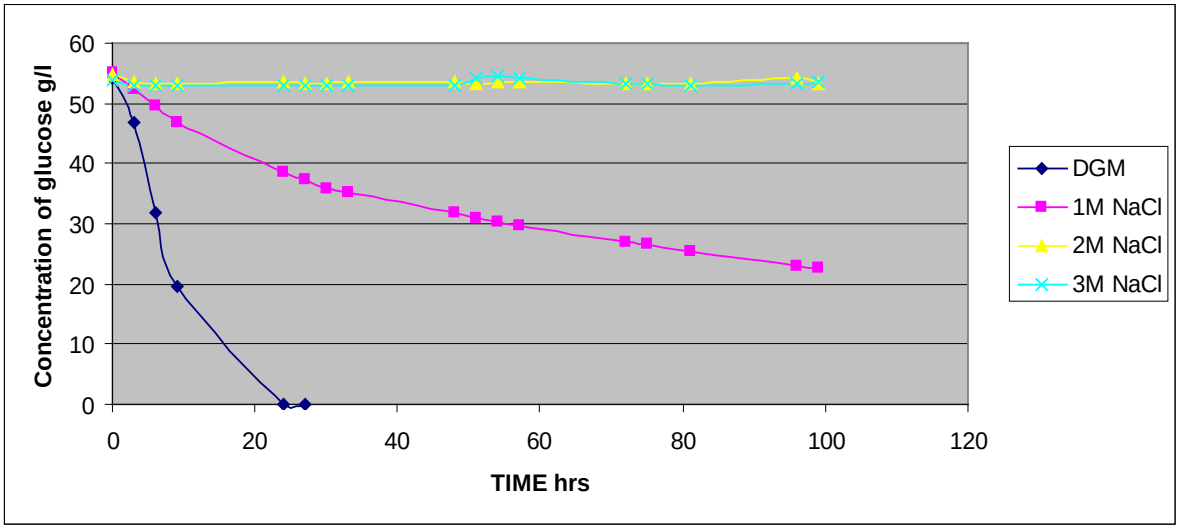

Figure 7-Free yeast cells at different osmotic stress

At first free yeast cells were cultivated in defined medium DGM, and DGM medium containing 1 M, 2 M and 3 M sodium chloride (NaCl) at anaerobic condition. As can be seen from figure 7 the glucose was completely consumed by the freely suspended cells in defined medium and correspondingly produced ethanol i.e. 55 g/l of glucose in 24 hours, 22 g/l of ethanol was produced as shown in figure 9. When freely suspended cells were cultivated in defined medium with 1 M sodium chloride to provide a higher osmotic stress, there was difference in glucose consumption when compared to the cultivations in defined medium without any stresses. Fermentation was observed in cultivations in 1 M sodium chloride containing medium, glucose consumed by free cells was 33 g/l (55 g/l - 22 g/l) after 100 hours. Yeast cells exposed to osmotic conditions leads to cell dehydration. To combat dehydration, specific gene induction occurs such as e.g. the glycerol pathway which leads to accumulation of compatible solutes e.g. glycerol and trehalose [26]. Fermentation did not occur when freely suspended cell where exposed to higher osmolarity at 2 M and 3 M NaCl present in the medium (Fig. 7).

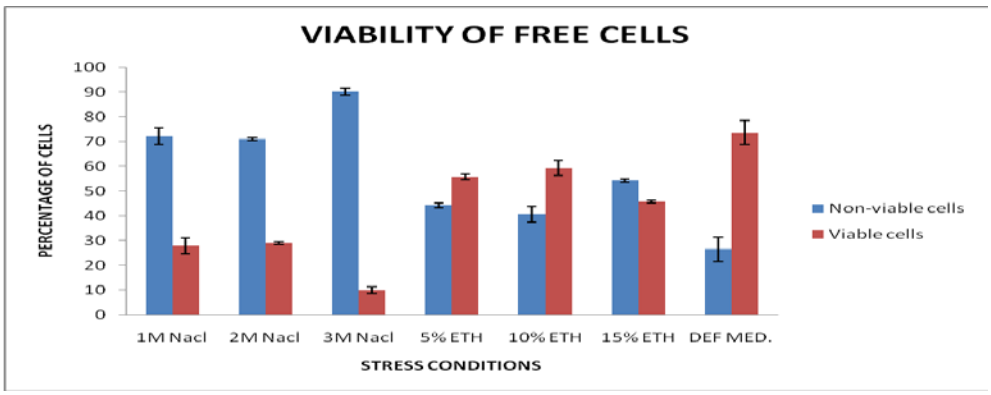

Figure 8 Viability of free yeast cells at different osmotic stress and ethanol stress

Figure 8 show the cell viability in the different media containing 1 M, 2 M and 3 M NaCl, and the cell viability was 25% (1 M NaCl), 20% (2 M NaCl) and 5% (3 M NaCl). This show that yeast cells cannot tolerate the high osmotic pressure occurring at 2 M and 3 M NaCl concentrations. A reason why the cells can withstand a the lower levels of 1 M NaCl but not the higher concentration can be a specific gene is induced during mild osmostress condition [28] which indicates that free cells tolerates only mild stress conditions. In defined medium free yeast cells consumption of glucose is faster than in the medium with 1 M NaCl, i.e. rate of glucose consumption is 6.9 times faster. As the osmotic pressure increases in medium with 2 M and 3 M NaCl there is no change in glucose consumption and ethanol production.

