RIBOFLAVIN PRODUCTION BY ENCAPSULATED *CANDIDA FLARERI*

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CHAPTER I
INTRODUCTION

1.1 Background

Vitamins belong to the group of nutrients which are necessary for growth. Riboflavin is an important B vitamin for maintaining human health (Shimizu, 2001). There is a large need for extra vitamins, and the quantities derived from plant and animal food sources are not enough due to food shortage or disease (Chandra et al., 1996). Added vitamins are now either prepared chemically or biotechnologically via fermentation processes. Some vitamins, like riboflavin, are currently produced almost exclusively via fermentation because several of the stages in the chemical process involve the use of toxic reagents (Figure 1.1). The waste products, therefore, require stringent environmental control and may need special forms of effluent treatment (Shimizu, 2001).

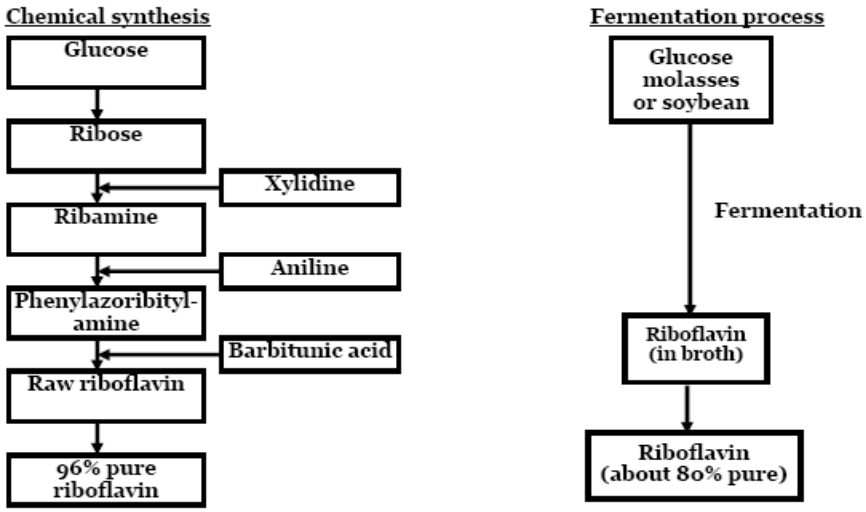

Figure 1.1. Chemical and fermentation processes for riboflavin synthesis (adapted from the 2001 Competition Commission report titled: BASF AG and Takeda Chemical Industries Ltd).

Improved fermentation processes using immobilized systems for the synthesis of vitamin compounds are gaining importance because the recovery and purification of these vitamin compounds from suspended systems remains highly complex. The major advantage of immobilization is the simplification of product recovery because fewer operations are required for biomass separation/product recovery (Raymond et al., 2004).

Problems with mass transfer are common in immobilized systems, in fact the success of an immobilization material is often measured in terms of the extent to which it does not restrict the diffusion of various solutes into and out of the gel. One of the most important factors that affects mass transfer, other than capsule size, is the pore size of the gel membrane (Nguyen and Luong, 1986; Chai et. al., 2004). Thus mass transfer can be enhanced by making the beads more porous. Previous work has shown that the use of surfactants influenced the rate of drug release from alginate microspheres (Wan et al., 1993). The drawback of such an attempt is the increased possibility of cell/biocatalyst leakage.

1.2 Objectives

The main objectives of this study are to develop immobilization techniques, and then to compare riboflavin production efficiencies in suspended and immobilized reactor systems.

Specific objectives include:

- Develop capsule immobilization techniques.

The encapsulation technique consists of dropping a calcium chloride/chitin mixture into a Na-alginate solution. Therefore, a thickening agent is required for the first mixture.

Several thickeners were investigated (PEG6000, CMC, etc…) and the best type/concentration were chosen for the studies.

- Investigate the effect of nonionic surfactants on the diffusion properties of capsules.

The effects of 5 different non-ionic surfactants on the diffusion properties of capsules were investigated. Because of the significant differences between beads and capsules, the effects of surfactants were expected to vary significantly for beads and capsules.

- Compare fermentation efficiencies in suspended vs. attached growth systems.

Increasing dilution rates in continuous bioreactors generally increases production rate. However, as dilution rate approaches a certain percentage of the maximum specific growth rate (μ_{max}), cell wash-out occurs in suspended growth systems. This problem is not an issue in attached growth systems as long as dilution rate does not exceed diffusion rate of substrate. Fermentation efficiencies of the optimum attached growth system will be compared to that of a suspended growth system.

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CHAPTER II
LITERATURE REVIEW

2.1 Riboflavin Structure and Importance

Riboflavin, also known as vitamin B_2, is an easily absorbed micronutrient with a key role in maintaining health in humans and animals. It is the central component of the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and is therefore required by all flavoproteins (Lago and Kaplan, 1981). As such, vitamin B_2 is required for a wide variety of cellular processes. Like the other B vitamins, it plays a key role in energy metabolism and is required for the metabolism of fats, carbohydrates, and proteins. In addition to producing energy for the body, riboflavin also works as an antioxidant by scavenging damaging particles in the body known as free radicals (Lim et al., 2001).

Riboflavin is yellow or orange-yellow in color. Good sources of riboflavin are milk, cheese, leafy green vegetables, liver, yeast, almonds, and legumes such as mature soybeans. In addition, it is used as a food coloring and to fortify some foods like baby foods, breakfast cereals, and pastas, and processed cheese (Graham et al., 2005). It is difficult to incorporate riboflavin into many liquid products because it has poor solubility in water. Hence there is a need for riboflavin-5'-phosphate, a more expensive but more soluble form of riboflavin. It is the principal form in which riboflavin is found in cells (Bacher et al., 2000).

In humans, signs and symptoms of riboflavin deficiency include cracked and red lips, inflammation of the lining of mouth and tongue, mouth ulcers, cracks at the corners of the mouth, and a sore throat (Graham et al., 2005). A deficiency may also cause dry and scaling skin, fluid in the mucous membranes, and iron-deficiency anemia. The eyes may also become bloodshot, itchy, watery and sensitive to bright light. In animals, riboflavin deficiency results in lack of growth, failure to thrive, and eventual death.

Riboflavin was first isolated from milk in 1879 by Blyth (Chandra et al., 1996) and was called lactoflavin. It has the empirical formula $C_{17}H_{20}N_4O_6$ and the structural formula is shown below.

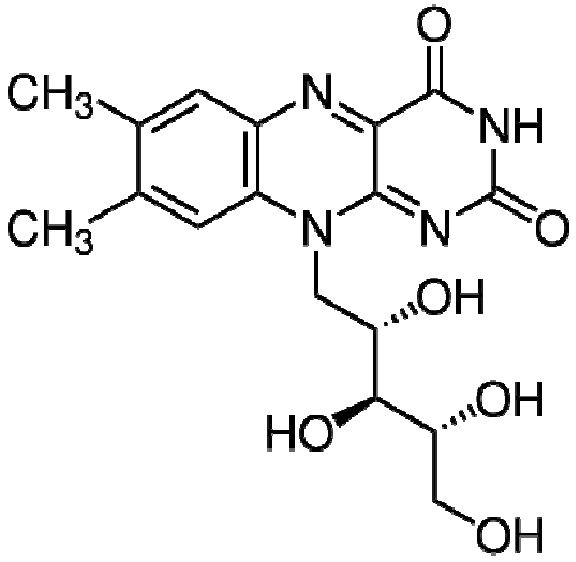

Figure 2.1 Riboflavin structure.

2.2 Riboflavin Production

Several organisms overproduce vitamins in huge amounts, and this has been exploited for industrial production. Most vitamins are now industrially produced and widely used as food or feed additives, medical or therapeutic agents, health aids, and cosmetic and

technical aids. Thus, vitamins are important products for which many biotechnological production processes, like microbial, enzymatic, as well as organic chemical synthetic transformation have been reported (Bacher et al., 2000). Some biological processes are now used for large-scale production. This is mainly due to the recent advances in metabolic and genetic engineering.

Chemical riboflavin production from ribose is currently being replaced by biological processes, which have the advantages of reducing costs of operation and the ability to use renewable substrates such as plant oil or sugar instead of mineral oil. Riboflavin can be produced either on an industrial or a laboratory scale by a wide variety of microorganisms (Buzzini and Rossi, 1997). The yeast *Candida famata* is an example of a natural overproducer of riboflavin via aerobic fermentation of organic substrates. However, the wild-type *C. famata* is very sensitive to iron inhibition. Therefore an iron-resistant industrial-scale strain has been developed by several companies, and riboflavin concentrations in the range of 20-30 g/L have been reported using this yeast (Lim *et al*., 2001). A recent more competitive process for riboflavin production employed by Roche Vitamins AG is the process employed by using *Bacillus subtilis*. Currently, the yearly riboflavin production by Roche Vitamins AG is 3000 t with *B. subtilis* at a site in Germany (F.Hoffmann-La Roche Ltd, 2007).

2.3 Microbial Production of Riboflavin

Riboflavin can be synthesized in large enough amounts by some fungi and bacteria to be exploited successfully on an industrial scale. Table 2.1 shows several riboflavin-producing microorganisms.

Clostridium acetobutylicum, which had a productivity of about 100 mg/L, was one of the first organisms used to produce riboflavin. Some organisms of the genus *Candida*, like *Candida flareri*, were able to produce about 600 mg/L of riboflavin. In order to produce higher levels of riboflavin using these yeasts, it was necessary to overcome iron inhibition. Unlike in yeast, iron inhibition is not a factor for the two molds, *Eremothecium ashbyii* and *Ashbya gossypii,* which are natural overproducers of riboflavin, and currently produce riboflavin levels exceeding 20 g/L (Lim et al., 2001).

Table 2.1 Maximum batch riboflavin concentrations of several natural strains

	Strains	Carbon source	Riboflavin (g/L)
Bacteria	*Clostridium acetobutylicum*	-	0.1
	Bacillus subtilis	Glucose	0.1
Yeast	*Candida flareri*	Glucose, fructose	0.6
	Candida guilliermondii	Liquid brewery waste	0.2
Fungi	*Eremothecium ashbyii*	Fructose, sucrose	0.2
		Glucose, molasses	20
		Molasses	3.3
	Ashbya gossypii	Glucose	1.0
		Corn oil, soybean oil	5.0
		Soybean oil, bone fat	20

Adapted from Lim et al., (2001)

Since the first description of the riboflavin production process (Guilliermond et al., 1935), much research has been conducted to develop defined synthetic media that maximizes riboflavin production. The earliest attempts were by Schopfer (1944) and Schopfer and Guilloud (1945) which resulted in the development of a synthetic medium for *Eremothecium ashbyii* and permitted growth and riboflavin production to occur under chemically defined conditions. With the determination of the nutritional requirements of

several riboflavin producers, it became possible to perform studies on the mechanism of riboflavin biosynthesis by means of precursors that modify riboflavin yield. Early studies on increasing riboflavin yields found that the biochemical key to riboflavin overproduction appears to involve resistance to the repressive effects of iron (Lim et al., 2001).

Natural overproducers of riboflavin can be divided into three categories based on the degree of accumulation of the vitamin: weak overproducers, moderate overproducers, and strong overproducers. Weak overproducers include the bacterial strain *Clostridium acetobutylicum*, moderate overproducers include several Candida species, and strong overproducers include the two molds *Eremothecium ashbyii* and *Ashbya gossygii.*

Most bacteria do not overproduce riboflavin (Demain, 1972), however some strains of Clostridia are weak overproducers. For example, early work by Hickey (1945) employed *Clostridium acetobutylicum* to produce riboflavin yields of 100 mg/L. The process was anaerobic and extremely sensitive to iron, where significant inhibition of vitamin overproduction occurred at iron levels of 1 ppm.

The moderate overproducing group, as listed by Demain (1972), is composed of yeasts including *Candida flareri* (considered synonymous to *C. famata* by ATCC), *Candida guilliermondii*, *Candida robusta, Candida ghoshii, Debaryomyces subglobosus,* and *Torulopsis famata.* These yeasts have been reported to produce approximately 600 mg riboflavin/L (Buzzini and Rossi, 1997). Carbon sources for these overproducers are glucose, fructose, mannose, sucrose, xylose, arabinose, and acetate. Nitrogen sources are preferably organic; the compounds most frequently used are asparagine, urea, glycine, and alanine. These organic nitrogen sources are preferred by some yeast strains while

being useless, or sometimes detrimental, to others. For example, adenine is the appropriate nitrogen source for *C. ghoshii*, but is inhibitory to *C. flareri* (Goodwin and McEvoy, 1959). With the later organism, guanine and xanthine are preferred. Like the bacteria, yeasts are sensitive to iron toxicity while cobalt and zinc stimulate overproduction without increasing growth, possibly by reversal of iron inhibition (Fedorovych et al., 2001).

The most active overproducers known are the two yeast-like ascomycetes molds *Eremothecium ashbyii* and *A. gossygii.* Fermentation by either organism occurs optimally at 26-28°C, and the process is aerobic and unaffected by iron. The overproduction of riboflavin by *E. ashbyii* was discovered by Guilliermond et al. (1935). Since then, much work has been done on this strain. In the early 1950s, media that produced riboflavin levels of 200 mg/L were developed (Pfeifer et al., 1950). Currently, yields of 1.5 to 2.5 g/L are possible with crude sources of media. Riboflavin overproduction by *E. ashbyii* can be carried out using either carbohydrates or lipids as the carbon source. Overproduction occurs in chemically-defined media with asparagine as the amino acid of choice.

