Robust Encapsulation of Yeast for Bioethanol Production

By

Sainath Namthabad

Ramesh Chinta

This thesis comprises 30 ECTS credits and is a compulsory part in the Master of Science with a Major in Resource Recovery - Industrial Biotechnology, 120 ECTS credits

No. 6/2012
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Sainath Namthabad (S104427@student.hb.se)
Ramesh Chinta (S104438@student.hb.se)

Master thesis
Subject Category: Industrial Biotechnology

University of Borås
School of Engineering
SE-501 90 BORÅS
Telephone +46 33 435 4640

Examiner: Mohammad Taherzadeh
Supervisor: Päivi Ylitervo
Address: University of Borås, School of Engineering
501 90 BORÅS
Abstract:

In the future the demand for ethanol is expected to increase greatly due to the rising energy requirements in the world. Lignocellulosic materials are a suitable and potentially cheap feedstock for sustainable production of fuel ethanol, since vast quantities of agricultural and forest residues are available in many countries. However, there are several problems involved in the utilization of lignocellulosic raw materials as sugar source. The most common way of releasing the simple sugars in the material is by dilute acid hydrolysis. This procedure is relatively simple and cheap, but in addition to the sugars it creates inhibitory compounds. These inhibitors make it very hard for the yeast to ferment the hydrolyzate and detoxification is often necessary. One way to overcome this problem is to encapsulate the yeast. Encapsulation is an attractive method since it improves the cells stability and inhibitor tolerance, increases the biomass amount inside the reactor, and decreases the cost of cell recovery, recycling and downstream processing. However, the method does not yet permit long-term cultivation since the capsules used so far are not robust enough. Therefore more studies have to be conducted in order to find methods which produce mechanically robust capsules. The main goal of this paper is to find a suitable method to produce robust capsules using different concentration of the chemicals at different pH and also implementing some modifications such as addition of cross-linkers in preparation procedure. In this paper comparison of three different encapsulation techniques were studied based on the mechanical robustness of the capsules. The three different techniques were calcium mineralized alginate-chitosan capsules, alginate capsules coated with 2% chitosan (2% AC) and genipin crosslinked alginate-chitosan (GCAC) capsules. The results indicate that GCAC capsules are most robust and were good enough for prolonged use since most of the capsules were not deformed in mechanical strength test. There were slight differences in the diameter and membrane thickness before and after swelling. No negative influence was observed on the yeast growth when applying the cross-linker. The results of this study will hopefully add valuable information and helps in further studies using other cross-linkers to prepare robust capsules.

Keywords: Microencapsulation, Cell immobilization, Entrapment, Biomineralization, Genipin, Bioethanol, Lignocellulosic hydrolyzates
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<tr>
<td>AC</td>
<td>Alginate-chitosan</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethylcellulose</td>
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<tr>
<td>DDGS</td>
<td>Dried Distiller’s Grains with soluble</td>
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<tr>
<td>DEAE</td>
<td>Diethylaminoethanol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GCAC</td>
<td>Genipin crosslinked alginate-chitosan</td>
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<tr>
<td>HMF</td>
<td>Hydroxymethylfurfural</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LHW</td>
<td>Liquid hot water</td>
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<tr>
<td>MSW</td>
<td>Municipal solid wastes</td>
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<tr>
<td>MTBE</td>
<td>Methyl tertiary butyl ether</td>
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<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
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<tr>
<td>SSF</td>
<td>Saccharification and fermentation</td>
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1. Introduction

One of the current problems of the world is the random and progressive depletion of the energy resources. The non-renewable fuels (fossil fuels) such as oil and natural gas have been the fundamental sources of energy during the 20th and early 21st century (Abril and Abril, 2009). The non-renewable fuels consumption is increasing at a faster rate day by day. The speculation in the stock exchanges and the permanent oil crisis in the Middle East countries, the largest producers of oil are the main reasons for the elevated prices of oil. This would result in the stagnation of the world economy if the oil maintains at high prices. In addition, the intensive usage of the fossil fuels has led to increase in the production of a wide variety of polluting gases (greenhouse gases) released into the atmosphere which eventually caused severe changes in the global climate (Sánchez and Cardona, 2008).

One of the solution to these problems is the development and utilization of technologies based on the alternative sources of energy such as renewable energy resources which helps the mankind in finding a solution for their energy requirements in an eco-friendly way (Sánchez and Cardona, 2008).

Among different alternative solutions, utilization of renewable sources such as solar energy converted by photosynthesis in the plants in the form of vegetal biomass (bioenergy) as the large part of the bioenergy is represented in energy crops and the lignocellulosic residues (Abril and Abril, 2009). The conversion of these feedstocks into biofuels is an important and good choice for alternative energy sources. The use of these alternative energy sources also helps in the reduction of polluting gases production and also reduces the costs for energy consumption. In addition, the use of biofuels also has some important social and economic effects. The agriculture sector also boosts with the development of energy crops used for the production of biofuels as it would leads to new jobs and money (Sánchez and Cardona, 2008).

1.1. Ethanol

Ethanol (ethyl alcohol, bioethanol) is the most widely employed liquid biofuels either as a gasoline enhancer or as a fuel. It has a lot of advantages when it is used as an oxygenate. One of them is the higher oxygen content of the ethanol which reduces the higher usage of the required additive. The increased percentage of oxygen helps in high oxidation of the hydrocarbons present in the gasoline and simultaneously reduction in the emission of carbon
monoxide and aromatic compounds. Ethanol can be an alternative additive to conventional additive methyl tertiary butyl ether (MTBE) which is highly toxic and known to contaminate water. Ethanol has higher octane booster properties and it is also non toxic (Thomas and Kwong, 2001, Bishnu et al., 2011).

In many countries using ethanol as fuel by addition to gasoline has been implemented and it has increased remarkably as many countries are trying to reduce oil imports and lower the production of polluting gases in order to improve the air quality. The ethyl alcohol production globally has reached about 110000 million liters (28946.39 million gallons) (Lichts, 2011), the largest producers being the USA and Brazil. On an average 73% of the ethanol produced globally is used as fuel ethanol, 10% as beverages and the rest 10% is used as industrial alcohol (Sánchez and Cardona, 2008)

The fuel ethanol can be produced from a variety of energy crops and lignocellulosic biomass and the complexity of production depends on the feedstock selected as the raw material. Many different technologies have been described for producing fuel ethanol from variety of sucrose containing feed stocks mainly sugar cane, starchy materials and lignocellulosic materials. The major complexity of the production is the biomass processing and a considerable wide research is going on the conversion of lignocellulosic biomass into fermentable sugars which involves many different conversion steps (Cardona and Sánchez, 2007).

The processing of biomass ranges from simple conversion of sugars by fermentation to the multi-stage conversion of the lignocellulosic biomass into ethanol. A wide diversity of technological alternatives requires thorough and proper analysis of the processes along with the development and design of the involved process operations. Process integration plays a key role for reducing the costs in the ethanol production industries and increasing bioethanol competition compared to gasoline. A large number of reviews have been published on the fuel ethanol production from many feed stocks and especially from lignocellulosic biomass (Lynd, 1996, Lee, 1997, Lin and Tanaka, 2006)

In many countries the main feedstock for ethanol production is sugarcane in the form of cane juice or its by-product molasses. In Brazil about 79% of the ethanol is produced from sugar
cane juice and the rest is produced from cane molasses (Wilkie et al., 2000). In India, the major raw material is molasses (Ghosh and Ghose, 2003).

1.2. Microorganism

The most widely employed organism for ethanol production is *Saccharomyces cerevisiae* (Baker’s yeast) apart from other microorganisms (Table 1). The organism has a capability of hydrolyzing sucrose present in the cane juice into two easily assimilable hexoses, glucose and fructose. Aeration plays a crucial role for growth and the ethanol production by *S. cerevisiae*. Though the organism *S. cerevisiae* as the ability to grow under anaerobic conditions, it requires small amounts of oxygen in order to synthesize fatty acids and sterols. In order to cover the need of the oxygen by the organism it can be supplied in the medium by addition of some chemicals such as urea, hydrogen peroxide (Narendranath et al., 2000).