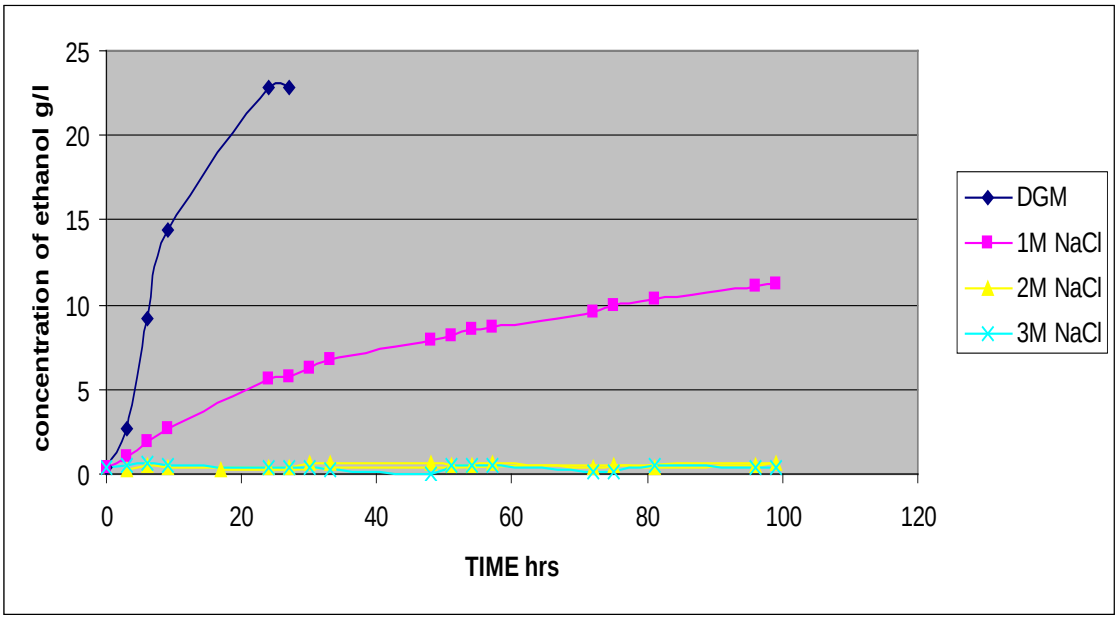

Figure 9-Free yeast cell at different osmotic stress - Ethanol concentration Vs Time

In defined medium with 1 M sodium chloride, the free cells produce 12 g/l (figure 9) of ethanol after a glucose consumption of 33 g/l (55 g/l – 22 g/l from figure 7) at 100 hours. As osmotic pressure increases such 2 M and 3 M sodium chloride ethanol production was nil. The results obtained by *Zhi-Jie Suna et al.* free cells with (*S. cerevisiae* Y800) showed almost no cell growth in medium containing 1 M NaCl [29].

As reported by *Eva Albers and Christer Larsson* [25] increasing the osmotic pressure with NaCl inhibitory effects were shown at much lower concentrations. The strains of *Saccharomyces cerevisiae*, and strain used in ethanol production from lignocellulosic material, abbreviated MoDo and X2180 strains were used for the experiment; MoDo and X2180 strains was the ones most resistant to NaCl. In YPD medium, growth of these strains was supported at a rate close to 0.1 h^{-1} even in media containing 100 g/l (1.7 M) of NaCl.

In lignocellulosic medium, the MoDo strain tolerates stress up to a NaCl concentration of 60 g l^{-1} (1.02 M) with the highest growth rate as well as the shortest lag phase. At higher sodium chloride concentrations there was a sharp increase in the length of the lag phase for the MoDo strain, and the growth rate fell to below 0.02 h^{-1} .

Relative rate calculation

In this experiment comparison of free yeast cells and encapsulated yeast cells are done by the relative rate method. According to *Johan westman et al.* (26) relative rate calculations for the glucose consumption and ethanol production are calculated.

((Io-Ia)/Ho)/((Ii -Ia)/Ho))

Io = Initial glucose concentration in defined medium (50 g/l)

Ia = Glucose concentration at chosen point in defined medium (30 g/l)

Ho = Cultivation hours at chosen point in defined medium (8 hrs)

Ii = Initial glucose concentration in medium with 1 M NaCl (50 g/l)

Ia = Glucose concentration at chosen point in medium with 1 M NaCl (30 g/l)

Ho = Cultivation hours at chosen point in medium with 1 M NaCl (55 hrs).

((50-30)/8)/((50-30)/55)) = 6.875 for 1 M NaCl medium compared with control.

ALGINATE CHITOSAN ALGINATE (ACA) ENCAPSULATED YEAST AT DIFFERENT OSMOTIC STRESS

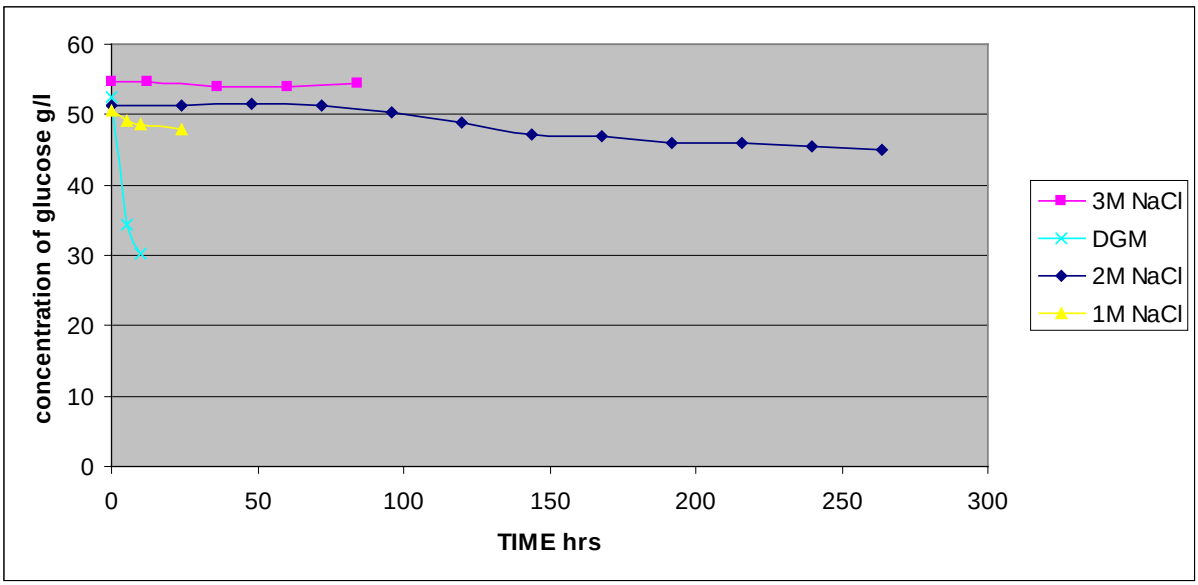

Figure 10- ACA capsules at different osmotic stress - Glucose concentration Vs Time

When it comes to encapsulated cells with alginate chitosan alginate (ACA) capsules, figure 10 shows that the rate of glucose consumption in defined medium is 2 times faster than in medium with 1 M NaCl and from figure 7 free cells relative rate of glucose consumption between control and 1M sodium chloride was 6.8 times faster . When comparing the relative rate between ACA capsules and free cells, ACA capsules got appreciable relative rate. However, in medium with 2 M NaCl there was an initial lag phase but not in freely suspended cells. Glucose consumption was observed in medium with 3 M NaCl by using encapsulated yeast cells but comparatively low i.e. 1 g/l (54 g/l – 53 g/l) from 0 to 50 hour.