The ability of *A. gossypii* to overproduce riboflavin was discovered in 1946 (Wickerham et al., 1946). After the discovery of *A. gossypii*, industrial production of riboflavin by *E. ashbyii* was replaced by *A. gossypii*. In early studies, some defined media were established using Tween 80 and purine, and riboflavin levels of 370 mg/L were produced with these media (Lim et al., 2001). In later more improved media, the production of riboflavin reached 1 g/L. Apart from these media, more complex media for industrial application were studied, and using soybean oil as a carbon source and collagenous

protein and corn steep liquor as nitrogen sources, yields of more than 5 g/L are currently being achieved (Lim et al., 2001).

2.4 Riboflavin Biosynthesis

The metabolic pathways of the three kinds of riboflavin overproducers, yeasts, gram-positive bacteria, and filamentous fungi, are shown in Fig. 2.2 (Lim et al., 2001). *C. famata* has a simple pathway from glucose to guanosine triphosphate (GTP), which is the entrance of riboflavin biosynthesis. *Bacilli* cells follow a different pathway from yeast, and convert glucose to guanosine monophosphate (GMP), which is also a precursor of GTP. *Ashbya gossypii* uses plant oil, which is obtained by breaking down fatty acids, and the resulting fatty acids enter to the peroxisomes. Then, several pathways are possible depending on the specific strain (Figure 2.2)

The resulting metabolites from carbon sources in several riboflavin overproducers enter the riboflavin biosynthetic pathway. Biosynthesis starts from GTP and the final product is riboflavin.

2.5 Industrial Production of Riboflavin, Synthetic vs. Microbial Fermentation

Synthetic riboflavin production is a mineral-oil-based process. The process is described in detail by Stahmann et al. (2000). It starts with D-ribose reacting with 3,4-xylidine in methanol. This reaction step produces riboside. The riboside formed is hydrogenated to give N-(3,4-dimethylphenyl)-D-1'-ribamine. This transient product is coupled with a phenyl diazonium halogenide, which produces an azo compound which is used in a cyclo-condensation with barbituric acid to give riboflavin. The main disadvantages of the procedure are: (1) it has a maximum yield of about 60% (from

substrate), thus generating a lot of waste, (2) it requires organic solvents, and (3) it requires 25% more energy in comparison to the microbial process.

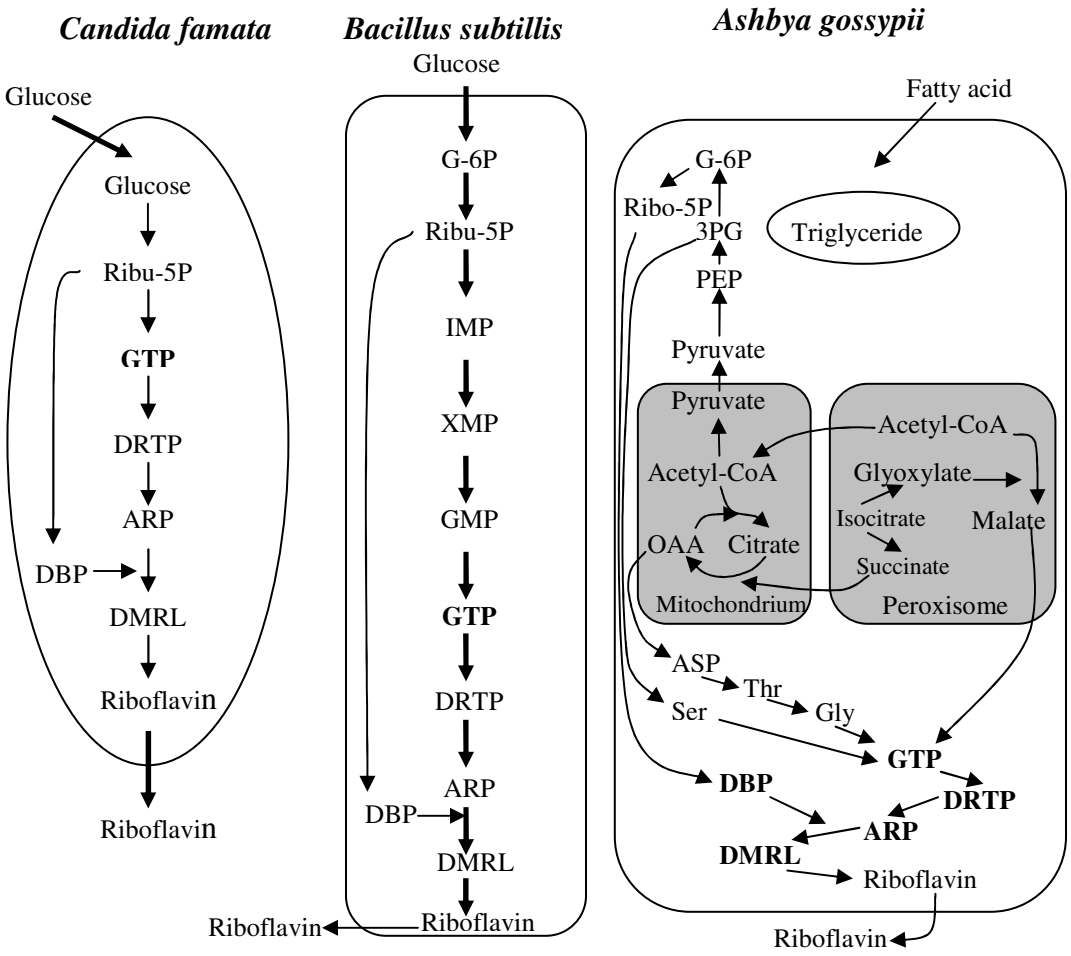

Figure 2.2 Proposed models for the metabolic pathways of riboflavin production for *A. gossypii, B. subtillis*, and *C. famata*. Adapted from Lim et al., (2001). Abbreviations: G-6P, Glucose-6-phosphate; 3PG, 3-phosphoglycerate; PEP, Phosphoenolpyruvate; Ribu-5P, Ribulose-5-phosphate; OAA, Oxaloactate; Asp, Aspatate; Thr, Thre- onine; Gly, Glycine; Ser, Serine; GTP, Guanosine triphos-phate; GMP, Guanosine monophosphate; XMP, Xanthine monophosphate; IMP, Inosine monophosphate; DRTP, 2, 5-diamino-6-ribosylamino-4 (3H)-pyrimidinedione 5-phosphate; ARP, 5-amino-6-ribitylamino-2, 4 (lH, 3H)-pyrimidine; DBP, L-3, 4-dihydroxy-2-butanone-4-phosphate; DMRL, 6, 7-dimethyl-8-ribityllumazine.

The first commercial fermentations for riboflavin production were established using *Clostridium acetobutylicum* (Meade et al., 1947). In 1965, commercial riboflavin production by fermentation was started by three companies, but a few years later all three plants were shut down because it was economically uncompetitive with the chemical process (Lago and Kaplan, 1981). Merck & Co., Inc. started riboflavin production by employing *A. gossypii* in 1974. Currently, yields of up to 15 g/L are being achieved using mutants of *A. gossypii* and *E. ashbyi* (Stahmann et al., 2000). A production plant with *A. gossypii* was started by BASF (Germany) in 1990, and in 1996 the chemical process was finally shut down (BASF 1996).

In 2000, La Roche Ltd replaced its chemical production by a biotechnical process with genetically enhanced *B. subtilis.* The plant is currently producing 3000 tons riboflavin/year and, according to Hoffmann-La Roche, is saving half the costs in comparison to the chemical process.

While the microbial fermentation process has many potential advantages, one major disadvantage is the purity of the final product. The 80% pure vitamin B2 produced by fermentation is suitable for use only in animal feed without further refining. BASF reported that the cost of purification could exceed 40% of total operating costs in its plants (Competition Commission, 2001). On the other hand, only about 2% of impurities need to be removed using the chemically-produced vitamin B2. Therefore, the purification plant can be significantly smaller. Thus, manufacturers using the chemical production route to produce vitamin B2 have a significant advantage if they produce the food-grade product rather than feed-grade vitamin B2.

2.6 Recovery of Riboflavin from Fermented Media

A typical recovery process is described in detail by Perlman (1979). After the fermentation process has been completed, the solids are either dried to a feed-grade product for animal-feed supplementation or processed to a U.S. pharmaceutical-grade product for human consumption. For either case, the first step is to adjust the pH of the fermentation broth to 4.5. If a feed-grade product is required, the broth is concentrated to 30% solids and dried on double-drum driers.

If a pharmaceutical-grade product is needed, insoluble riboflavin must be re-solubilized before the purification step. The fermented broth is heated for 1 hr at 121°C to solubilize all the riboflavin. The suspended matter is removed by centrifugation and riboflavin is recovered by conversion to the less soluble form. Two patented methods have been described for the purification step, a chemical method (Hines, 1945a) and a microbiological method (Hines, 1945b). In the microbiological method, the medium containing dissolved riboflavin is mixed with necessary nutrients and adjusted to pH 5.5-7.5. The temperature is maintained at 8-47°C, depending on the bacterial species used. The fermentation is allowed to continue for 18-24 hrs and the precipitate formed (riboflavin) is centrifuged and collected.

In the chemical method (Hines, 1945a), the solution containing riboflavin is filtered prior to any treatment. The solution is then adjusted to a pH of 5.0-5.5 before a reducing agent mixture is added to the solution. The mixture contains compounds such as $Na_2S_2O_4$, $TiCl_3$, $SnCl_2$, VSO_4, $CrCl_2$, and $CrSO_4$ and the temperature of the solution is kept at 20-30°C. The riboflavin precursor is thus precipitated and the supernatant liquor is decanted. The residue is filtered and the filtrate extracted by any well-known method

(e.g. 75% isopropyl alcohol). Recovery of riboflavin gives a yield of 70-90.5%. The precipitated riboflavin is then dissolved in water, a polar solvent, or an alkaline solution and recovered by re-crystallization from the aqueous or polar solvent, or by acidification of the alkaline solution.

2.7 Cell Immobilization Technology

Immobilized cell technology has been widely used in a variety of industrial applications and laboratory experiments. Immobilization is the process of confining cells in a region or matrix. Various types of cells can be immobilized; examples are bacteria, yeast, fungi, plant cells, and animal and insect cells. The major advantage of cell immobilization is the simplification of product recovery because fewer operations are required for biomass separation/product recovery (Raymond et al., 2004). Therefore, the application of cell immobilization is a practical solution to reduce the cost of complicated and expensive product recovery. Other advantages include the retention of catalytic activities for repeated use and an increase in cell densities while maintaining activity over a long period of operation (Buzzini and Rossi, 1997). Moreover, the immobilized cells are retained easily in the bioreactor and, consequently, utilized continuously.

Some immobilized systems have been developed to eliminate inhibition caused by high concentrations of substrate and product and to enhance productivity and yield of ethanol production. Accordingly, Takamitsu et al. (1993) showed that ethanol production in an immobilized cell reactor using *Zymomonas mobilis* was doubled compared to a similar suspended reactor system. The work done by Najafpour et al. (2004) aimed at obtaining high ethanol production with high yield of productivity and, consequently, lowering the high operating costs. Their results showed that continuous ethanol

production in an immobilized cell reactor (ICR) fed with high sugar concentration was successful. Moreover, glucose concentration as high as 150 g/L did not cause inhibition while 50 g/L glucose was enough to significantly inhibit a suspended system. Also, ethanol production increased 5-fold when glucose concentration was doubled from 25 to 50 g/L, and a similar behavior was not observed in the suspended system. The results indicated that the immobilization of *Saccharomyces cerevisiae* possesses the capacity not only to utilize high concentrations of sugar but also to yield higher ethanol productivities during the course of continuous fermentation.

In addition, work done by Najafpour (1990) showed that if intact microbial cells are directly immobilized, the removal of microorganisms from downstream product can be omitted and the loss of cellular activity can be kept to a minimum level. Immobilized systems were developed as an alternative to expensive and complicated chemical processes, for example chemical production of xylitol requires high temperature and pressure and highly purified xylose which makes the process very costly (Cunha et al., 2007). As an alternative to the chemical process, bioproduction of xylitol from sugarcane bagasse hydrolysate was investigated (Cunha et al., 2007). They used polyvinyl alcohol (PVA) hydrogel entrapped *C. guilliermondii* cells in repeated batches with cell recycling. Their results showed that hydrogel beads exhibited good mechanical strength which makes them suitable for long-term use.

The immobilization matrix can be formed into geometries that provide improved mass transfer within the biological reactor, thus increasing the efficiency of nutrient supply and product removal. A particularly attractive geometry is the spherical configuration provided by hollow capsule membranes. The advantage of hollow capsules

is the decreased diffusion barrier provided by the interior, which should give rise to rapid equilibrium and more uniform distribution of substrate in the capsules (Chinnayelka and McShane, 2004).

Application of immobilized cell systems ranges from wastewater treatment to the production of therapeutics. These systems have been used to solve problems encountered in conventional bioreactors using suspended cell culture, including low biomass concentrations, low biomass productivity and product formation, inefficiency in continuous production, low stability to sudden fluctuation, and limited dilution rate in the case of continuous operation (Raymond et al., 2004).

In general, there are three major groups of immobilization techniques:

- Carrier-Binding: the binding of cells or enzymes to water-insoluble carriers.
- Cross-Linking: intermolecular cross-linking of cells or enzymes by bi-functional or multi-functional reagents.
- Entrapping/Encapsulating: incorporating cells or enzymes into the lattices of a semi-permeable gel or enclosing the enzymes in a semi-permeable polymer membrane.