Other yeast such as *Schizosaccharomyces pombe* have advantages like tolerance for high solids content and high amounts of salts (high osmotic pressures) (Bullock, 2002). The most promising bacterial microorganism employed in the production of ethanol microorganism is *Zymomonas mobilis*. It has low energy efficiency with high ethanol yield (97% theoretical). But the range of fermentable sugars by the organism is too narrow as it can ferment only glucose, fructose and sucrose (Claassen et al., 1999). The other main disadvantage of using this bacterium for production of ethanol is that it increases the viscosity of fermentation broth and this is due to the formation of levan, a polysaccharide made up of fructose units. Sorbitol is also formed due to reduction of fructose which eventually decreases the efficiency of converting sucrose into ethanol (Lee and Huang, 2000).

| *Bacillus stearothermophilus* |
| *Candida shehatae* |
| *Chalara parvispora* |
| *Clostridium thermohydrosulfuricum* |
| *Clostridium thermocellum* |
| *Mucor indicus* |
| *Pachysolen tannophilus* |
| *Pichia stipitis* |
| *Thermoanaerobacter ethanolicus* |
| *Thermoanaerobacter thermosaccharolyticum* |

Table 1: Microorganisms employed in Ethanol Production (Sánchez and Cardona, 2008)
The major problem for the ethanol fermentation is the high osmolarity of the media which is primarily based on the cane molasses as raw material. Its high osmolarity is due to high concentrations of sugars and salts present in the fermentation medium (Morimura et al., 1997).

A wide variety of studies have been carried out to obtain *S. cerevisiae* strains that are tolerant to high concentrations of salt and high temperatures. Protoplast fusion and flocculation techniques has been used for obtaining strains of *S. cerevisiae* capable of growing at 35 °C and molasses concentration of 22 % (w/v). By maintaining these conditions and performing repeated batch culture at laboratory scale, high ethanol concentrations of 91 g/L and productivities of 2.7 g/(Lh) have been observed (Morimura et al., 1997).

By conditioning or modifying of the molasses by adding different types of compounds can neutralize the inhibitory effects of the medium. This also improves the production of ethanol. The molasses should also be supplemented with the nutritional factors that promote the yeast growth. The conditioning of beet molasses by agents such as EDTA (ethylenediaminetetraacetic acid), ferrocyanide and zeolites has been demonstrated (Ergun et al., 1997). It has been observed that zeolite acts as pH regulator in the fermentations with higher concentrations of glucose (Castellar et al., 1998). Addition of enzymes such as amylases, cellulases and amylpectinases will convert non fermentable sugars into assimilable compounds leading to improved ethanol fermentation (Acevedo et al., 2003). In order to stop bacterial growth and increase the ethanol yields addition of minimum inhibitory concentrations of hop acids such as alpha acid, beta acids, isoalpha acids, rho-isoalpha acids, tetrahydro-isoalpha acids and hexahydro-isoalpha acid to molasses, avoids the need for antibiotics (Maye, 2006).

**1.3. Ethanol from different feedstocks**

**1.3.1. Ethanol from Starch**

Starch is one of the high yield feedstocks used for ethanol production by fermentation after its hydrolysis. The hydrolysis was traditionally done by acids but it has been replaced by specific enzymes, due to absence of secondary reactions. Generally amylases are used as the catalysts for the reaction. Alpha-amylase obtained from *Bacillus licheniformis* or other engineered strains of *E. coli* and *Bacillus subtilis* have been used in the first step of starch
hydrolysis usually at high temperatures (90 °C - 110 °C) (Apar and Özbek, 2004). The product formed in the first step is called as liquefaction which contains dextrines and small amounts of glucose. This liquefaction is subjected to saccharification at lower temperatures (60 °C - 70 °C) by glucoamylase obtained generally from *Aspergillus niger* or *Rhizopus* species (Pandey et al., 2000, Shigechi et al., 2004).

### 1.3.2. Ethanol from Corn

Corn is widely used in the production of ethanol in the USA. It is milled in order to extract starch. The obtained starch is treated enzymatically to obtain glucose syrup which is then fermented to ethanol. Usually there are two types of milling of the corn, wet and dry milling. In the wet milling process corn grain is fractionated into its components which are sold as by-products and the starch is converted into ethanol. During dry milling the grains are not fractionated and all the components enter into the process and accumulate into a concentrated distillation co-product called as Dried Distiller’s Grains with soluble (DDGS). The remaining steps such as liquefaction, saccharification and fermentation steps are the same in both methods. Usually fermentation is performed using the *S. cerevisiae* at 30-32°C with the addition of ammonium sulphate or urea as nitrogen sources. In order to provide an additional source to the yeast proteases can be added (Bothast and Schlicher, 2005).

Many new methods have been demonstrated and implemented which involves high degrees of integration of simultaneous saccharification and fermentation (SSF) mostly in dry milling processes (Cardona and Sánchez, 2007). The cascade process is also employed in the wet milling, which involves separate saccharification and fermentation wherein the hydrolysis of dextrines by glucoamylases, yeast propagation, pre-fermentation and fermentation are carried out with the help of cascade system. The rise in the production capacity of ethanol in the USA is mainly represented by dry mill ethanol production plants (Cardona and Sánchez, 2007).

### 1.3.3. Other starchy materials

Besides corn, ethanol can also be produced from wheat (Soni et al., 2003), rye, barley, triticale (Wang et al., 1997), and sorghum(Zhan et al., 2003). Some kinds of pretreatments have proven to be useful. The starch content of the feedstock can be increased by pearling of wheat, rye and triticale grains (Wang et al., 1997). In addition, a technology called very high
gravity (VHG) technology has been tested with promising results for oats, barley, rye and triticale. In Malaysia ethanol has been produced from Sago palm (Abd-Aziz, 2002). The ethanol production from bananas and banana wastes using commercial a-amylase and glucoamylase has also been studied (Hammond et al., 1996).

Apart from the above mentioned crops one of the best crops for producing fuel ethanol is the sweet sorghum which produces grains. The grains of the sweet sorghum contains high content of starch, stem stalks containing high sucrose content and leaves and bagasse with high content of lignocellulose. Moreover, sorghum crop can be cultivated in both temperate and tropical countries as it requires less water i.e.; 1/3 of the water needed for cane cropping and half. It is also tolerant to flooding, drought and salinity (Cardona and Sánchez, 2007).

1.4. Ethanol from lignocellulosic biomass

Lignocellulosic biomass is an attractive feedstock for ethanol production as it is the most abundant biopolymer on the earth. Lignocellulosic biomass comprises about 50% of the world’s biomass and it is estimated that about 10-50 billion ton is produced annually (Claassen et al., 1999). A lot of lignocellulosic materials have been tested for production of bioethanol. Generally the prospective lignocellulosic materials used for fuel ethanol production can be divided into six main groups in the following way:

a) Crop residues (wheat straw, rice straw, rice hulls, barley straw, cane and sorghum bagasse, olive stones and pulp)
b) Hardwood (Aspen, poplar)
c) Softwood (pine, spruce)
d) Cellulosic wastes( waste paper, newsprint and recycled paper sludge)
e) Herbaceous biomass (alfalfa hay, switch grass, reed canary grass, coastal Bermuda grass, timothy grass) and
f) Municipal solid wastes (MSW).