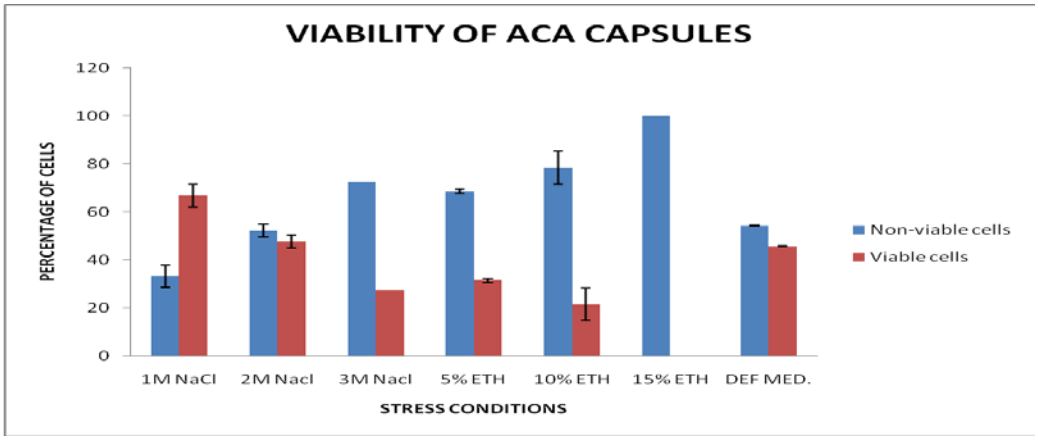

Figure 11-viability of yeast cells in ACA capsules under stress conditions

Figure 11 show the viability of encapsulated cells cultivated in medium with 1 M, 2 M and 3 M NaCl, and the viability of the encapsulated yeast cells were 70%, 50% and 25% respectively. This indicates that yeast cells are more viable when encapsulated under stress condition than free yeast cells, this shows the proof that encapsulation played major role to combat with osmotic pressure and act as a barrier for yeast cell which can be promising for ethanol production at stressful conditions. Thus, encapsulated yeast cells can be cultivated at stressful conditions which could be a good option to overcome stress conditions for ethanol production.

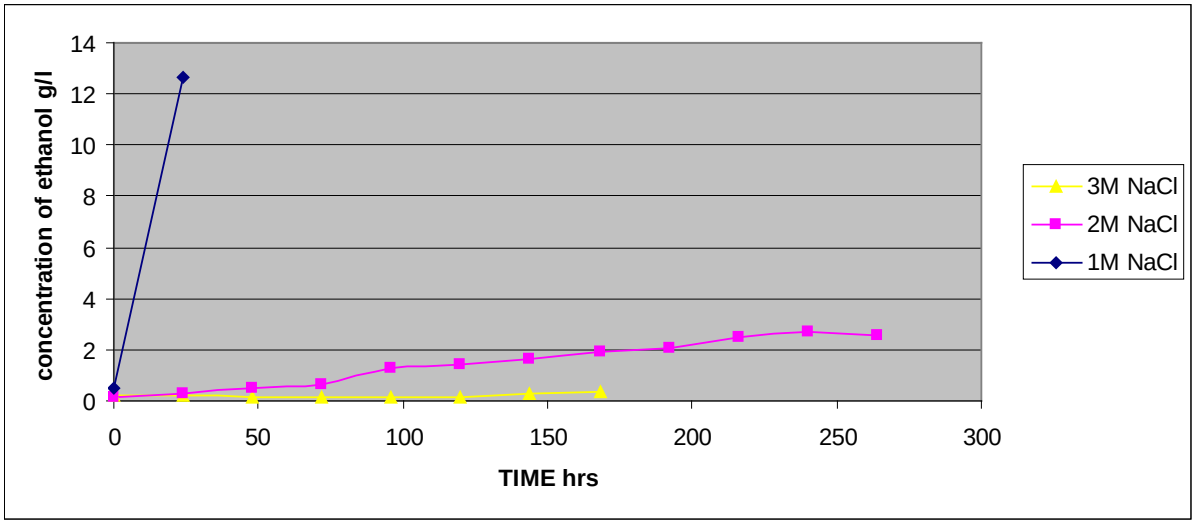

Figure 12- ACA capsules in osmolarity stress-Ethanol concentration Vs Time

The relative rate of ethanol production was calculated as described before. Relative rate between defined medium and medium with 1 M NaCl

((0-10)/8) / ((0-10)/80) = 10

Consequently, the ethanol production in defined medium with free yeast cells was 10 times faster than with free cells in medium with 1M NaCl. According to figure 12 the cultivation of encapsulated cells in 1 M NaCl containing medium resulted in 12.9 g/l ethanol at around 24 hours, this ethanol concentration is higher when compared with free yeast cells under the same stress condition. In 2 M NaCl containing medium the encapsulated cell produced 1.8 g/l of ethanol after 100^{th} hour and in medium with 3 M NaCl medium ethanol production was nil.

From the point of Zhi-Jie Suna et al. [29] theoretically explains that under osmotic stress, the encapsulated Y02724 was only physically protected by liquid matrix (LM), solid matrix and/or membrane. The same paper later concludes that the protection effect of membrane, liquid matrix, or solid matrix was insignificant, because there was no difference in growth between MB (without membrane/with gel matrix), LCM (with membrane/with liquefied matrix) and free cells. Also the relative growth of encapsulated cells was always higher than free cells at the different stress conditions. The encapsulation cells however did grow in 1.25

M NaCl, but free cells almost stop growing in 1 M NaCl. From our data encapsulation of yeast cells performed well compared to *Zhi-Jie Suna et al.* [29] experimental data.

According to Johan Westman et al [26]. mild stress level that the cells sensed under encapsulation was unknown. However it was hypothesized, that encapsulated yeast cells are protected by the cells in the outer layers, whereas cells at the core are less exposed to the stress condition than the cells at the edges of the capsules. Hence the encapsulated cells are more or less comfort in stress condition [26].