Carrier-Binding is the simplest way of preparing immobilized cells or enzymes (Martinsen et al., 1992). The method relies on non-specific physical interaction between the enzyme and the surface of the matrix. A major advantage of adsorption is that usually no reagents and only a minimum of activation steps are required. This method has a disadvantage that the adsorbed enzyme may leak from the carrier during use due to weak binding between enzyme and carrier. Another disadvantage is non-specific adsorption of other proteins or other substances as the immobilized enzyme is used.

Cross-linking is the intermolecular immobilization of cells or enzymes either to other protein molecules or to functional groups on an insoluble support matrix (Martinsen et al., 1992). Since the cell or enzyme is covalently attached to the support matrix, very little desorption will occur using this method. Generally, cross-linking is best used in conjunction with one of the other methods since using this method alone usually results in relatively low enzymatic activity.

Another method of immobilization is the confining of enzymes or cells within the lattices of polymerized gels. This allows the free diffusion of low molecular weight substrates and reaction products (Nguyen and Luong, 1986). Entrapping/encapsulating methods differ from the carrier-binding and cross linking in that cells or enzymes do not bind to the gel matrix or membrane. This results in a wide applicability provided that suitable conditions for the immobilization of various enzymes are chosen. Polyacrylamide, calcium alginate, and gelatin are three media commonly used in entrapment techniques. Although many other materials for entrapment exist, all these gels can be formed with a simple set of equipment and share similar procedures.

Various methods for the entrapment/encapsulation of cells have been reported. Examples are immobilization by polyacrylamides, polysaccharides, and proteins (Bucke and Brown, 1983). Table 2.2 shows examples of cell entrapment in polymeric beads.

Immobilization by polyacrylamide was the first gel to be used to entrap living microbial cells (Bucke and Brown, 1983). The entrapment of cells is achieved by stirring cell preparations into the polyacrylamide solution and adding a polymerizing agent (e.g. tetramethyl ethylene diamine) to initiate polymerization. The resultant gel may be shaped

as required. Gel strength and pore size may be controlled by varying the concentration of the acrylamide monomer and polymerizing agent.

Table 2.2 Examples of cell entrapment in polymeric beads[1].

Support material	Cell	Reactant/Product
Agarose	Hybridoma cell	Antibody
Calcium alginate	Hybridoma cell	Antibody
	Saccharomyces carlabergensis	Glucose/ethanol
	Leuconostoc mesenteroids	Dextransucrase
	Saccharomyces cervisiae	Molasses/ethanol
	Asperigillus niger	Citric acid
	Candida famata	Riboflavin
Kappa-carrageenan	*Penicillium chrysogenum*	Penicillin
PVA/boric acid/Ca-Al	*Pseudomonas*	Phenol degradation
BIX12-alginate hybrid	Hybridoma cell	Monoclonal antibody
Carrageenan	*Bacillus subtilis*	Alpha-amylase

[1] Adapted from Park and Chang (2000)

The polysaccharide most commonly used for cell immobilization is alginic acid. This is a copolymer of D-mannuronic and L-guluronic acids. A wide range of alginic acids of varying compositions and molecular masses are available commercially. Gels are formed with divalent cations (e.g. calcium and barium) that cross-link guluronic acid units of different molecules. Cell preparations are stirred into solutions of sodium alginate and the mixture is extruded into a solution of a divalent cation salt. The gelation reaction is instant but it is necessary to leave the gel bodies in the calcium salt solution for at least 20 min to allow for complete gelation of the alginate.

Collagen can be used to immobilize cells. Cells are mixed with dispersed collagen fibers at an appropriate pH. The mixture is dried into a film, which is strengthened by cross-linking with glutaraldehyde. The resulting collagen/cell complex is very similar in

general terms to whole mammalian tissues in which cell masses are connected and supported by collagen.

The major disadvantages of cell entrapment are cell leakage and subsequent growth in the medium solution and the fact that cells usually grow on the surface and in the pore spaces of the matrix where the space available for cell growth is limited. To overcome these problems, cells can be encapsulated instead. Liquid core capsules have some important advantages for microbial cell immobilization over conventional bead entrapment. The liquid core gives a larger space for cell immobilization, cells are free to grow, and mass transfer resistance is reduced inside the capsule. There are several techniques for encapsulation of cells. The most popular techniques are coacervation and liquid droplet forming methods.

Coacervation is the phenomenon when a liquid phase separates from a solution, usually a polymer, and coats a liquid core. The phase separation occurs as a result of changing some parameters of the system such as temperature, pH, or the addition of a chemical. The liquid core droplet is called coacervate. The coating material is solidified by several means including cross-linking.

Klein et al. (1983) developed the liquid droplet forming method by reversing the procedure of the calcium alginate bead method. Cells are mixed in a solution of calcium chloride and dropped into a stirred sodium alginate solution. A calcium alginate membrane is formed instantaneously on the surface of the droplet. Wall thickness, pore size, and mechanical strength of the capsules can easily be controlled by variation of the concentrations of alginate and calcium. Table 2.3 shows examples of cell encapsulation using encapsulation techniques.

Problems with mass transfer are common in immobilized systems. In fact the success of an immobilization material is often measured in terms of the extent to which it does not restrict the diffusion of various solutes into and out of the gel. Thus, it is important to know how the properties of the immobilizing material affect the transport of various solutes.

Table 2.3 Applications of various encapsulation techniques[1].

Method/material	Cell
Pre-gel dissolving	
Alginate/poly-L-lysine/alginate	*E. Coli*
Alginate/poly-L-lysine/polyethyleneimine	Rat pancreatic tissue
Liquid droplet forming	
Polyacrylate	Hybridoma cell
Calcium alginate	Monkey kidney cell
Interfacial polymerization	
Chitosan/hexamethylene diisocyanate	*Lactobacillus casei*
Gelatine/toluene2,4diisocyanate	*Lactococcus lactis*
Coacervation	
Collodion	*Erythrocyte hemolysate*

[1] **Adapted from Park and Chang (2000)**

2.8 Characteristics of Calcium Alginate Capsules

An important aspect in immobilized cells is the choice of the support material and the immobilization procedure. Materials and procedures should be compatible with the biocatalyst and the process, i.e. the immobilization procedure should be mild, diffusion of substrates and products in the support material should be relatively easy and the support material should be stable during the process.

Successful application of an immobilization material is generally measured in terms of its restriction to the diffusion of solutes into and out of the gel. Thus, it is important to

know how the properties of the immobilizing material will affect the transport of solutes through the gel.

Alginate is used because of its non-toxicity and ability to form hydrogels under mild conditions. Alginates are linear chains of (1-4)-linked monomers of β-D-mannuronic acid and α-L-guluronic acid extracted from seaweed. There are different sequences and compositions of these monomers in the various available types of alginates. The monomers occur in the alginate chain in blocks. The regions are referred to as M blocks for poly(mannuronic acid), G blocks for poly(guluronic acid), and MG blocks for poly(mannuronic-guluronic acid) (Figure 2.3). The differences in the nature of the linkage between M blocks and G blocks are reflected in the conformation of these sections in the polymer chain. The M block section is flat while the G block section is buckled (Clare et al. 1993), which causes the alginate polymer to exhibit different chain flexibility in solution.

The difference in flexibility comes from the restriction about the carbon-oxygen bonds joining the monomers (Amsden and Turner, 1999). Moreover, alginate with high M content is more flexible in solution than alginate with high G content (Whittington, 1971). Also, the greater the content of guluronic acid monomers in the alginate, the stronger and more brittle the gel is (Clare et al. 1993).

Work done by Martinsen et al. (1992) showed that the greater the M content of the polymer, the greater the retardation to movement through the gel the solute experiences. In contrast, the work done by Amsden (1998) showed that the greater the G content of the gel, the greater the restriction to solute transport.

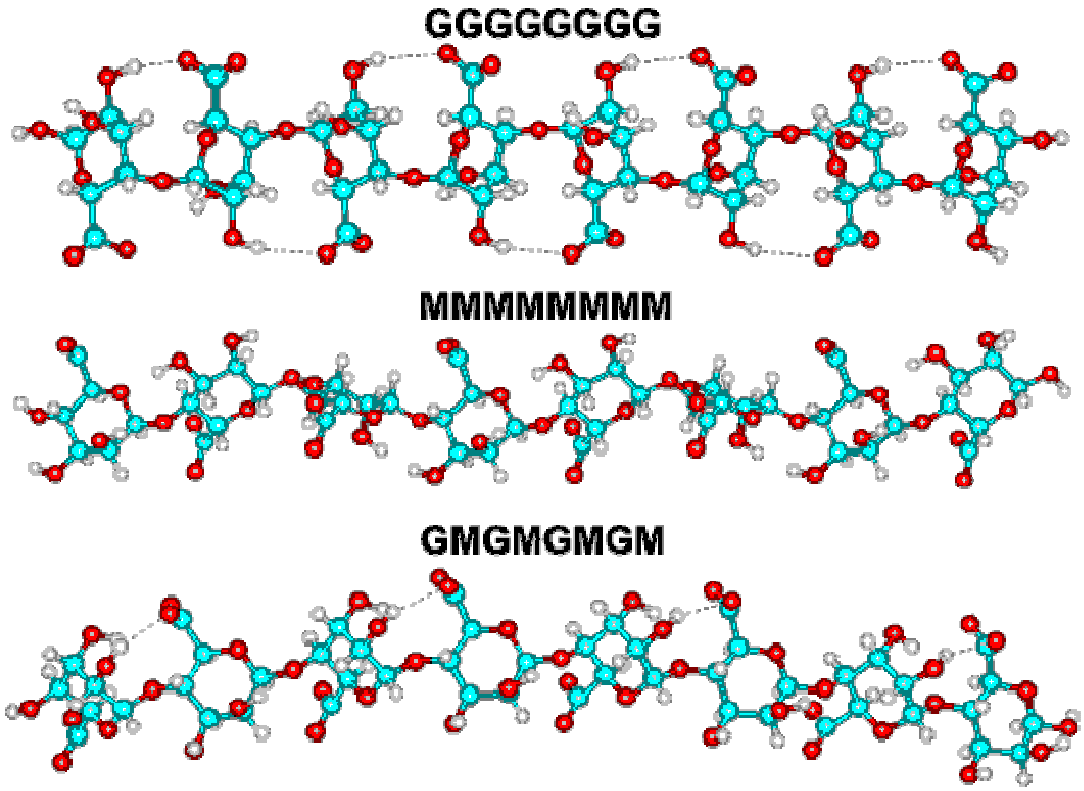

Figure 2.3. Structure of alginate. MM are mannuronic- mannuronic acid blocks, GG are guluronic- guluronic blocks, and GM are mannuronic-guluronic blocks. Adapted from Amsden and Turner (1999)

The restriction to solute transport in terms of alginate structure was explained more thoroughly in the work of Amsden and Turner (1999). The effect of alginate type, and thus alginate polymer chain flexibility, on solute diffusivity was investigated. It was found that diffusivity within the alginate gels decreased as the polymer flexibility decreased (which decreases with greater polymer G content). Hence, the greater the polymer flexibility, the greater the solute diffusivity within the gel.

2.9 Other Applications of Alginate Gels

In addition to their use in cell immobilization, alginate gels have wide applications in numerous fields. For example, highly purified forms of alginate are used in the manufacturing of antacid tablets such as the non-prescription medication Gaviscon®

which is produced under patent by GlaxoSmithKline Inc. When taken by mouth the combination of the alginic acid and bicarbonate creates a foam barrier that floats on the stomach acid.

In the textile industry, alginate is used as a holding fiber. When the cloth is finished, the alginate is dissolved leaving an open area. It is also used in the food industry, such as thickening soups and jellies. Calcium alginate is also used in different types of medical products, including wound dressings. For example, calcium alginate dressings gave significant protection against infection by pathogenic isolates of *Agrobacterium tumefaciens* (Deacon et al., 1988). Calcium alginate acted by physically excluding the pathogen from wound surfaces.

2.10 Calculation of Diffusion Coefficient

Diffusion is the process by which concentration gradients in a solution spontaneously decrease due to the Brownian motion of the individual molecules (Pfeifer et al., 1950).

The substrate conversion rate of most immobilized cells depends not only on the availability of substrates and microbial kinetics, but on the diffusion rate of metabolites through the immobilized cell system as well (Pu and Yang, 1988). Therefore, characterization of the internal mass transfer properties may be considered essential for the modeling, design and scale-up of immobilized cell systems.

Microorganisms that are entrapped in gel beads behave similarly to suspended cells (Nguyen and Luong, 1986). Immobilization by entrapment often does not affect the immobilized cells and, therefore, cells exhibit the same kinetics as suspended cells. Observed differences are generally caused by diffusion limitations (Westrin, 1990.). As a

result, the kinetics of immobilized cells are apparently different from suspended cells. These differences are usually attributed to mass transfer phenomena.

Resistance to mass transfer is, in fact, one of the most common problems imposed by the gel membrane around the liquid core, especially when diffusion of large substrates is involved (Nguyen and Luong, 1986). Mass transfer of a substrate to a reaction site or of product away from it may be impeded and reactor performance may suffer significantly. The diffusivities of various solutes in calcium alginate beads/capsules have been studied by several researchers (Nguyen and Luong, 1986; Pu and Yang 1988;Chai et. al., 2004). For example, Pu and Yang (1988) determined the diffusion coefficient of sucrose and yohimbine in calcium alginate beads. The results showed that cell-free beads showed little mass transfer resistance to both solutes. Therefore, the effective diffusion coefficient was affected significantly by the concentration of cells in the beads since, according to their results, diffusivity decreased sharply as the concentration of cells increased.