The composition of most of the above mentioned lignocellulosic materials mainly consists of three components namely cellulose, hemicelluloses and lignin (Sun and Cheng, 2002).
A considerable number of studies and research have been carried out across the world for developing large scale production of fuel ethanol from lignocellulosic biomass (Bishnu et al., 2011, Abril and Abril, 2009). The major limiting factor of the lignocellulosic feedstock lies in its processing which is related to the nature and composition of the lignocellulosic biomass. The ethanol is produced from lignocellulosic biomass after the polymers present in the biomass is broken down into fermentable sugars. But this degradation process of the polymers is complicated, energy consuming and not completely developed (Abril and Abril, 2009).

1.4.1. Pretreatment of the lignocellulosic biomass

The pretreatment of feedstock is the major hurdle while processing lignocellulosic biomass. The lignocellulosic material consists of a matrix of two polymers, cellulose and lignin which are bound together by hemicellulose chains. In the pretreatment step, the matrix should be broken to increase the content of amorphous cellulose by reducing the crystallinity of the cellulose. Amorphous cellulose is most suitable for enzymatic degradation. The other reason of pretreatment is the hydrolysis of hemicellulose and release or degradation of lignin (Lynd, 1996). The pretreatment should also improve the formation of sugars during the subsequent enzymatic hydrolysis and avoid the formation of inhibitors which affect the subsequent fermentation process. Several physical, chemical, biological methods have been designed and proposed for the pretreatment of lignocellulosic biomass (Sun and Cheng, 2002).

The physical methods employed for pretreatment includes chipping, grinding, milling which helps in reducing the crystallinity of the cellulose, so that the cellulases can access the biomass and convert the cellulose. But these methods are not preferred because of the high energy and capital costs (Ghosh and Ghose, 2003).

A wide variety of physical-chemical methods has been developed and employed for pretreatment. Using physical-chemical methods is more effective than using the physical methods. The methods include e.g. steam explosion during which hemicelluloses and lignin are converted into soluble oligomers because of autohydrolysis reaction (Shahbazi et al., 2005, Söderström et al., 2003). One method is the thermohydrolysis or liquid hot water (LHW) method. This method is similar to dilute acid pretreatment and does not produce any
toxic wastes and inhibitors (Laser et al., 2002). Other methods include Ammonia fiber explosion method which is similar to the steam explosion method (Sun and Cheng, 2002).

In chemical pretreatment of lignocellulose biomass different chemical agents such as acids, alkalis, peroxide and organic solvents are employed. Acids such as $\text{H}_2\text{SO}_4$ and $\text{HCl}$ have been widely used. The dilute sulphuric acid hydrolysis has been successfully developed and found improvement in cellulose hydrolysis. But the cost of dilute acid pretreatment is higher than some other physical-chemical methods (Sun and Cheng, 2002).

Saha et al., used dilute acid pretreatment method and employed it in a two stage way. First, hemicellulose was depolymerized at 140 °C for 15 min in order to avoid the formation of carboxylic acid and furan compounds. The second step was carried out at 190 °C for 10 min in order to make cellulose more accessible to hydrolysis by enzymes. If the dilute acid pretreatment is performed at low temperatures the formation of furfural and hydroxymethylfurfural (HMF) can be avoided. The formation of furfural and hydroxymethylfurfural (HMF) results in lower sugar yields (Saha et al., 2005b, Saha et al., 2005a).

One of the major problems faced during the pretreatment and hydrolysis of the lignocellulose biomass is the variability in the lignin and hemicelluloses content. The variability depends on the type of plant biomass, crop age and harvesting method. Therefore none of the above mentioned pretreatment methods can be applied in a standard way for all kinds of feedstocks (Claassen et al., 1999).

1.4.2. Detoxification of lignocellulosic hydrolyzates

In addition to fermentable sugars, a wide variety of compounds that can seriously inhibit the fermentation process are formed during the pretreatment and hydrolysis steps of lignocellulosic biomass. The inhibitors are formed as a result of the hydrolysis of extractive components, organic (acetic acid) and sugar acids (glucoronic and galacturonic) that are esterified to hemicellulose and solubilized phenolic derivatives. Some inhibitors such as furfural and HMF are degradation products of soluble sugars. Other inhibitors such as cinnamaldehyde, p-hydroxybenzaldehyde, syringaldehyde are produced due to degradation of lignin and some metal ions are produced as a consequence of corrosion (Lynd, 1996,
Palmqvist and Hahn-Hägerdal, 2000b). Therefore, detoxification is usually necessary prior to the fermentation. These detoxification methods can be physical, chemical or biological. These various methods cannot be compared directly as they vary in the degree of neutralization of inhibitors (Palmqvist and Hahn-Hägerdal, 2000a). Moreover, the fermenting microorganisms may have different levels of tolerances to the inhibitors.

The ethanol fermentation is directly influenced by the presence of these inhibitors in the feed streams. In continuous or fed-batch fermentation processes a low feed rate gives low concentrations of the inhibitors in the broth. In the continuous culture, the growth rate is diminished by the presence of inhibitors and the productivity of the process is linked to the dilution rate directly (Taherzadeh, 1999, Nilsson et al., 2001).

A large number of studies have been carried out on *S. cerevisiae* and xylose-fermenting yeast to assess the effect of toxic substances on the growth and ethanol production. The effect of inhibitors such as acetic acid, furfural and p-hydroxybenzoic acid on the growth and ethanol productivity of *S. cerevisiae* and *C. shehatae* has been studied immensely (Palmqvist et al., 1999). Genetically modified *E. coli* capable of assimilating xylose and recombinant microorganisms able to ferment lignocellulosic hydrolyzates have been investigated (Zaldivar et al., 1999, Zaldivar et al., 2000).

### 1.4.3. Immobilization of yeast cells

One way to overcome the effect of inhibitors present in the lignocellulosic hydrolyzates on fermentation is cell immobilization. Immobilization has been reported to improve the yeast cells stress tolerance (Talebnia et al., 2005, Pourbafrani et al., 2007). When the cells are immobilized they are confined close to each other. The cellular metabolism and the growth pattern of the cells are affected when they grow in limited spaces (Van Iersel et al., 2000). Moreover, immobilized cells are protected from harsh environmental conditions such as temperature, pH, organic solvents and toxic compounds. The handling of immobilized cells is very easy as they can be recovered from the solution by sedimentation (Park and Chang, 2000, Talebnia and Taherzadeh, 2006, Talebnia and Taherzadeh, 2007). The continuous process can be operated with high cell density without any loss of the cells at high dilution rates. This also plays an important role in increasing the productivity of the bioreactor (Park and Chang, 2000).
Cell immobilization confers many advantages such as increase in uptake of substrate, easy recycling of the cells and their reuse, improved product yields, easier product recovery and lower risk of contamination by inhibitors and microbes (Kourkoutas et al., 2004).

2. Cell immobilization

Cell immobilization is defined as the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity. It usually mimics the conditions that occur when cells grow on any surface or within any natural structures (Karel et al., 1985). The concept of cell immobilization was developed from the concept of enzyme immobilization for the purpose of simplifying the complicated bioreactions catalyzed by a number of intracellular enzymes (Bai et al., 2008). The interest in development of cell immobilization methods has been increasing recently as it offers numerous advantages such as high cell stability, increased fermentation productivity, feasibility of continuous processing, low recovery and recycling costs and easy downstream processing. Cell immobilization also protects the cells from harsh environmental conditions such as pH, temperature, organic solvents and other toxic compounds (Kourkoutas et al., 2004). Cell immobilization is considered as one of the ways to increase the bioreactor productivity in fermentation processes for e.g. ethanol production (Park and Chang, 2000).

2.1. Cell immobilization techniques

A large number of fermentation and biotechnological processes have been implementing immobilization techniques and therefore several techniques and support materials have been proposed. Based on the physical mechanism employed these techniques can be broadly divided into four categories as shown in Fig 1:

a) Attachment or adsorption on solid carrier
b) Entrapment within porous matrix
c) Self aggregation by flocculation (natural) or by cross linking agents (artificially induced)
d) Cell containment behind barriers.