ALGINATE CHITOSAN (AC) ENCAPSULATED YEAST AT DIFFERENT OSMOSTRESS

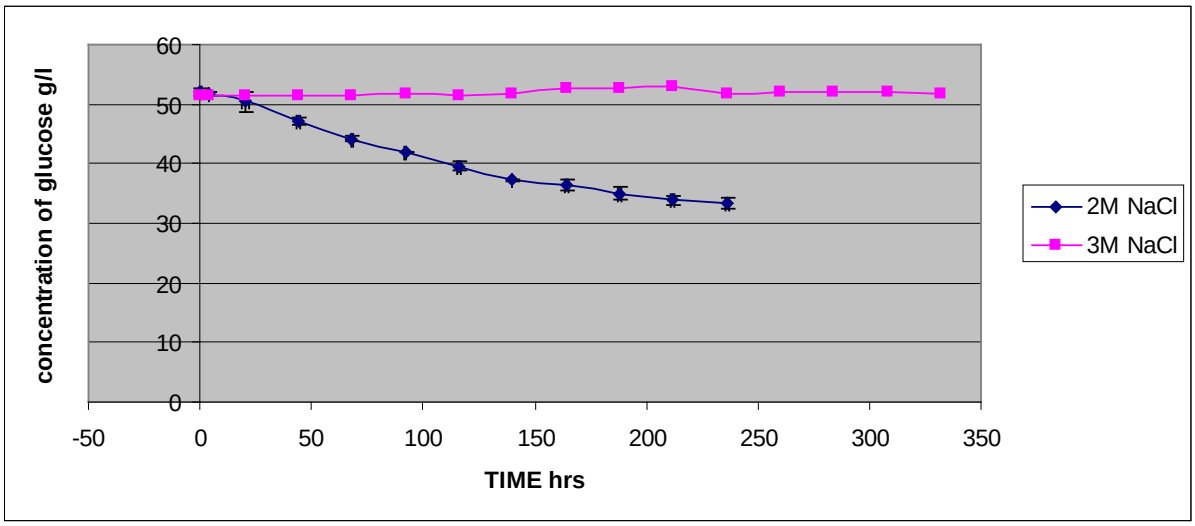

Figure 13-AC capsules under osmalarity stress –Glucose concentration Vs Time

Experiments were also conducted with AC encapsulated cells to observe if there is any difference with that of ACA encapsulation in the cells stress tolerance. Yeast cells were encapsulated in alginate chitosan complex to observe their performance at high stress conditions at 2 M and 3 M NaCl concentrations. As shown in figure 13 the encapsulated yeast cells consumed 22 g/l in 230 hours and a longer lag phase was observed and subsequently, ethanol production was 6.2 g/l after 230 hours (figure 14). In 3 M NaCl containing media, there was no glucose consumption observed from yeast cells, this proofs that these encapsulated yeast cells cannot tolerate very high osmotic stress conditions (3 M NaCl).

When comparing the two different capsule types ACA and AC capsules one could conclude that glucose consumption at 100th hour in 2M NaCl stress media from figure 13 AC capsules, was 20 g/l (53 g/l - 33 g/l) and from figure 10 ACA capsules glucose consumption was 2 g/l (52 g/l – 50 g/l) the glucose consumption rate was thereby better in AC capsules than in ACA capsules.

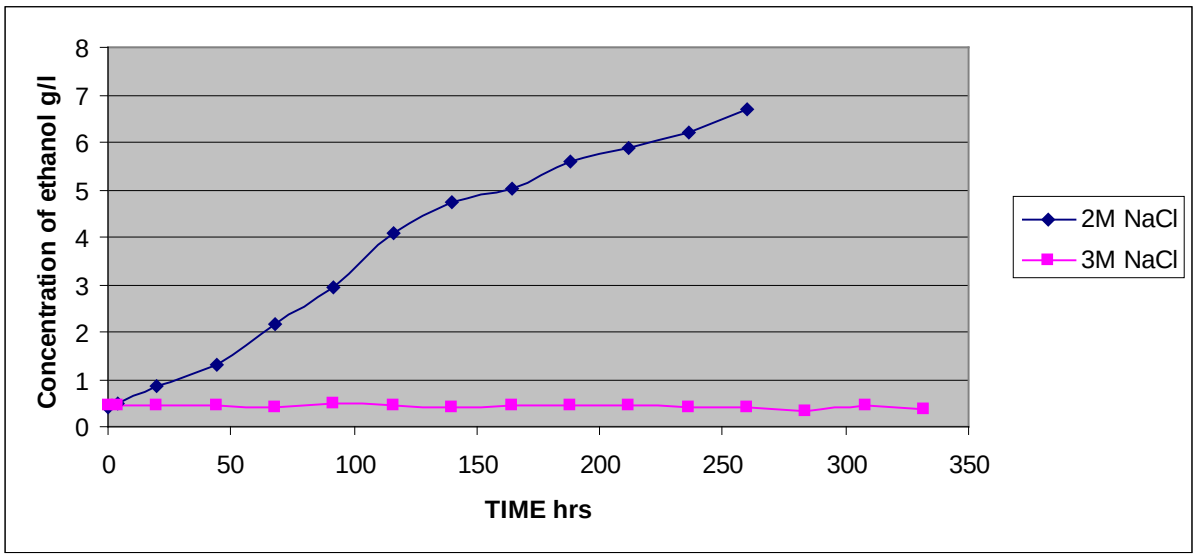

Figure 14-ACcapsules under osmalarity stress –Ethanol concentration Vs Time

FREE CELLS CULTIVATED AT DIFFERENT ETHANOL STRESS

During anaerobic fermentation yeast produce the toxic substance ethanol. Higher levels of ethanol can be toxic to yeast cells. Inorder to overcome problems with ethanol toxicity encapsulation was studied as a way to reduce the toxic effect of ethanol. Free cells was used as a comparison in the study.

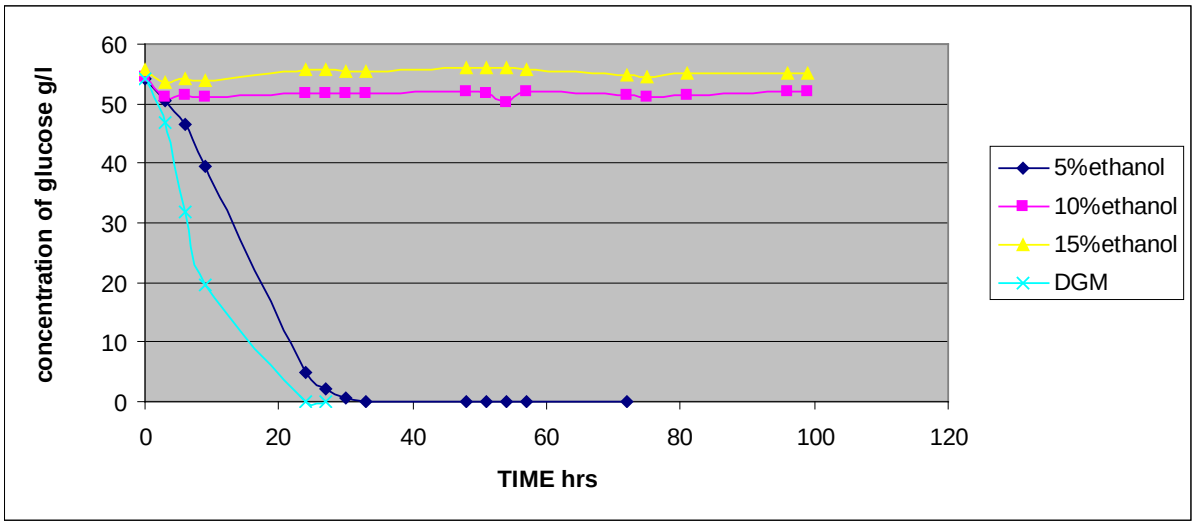

Figure 15-Free yeast cells under ethanol stress

Freely suspened yeast cells were subjected to ethanol stress condition at different concentrations i.e 5%, 10%, and 15% ethanol. Figure 15 shows the glucose consumption of free cells in defined medium compared with that of glucose consumption in 5% ethanol containing medium difference was very small. The relative rate of ethanol consumption between free cells in defined medium and 5% ethanol stress condition medium was 2.4 times (Relative rate ((55-40)/5)/((55-40)/12) = 2.4) faster than in 5% ethanol. This shows that free cells at 5% ethanol are able to survive and consume glucose but when it comes to viability from figure 16 60 % free cells are viable in 5% ethanol levels compared to unstressed free cells in defined medium where the viability was 80%.