One of the most important factors that affect mass transfer, other than capsule size, is the pore size of the gel membrane (Nguyen and Luong, 1986; Chai et. al., 2004). Thus mass transfer can be enhanced by making the beads more porous. The drawback of such an attempt is the increased possibility of cell/biocatalyst leakage. The work done by Wan et al. (1992) showed that the hydrophile-lipophile balance (HLB) of some surfactants influenced the rate of drug release from alginate microspheres. However, no definite conclusion about the effect of HLB on diffusivity could be drawn.

Consequently, quantifying diffusivity (i.e. diffusion coefficient) is essential for predicting immobilized reactor system performance. Several experimental approaches may be used for the determination of diffusion coefficients of immobilized cell supports.

An ideal method for the measurement of diffusion coefficients in immobilized cell systems does not exist, so depending on the shape of the immobilized cell support and the required precision, the best method has to be chosen. These methods are grouped as steady-state concentration gradient methods, non-steady-state concentration gradient methods, and instrumental methods (Westrin et al., 1994).

The steady-state diaphragm cell is an example of the steady-state concentration gradient method (Westrin et al., 1994). In this method, a diffusional steady-state flux is set up through a gel diaphragm separating two liquid-filled, stirred compartments. The steady-state approach has a constant upstream and a zero downstream diffusant concentration, while the pseudo-steady-state diaphragm cell allows concentration variation in the compartments. The time-lag diaphragm cell, the uptake/release from gel bodies dispersed in a stirred solution, the concentration profile in a semi-infinite slab, and the finite couples methods are all examples of the non-steady-state concentration gradient methods (Westrin et al., 1994).

The time-lag diaphragm cell method uses similar equipment as the steady-state diaphragm cell method. The upstream diffusant concentration is constant, while the downstream compartment and the gel diaphragm are initially free from diffusant.

In the uptake/release from gel bodies dispersed in a stirred solution method, the decrease or increase in diffusant concentration in the liquid phase is measured, and the diffusion coefficient D is obtained. Applications include uptake or release from gel beads and cylinders or discs dispersed in a finite, stirred solution (Westrin, 1990).

Methods that use a concentration profile in a semi-infinite slab focus on the concentration profile inside an initially diffusant-free gel slab in contact with a liquid

solution containing the diffusant. Unlike all the methods presented above, the method of finite couples employs one single measurement of the mean concentration in an initially diffusant-free slab placed in contact with an initially diffusant-containing slab.

Instrumental methods used to calculate diffusion coefficients are often expensive, complicated, and require highly skilled technicians. Examples of such methods are: the Fourier transform pulsed-gradient spin-echo (FT-PGSE) method, the dynamic light scattering method, and the holographic relaxation spectroscopy method. These methods do not measure diffusion coefficient directly; rather they measure a parameter, like the frequency of scattered light in the dynamic light scattering method, and the diffusion coefficient is deduced.

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CHAPTER III
EFFECT OF NONIONIC SURFACTANTS ON THE DIFFUSIONAL PROPERTIES OF HYDROGEL CAPSULES

3.1 Abstract

Calcium alginate capsules were prepared using nonionic surfactants having various hydrophile-lipophile balance (HLB) values. The effect of the various nonionic surfactants on the diffusion of glucose and riboflavin through hollow capsules was investigated using the uptake release from gel bodies method developed by Nguyen and Luong (1986). The addition of surfactants increased the average thickness of the capsule membranes from 97 μm for control capsules to 148 μm for capsules made with a surfactant mixture having an HLB of 1.8. Moreover, the addition of surfactants increased the average diameter of capsules from 0.29 cm for controls to 0.35 cm for capsules made with a surfactant mixture having an HLB of 5.94. The addition of a surfactant with high HLB (16.7) increased the combined diffusion coefficient of glucose by 66% compared to controls. In contrast, the addition of a surfactant with low HLB (1.8) increased the combined diffusion coefficient of riboflavin by 65% compared to controls. The results show that diffusion through the membrane is the controlling factor for all cases tested and the increase in diffusion coefficients of glucose and riboflavin could be due to the increase in membrane porosity.

3.2 Introduction

Riboflavin (Vitamin B2), a water-soluble vitamin, is essential for converting food into energy and for the metabolism and growth of humans and animals. Riboflavin can be produced commercially by either chemical synthesis or fermentation processes. The microbial method has become the preferred method in recent years due to advances in metabolic and genetic engineering (Özbaş and Kutsal, 1991). Immobilization of cells and enzymes has also gained increasing interest in recent years. As a result, different methods of immobilization have been investigated. Immobilization based on chemical binding or physical retention are the two main types of immobilization, each with its own advantages and limitations. Various methods for immobilization by physical retention exist in the literature (Hartmeyer, 1988; Brodelius & Mosbach, 1982, Cheetham et al., 1979). Among them, immobilization by calcium alginate is generally preferred because of the mild immobilization conditions, rapid gelation, low cost, and simplicity of preparation.

Cells can be immobilized by calcium alginate either by entrapment inside a lattice-type structure or by encapsulation inside a spherical shell that consists of a cell-free calcium alginate membrane. The major advantage of encapsulation over entrapment on a solid matrix is that cells are free to grow inside the liquid core structure while allowing a more uniform substrate/cell contact (Chai et. al, 2004). One common problem imposed by the gel membrane around the liquid core is the resistance to mass transfer, especially when diffusion of large substrates is involved (Nguyen and Luong, 1986). Mass transfer of a substrate to a reaction site or of product away from it may be impeded and reactor

performance may suffer significantly. Consequently, quantifying diffusion is essential for predicting immobilized reactor system performance.

One of the most important factors that affect mass transfer, other than capsule size, is the pore size of the gel membrane (Nguyen and Luong, 1986; Chai et. al., 2004). Thus mass transfer can be enhanced by making the beads more porous. The drawback of such an attempt is the increased possibility of cell/biocatalyst leakage. Work done by Wan et al. (1993) showed that the hydrophile-lipophile balance (HLB) of some surfactants influenced the rate of drug release from alginate microspheres. However, no definite conclusion about the effect of HLB on diffusivity could be drawn.

The main purpose of this work was to examine the effect of HLB of various surfactants on the diffusivity of riboflavin and glucose through hollow calcium alginate/chitosan capsules. Diffusion coefficients of riboflavin and glucose through hollow capsules have not been previously reported.

3.3 Experimental Methods

Experimental Setup

Batch experiments were performed to evaluate the effects of nonionic surfactants on diffusion of glucose and riboflavin through calcium alginate capsules. Parameters of interest were diffusion rates of glucose and riboflavin, membrane thickness and diameter of capsules, and surface texture of capsules. Riboflavin was added to the $CaCl_2$/Chitosan/PEG mixture prior to mixing with cells and riboflavin release rates were measured. These data were used to calculate the diffusion coefficient of riboflavin from capsules. Finally, reduction of glucose concentration in solution was measured with time as well and these data were used to measure glucose diffusion into the capsules.

The nonionic surfactants used in all experiments were Tween 20, Tween 85, and Span 85 plus mixtures of Span 85 and Tween 85. Table 3.1 shows the surfactants used.

Table 3.1 Nonionic surfactants used in the diffusion experiments and their corresponding hydrophile-lipophile balance (HLB) values. 20 mL of each mixture was added to 1000 mL Na-alginate to make a 2% v/v concentration.

	HLB			
	1.8	**6**	**11**	**16.7**
% w/w of mixture	**100% Span 85**	**55% Span 85 + 45% Tween 85**	**100% Tween 85**	**100% Tween 20**

Yeast Culture Maintenance

Candida famata (ATCC ID# 20850) obtained from the American Type Culture Collection, was maintained on malt extract/agar plates and subcultured every 2 weeks to insure viability.

Capsule Preparation

Work done by Pu and Yang (1988) showed that cell-free beads showed little mass transfer resistance to both solutes. According to their results, the effective diffusion coefficient was affected significantly by the concentration of cells in the beads. Therefore, a cell concentration of 10% (w/v) was used in our experiments.

To prepare the capsules, procedures were similar to those of Yu et al. (2004). After harvesting by centrigufation (1500xg), cells were mixed with a calcium chloride/chitosan/PEG/riboflavin solution. Chitosan (Fisher Scientific PA, U.S.A.) was dissolved to a final concentration of 3% w/v in a 2% w/v $CaCl_2$ (Sigma-Aldrich MO, U.S.A.) solution containing 30% w/v PEG 6000 (Fisher Scientific PA, U.S.A.) and the pH was adjusted to 5.5 with a few drops of 9.5 M NaOH solution. PEG 6000 was used to adjust the viscosity of the $CaCl_2$/chitosan and ensure that the capsules were spherical in

shape. Riboflavin (50 mg/L) was added to the final solution prior to extrusion. The 2% w/v alginic acid solution was prepared by dispersing 20 g of alginate (Sigma-Aldrich MO, U.S.A.) in enough deionized water to make 1L. The $CaCl_2$/chitosan/PEG solution containing the cells was extruded dropwise via a 30 mL syringe equipped with a 26G needle into the stirred alginate solution containing 2% (v/v) of a nonionic surfactant. After a reaction period of 20 min, the beads were collected and washed with deionized water and hardened for 15 min in 1% $CaCl_2$ solution.

Capsule Measurement

The average diameter of the capsules was measured by lining up 20 capsules along a ruler (Wijffels et al., 1995). To determine the membrane thickness, the capsules were freeze-dried, cut in half using a blade scalpel (Fisher Scientific PA, U.S.A.), transferred to a scanning electron microscope (JEOL-JSM 6360, Japan), and the membrane thickness was measured in three different locations. To investigate the effect of surfactants on porosity, three capsules were randomly taken and prepared for SEM as mentioned above. Photos of the surface texture (×30) as well as membrane thickness (×200 to ×220) were taken at various locations.

Riboflavin Concentration

To determine the initial concentration of riboflavin (Fisher Scientific PA, U.S.A.) in the capsules (the initial concentration of riboflavin, C_0, was different in each case depending on encapsulation efficiency), capsules were dissolved in a 5% w/v solution of sodium hexametaphosphate (Buzzini and Rossi, 1997). The solution was then centrifuged at 1750 g, and a 2 mL sample was collected and filtered through a 0.2 μm membrane filter (Whatman Inc. NJ, U.S.A.). The concentration of riboflavin was measured by

absorbance at 446 nm where 0.5 mL was withdrawn from the flask and transferred to a 2 mL centrifuge tube containing 0.5 mL deionized water. The tube then was centrifuged for 5 min at 1500 rpm and the supernatant was carefully withdrawn and filtered into a 2 mL cuvette. Absorbance readings were taken at 446 nm using a UV-vis spectrophotometer (Cary 50 Bio, Varian Inc., U.S.A.) and concentration of riboflavin was determined using a calibration curve.

Glucose Concentration

To determine the concentration of glucose in solution, samples were prepared as above. The concentration was determined by a liquid chromatograph (Agilent technologies, U.S.A.) equipped with a refractive index detector (RID-G1362A). Separation was achieved with a Biorad Aminex® HPX-87P ion exchange column (85°C). Mobile phase was deionized water at 0.6 mL/min.

Diffusivity Measurements

Diffusivity measurements were conducted at 30°C in 250 mL Erlenmeyer flasks equipped with three baffles. Temperature was maintained at 30°C using a Fisher Isotemp® incubator (Fisher Scientific PA, U.S.A.). The flask containing 30 g/L glucose (50 mL liquid volume) was placed on an orbital shaker at 200 rpm. A determined number of capsules was suspended in the substrate solution such that the number of capsules chosen gave a capsule-to-liquid ratio of 0.35 w/v. Due to the concentration gradients, glucose diffused into the capsules and riboflavin diffused out. The diffusion coefficients of glucose and riboflavin were determined from their change in the concentrations.

The concentration of a solute due to its diffusion into a solute-free spherical solid bead ($C_{(t)}$) is given by Crank (1956) as follows:

$$C(t) = \frac{\alpha C_0}{1+\alpha}\left(1 + \sum_{n=1}^{\infty} \frac{6(1+\alpha)e^{\left(\frac{-D_e q_n^2 t}{r^2}\right)}}{9(1+\alpha)+q_n^2\alpha^2}\right)$$

where r is the radius of the sphere, t is time, D is the diffusion coefficient, α is the ratio of the liquid volume to bead volume, and q_n is the positive nonzero root of:

$$\tan q_n = \frac{3q_n}{3+\alpha q_n^2}$$

Similarly, the concentration of a solute due to its diffusion from the bead into a solute-free solution is given by:

$$C(t) = \frac{C_0}{1+\alpha}\left(1 - \sum_{n=1}^{\infty} \frac{6\alpha(1+\alpha)e^{\left(\frac{-D_e q_n^2 t}{r^2}\right)}}{9(1+\alpha)+q_n^2\alpha^2}\right)$$

When it comes to hollow capsules, however, the diffusivity through their membranes (D_1) is different than that through the liquid core inside the capsule (D_2). Diffusion in this case is more complex. Therefore, the capsule is assumed as a solid sphere and the diffusion coefficient (D) in equations 1 and 3 can be substituted by a combined diffusion coefficient D_m (Chai et al., 2004). D_m is defined as the combination of diffusivity in the membrane and diffusivity in the liquid core and the relationship between D_m, D_1, and D_2 is given as (Chai et al., 2004):

$$D_m = \frac{r_b}{\frac{r_b - r_a}{D_1} + \frac{r_a}{D_2}}$$

Where r_a and r_b are the internal and external radii respectively. It is assumed also that the diffusion process in the liquid core of the capsules is considered as that in pure water hence, $D_2 = D_w$. Using this assumption, D_1 can be calculated after D_m has been

determined. The values of D_w for glucose and riboflavin are 3.77×10^{-4} and 4.34×10^{-4} cm^2/min respectively (Yamamoto et al., 2005).