All the above methods have a similar purpose i.e.; to retain high cell concentration within the bioreactor which leads to increased volumetric productivity and lower the fermentation costs (Pilkington et al., 1998)
2.1.1. Attachment or Adsorption on solid carrier

This method involves attachment of the cells to solid material which acts like a support. Usually adsorption is reversible, and the cells are bound to the support by interactions such as electrostatic, ionic and hydrogen bonds. There are two types of whole cell adsorptive carriers: a) adsorptive carriers with small pores which allow adsorption only onto the external surfaces...
b) adsorptive carriers with large pores which allow adsorption onto internal surfaces. A wide variety of carriers such as DEAE (diethylaminoethanol) cellulose, porous glass, silicon carbide, volcanic rock, sponge, diatomaceous earth, wood blocks and gluten pellets have been used for immobilization of yeast cells (Pilkington et al., 1998).

Usually the thickness of the bio-film formed on the surface of the carriers may range from monolayer to layers of cells. The strength of attachment of cells mainly depends on cell and carrier type. The support materials used in this method can be regenerated. Usually these adsorptive matrices do not have a diffusion barrier between the cells and the surrounding medium and this may leads to significant cell leakage. This kind of cell leakage is not so desirable for the processes involving cell free effluents. One of the problem with adsorption is cell desorption which may occur because of changing factors such as environmental ionic strength, temperature, pH and other physical stresses. Compared to the gel entrapment method, the biomass loading is low and mass transfer is limited in the adsorption attachment method (Pilkington et al., 1998).

### 2.1.2. Entrapment within porous matrix

This method involves entrapment of cells into a porous polysaccharide gels such as calcium alginate, k-carrageenan, agar, chitosan and other polymer matrices like gelatin, collagen and polyvinyl alcohol (Park and Chang, 2000, Kourkoutas et al., 2004). Usually in this method the porous material is formed inside the culture of cells or the cells are allowed to penetrate in the matrices until their mobility is being obstructed by presence of the other cells. This method leads to inclusion of cells in a rigid network and prevents the diffusion of cells into the surrounding environment (Kourkoutas et al., 2004). The polymeric beads formed are usually spherical with diameters ranging from 0.3 to 3.0 mm. The immobilization of yeast cells using entrapment is a relatively simple method and high biomass concentration can be achieved (Pilkington et al., 1998).

The entrapped cells can grow since nutrients diffuse in through the matrix and in the same way metabolites can be transferred into the surrounding medium. The diffusion limitations are imposed by the porosity of the matrices and by the impact of accumulated biomass. In this method the cells near the surface behave differently when compared to the partially
starved cells present inside the beads and this leads to a development of non-homogenous cell population (Freeman and Lilly, 1998). One of the major problems with the entrapment method is that cells present on the outer surface of the beads multiply and gets released from the matrix of resulting in the presence of both free and immobilized cells (Kourkoutas et al., 2004). In order to avoid this problem double layer beads have been developed which have an internal core containing the cells and an external layer which prevents the cells from escaping from the inner core (Tanaka et al., 1989, Ramon-Portugal et al., 2003).

The concentration gradients of the metabolites within the polymeric beads occur due to the diffusion limitations caused by the gel matrix and the high biomass loading. However, it can be overcome by understanding the mass transfer phenomena within the entrapment matrices and providing different conditions at the gel surface and in the bead centre. Loss of mechanical integrity of the gel usually occurs by dissolution or by compression, abrasion or accumulation of gas internally. In order to improve the mechanical strength of the gel, alginate gel beads have treated with stabilizing agents such as sodium meta-periodate and glutaraldehyde (Pilkington et al., 1998).

### 2.1.3. Aggregation by flocculation

Cell flocculation is defined as “the formation of an open agglomeration that relies upon molecules acting as bridges between separate particles” (Stewart and Russell, 1986). It is also defined as “the property of the cells in suspensions to adhere in clumps and sediment rapidly” (Jin and Alex Speers, 1998). It can be treated as an immobilization technique and can make potential use of it in reactors like packed-bed, fluidized bed and continuous stirred tank reactors. The yeast cells naturally have the property of flocculation and this can be exploited in beer brewing and other fermentation processes. In order to enhance flocculation in cell cultures that do not naturally posses the property artificial flocculating agents and cross linkers can be used (Kourkoutas et al., 2004).

In order to maximize the bioreactor efficiency flocculation should be controlled. Many factors such as genetic structure of the strain, cell wall structure, surface charge, growth phase, pH of medium, cation composition of the medium, incubation temperature influence the natural flocculation characteristics of yeast and are of much importance in fermentation productivity, its recovery and removal in the fermentation process. Slow sedimentation rates
occur due to weak flocculation activity and results in wash out of the cells from the bioreactor leading to lower fermentation rates (Pilkington et al., 1998).

2.1.4. Cell containment behind a barrier
In this method cells are usually retained by a membrane or entrapped in a microcapsule or may be attached to the surface. Immobilization can also be done by forming a barrier by a liquid-liquid interface between two immiscible fluids. This method seems to be ideal when cell free product and minimum transfer of molecules is necessary. The mass transfer limitations and membrane fouling caused by the cell growth are the major disadvantages of this method (Kourkoutas et al., 2004).

Among all the above immobilization techniques, calcium-alginate gel encapsulation has been widely used as the gelling process is very easily performed. Moreover the process is done at mild conditions and the alginate used is inert and non-toxic to the cells. But as discussed earlier growing cells entrapped in the gel bead leads to some problems such as cells present in the surface of bead proliferate resulting in increased mass transfer resistance and leak by breaking the surfaces. Moreover, the pore size in the gel matrix is in the order of about 10 nm reducing the space for the trapped cells to proliferate resulting in low densities of the cells in the trapped gel beads. In order to overcome this problem other immobilization techniques has been developed such as encapsulation (Koyama and Seki, 2004).

2.1.4.1. Microencapsulation
Encapsulation of cells is very attractive and has several advantages over the conventional entrapment method. In encapsulation the cells are enclosed within a liquid-core capsule surrounded by a semi-permeable membrane formed by e.g. alginate polymer network. The liquid core of the capsule provides larger space for cellular growth resulting in higher density of cells, reduces mass transfer resistance compared to entrapment in porous beads (Cheong Soo et al., 1993, Koyama and Seki, 2004, Park and Chang, 2000). The semi-permeable membrane formed by the alginate polymer network plays a crucial role in the diffusion of substrate and product. The membrane of the capsule is permeable to nutrients, substrate, product and some enzymes but cells and large proteins cannot pass through the membrane (Bickerstaff, 1996).
The membrane of the capsule also protects the cells from toxic compounds present in the medium and provides a comfortable environment to the cells inside. Moreover, the capsules are mechanically robust enough for limited use (Park and Chang, 2000). Cell stability and inhibitor tolerance in lignocellulosic hydrolyzates can also be improved by encapsulation (Talebnia et al., 2005, Talebnia and Taherzadeh, 2006). Much higher cell concentrations can be achieved inside the capsules than in the gel core beads (Park and Chang, 2000). Other benefits of encapsulation such as high invertase activity of recombinant S. cervisiae compared to free cells (Chang et al., 1996), long-term stability of the whole cell enzymes (β-galactosidase in E. coli) (Oh and Park, 1998) and increased ethanol productivity by encapsulated S. cervisiae (Mei and Yao, 2002) has been reported.