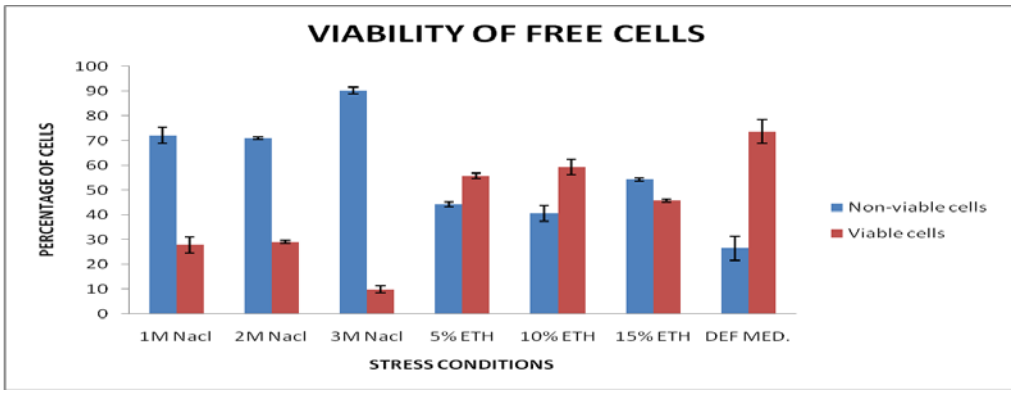

Figure 16- viability of free yeast cells under stress conditions

There was a negligible difference between free cells in defined medium and in 5% ethanol containing medium. Besides, the concentration of ethanol increases at 10% and 15% ethanol there is no lag phase observed from graph, percentage of nonviable cells are observed more than viable cells as shown in figure 16.

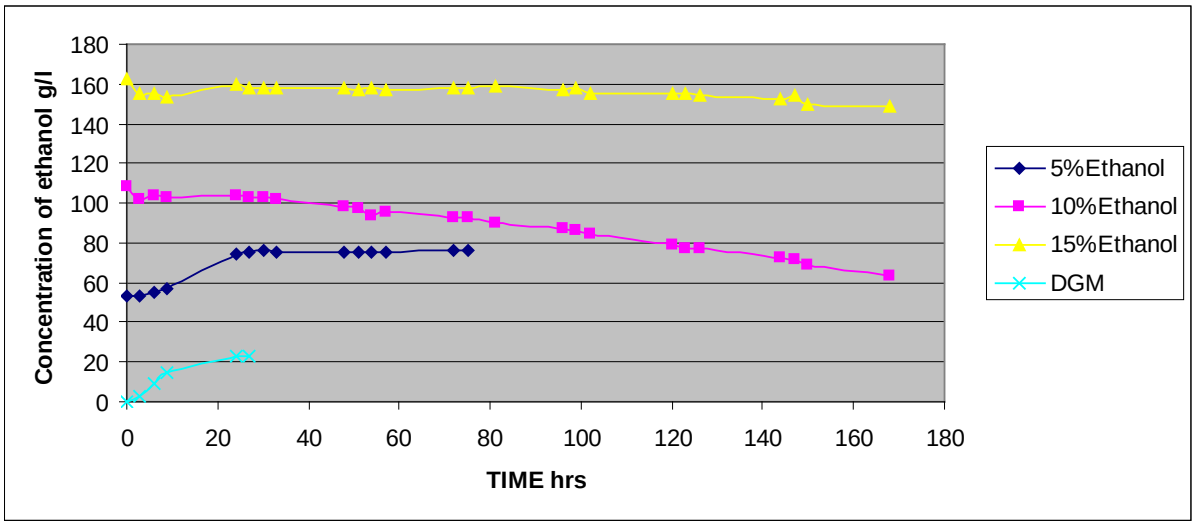

Figure 17-Free yeast cells under ethanol stress-Ethanol concentration Vs Time

Similar experiment have been conducted by Eva Albers et al. [27], the author explains that in lignocellulosic media considering strains of *Saccharomyces cerevisiae* the JBA strain (Jästbolaget AB, Rotebro, Sweden) showed lowest growth rate and longest lag phase. MoDo strain (CCUG53310, Culture collection university of Goteborg) was the most resistant up to an ethanol concentration of 4%. There might be something of a threshold value at 4–6% of ethanol for all strains tested as there was a drastic reduction in growth rate and prolongation of the lag phase in this interval.