Statistical Analysis

Statistical comparison was performed using *Student's t-test* for the comparison of two means. A significance level of 0.05 was chosen. Microsoft Excel® was used to calculate the means and standard deviation.

3.4 Results and Discussion

Diffusion Measurements

Diffusion of glucose and riboflavin through capsules is shown in Figures 3.1 and 3.2 respectively. Each experiment was run in triplicate. Figures 3.1 and 3.2 show that concentration of the bulk solutions decreased sharply immediately after the addition of capsules, then after 10 minutes the decrease gradually leveled off. Figure 3.1 also shows that the relative glucose concentration was the lowest in the flasks containing Tween 20 (HLB 16.7).

Diffusion coefficients were calculated using Mathematica 5.2 (2005). The average values of D_m for glucose and riboflavin are shown in Figures 3.3 and 3.4 respectively. Figure 3.3 shows that capsules made with Tween 20 (HLB 16.7) had the highest glucose diffusivity.

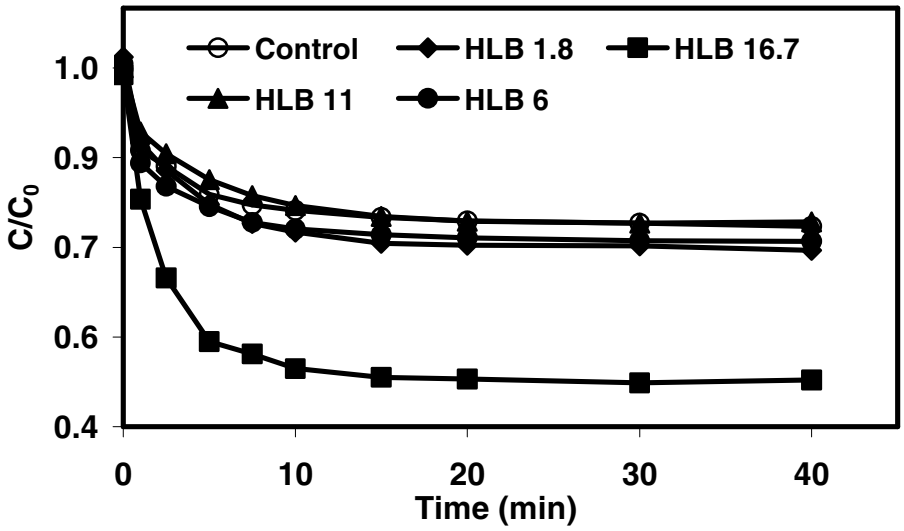

Figure 3.1. Diffusion of glucose into capsules made with surfactants having different HLB values. Values shown are the average of three experiments. C is glucose concentration in solution at time t. C_0 is glucose concentration at time 0.

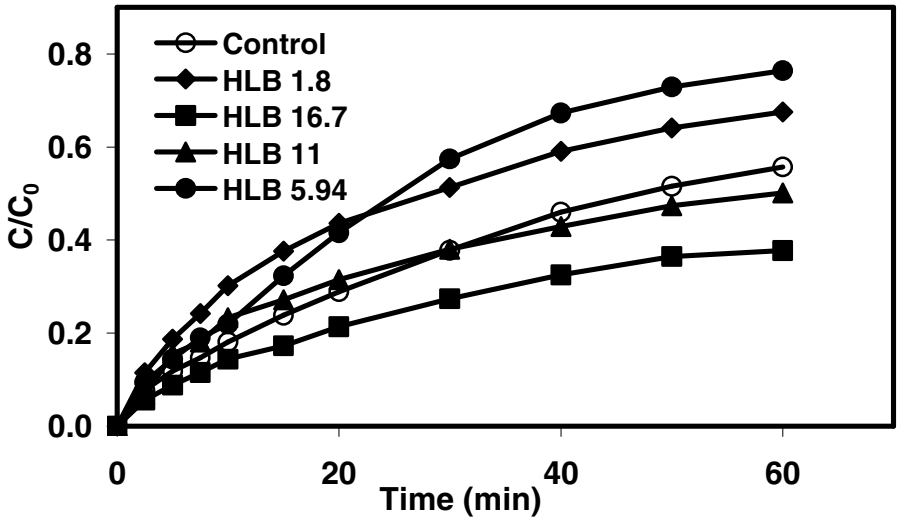

Figure 3.2. Diffusion of riboflavin away from capsules made with surfactants having different HLB values. Values shown are the average of three runs. C is riboflavin concentration in solution at time t, C_0 is riboflavin concentration at time 0.

For riboflavin (figure 3.4), the highest diffusivity was observed in capsules made with Span 85 (HLB 1.8). In the case of riboflavin, the combined diffusion coefficient ranged from 15.1% (HLB 16.7) to 80.8% (HLB 1.8) of its diffusion in water (4.34×10^{-04}

cm^2/min, (Yamamoto et al., 2005) with the control having a statistically higher diffusion coefficient than that of HLB 16.7.

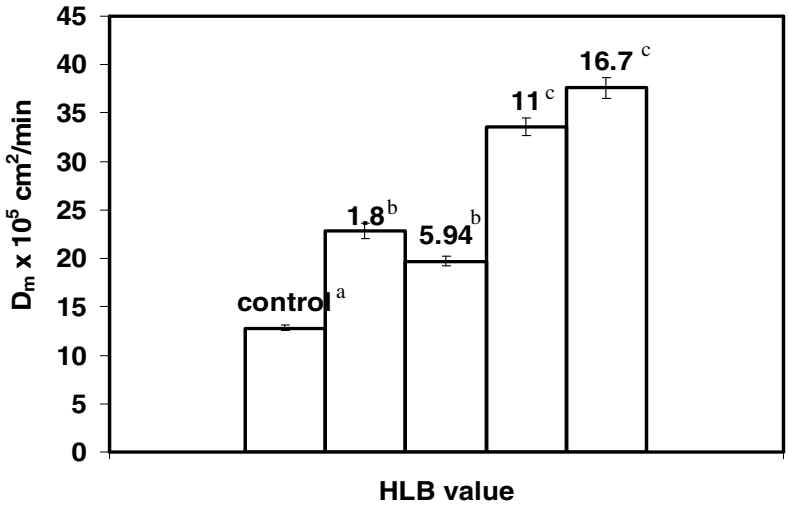

Figure 3.3. Effects of hydrophile-lipophile balance on combined diffusion coefficient of glucose. Data points are averages of triplicates. Error bars = standard deviation. Superscripts having the same letter are not significantly different ($\alpha = 0.05$).

Tables 3.2 and 3.3 show the combined (D_m) and membrane (D_1) diffusion coefficients of glucose and riboflavin. Diffusivity through the membrane (D_1) for glucose (Table 3.2) and riboflavin (Table 3.3) is much lower than the combined diffusion, which means that diffusion through the membrane is the controlling factor for both solutes.

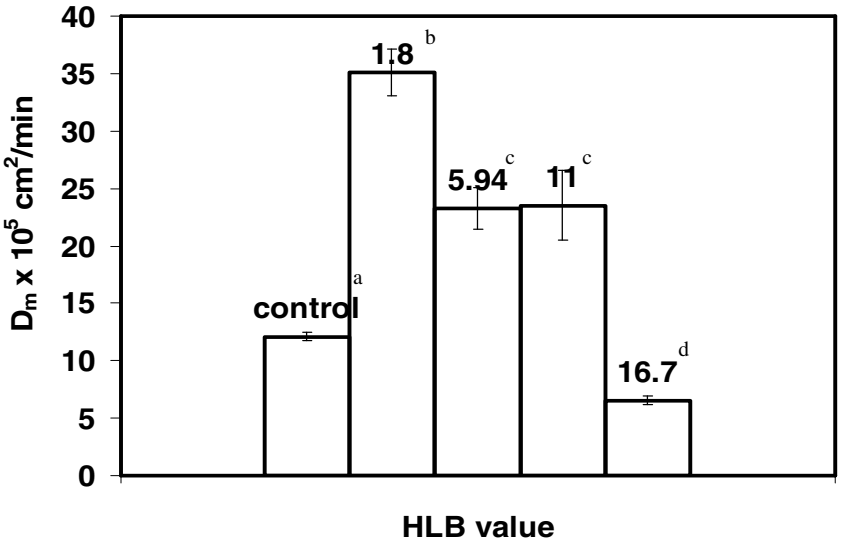

Figure 3.4. Effects of hydrophile-lipophile balance on combined diffusion coefficient of riboflavin. Data points are averages of triplicates. Error bars = standard deviation. Superscripts having the same letter are not significantly different ($\alpha = 0.05$).

Table 3.2. Diffusivity of glucose into Ca-alginate capsules made with surfactants having various hydrophile-lipophile balances. Values shown are the average of triplicates. D_1=diffusion coefficient through membrane (cm^2/min), D_m=combined diffusion coefficient (cm^2/min)

	HLB level				
	control	1.8	5.94	11	16.7
D_m	1.28E-04	2.29E-04	1.97E-04	3.35E-04	3.76E-04
D_1	8.59E-06	2.15E-05	1.49E-05	2.58E-05	2.86E-05

Table 3.3. Diffusion of riboflavin from Ca-alginate capsules made with surfactants having various hydrophile-lipophile balances. Values shown are the average of triplicates. D_1=diffusion coefficient through membrane (cm^2/min), D_m=combined diffusion coefficient (cm^2/min)

	HLB level				
	control	1.8	5.94	11	16.7
D_m	1.21E-04	3.51E-04	2.32E-04	2.35E-04	6.56E-05
D_1	1.10E-05	1.02E-04	4.00E-05	3.36E-05	6.05E-06

As the $CaCl_2$/chitosan mixture is extruded drop-wise into Na-alginate solution, Ca^{2+}/chitosan immediately reacts with alginate molecules at the droplet surface. As more

contact time is allowed, more Ca^{2+}/chitosan diffuses outward and reacts to form the insoluble membrane. The presence of surfactants in the Na-alginate solution during the formation of capsules may have caused more or less resistance (depending on the HLB number of the surfactant) to subsequent Ca^{2+}/chitosan, resulting in a Ca^{2+}/chitosan gradient as the reaction progressed outward. As the resistance increases (with different HLB number) the gradient increases and the structure becomes less uniform and more porous.

Capsule Measurement

The average values of membrane thickness for capsules prepared with various nonionic surfactants are shown in Figure 3.5. For all cases tested, capsules made with surfactants had thicknesses larger than the control with the largest being for HLB 1.8. A statistical comparison of means showed that the thicknesses of all capsules made with surfactants were significantly larger than the control ($\alpha = 0.05$).

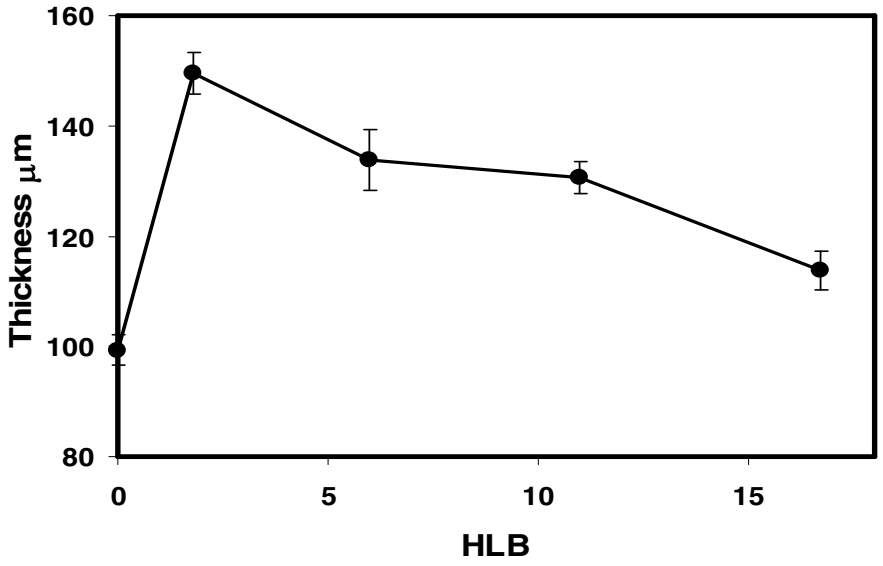

Figure 3.5. Effects of hydrophile-lipophile balance on thickness of calcium alginate capsules. Data points are the average of triplicate measurements. Error bars = standard deviation.

Figure 3.6 shows the diameter of capsules prepared with various nonionic surfactants. The addition of surfactants increased the average diameter of capsules with the largest being for HLB 5.94.