The encapsulation of the cells is mainly done by the following techniques

a. Coacervation

In this technique, a liquid core droplet called coacervate is formed when some parameters such as pH, temperature or composition of the liquid phase are adjusted. The coacervate is formed when a liquid phase of pre-membrane component get separated from the polymeric solution and wrap around the liquid core as a uniform layer. The pre-membrane component has to be solidified by means of cross-linking or solvent removal methods. Usually when the core material is water soluble the pre-membrane component is hydrophobic and it is hydrophilic when they are insoluble. This technique is efficient but it is expensive (Park and Chang, 2000).

b. Emulsion/Interfacial polymerization

This technique involves dissolving monomers in the immiscible phases. The water soluble monomer is dispersed in the organic phase by stirring in the form of aqueous phase. The formation of capsule membrane results from the addition of other organic solvent-soluble monomer to the continuous organic phase. For example polymer membrane such as polyamide, nylon, polyterephthaloylchloride, polyester, polyphenylester is produced by the reaction between water-soluble monomer, such as polyamine, 1,6 hexamethylenediamine, piperidine, L-lidine, polyphenol, 2,2-bis (4-hydroxyphenyl)-propane, and organic solvent soluble monomer, such as sebacoyl chloride, terephthaloylchloride, bischloropormate, 2,2-dichloroether. The most important issue is to use mild conditions and
non toxic monomers as many of the employed conventional monomers are toxic to living cells. It was found that replacement of the monomers with nontoxic chitosan gave good results (Park and Chang, 2000).

c. Pregel dissolving: two-step method

This method was developed by Lim in 1980 (Lim and Sun, 1980, Lim, 1982). In this method, the calcium alginate beads containing cells are prepared by the conventional calcium alginate beads preparation method (Fig 2). Thereafter a polyelectrolyte membrane complex is created on the surface of e.g. calcium alginate beads by letting the carboxyl group of the calcium alginate binds to e.g. acid-reactive amine or imine groups in poly-L-lysine or polyethylenimine. Thereafter the bead is retreated with sodium alginate in order to bind the residual poly-L-lysine on the surface of the bead. The liquidcore capsule is formed by dissolving the calcium alginate core in a sodium citrate solution. The capsules prepared by this method have been widely used for immobilizing animal and microbial cells (Park and Chang, 2000).

![Diagram](image-url)  

Fig 2: Pregel dissolving: two step method (Park and Chang, 2000)
d. Liquid droplet forming: one-step method

In this method the calcium alginate beads containing cells are prepared in the reverse procedure to the conventional calcium alginate beads preparation method (Klein et al., 1983). The calcium chloride solution containing microbial cells is added drop wise into a swirling sodium alginate solution (Fig 3). This results in the formation of a calcium alginate membrane on the surface of the aqueous droplet by ionic interaction. A wide variety of biomaterials such as living tissue, cells and enzyme can be immobilized inside the capsule. During the encapsulation process the material does not come in contact with ionic polymer which is converted to the capsule membrane. Different parameters such as pore size, wall thickness, surface charge and mechanical strength of the capsules can easily be controlled by alteration of the concentrations of alginate, calcium and gel-forming polymer. Many studies involving encapsulation of microbial cells such as yeasts, bacteria, and fungi by means of this one-step method since 1993 have been studied (Chang et al., 1996, Cheong Soo et al., 1993, Oh and Park, 1998).

![Diagram of liquid droplet forming: one-step method](image)

Fig 3: Liquid droplet forming: one step method (Park and Chang, 2000)
Liquid-core alginate membrane capsules are prepared by adding calcium chloride solution containing suspended cells dropwise into an agitated alginate solution. Usually a thickening agent such as carboxymethylcellulose (CMC), dextran, xanthan gum or sucrose is added to the calcium chloride solution in order to prevent the deformation of the capsules caused due to shear stress resulting from the agitation of the alginate solution. Studies show that high molecular weight thickeners cannot pass through the alginate membrane into the outer solution, resulting in relatively viscous liquid-core. But low molecular weight thickener, e.g. sucrose caused osmotic damage to the cells during the preparation of capsules (Koyama and Seki, 2004).

In many studies microencapsulation has been carried out by using either natural or synthetic polymers such as calcium alginate (Cheong Soo et al., 1993), carragennan-oligochitosan (Bartkowiak and Hunkeler, 2001), alginate-poly-L-lysine (Lim and Sun, 1980), chitosan-CMC (Yoshioka et al., 1990), alginate-oligochitosan (Bartkowiak and Hunkeler, 1999), alginate-aminopropylsilicate (Sakai et al., 2002) and polyamide (Green et al., 1996).

Alginate/poly-L-lysine (PLL) microcapsule has been widely studied for live cell encapsulation (Chandy et al., 1999, Thu et al., 1996). Alginate (a linear copolymer of (1-4) linked β D-mannuronic acid and α L- guluronic acid), a naturally occurring polysaccharide isolated from brown algae is widely used in encapsulation due to its excellent properties. Alginate is biocompatible, non toxic and forms gels at mild conditions. Alginate is usually used as an inner polyanionic polymer. Coating capsules with poly-L-lysine (PLL) result in a reduced membrane porosity, however the major disadvantage of PLL-alginate is the fragility and the short term durability of these capsules (Chen et al., 2007).

In the field of encapsulation chitosan has been alternative to PLL for microcapsule coating. Chitosan is a linear polysaccharide of (1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. It is also the most abundantly available cationic polysaccharides. Chitosan can be obtained by the alkaline deacetylation of chitin. Due to low toxicity, good biocompatibility and controllable biodegradability it has been used as an active agent in encapsulation (Chen et al., 2007, Chen et al., 2009). It has been reported that chitosan has been used in liposome formulation due to its high affinity for cell membranes (Prabaharan and Mano, 2005). It is also reported as a suitable substrate for biomimetic polymers because of its similar structure.
to the glycosaminoglycans found in the tissues. When alginate and chitosan are used for encapsulation, the alginate-chitosan (AC) membrane is formed because of the electrostatic interactions between the two oppositely charged polysaccharides. Encapsulation of live microbes, cells, DNA, proteins and enzymes in alginate-chitosan (AC) capsules has been widely studied (Chen et al., 2009).

It has been showed by previous studies that the stability of the AC capsule membrane is limited and requires significant improvement. The chemistry of the microcapsule membrane has to be improved in order to permit long term use of the capsules. One way to increase the capsular strength and resistance to chemical and mechanical stresses is to covalently cross-linking e.g. chitosan with bi-functional reagents such as glutaraldehyde, carbodomide and epoxy compounds. The treatment leads to the formation of a crosslinked three dimensional (3D) network in the capsular structure. However, synthetic cross-linkers are cytotoxic and often have devastating effects on the live cells, microbes and other tissues to be encapsulated. In recent years it has been reported that genipin, an iridoid glucoside extracted from Gardenia fruits can be used as an alternative cross-linker. Genepin have a low cytotoxicity to live cells but allowing for effective cross linking at mild conditions resulting in capsules with increased capsular strength and resistance to mechanical stress (Chen et al., 2009, Chen et al., 2006).
3. **Aim of the work**

The main aim of the project was to compare three different yeast encapsulation methods for ethanol production and increase the robustness of the capsules for longer usage and durability by altering some parameters such as pH and concentration of the chemicals. The three different encapsulation methods investigated were

1. Calcium phosphate mineralized AC capsules
2. Alginate capsules treated with 2% chitosan solution
3. Genipin (a natural crosslinker) crosslinked alginate-chitosan (GCAC) capsules

In all the above methods sodium alginate and chitosan were used as many of previous encapsulation studies are based on preparation of the capsules by sodium alginate and chitosan. In order to improve the robustness of the capsules slight modifications were made as such coating the sodium alginate capsules with chitosan solution of different concentrations and also different pH’s. The pH of the chitosan solution ranged from 5-7 and the concentration of the chitosan solution ranged from 0.2 % (w/v) – 2% (w/v) in 2 % (v/v) acetic acid solution. Apart from the modification by using different chitosan solutions, genipin, a natural crosslinker was used to crosslink amino groups present in the chitosan in order to check whether it increase the robustness of the capsules in order to make them for long use. The genipin solution of different concentrations was used and crosslinked at different pH ranging from 7-8.