ALGINATE CHITOSAN ALGINATE (ACA) ENCAPSULATED CELLS AT DIFFERENT ETHANOL STRESS

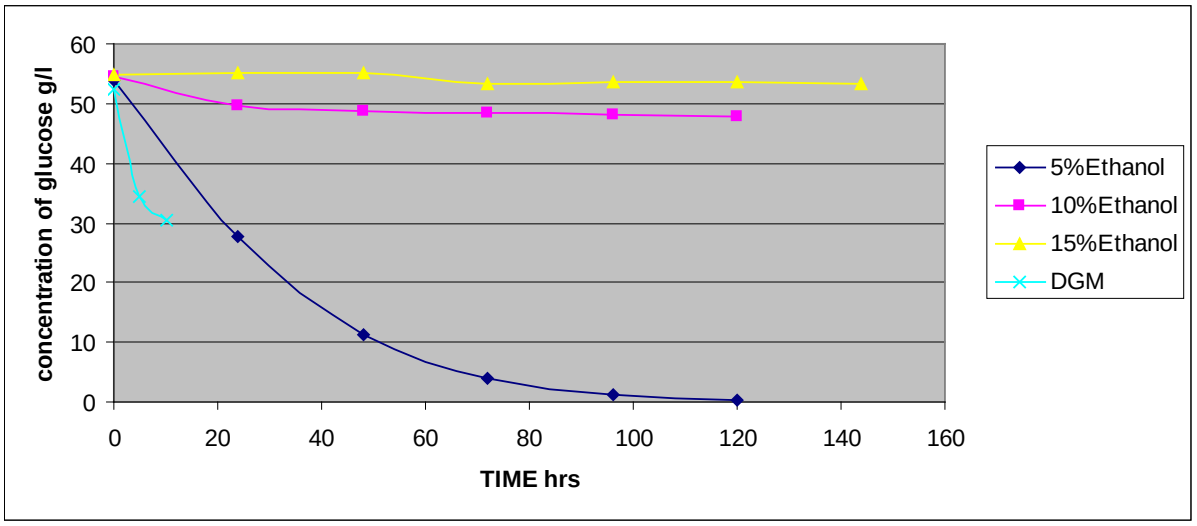

Figure 18-ACA capsules at different ethanol stress

Now considering encapsulated yeast cells in defined medium and at different ethanol stress condition such as 5%, 10%, and 15% ethanol concentration. While experiment were performed yeast cell leakage from the capsules was noted in defined medium at 18th hour. No leakage of yeast cells was observed from the capsules at different ethanol stress condition. This can be becasuse of various reasons such as yeast cells under stress condtion cannot grow as faster as in defined medium. Encapulated yeast cells shown appriecible glucose consumption in 5% ethanol medium. As can be seen from figure 18 25 g/l glucose was consumed by encapsulated yeast cells in defined medium at 10th hour and 15 g/l glucose was consumed by encapsulated yeast cells in 5% ethanol at 10th hour. At a higher ethanol concentration of 10% a very longer lag phase observed. At 15% ethanol concentration, lag phase is seen at 50th hour which is slower than 10% ethanol concentration which is negligible but viability at 15% is zero. One can thereby conclude that the yeast cells cannot tolerate higher concentration of ethanol even upon encapsulation when comared to suspended yeast.

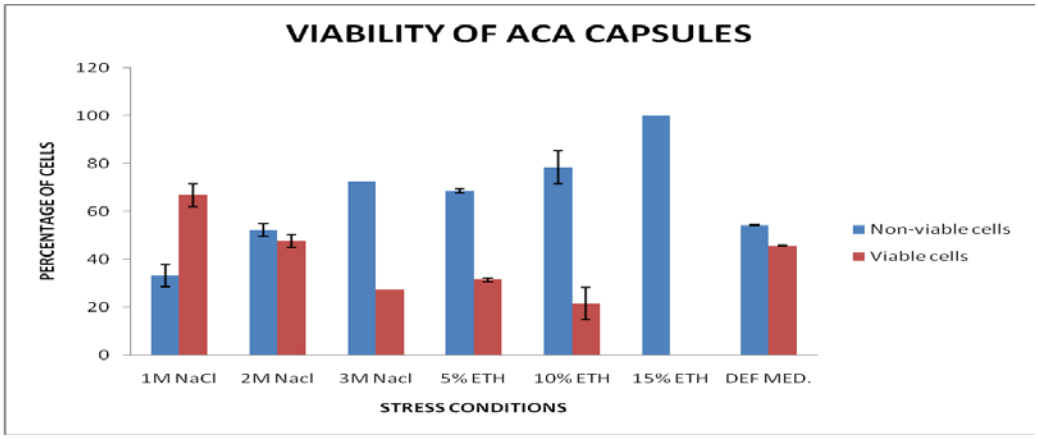

Figure 19-viability of ACA capsules under stress condition

Figure 19 depicts that the viabiltiy of the cells in the capsules at 5% and 10% ethanol concentrationwas 30% and 20%, this show that the percentage of viable cells in ethanol stress condition was very low. From above results it can be concluded that encapsulated yeast cells should preferably be cultivated below a ethanol concentration of 5% to achieve a good ethanol production.

Ethanol production corresponding to glucose consumption in ethanol stress medium.

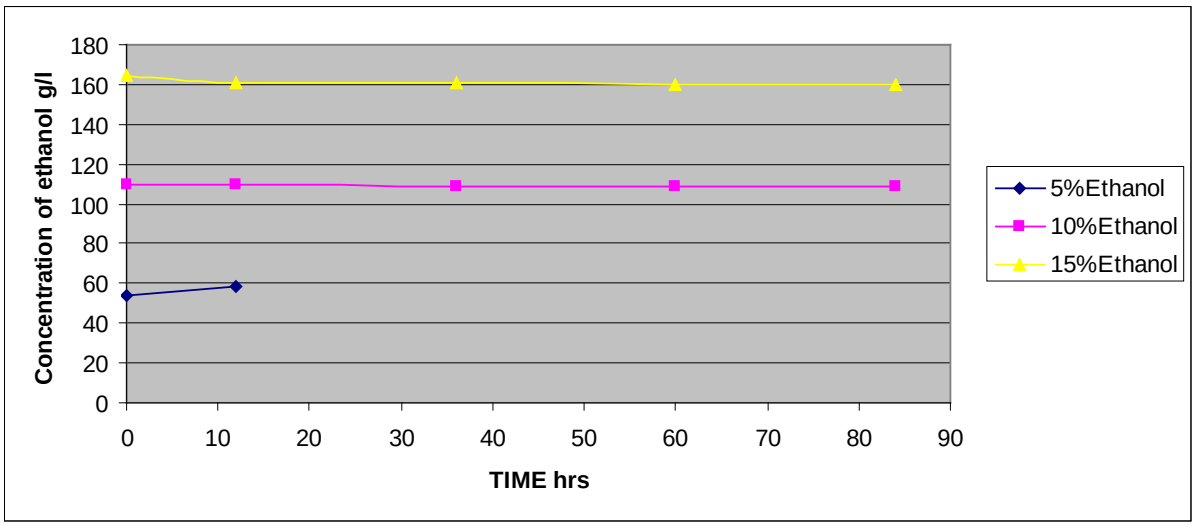

Figure20-ACA capsules at different ethanol stress-Ethanol concentration Vs Time

In defined medium free yeast cells produces 22 g/l ethanol in 24 hours, in 5% ethanol concentration it produces 24 g/l ethanol (55 g/l - 79 g/l) in 24 hours (figure 15) which is more than the defined medium. At higher ethanol concentration i.e 10% and 15% there is a decline in ethanol concentration as there is no glucose consumption. This is hypotheses to be a result of ethanol evaporation from the given medium.

Ethanol can stress the physiology of the yeast and can cause effects like growth inhibition, affect membrane fluidity, alteration of vacuolar morphology and endocytosis, change in

membrane lipid composition, induction of heat shock proteins (HSP) genes and inhibition of some enzymatic activities [28]. Ethanol dependent stress responses may be indirectly induced by the perception of damage or water availability rather than through a specific sensing system according to *Hallsworth, et al.* [28]. Many researches has concentrated on studying the changes in lipid composition and protection effects of some of the membrane components against ethanol stress. *Thomas et al.* [28] showed that the enrichment of the plasma membrane with double unsaturated C18 fatty acid linoleyl resulted in a higher resistance to ethanol than when the cell membrane was enriched with the mono-unsaturated C18 fatty acid oleyl. The author explains that enrichment with combinations of ergosterol, sterol naturally produced by *S. cerevisiae*, and specific fatty acids had the same beneficial effects. Ethanol concentration lowered the rate of solute accumaltion of glucose and amino acids like lysine and arginine [28].