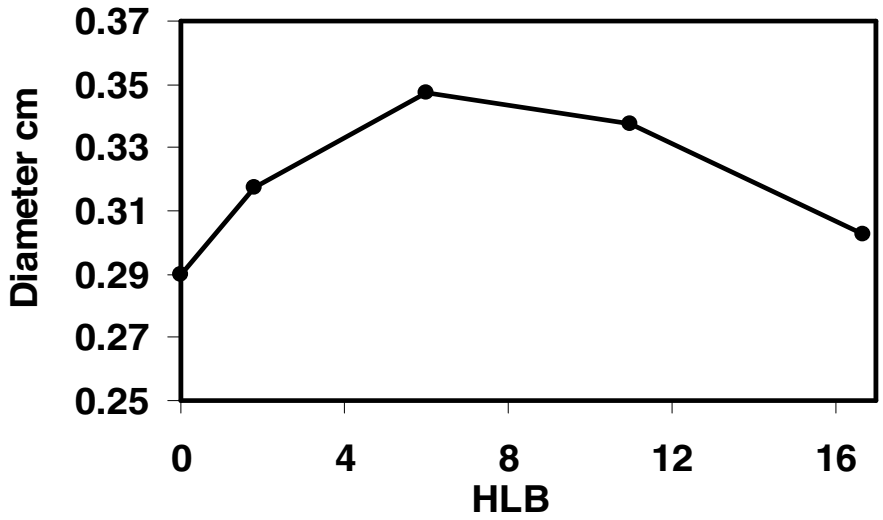

Figure 3.6. Effect of hydrophile-lipophile balance (HLB) on diameter of calcium alginate capsules. Each data point is obtained by lining up 20 capsules along a ruler.

Capsule Structure

Figure 3.7 shows SEM photographs of capsules made with and without surfactants. Figures 3.7(a) and 3.7(b) are for whole capsules, and it can be clearly seen that the control (3.7a) has a smoother surface while Figure 3.7(b) (with surfactant) has an irregular porous structure. Figures 3.7(c) and 3.7(d) show cross-sections of capsules with and without surfactants. It appears that the membrane of the control (3.7c) is denser and more uniform while that of 3.7(d) (with surfactant) is less uniform.

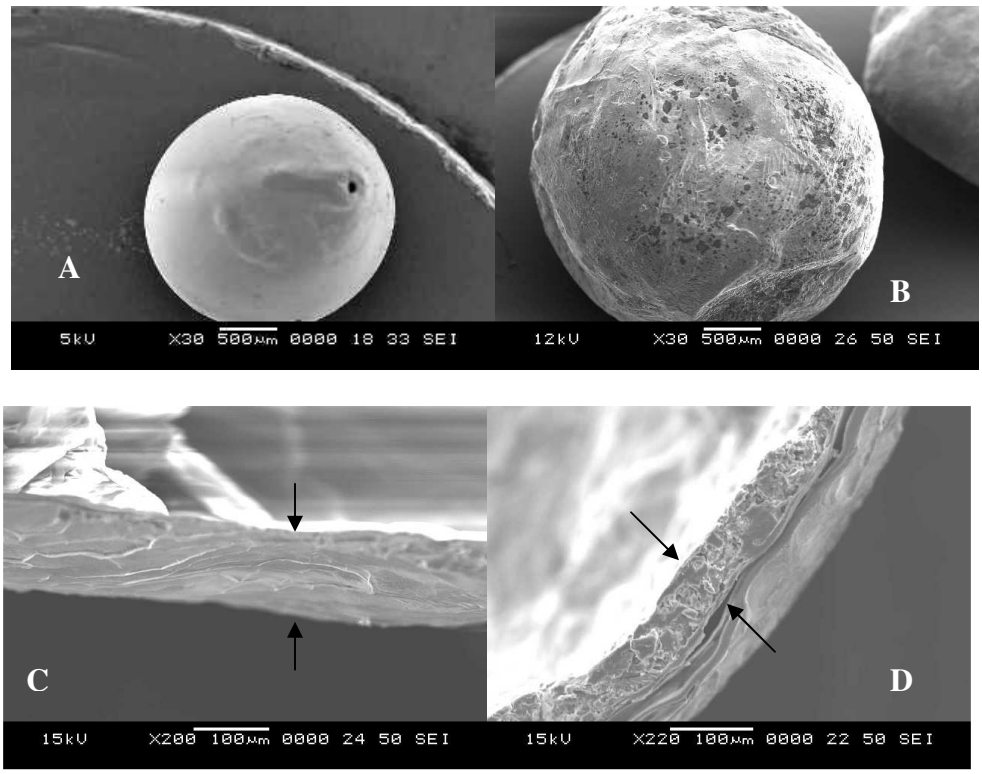

Figure 3.7. SEM photograph of Ca-alginate capsules made with and without added surfactant having HLB 16.7. (a) Whole capsule control, (b) whole capsule with surfactant, (c) cross-section control, (d) cross-section of capsule with surfactant.

Similar observations were made for capsules containing surfactants with other HLB levels as well. Since a more porous network structure will result in larger pores, this might explain the increase in glucose and riboflavin diffusion.

3.5 Conclusion

Results indicate that the diffusion coefficient of glucose through a calcium alginate membrane was highest (6.05×10^{-6} cm^2/min) in capsules made with Span 85 (HLB 1.8). In contrast, the diffusion coefficient of riboflavin through a calcium alginate membrane was highest (8.59×10^{-6} cm^2/min) in capsules made with Tween 20 (HLB 16.7). Reported diffusion coefficients of glucose through hollow capsules in the literature are consistent but slightly higher than our data. This could be due to the addition of chitosan in our experiments.

The addition of surfactants that are more oil soluble (low HLB) during capsule preparation increased the diffusion coefficient of riboflavin away from capsules, while the addition of surfactants that are more water soluble (high HLB) increased the diffusion coefficient of glucose into the capsules. The effects of surfactants on the production of microcapsules have been previously investigated (Wan et al., 1993), and their study showed that the use of surfactants increased the rate of drug release. These results agree with those of Wan et al. (1993).

The addition of surfactants in general increased the average thickness of capsule membranes. The thickest membrane (148.6 μm) was for capsules made with Span 85 (HLB 1.8), which is an increase of 34% over controls (97.5 μm). Similarly, the addition of surfactants increased the average diameter of capsules. The largest diameter (0.348 cm) was for capsules made with a mixture of 55% Span 85 + 45% Tween 85 (HLB 6). This is an increase of 16% over controls (0.29 cm). Finally, the addition of surfactants resulted in capsules that have an irregular porous surface and a less uniform membrane. In contrast, the controls had a smoother surface and a more uniform membrane. Taking all these observations into consideration, although all surfactants tested increased membrane thickness and diameter of capsules, their addition may have rendered the membrane more permeable by making the capsules more porous, thus increasing solute diffusion through it.

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CHAPTER IV
CONTINUOUS RIBOFLAVIN PRODUCTION BY *CANDIDA FLARERI* IMMOBILIZED IN SURFACTANT-MODIFIED CALCIUM ALGINATE CAPSULES

4.1 Abstract

The performance of three immobilized yeast bioreactors producing riboflavin using hollow calcium alginate capsules was investigated. Capsules were prepared with and without the addition of nonionic surfactants. Both riboflavin production and glucose consumption occurred at faster rates with immobilized cells than when free cells were used. The utilization rate of glucose for capsules made with HLB 1.8 and HLB 16.7 were 16.5 and 15.5 g/L.d, respectively, while that of freely suspended cells was found to be 6.1 g/L.d. The effect of increasing dilution rates on reactor performance was studied as well. The highest riboflavin level in the suspended cell system was 720 mg/L with a dilution rate of 0.02 h^{-1}. However, riboflavin production decreased sharply when dilution rate increased to 0.03h^{-1}. For the control system (capsules made without addition of surfactants), higher riboflavin production (770 mg/L) at a dilution rate of 0.02 h^{-1} was observed and no significant decrease in the productivity of riboflavin was observed at higher dilution rates. Riboflavin productivity for systems made with Span 85 (HLB 1.8) and Tween 20 (HLB 16.7) was higher than the control system. Results suggest that the reactors having capsules made with Span 85 (HLB 1.8) and Tween 20 (HLB 16.7) were superior to the free cell and control systems at higher dilution rates.

4.2 Introduction

The major limiting factor in the development of continuous biological reactors is the inability to maintain stable and viable cell cultures for significant periods of time (Shin et al., 2004). In the bioprocessing industry, a major problem is the low cell density and low productivity observed with conventional cell suspension culture. Furthermore, many products of cells, especially mammalian cells, are produced and secreted in the stationary phase of the culture (non-growth associated) (Cunha et al., 2007). The application of cell immobilization on or within a solid matrix has several advantages in biochemical reactors. In general, these systems improve stability, increase cell densities more than is possible in normal suspension cultures, and enable long-term continuous processes resulting in potentially high reactor productivities (Mandavilli, 2000).

Takamitsu et al., (1993) showed that ethanol production in an immobilized cell reactor using *Z. mobilis* was doubled compared to a similar suspended reactor system. In addition, work done by Najafpour (1990) showed that if undisturbed microbial cells are directly immobilized, the recovery of microorganisms from downstream product can be eliminated and the loss of cellular activity can be kept to a minimum level.

The substrate conversion rate of most immobilized cells depends not only on the availability of substrates and microbial kinetics, but on the diffusion rate of metabolites through the immobilized cell system as well (Najafpour, 1990). Mass transfer of a substrate to a reaction site or of product away from it may be impeded and reactor performance may suffer significantly.

Increasing dilution rates in continuous bioreactors generally increases production rate (Takamitsu et al., 1993). However, as dilution rate approaches a certain percentage of the

maximum specific growth rate (μmax) cell wash-out occurs in suspended growth systems. This problem is not an issue in attached growth systems as long as dilution rate does not exceed the diffusion rate of substrate (Mandavilli, 2000). Thus, if substrate diffusion rate can be enhanced, greater dilution rate can be achieved and, in turn, better productivity can be obtained.

The purpose of this study was to develop an immobilized yeast bioreactor to produce riboflavin using calcium alginate capsules. Some of the capsules were prepared with added surfactants, since previous work has shown that the addition of nonionic surfactants to capsules increases substrate and product diffusivity. Surfactants were chosen with relatively low or high hydrophile-lipophile balance (HLB) values. Three different immobilized systems were investigated: a control, a system with capsules made with the addition of Span 85 (HLB 1.8), and a system with capsules made with the addition of Tween 20 (HLB 16.7). Fermentation efficiencies of the immobilized systems were compared, along with a suspended growth system.

4.3 Materials and Methods

Experimental Setup

Four different reactor systems were compared in both batch and continuous modes; three immobilized systems and one suspended cell system. Calcium alginate capsules were prepared with two different types of surfactant, plus a control with no surfactant. During each run, dilution rate was incrementally increased and glucose uptake and riboflavin production were monitored. The increased diffusivity of capsules should enable the reactor to operate at relatively higher dilution rates without loss of efficiency.

Organism

Cells of the yeast *Candida flareri* ATCC # 20850, provided by the American Type Culture Collection, were used in this study. The strain was maintained on YM Agar (Fisher Scientific, U.S.A.) plates, which were stored at 4 ºC after growth and subsequently transferred to a freshly prepared agar plate every 3 weeks.

Growth Medium and Growth Reactor

A loopful of colony from the agar plate was aseptically transferred to 250 mL Erlenmeyer flasks containing 100 mL of YPD Broth (Fisher Scientific, U.S.A.). The inoculum was cultivated in a rotary shaker at 200 rpm at 26 ºC for 3 days before harvesting. Afterward, the cells were collected by centrifugation (2000 ×g for 15 minutes), rinsed with sterile distilled water, centrifuged, and resuspended in sterile distilled water to yield a suspension with high cell concentration. Yeast harvested from the flasks was used to inoculate a growth reactor. This strategy ensured that a source of viable yeast was readily available.

Figure 4.1 shows the growth reactor configuration. The process was carried out in a 5L glass bottle with 3L liquid volume. Agitation speed (200 rev/min) was provided by a magnetic stirrer (Fisher Scientific, U.S.A.), temperature (26 °C) was maintained by heat tape and temperature sensors, humidified sterile air flow was provided through a glass fiber diffuser, and antifoam (0.1% w/v of antifoam solution B, Fisher Scientific, U.S.A.) was added manually.

Production Medium

Production medium was similar to that of Buzzini & Rossi (1997). The medium contained the following in 1 L: 30 g glucose, 0.5 g KH_2PO_4, 0.5 g $CaCl_2.2H_2O$, 0.5 g

$MgSO_4.7H_2O$, 2 g $(NH_4)_2SO_4$, 0.1 g KI, 2 g asparagine, 2 g glycine, 0.01 mg B, 0.01 mg Mn, 0.07 mg Zn, 0.01 mg Co, 0.01 mg Mo, 0.01 mg Fe, 1 µg biotin. Final pH of the medium was adjusted to 6.5 with a few drops of sulfuric acid.