After the preparation of the capsules, first the mechanical robustness was tested then permeability of the capsules was investigated and there after fermentation studies were performed with the robust encapsulated yeast capsules of the above methods and the results were analyzed.
4. Materials and methods

4.1. Microorganism and Media

The yeast strain *S. cerevisiae* CBS 8066 was used in the experiments and was maintained on YPD agar plates containing yeast extract (10 g/L), D-glucose (20 g/L), soya peptone (20 g/L) and agar (20 g/L) and preserved at 4 °C. The yeast culture was grown in defined medium containing (NH₄)₂SO₄, KH₂PO₄, MgSO₄ 7H₂O, glucose, trace metals and vitamins. The media was prepared by dissolving 20 ml of (NH₄)₂SO₄ stock solution (175 g/L), 20 ml of KH₂PO₄ stock solution (375 g/L) and 5 ml of MgSO₄ 7H₂O stock solution (150 g/L) and 10 ml of trace metals were added and made up to 500 ml using distilled H₂O (Taherzadeh et al., 1996). Glucose solution (50 g/L) was prepared and autoclaved separately and after autoclaving both the solutions were added in aseptic conditions and to this 1ml vitamin solution was added in sterile conditions. Afterwards 100 ml of overnight culture was taken into two 50 ml tubes and were centrifuged at 4500 rpm for 5 minutes and the pellet consisting of yeast cells was mixed in the solution containing 1.3 % (w/v) CaCl₂ and 1.3 % (w/v) CMC and used in preparing the yeast encapsulate capsules.

4.2. Preparation of AC capsules

The AC capsules were prepared by the one step liquid droplet forming method. A sterile solution containing 0.6 % (w/v) sodium alginate and 0.1 % (v/v) Tween 20 was used in order to form capsules. The capsules were formed by adding droplets of CMC/CaCl₂ solution through two needles to the stirred sodium alginate solution using a peristaltic pump. The formed sodium alginate capsules were allowed to form for 10 min and washed with distilled water and hardened in 1.3 % (w/v) CaCl₂ solution for at least 20 min. The resulting Ca-alginate capsules were thereafter transferred into and treated with 0.2 % (w/v) low molecular weight chitosan solution and 2 % (w/v) chitosan in 2 % (v/v) acetic acid solution. The volume ratio of capsules and chitosan solution was 1:5 and placed in shaker bath at 37 °C and 130 rpm for 24 hours. This coating of chitosan was performed in a 1 L Erlenmeyer flask. Afterwards the capsules were rinsed with 0.9 % NaCl solution in order to remove the excess chitosan. Again they were washed with sterile distill water (Päivi et al., 2011).
4.2.1. Calcium mineralized AC capsules

An earlier described biomineralization method (Lévêque et al., 2002) was used to prepare calcium phosphate mineralized capsules. After the AC capsules were prepared they were let to harden in 1.3 % CaCl$_2$ solution. Afterwards the capsules were washed with sterile water. The AC capsules were treated with Na$_2$HPO$_4$ solution of different concentrations such as 0 mM, 30 mM, 50 mM, 100 mM and 300 mM each at different pH (5, 6.5 and 8) respectively for 15 min. The calcium phosphate mineralized AC capsules are formed by the in situ precipitation of calcium phosphate within the membrane by counter diffusion of Ca$^{2+}$ and HPO$_4^{2-}$ ions.

4.2.2. Alginate capsules treated with 2% chitosan solution

The AC capsules were prepared in the same way as explained above but with slight modifications. The chitosan coating was done by 2 % (w/v) chitosan in 2 % (v/v) acetic acid solution at different pH such as 3, 4, 5 and 6 respectively for 30 min. The volume fraction of the alginate capsules and chitosan solution was 1:5.

4.2.3. Quantification of chitosan present in the capsules

The capsules which were coated with 2% chitosan at different pH were air dried at room temperature and were immersed in the acid orange II dye (pH 3) of 500 µM concentration and incubated for 12 hours on a shaker at 130 rpm. Afterwards the reacted capsules were washed thoroughly with water until they become colorless. Then the dye reacted with the amino groups of the chitosan in the capsules was removed by incubating the capsule in distilled water (pH 12) for 5 hours. The pH of the water was adjusted by using 1 N NaOH. Afterwards the supernatant of the solution was collected and the pH was adjusted to 3 and optical density was measured at 480 nm. The concentration of the chitosan was calculated from the standard curve of the dye (Wu et al., 2007).

4.2.4. Genipin crosslinked alginate-chitosan (GCAC) capsules

The AC capsules prepared were crosslinked with genipin by treating the capsules in genipin solution of different concentrations such as 70 µg/ml, 80 µg/ml, 90 µg/ml and 100 µg/ml solutions at different pH (7, 7.5 and 8) respectively for 24 hours. The volume ratio of AC capsules and genipin solution was 1:10.
4.3. **Measurement of diameter and membrane thickness of capsules**

In order to measure the diameter and membrane thickness of the capsules around 10 capsules were taken in a small plate and observed under the light microscope. Afterwards 5 ml of capsules were kept in 5 ml of 0.9 % NaCl solution for one week in order to measure how much the capsules swell. After one week the capsules were observed in order to see the changes in the diameter and membrane thickness after swelling. In this way measurement of diameter and membrane thickness was performed twice, before and after swelling of the capsules.

4.4. **Mechanical strength test**

In order to measure the mechanical strength of the prepared capsules, 20 capsules each of calcium phosphate mineralized AC capsules, alginate capsules treated with 2 % chitosan, and GCAC capsules respectively were taken and added to 5ml of 0.1M sodium citrate buffer solution in a beaker and were kept for stirring on a magnetic stirrer at 600 rpm for 3 hours. After 3 hours of stirring, the capsules were observed with microscope, any breakage or deformation of the capsules was noted and the broken and deformed capsules were counted.

4.5. **Permeability test**

The permeability testing was performed in order to examine the diffusion through the capsules membrane. The measurements were done by adding 10 ml of capsules (consisting of 40-50 capsules) in 50 ml of 1 mg/ml albumin and hemoglobin protein solutions respectively. 1ml of protein sample was collected for every 20 minutes starting from the 0th min up to 4 hours until uniform conditions was achieved. Then the concentration of the protein was quantified by Bradford assay method.

4.6. **Fermentation studies**

After performing the above tests, yeast cells were encapsulated in the capsules prepared at the best promising conditions for both alginate capsules coated with 2 % chitosan and GCAC capsules. Prior to fermentation, 100 ml of yeast containing capsules were added to a flask containing 500 ml defined media and were incubated for 24 hours at 37 °C at 130 rpm in a water bath in order to accumulate some biomass (pre-cultivation). The pre-cultivation was again followed for another 24 hours. Then 10 ml (around 50 capsules) from both alginate
capsules coated with 2% chitosan and GCAC capsules respectively were added to small flasks (250 ml conical flasks) containing 100 ml of defined medium and aerobic fermentation was done at 30 °C at 130 rpm in a water bath. The fermentation was also done in small flasks containing media with ethanol (23 g/L), furfural (2 g/L) and low pH (pH 3). 1 ml of sample was collected from the medium for every 2 hours (0th, 2nd, 4th, 6th, 8th, 24th hours) to determine the amount of ethanol produced and the amount of glucose consumed. After the fermentation was completed the collected samples were analyzed by HPLC (Aminex HPX-87H column) and the results of two different types of capsules were compared.

4.7. Biomass measurements

The cell dry mass concentration inside the capsules was determined by separating the yeast biomass from the capsule membrane. Each time, 10 capsules were crushed and the biomass was removed from the capsule membranes with distilled water. The biomass samples were centrifuged and washed once with distilled water. The samples were dried at 105°C for 24 h and weighed after temperature equilibration in a desiccator.