Studies revel that specific lipids does not support stress tolerance, the ratio of different lipid components helps in tolerance. According to F.F.Bauer et al. [28], the molecular response to ethanol stress induces a set of Hsps which are also activated as a response to heat shock. HSP30 and HSP12 genes, HSP104 contribute to heat shock as well as to ethnaol tolerance. High ethanol concentrations lead to problems in protein denaturation, it needs the induction of anti-oxidant enzymes, because high ethnaol cause to the accumulation of some reactive oxygen speacies [28].

Glycerol production

This experiment were designed to observe glycerol production because when yeast cells are encountered with osmotic stress, cell dehydration occurs, under these conditions yeast cells adapt to the environment by accumulation of solutes such as glycerol. Glycerol production by yeast cells plays a major role at osmotic conditions; it balances solute concentration in the yeast cells during stress conditions and acts as a protectant therefore the glycerol levels were studies during the cultivations in this project. Figure 21 show glycerol concentration during cultivations with encapsulated yeast cells in AC capsules at osmoticconditions. When 1 M NaCl was present 4.1 g/l glycerol had been produced after 10^{th} hour, in medium with 2 M NaCl the glycerol concentration was 0.3 g/l after 100^{th} hour, almost no glycerol was produced at the highest NaCl concentration. Figure 22 show results from cultivations with AC encapsulated yeast cells the glycerol production at an ethanol concentration of 5% was 2.3 g/l after 100^{th} hour, at an ethanol concentration of 10% 0.2 g/l glycerol had been produced after 100^{th} hour. Figure 23 show results from cultivations with ACA encapsulated yeast cells here the glycerol production at 2 M NaCl concentration was 2.2 g/l after 100^{th} hour. These data clearly show that glycerol production plays major role in the survival of yeast cell in stress condition.