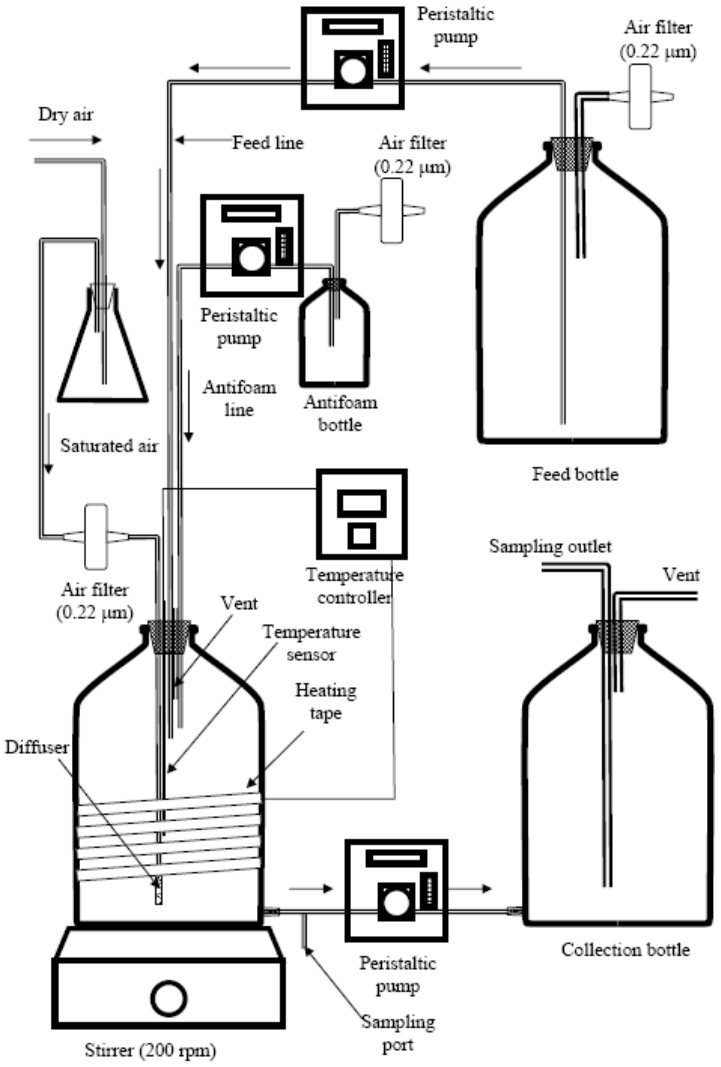

Figure 4.1. Growth reactor set-up

Preparation of Hollow Calcium Alginate Capsules

To prepare the capsules, procedures were similar to those of Yu et al. (2004). After harvesting, cells (10% w/v) were mixed with a calcium chloride/chitosan/PEG solution. Chitosan (Fisher Scientific PA, U.S.A.) was dissolved to a final concentration of 3% w/v in a 2% w/v $CaCl_2$ (Sigma-Aldrich MO, U.S.A.) solution containing 30% w/v PEG 6000 (Fisher Scientific PA, U.S.A.) and the pH was adjusted to 5.5 with a few drops of 9.5 M NaOH solution. PEG 6000 was used to adjust the viscosity of the $CaCl_2$/chitosan and ensure that the capsules were spherical in shape. The 2% w/v sodium alginate solution was prepared by dispersing 20 g of alginate (Sigma-Aldrich MO, U.S.A.) in enough deionized water to make 1L.

Three types of capsules were prepared and individually tested. The first type (control) was made without the addition of surfactants. The second and third types were capsules made with the addition of Span 85 (HLB 1.8) and Tween 20 (HLB 16.7) to the sodium alginate solution (2% v/v final concentration). The $CaCl_2$/chitosan/PEG solution containing the cells was extruded dropwise via a 30 mL syringe equipped with a 26G needle into the stirred alginate solution containing 2% (v/v) of a nonionic surfactant. After a reaction period of 20 min, the beads were collected and washed with deionized water and hardened for 15 min in 1% $CaCl_2$ solution. Each reactor was loaded with 10% w/v capsules.

Reactor Design and Operation

The general reactor design is shown in Figure 4.2. The process was carried out in a 3L glass fermenter with 2L liquid volume. Aeration was achieved via a fritted disk (40 μm bubble size) with mixing provided by the action of bubbles rising from the bottom

since density of capsules was close to that of water. Foam level, pH, and temperature were controlled using a BioFlo® 110 Modular Controller (New Brunswick Scientific, N.J., U.S.A.) and data were collected using the BioCommand® Plus software (New Brunswick Scientific, N.J., U.S.A.). At the beginning of each experimental run, the whole set-up was autoclaved for 15 min at 121 ºC and 15 psi. Each reactor was first operated in batch mode for 5 days until all glucose was utilized.

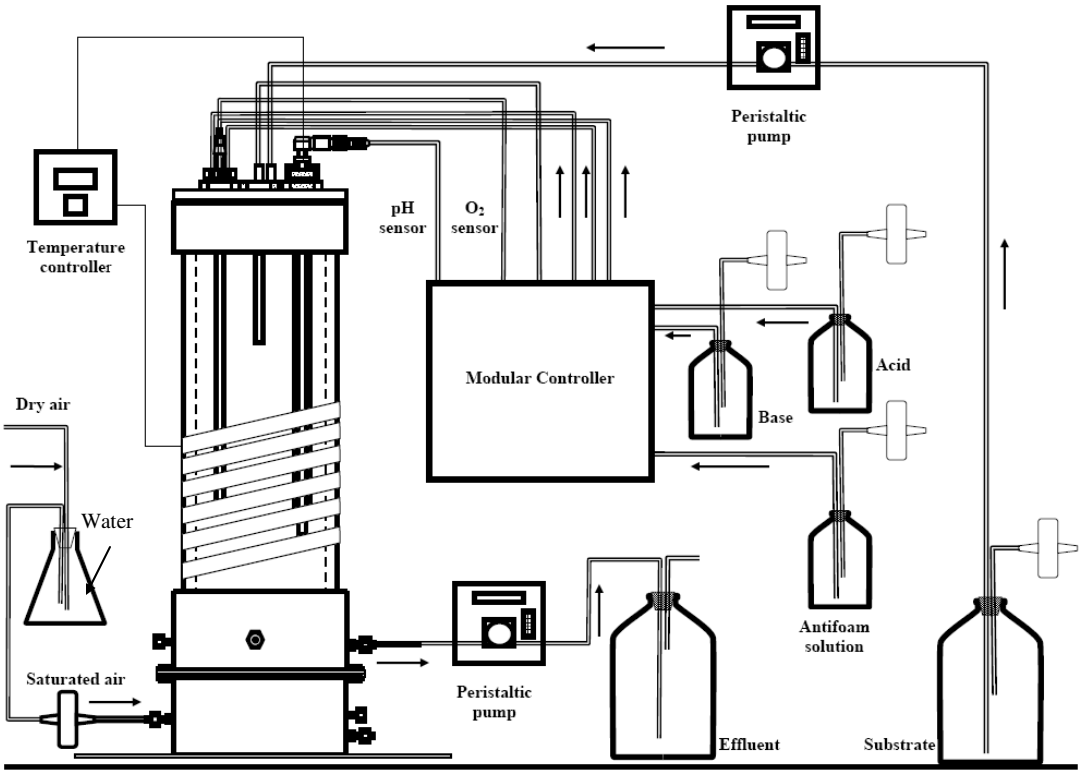

Figure 4.2. Production reactor set-up.

After the first 5 days, the fermenter was fitted with two peristaltic pumps connected to substrate and waste tanks, respectively, which provided both glucose feeding and effluent withdrawal. Dilution rate was varied (0.01, 0.02, 0.03, and 0.04 h^{-1}) to investigate the performance of the reactor under various operating conditions. Dilution

rates were increased only when steady state vitamin B2 (riboflavin) production was observed (when variation in riboflavin concentration was less than 3%).

Analytical Methods

A flowchart showing sample analysis and related procedures is shown in Figure 4.3. During each run, concentration of cells immobilized in the capsules and broth, glucose, dissolved O_2, riboflavin concentration, pH, and temperature were measured at specified intervals (once every 12 hrs for the first two days and once daily after that).

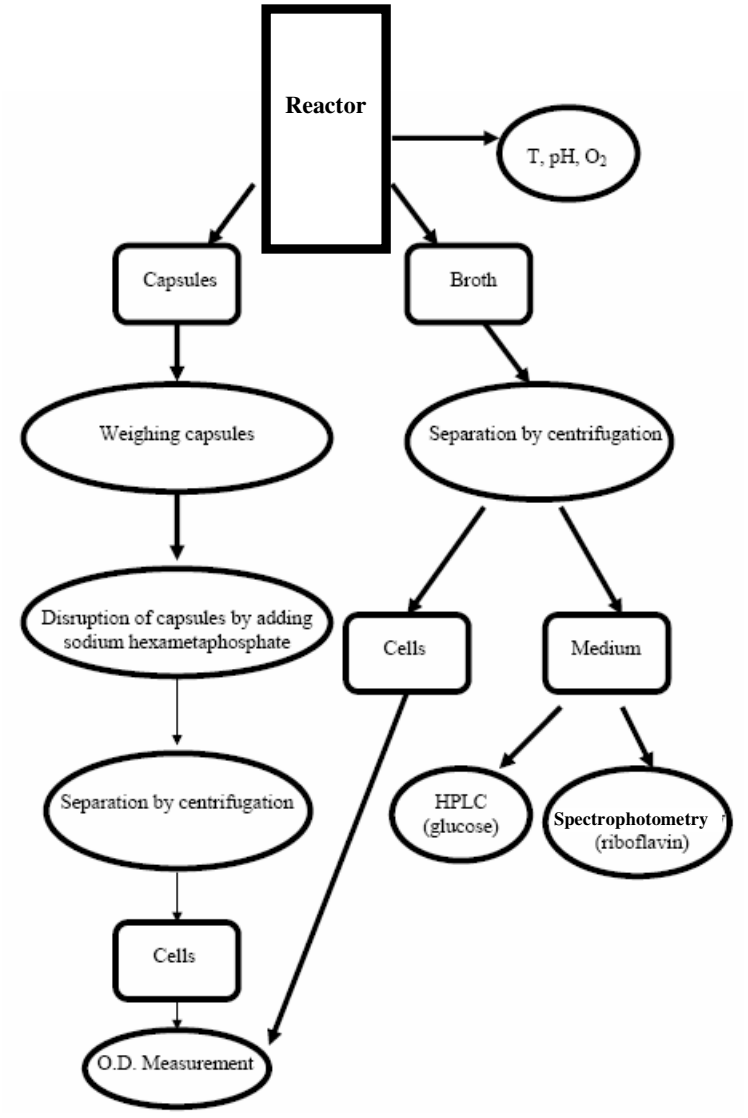

Figure 4.3 Flowchart showing procedures related to sample analysis.

Cell Measurement

Biomass concentration was determined by dry weight measurement (Buzzini and Rossi, 1997). Capsules were collected from the vessel and weighed. The capsules were then dissolved in 5 mL of 5% w/v solution of sodium hexametaphosphate $(NaPO_3)_6$ solution. Cells were harvested by centrifugation at 2000 ×g for 10 min at 4 ºC and washed twice and resuspended in 1mL deionized water. Cell concentration was determined by a UV-VIS light spectrophotometer (Cary 50 Bio, Varian Inc., Walnut Creek, CA) at 620 nm using a calibration curve obtained through correlation between cell dry weight and optical density. Cell concentration in the broth was estimated by the same method.

Riboflavin Measurement

To determine the concentration of riboflavin in solution, 1 mL samples were withdrawn from the vessel at the specified intervals. Samples were then centrifuged at 2000 ×g for 10 min at 4 ºC, and the supernatant was carefully withdrawn and filtered through a 0.2 μm membrane filter (Whatman Inc. NJ, U.S.A.) into a 2 mL cuvette. The concentration of riboflavin was measured by the colorimetric method (Buzzini and Rossi, 1997). Absorbance readings were taken at 446 nm using a UV-vis spectrophotometer and concentration of riboflavin was determined using a calibration curve prepared with riboflavin standards (Sigma-Aldrich MO, U.S.A.).

Glucose Measurement

To determine the concentration of glucose in solution, samples were prepared as above. The concentration was determined using the Agilent 1200 liquid chromatography system (Agilent Technologies, U.S.A.), equipped with a refractive index detector (RID-

G1362A). Separation was achieved with a Biorad Aminex® HPX-87P ion exchange column (85°C). The mobile phase was deionized water at 0.6 mL/min.

Kinetics

In a continuous suspended growth process, the net growth rate is equal to the dilution rate. The principal advantage of continuous culture is that the rate of dilution controls the rate of growth via the concentration of the growth-limiting nutrient in the medium. As long as the dilution rate is lower than the maximum growth rate attainable by the yeast culture, the cell density will increase to a point at which the cell division rate balances the cell death rate. This steady-state cell density is also characterized by a constancy of all metabolic and growth parameters. On the other hand, if the dilution rate exceeds the maximum cell growth rate (in suspended systems), then cells are removed faster than they are produced and total washout of the entire cell population eventually occurs. Therefore, it is necessary to quantify the maximum growth rate attainable by the yeast culture. This can be achieved by operating the reactor in batch mode first. Once the kinetic parameters are quantified, the reactor can be switched to continuous mode.

There are many models that relate specific growth rate to nutrition. The Monod equation is one such expression, which relates limiting nutrient concentration to a population's growth rate. The expression is: $\mu = \mu_{max} \frac{S}{K_s + S}$

where S = concentration of limiting nutrient (g/L)

μ = specific growth rate coefficient (h^{-1})

μ_{max} = maximum specific growth rate (h^{-1})

K_S = half saturation coefficient (g/L)

Once the substrate uptake vs. time data are collected, the above expression can be used to fit the data and calculate μ_{max}.