4.8. Yeast Vitality

In order to estimate the vitality of the yeast in the suspension, methylene blue staining technique was used (Smart et al., 1999).
5. Results

In order to find a robust encapsulation method for yeast, three different kinds of encapsulation methods were tested as explained above. The diameter and the membrane thickness of the capsules were also measured using a light microscope. Afterwards they were subjected to mechanical strength and permeability tests. After the tests the robust capsules were used for the fermentation in order to find the influence of the inhibitors on the growth of the yeast and ethanol production. Then finally the biomass concentration and yeast vitality inside the capsules was observed. The results of the above observations are as follows.

A. Measurement of capsule diameter and membrane thickness:

a) Calcium phosphate mineralized AC capsules: The diameter of the calcium phosphate mineralized AC capsules before swelling at different pH and different concentrations of Na$_2$HPO$_4$ was found to be in the range of 3.8 mm - 4.5 mm (Fig 4). The diameter of the capsules slightly increased after keeping them in 0.9 % NaCl solution and was found to be in the range of 3.9 mm - 4.4 mm (Fig 5). The capsules prepared at higher pH e.g. pH 8, and higher concentrations of Na$_2$HPO$_4$ e.g. 300 mM was found to swell and break therefore the diameter could not be measured. The error bars in the graphs denotes the standard deviation among the measured values for the two sets of 5 capsules each.

![Biomineralized capsules diameter vs Conc](image)

Fig 4: Diameter of the calcium phosphate mineralized AC capsules before swelling
Fig 5: Diameter of the calcium phosphate mineralized AC capsules after swelling

The membrane thickness of the calcium phosphate mineralized AC capsules prepared at different pH and different concentrations of Na₂HPO₄ was found to be in the range of 0.35 mm - 0.55 mm before swelling (Fig 6). Not much difference in the membrane thickness of the capsules was found after keeping them in NaCl solution and was in the range of 0.34 mm - 0.54 mm (Fig 7). It was observed that in the capsules prepared at pH 6.5 and pH 8 with concentrations of Na₂HPO₄ of 100 mM and 300 mM the membranes were barely visible before and after swelling and the membrane thickness could not be measured whereas the membranes were visible in the capsules prepared with concentration of Na₂HPO₄ of 0 mM, 30 mM and 50 mM before and after swelling. This may be due to the higher concentrations of Na₂HPO₄ which lead to higher calcium phosphate precipitation at different pH of the solutions.
Fig 7: Membrane thickness of calcium phosphate mineralized AC capsules after swelling

**b) AC capsules without crosslinker:** The diameter of the alginate capsules treated with 2 % chitosan prepared without any crosslinker at pH 4 and pH 5 before and after swelling was found to be in the range of 3.4 mm - 4.1 mm (Fig 8). The capsules prepared at pH 3 and pH 6 shrunk and deformed therefore the diameter of these capsules could not be measured as most of the capsules become flat and broken.

Fig 8: Diameter of alginate capsules treated with 2 % chitosan (before swelling)

The membrane thickness of the alginate capsules treated with 2 % chitosan prepared without any crosslinker at pH 4 and pH 5 before and after swelling was found to be in the range of...
0.24 mm - 0.32 mm (Fig 9). The capsules prepared at pH 3 and pH 6 shrink and deformed therefore the membrane thickness of these capsules could not be measured.

Fig 9: Membrane thickness of alginate capsules treated with 2 % chitosan (before swelling)

a) Genipin crosslinked alginate-chitosan (GCAC) capsules: The diameter of the GCAC capsules prepared using different concentrations of genipin at pH 4 and pH 5 was found to be in the range of 3.6 mm - 3.7 mm and 3.9 mm – 4.0 mm respectively before swelling (Fig 10).

Fig 10: Diameter of GCAC capsules (before swelling)

After swelling the diameter was found to be slightly increased and found to be in the range of 3.6 mm - 3.7 mm at pH 4 and 3.9 mm - 4.0 mm at pH 5 respectively (Fig
The percentage of the swelling was nearly 1% and no other significant differences such as deformation or shrinkage of the capsules were observed in the capsules prepared at both pH.

Fig 11: Diameter of GCAC capsules (after swelling)

The membrane thickness of the GCAC capsules prepared using different concentrations of genipin at pH 4 and pH 5 was found to be in the range of 0.180 mm - 0.198 mm and 0.223 mm - 0.244 mm respectively before swelling (Fig 12).

Fig 12: Membrane thickness of GCAC capsules (before swelling)

After swelling the membrane thickness was found to be decreased and was in the range of 0.157 mm - 0.223 mm at pH 4 and 0.223 mm - 0.243 mm at pH 5 respectively (Fig 13). There was no deformation of the membrane and they were clearly visible in the capsules prepared at both pH.
Fig 13: Membrane thickness of GCAC capsules (after swelling)

B) Mechanical strength results:

a) **Calcium phosphate mineralized AC capsules:** After mechanical stirring it was found that none of the capsules were broken or deformed at pH 5. But at pH 6.5 and pH 8 the capsules were deformed at Na$_2$HPO$_4$ concentrations of 0 mM, 30 mM and 50 mM and broken at concentrations of 100 mM and 300 mM and this may due to the difference in calcium phosphate precipitation (Table 2).

<table>
<thead>
<tr>
<th>Conc of Na$_2$HPO$_4$ (mM)</th>
<th>No of capsules kept for stirring</th>
<th>No of capsules broken/deformed (pH 5)</th>
<th>No of capsules broken/deformed (pH 6.5)</th>
<th>No of capsules broken/deformed (pH 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>20</td>
<td>0</td>
<td>deformed</td>
<td>deformed</td>
</tr>
<tr>
<td>30 mM</td>
<td>20</td>
<td>0</td>
<td>deformed</td>
<td>deformed</td>
</tr>
<tr>
<td>50 mM</td>
<td>20</td>
<td>0</td>
<td>deformed</td>
<td>deformed</td>
</tr>
<tr>
<td>100 mM</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>300 mM</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2: Mechanical strength results of calcium phosphate mineralized AC capsules

b) **Genipin crosslinked alginate-chitosan (GCAC) capsules:** After mechanical stirring it was found that most of the GCAC capsules were broken at 70 µg/ml and 80 µg/ml concentration of genipin whereas only 50 % and 25% of them were broken at 90 µg/ml and 100 µg/ml concentration of genipin respectively at pH 7.5 and pH 8 (Table 3).
Table 3: Mechanical strength results of GCAC capsules

<table>
<thead>
<tr>
<th>Conc of genipin</th>
<th>No of capsules kept for stirring</th>
<th>No of capsules broken (pH 7)</th>
<th>No of capsules broken (pH 7.5)</th>
<th>No of capsules broken (pH 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 µg/ml</td>
<td>20</td>
<td>19</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>80 µg/ml</td>
<td>20</td>
<td>18</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>90 µg/ml</td>
<td>20</td>
<td>13</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>20</td>
<td>12</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

C) Quantification of chitosan: The amount of chitosan present in the alginate-chitosan membrane was quantified by using an acid orange II dye. Afterwards the amount of dye reacting with the free amino groups of chitosan was calculated (Fig 14) and based on this four different concentrations of genipin were used for preparation of GCAC capsules in order to check for robustness.

![Quantification of chitosan](image)

Fig 14: Amount of acid orange reacted to free amino groups present in the chitosan

D) Permeability test: The robust AC capsules coated with 2% chitosan and genipin GCAC from the mechanical strength were used in permeability test in 50 ml of 1mg/1ml hemoglobin and bovine serum albumin (BSA) proteins respectively

a) Permeability test of alginate-chitosan capsules: After testing the mechanical strength, robust AC capsules coated with 2% chitosan without any crosslinker prepared at pH 4 and pH 5 were selected and used in permeability tests with hemoglobin and bovine serum albumin (BSA) proteins respectively. The diffusivity of the protein was observed as below (Fig 15 and Fig 16).
b) Permeability results of GCAC capsules: After testing the mechanical strength, robust GCAC capsules prepared at pH 7.5 and pH 8 were selected as only around 25% of capsules were broken after mechanical stirring and used in permeability test with hemoglobin and albumin respectively. The diffusivity of the protein was observed as below (Fig 17 and Fig 18).
The hemoglobin concentration was found to be close to zero in 2% AC and GCAC capsules prepared at pH 4 and this may be due to the pore size in the capsules which allowed entire protein into the capsules and after few hours it rise as the concentration of the protein become uniform inside and outside the capsules.