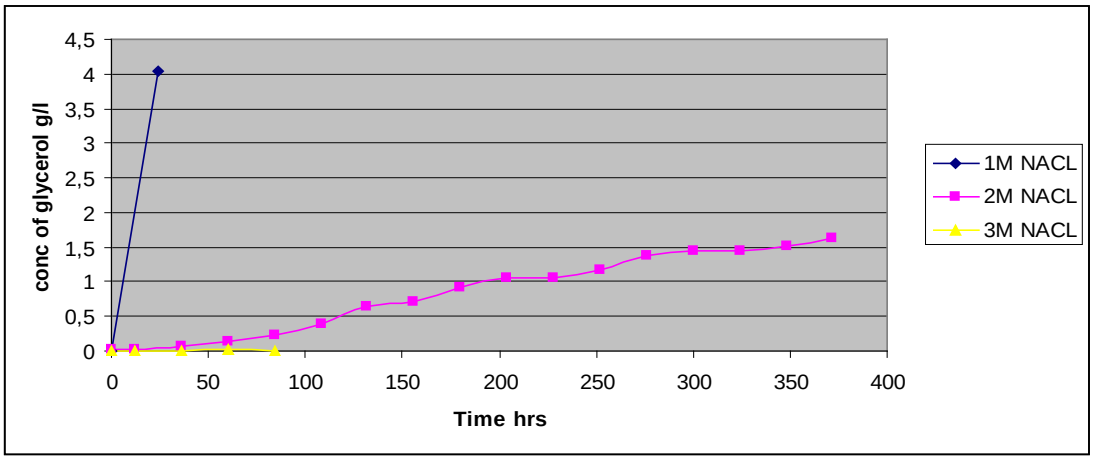

Figure 21-AC capsules – glycerol production in osmolarity stress

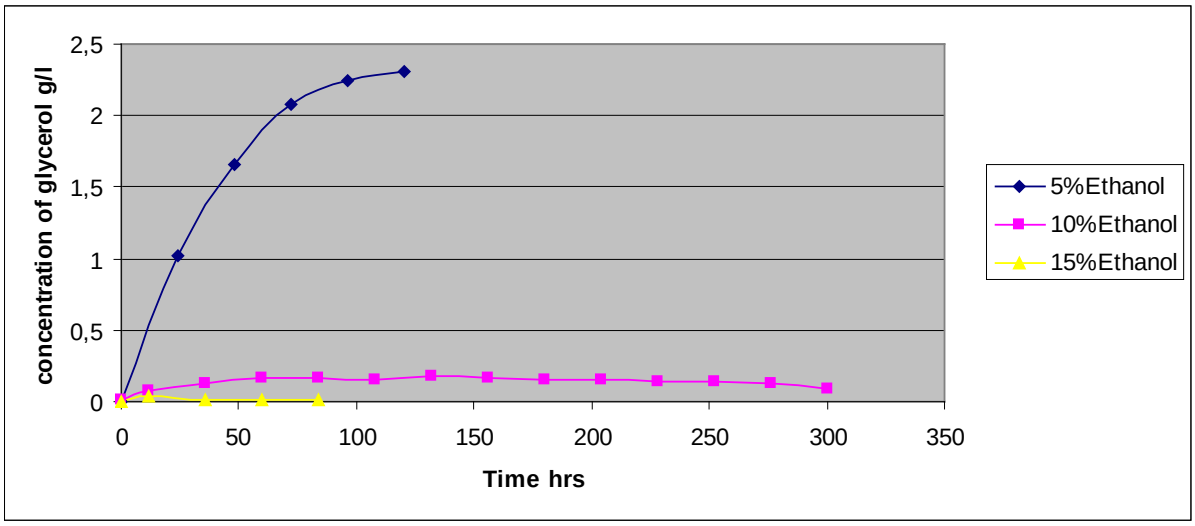

Figure 22-AC capsules-glycerol production in ethanol stress

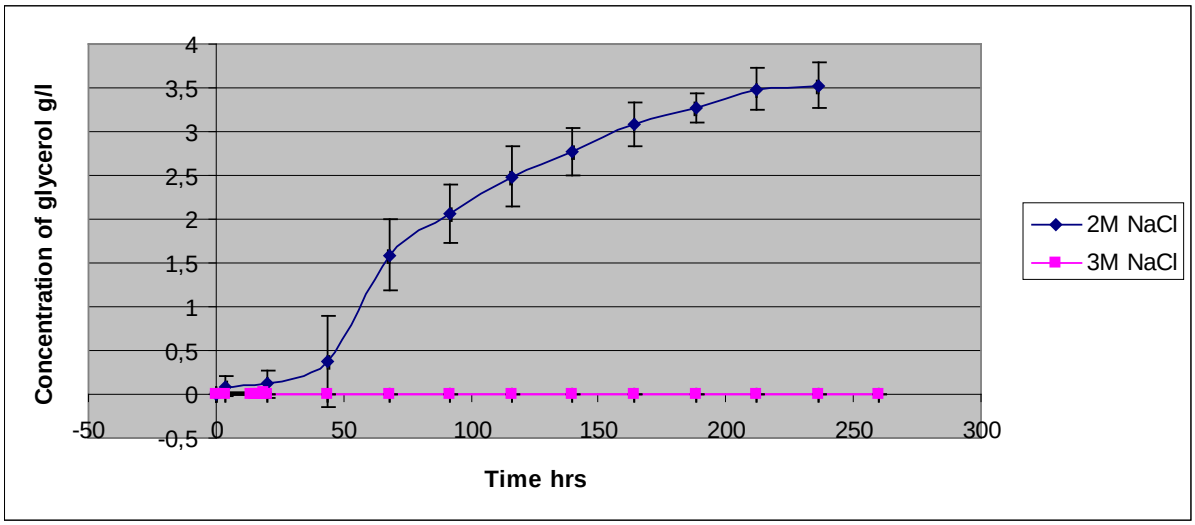

Figure 23- ACA capsules- glycerol production in osmolarity stress

SUMMARY OF THE RESULTS

Stresses	**Freely suspended yeast cells**	**Alginate chitosan capsules**	**Alginate chitosan alginate capsules**
Control **defined medium glucose consumption and ethanol production**	Glucose consumption is 55 g/l in 24 hours and 22 g/l of ethanol is produced		Glucose consumption is 25 g/l at 10^{th} hour
Osmotic stress at 1 M NaCl glucose consumption and ethanol production	Glucose consumption is 33 g/l at 100 hours, and 12 g/l of ethanol is produced		Glucose consumption is 2 g/l around 30^{th} hour and 12.9 g/l ethanol at around 30^{th} hour.
Osmotic stress at 2 M NaCl glucose consumption and ethanol production	nil	Glucose consumption is 22 g/l in 230 hours and ethanol production is 6.2 g/l at 230 hours	Glucose consumption is 2g/l at 100th hour and 1.8 g/l of ethanol produced.
Osmotic stress 3M NaCl , glucose consumption and ethanol production	nil	nil	Nil
Control Defined medium	Glucose consumption is 55 g/l at 24^{th} hour and 20 g/l ethanol produced .		Glucose consumption is 25 g/l glucose

Ethanol stress at 5%, glucose consumption and ethanol production	Glucose consumption is 55 g/l at 30th hour and 30 g/l ethanol is produced at 30th hour		Glucose consumption is 10 g/l at 10th hour and 5 g/l of ethanol 10th hour
Ethanol stress at 10%, glucose consumption and ethanol production	Nil		Nil
Ethanol stress at 15%, glucose consumption and ethanol production	Nil		Nil

DISCUSSION

Three different types of capsules i.e. AC, ACA, APTES and freely suspended yeast were prepared in the experiment and the results regarding how well the yeast cells consume glucose and ethanol production in the stress condition with the barrier of capsules was compared. There was difficulty initially to prepare capsules in the right way; many experiments failed because of no proper capsules were formed. It was very important to maintain absolute correct speed of dripping $CaCl_2$/CMC/yeast cells containing liquid into the sodium alginate solution; also the height which the droplets fall into the sodium alginate solution and even stirring speed of magnet in sodium alginate solution affected the capsule formation. Chances of leaking yeast cells in medium are more so need to be careful to avoid such problems. If any air bubble was trapped inside the capsule, the capsule will burst and cells will leak into the medium. Another problem was that the yeast cells releases carbon dioxide gas during fermentation, if it is produced too fast it accumulates inside the capsule and can break the capsule. To avoid leakage of yeast cells into the medium several different ways were tested but none of these helped in overcoming the problem. For example the concentration of tween 20 was increased and tween 80 was added instead of tween 20 but all these tests were unsuccessful.

Free cells cultivated in medium at higher stress conditions such as 2 M, 3 M NaCl and 10%, 15% ethanol resulted in no fermentation, this shows that freely suspended yeast cells are unable to survive at higher stress conditions but by using encapsulated yeast cells fermentation occurred in medium containing 2 M NaCl and 10% ethanol. From the results AC capsules containing yeast cells are well tolerated in 2 M NaCl and 10% ethanol stress rather than ACA capsules. It is possible that AC capsules can be used in further experiments to overcome the higher stress conditions by the yeast cells. The most important observation was

that higher stress condition 3 M sodium chloride and 15% ethanol concentration no glucose consumption occurred with free yeast cells. However, in AC encapsulation yeast cells lag phase was observed but after long hours the viability was better in the AC capsules than in ACA capsules and free yeast cells. There was no glucose consumption by any of these capsules in 15% ethanol stress condition. The encapsulation was not helpful at such high ethanol concentrations.

In the medium with 10%, 15% ethanol concentration after several hours of cultivation there was a decrease in the amount of ethanol in the medium occurred, this is probably due to ethanol evaporation because of its property, and ethanol is highly volatile in higher concentration. To prove this further experiment are needed.

CONCLUSION:

In this study different stress conditions was investigated during fermentation using ethanol and sodium chloride at different concentrations such as 5%, 10%, and 15% ethanol, 1M, 2M and 3M NaCl. The ethanol yield, consumption of glucose and production of glycerol was studied in both free cells (without any capsulation directly suspended the culture in the media) and encapsulated cells using alginate and chitosan as capsule matrix. Additionally alginate chitosan alginate capsules were used to find out if different membrane capsule had a influence on the stress tolerate stress of the yeast cells. It was shown that freely suspended yeast cells cannot tolerate higher stress conditions like 2M, 3M NaCl or 10%, 15% ethanol concentration. When alginate chitosan (AC) capsules and alginate chitosan alginate (ACA) capsules where compared a difference in stress tolerance of the yeast cells was observed. AC capsule encapsulated yeast tolerated osmotic stress better than ACA capsules in 2M of NaCl where as freely suspended yeast cells unable to tolerate 2M of NaCl . At 100^{th} hour in AC capsules glucose consumption was 12 g/l where as in ACA capsules glucose consumption at same 100^{th} hour was 2 g/l. At 10% ethanol concentration yeast inside ACA capsules showed 5 g/l of glucose consumption but in freely suspended yeast cells there is no glucose consumption as they cannot tolerate higher stress levels. Finally encapsulation of yeast cells tolerate mild stress levels which is significant towards further experiments. Future research should focus on elaborating if encapsulation can aid in improving the stress tolerance towards low pH and high temperatures of yeast cells and planned to even observe higher levels of pH, glucose and temperature stress levels in encapsulation yeast cells.

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