Fig 17: Diffusivity of hemoglobin protein into GCAC capsules

Fig 18: Diffusivity of BSA protein into GCAC capsules
E) **Fermentation studies results:** The fermentation of glucose was performed by encapsulating the yeast in robust alginate capsules treated with 2% chitosan solution at pH 4 and pH 5 and the robust GCAC prepared at pH 7.5 and pH 8 which withstand the mechanical stirring and the results were compared in both the cases. The fermentation of glucose was performed in small flasks of 250 ml containing 100 ml of defined media each containing inhibitors such as ethanol (23 g/L), furfural (2 g/L) and low pH (pH 3). The fermentation was also performed in absence of the above inhibitors (control). The below are the results of the fermentation after performing HPLC of the fermentation samples.

a) **Alginate capsules coated with 2% chitosan:** After performing the fermentation with yeast encapsulated alginate capsules coated with 2% chitosan for 24 hours, the samples collected for every four hours were analyzed using HPLC and it was observed that ethanol was produced in all the cases i.e.; in presence of inhibitors such as ethanol, low pH, furfural and the control (without any inhibitors) (as shown in Fig 19) which shows that there is no influence of the inhibitors on the fermentation and growth of yeast.

![Ethanol production in presence of inhibitors](image)

**Fig 19:** Production of ethanol in presence of inhibitors and absence of inhibitors by 2% AC capsules
b) Genepin crosslinked alginate-chitosan (GCAC) capsules: After performing the fermentation with yeast encapsulated GCAC capsules for 24 hours, the samples collected for every four hours were analyzed using HPLC and it was observed that ethanol was produced in all the cases i.e.; in presence of inhibitors such as ethanol, low pH, furfural and the control (without any inhibitors) (as shown in Fig 20) which shows that there is no influence of the inhibitors on the fermentation and growth of yeast.

![Ethanol production in presence of inhibitors](image)

**Fig 20**: Production of ethanol in presence of inhibitors and absence of inhibitors by GCAC capsules

**F) Biomass measurements**

The yeast cell dry mass concentration inside the capsules was determined by separating the yeast biomass from the capsule membrane and was measured and found to be in the following way (Table 4 and Table 5).

<table>
<thead>
<tr>
<th>GCAC capsules (in presence of)</th>
<th>Average Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pH</td>
<td>0.0173 g/10 capsules</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.0203 g/10 capsules</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.0115 g/10 capsules</td>
</tr>
<tr>
<td>Control</td>
<td>0.0205 g/10 capsules</td>
</tr>
</tbody>
</table>

*Table 4: Average dry weight of yeast in GCAC capsule*
Table 5: Average dry weight of yeast in 2% AC capsules

<table>
<thead>
<tr>
<th>2% AC capsules (in presence of)</th>
<th>Average Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pH</td>
<td>0.01885 g/10 capsules</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.02450 g/10 capsules</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.01800 g/10 capsules</td>
</tr>
<tr>
<td>Control</td>
<td>0.02000 g/10 capsules</td>
</tr>
</tbody>
</table>

**G) Yeast Vitality:** The vitality of the yeast cells was estimated by methylene blue staining and found that most of yeast cells were alive (data not shown) which indicates that there is no influence of the inhibitors on the growth of the yeast cells.

6. **Discussion**

In the present study, three different encapsulation methods were studied and compared in order to find a robust encapsulation method for yeast encapsulation. The study of calcium phosphate mineralized AC capsules revealed that the surface of the capsules was changed with increasing phosphate ion concentrations during the treatment. It was observed that during low Na$_2$HPO$_4$ concentration such as 0 mM, 30 mM, 50 mM the membrane around the capsules was clearly visible and at higher concentrations such as 100 mM and 300 mM it was barely visible which may be due to high levels of calcium phosphate precipitation. When these capsules were subjected to mechanical strength testing it was found that the capsules prepared at low concentration of phosphate such as 0 mM, 30 mM and 50 mM were robust enough at pH 5 and they were intact whereas those prepared at high concentrations of phosphate levels such as 100 mM and 300 mM at pH 6.5 and pH 8 were not robust as they got deformed and broke down during stirring in mechanical strength test. From this we studied that these capsules were robust enough at low concentration of phosphate levels and the capsules become brittle as phosphate concentration increases. The best capsules in this method were at pH 5 as the capsules were intact and were not deformed or broken. But these capsules were not much robust than the AC capsules as after few days they got dissolved in the 0.9% NaCl solution when kept for swelling test.

In another method alginate capsules were prepared by using 2% chitosan solution at four different pH levels such as pH 3, pH 4, pH 5 and pH 6. It was found that the capsules prepared at pH 3 and pH 6 got deformed and shrink upon chitosan treatment whereas the
capsules prepared at pH 4 and pH 5 were intact and robust. Upon mechanical strength testing the capsules prepared at pH 4 and pH 5 were robust enough and were not broken whereas the capsules prepared at pH 3 and pH 6 respectively were broken. The robust capsules in this method were found to be prepared at pH 4 and pH 5. In order to find out the concentration of reacting chitosan with the alginate quantification of chitosan was performed using acid orange II.

A new method for preparation of capsules was performed by slightly modifying the above method by including a natural crosslinker, genipin during the preparation of the capsules whether to check whether the capsules become more robust. Upon mechanical strength testing it was found that most of the capsules prepared at pH 7 were not robust enough as every capsule broke at all concentrations of genipin. The capsules prepared at pH 7.5 and pH 8 at a genipin concentrations of 90 µg/ml and 100 µg/ml were more robust as only 25% of the capsules kept for mechanical stirring broke and whereas most of the capsules prepared at genipin concentrations of 70 µg/ml and 80 µg/ml broke. The GCAC capsules were found to be more robust than the AC capsules and the calcium biomineralized AC capsules.

The robust capsules from the above two methods were subjected to permeability testing and checked for the diffusivity of hemoglobin and BSA proteins. Both the proteins diffused through the capsules membrane and in less than 4 hours the protein form a homogenous solution inside and outside the capsules.

7. Conclusion

From the results and observations it can be concluded that the alginate capsules prepared by 2 % chitosan treatment at pH 4 and crosslinked with genipin solution (100 µg/ml concentration) at pH 7.5 were the best capsules as considerable amount of ethanol production was seen and there was no leakage of the yeast cells into the medium. It was also observed that there was no effect of the presence of inhibitors such as ethanol, furfural and low pH on the fermentation and growth of the yeast cells inside the capsules. This clearly indicates that the genipin cross-linked alginate-chitosan capsules are robust enough for bioethanol production. Apart from these results a further study on the chemistry of the membrane formed on the capsules is required in order to study in depth mechanism of the robustness and integrity of the capsules. In this way it can be observed that addition of a crosslinker, genipin enables the capsules to be robust enough and does not influence on the growth of the yeast and glucose fermentation.
8. Future Work

The stress tolerance conditions of the encapsulated yeast can be studied using the 2 % AC and GCAC capsules. Further studies involving wide variety of polymers and crosslinkers can be done in order to produce robust capsules based on the above obtained results by modifying the concentration of the chemicals.

9. Acknowledgement

We are grateful to our supervisor and our colleagues in the Biotechnology group at School of Engineering, University of Borås for supporting us in order to carry out the research as part of our master thesis.
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