For a continuously fed bioreactor, the cells are continuously supplied with substrate at a growth limiting level, hence they remain in the exponential phase. The following expression describes change in cell concentration with time in continuous mode:

$$\frac{dX}{dt} = (\mu - D)X$$

where D = dilution rate (hr^{-1}) and X = cell concentration in the reactor

At steady-state, $\frac{dX}{dt} = 0$ and $\mu = D$

4.4 Results and Discussion

Batch Riboflavin Production

Four different reactors were studied, including a free (suspended) cell reactor, a reactor containing capsules made without addition of surfactant (control), a reactor containing capsules made with surfactant having HLB 1.8, and a reactor containing capsules made with surfactant having HLB 16.7.

Preliminary batch experiments were performed to investigate the optimum reactor operating conditions. Riboflavin production and glucose utilization profiles are shown in Figures 4.4 and 4.5, respectively.

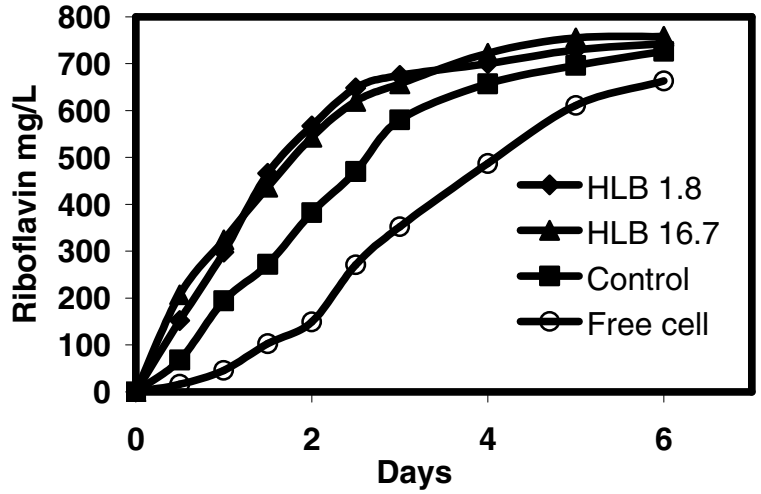

Figure 4.4. Batch riboflavin production for a reactor containing free (suspended) cells, a reactor containing capsules made without addition of surfactant (control), a reactor containing capsules made with surfactant having HLB 1.8, and a reactor containing capsules made with surfactant having HLB 16.7.

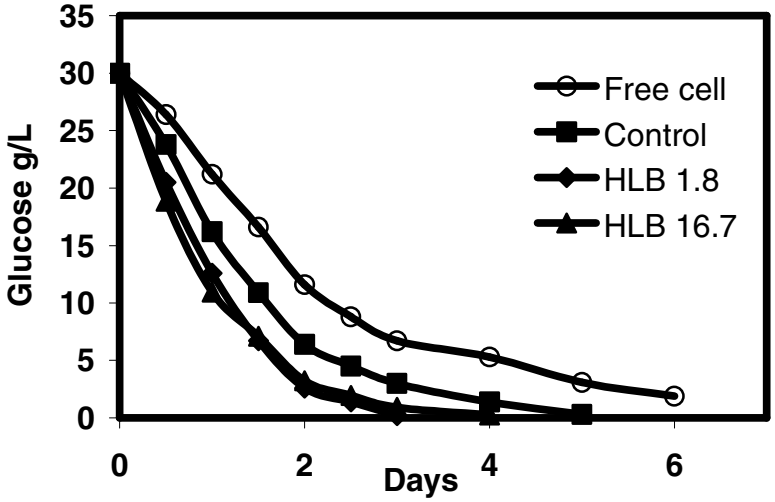

Figure 4.5. Batch glucose utilization for a reactor containing free cell, a reactor containing capsules made without addition of surfactant (control), a reactor containing capsules made with surfactant having HLB 1.8, and a reactor containing capsules made with surfactant having HLB 16.7.

Both riboflavin production and glucose consumption occurred at faster rates with immobilized cells than when free cells were used. Most of the 30 g/L glucose was

utilized in three days for the immobilized systems, but it took six days to utilize the glucose in the suspended cell system. More than 78% of glucose was utilized in the course of the first two days of fermentation for all immobilized systems while only 61% was utilized for the free cell system. Figure 4.6 shows initial substrate utilization rates for all cases studied. Higher initial glucose consumption rates were observed for the immobilized systems than for the free cell system.

The utilization rate of glucose by freely suspended cells was found to be 6.1 g/L.d, utilization rate for control was 11.6 g/L.d, and the rate increased to 16.5 and 15.5 g/L.d for capsules made with HLB 1.8 and HLB 16.7 respectively.

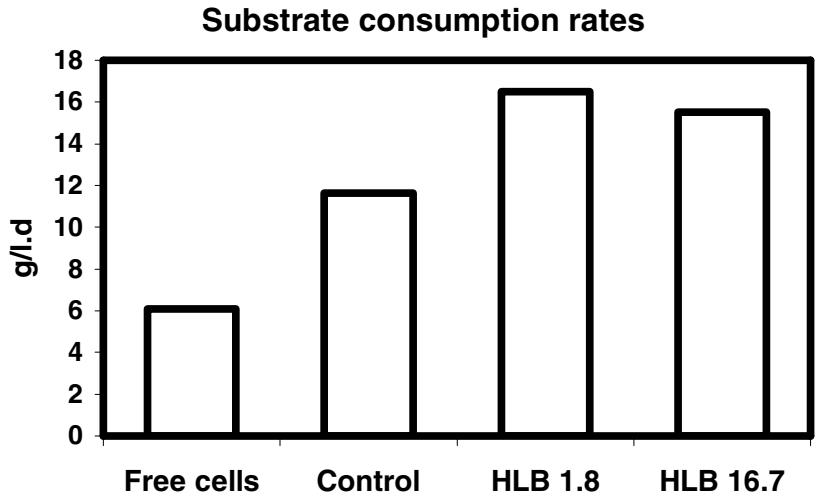

Figure 4.6. Initial substrate utilization rates for free cell reactor, a reactor containing capsules made without addition of surfactant (control), a reactor containing capsules made with surfactant having HLB 1.8, and a reactor containing capsules made with surfactant having HLB 16.7.

Table 4.1 shows the total glucose consumption (ΔS), riboflavin production (ΔP) and the yield coefficient ($\Delta P/\Delta S$) for the first 5 days of operation. Results indicate that immobilized cell systems had slightly higher yields than the free cell system. The highest yield was for the capsules made with Tween 20 (HLB 16.7).

Table 4.1 Batch yields of riboflavin on glucose. ΔS= glucose consumed (g), ΔP= riboflavin produced (g). $Y_{P/S}$= yield coefficient (g riboflavin/g glucose)

	ΔS (g)	ΔP (g)	$Y_{P/S}$
Free cell	28.1	0.663	0.0236
Control	29.7	0.726	0.0244
HLB 1.8	29.8	0.742	0.0249
HLB 16.7	29.7	0.758	0.0255

To quantify the maximum growth rate attainable by the yeast culture, the reactor was operated in batch mode before switching to continuous mode. The maximum specific growth rate (μ_{max}) was calculated as 0.031 h^{-1}. Complete calculation procedures are presented in appendix A.4.

Continuous Riboflavin Production

In order to investigate the possibility of improving the productivity of continuous bioconversion, the effect of increasing dilution rates was studied. Continuous glucose utilization and normalized continuous riboflavin production profiles are shown in Figures 4.7 and 4.8, respectively.

The continuous fermentation was started with a dilution rate of 0.01 h^{-1}, and after 3 days steady state riboflavin concentration was achieved. In the suspended cell system, riboflavin averaged a value of 74.9 mg riboflavin/g cell with the lowest dilution rate. Riboflavin production decreased to 73.6 and 63.4 mg riboflavin/g cell when the dilution rate increased to 0.02 and 0.03 h^{-1} respectively. However, riboflavin production decreased sharply to an average of 48.2 mg riboflavin/g cell when dilution rate increased to 0.04 h^{-1}.

For the control system, immobilized cells showed better utilization of substrate than the free cell system. Moreover, production of riboflavin decreased slightly from an average of 23.2 to 21.4 mg riboflavin/g cell with an increase in the dilution rate from 0.01

to $0.02h^{-1}$. However, no further decrease in riboflavin production and less fluctuation were observed at higher dilution rates. The systems containing capsules made with surfactants followed a similar trend as that of the control.

The variation of riboflavin productivity with time at different dilution rates is shown in Figure 4.9. In the suspended cell system, riboflavin productivity increased with increasing dilution rates up to 0.02 h^{-1}. However, when dilution rate increased to $0.03h^{-1}$ riboflavin productivity began to decrease sharply and a further increase in dilution rate resulted in a further loss of riboflavin productivity. For systems containing capsules made with surfactants, productivity was higher than the control system at dilution rates 0.03 and 0.04 h^{-1}. Moreover, these two systems were more stable at higher dilutions than the control, as loss of productivity at a dilution rate of $0.04h^{-1}$ for the control was observed. Superiority of the immobilized systems containing capsules made with surfactants is more evident at the higher dilution rates. The decrease in riboflavin productivity by free cells at higher dilution rates was probably due to cell washout.

Figure 4.10 shows the variation of the relative substrate concentration with dilution rate. For the free cell system, it was observed that as dilution rate increased, substrate utilization efficiency increased (noted by increase in relative substrate concentration). However, as dilution rate increased further than 0.02 h^{-1} utilization efficiency decreased sharply. The control system followed a similar trend as that of the free cell system although higher utilization efficiencies were achieved for the same dilution rates used.

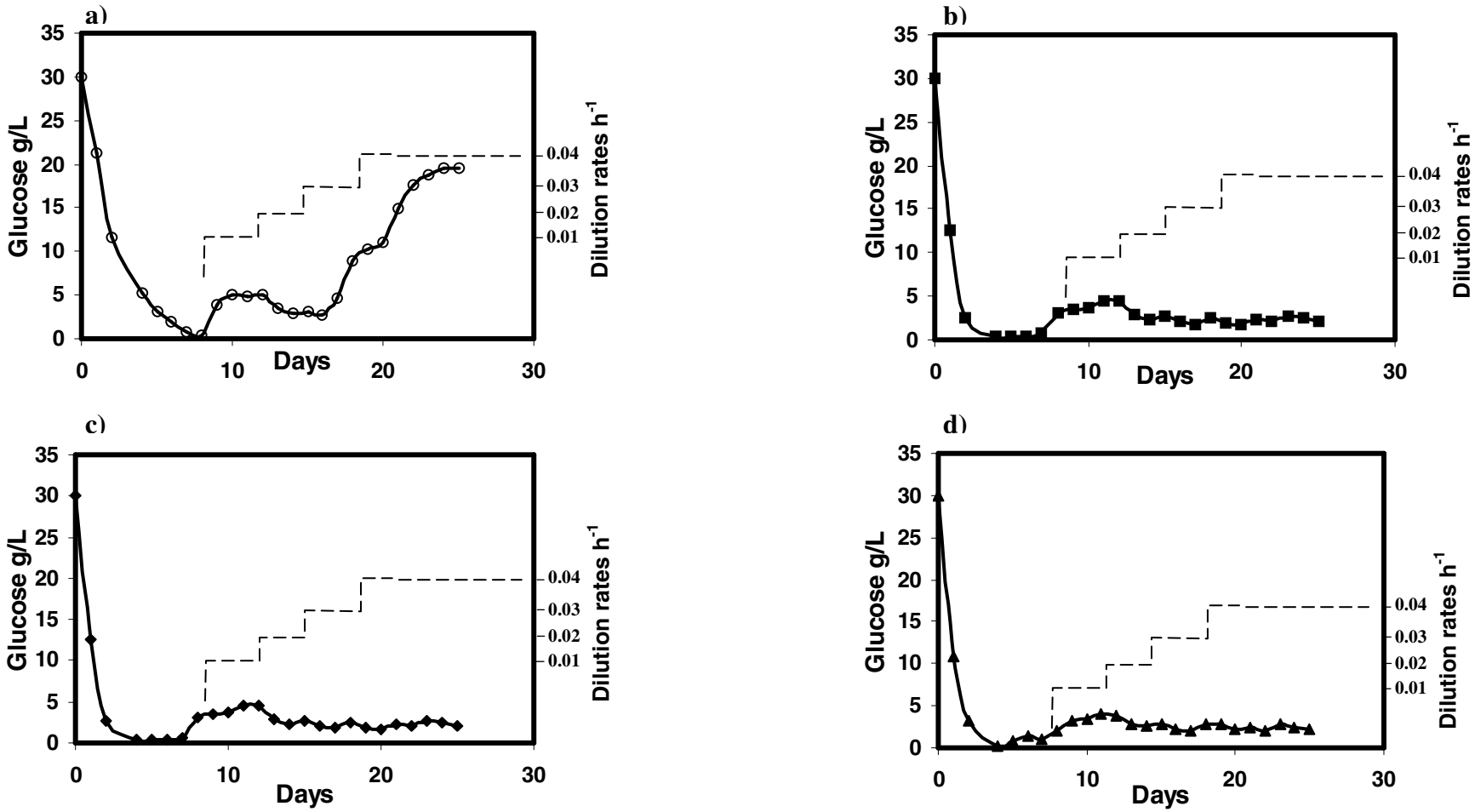

Figure 4.7 Continuous glucose consumption for a) a free (suspended) cell reactor, b) a reactor containing capsules made without addition of surfactant, c) a reactor containing capsules made with surfactant having HLB 1.8, and d) a reactor containing capsules made with surfactant having HLB 16.7. First 5 days were batch mode.

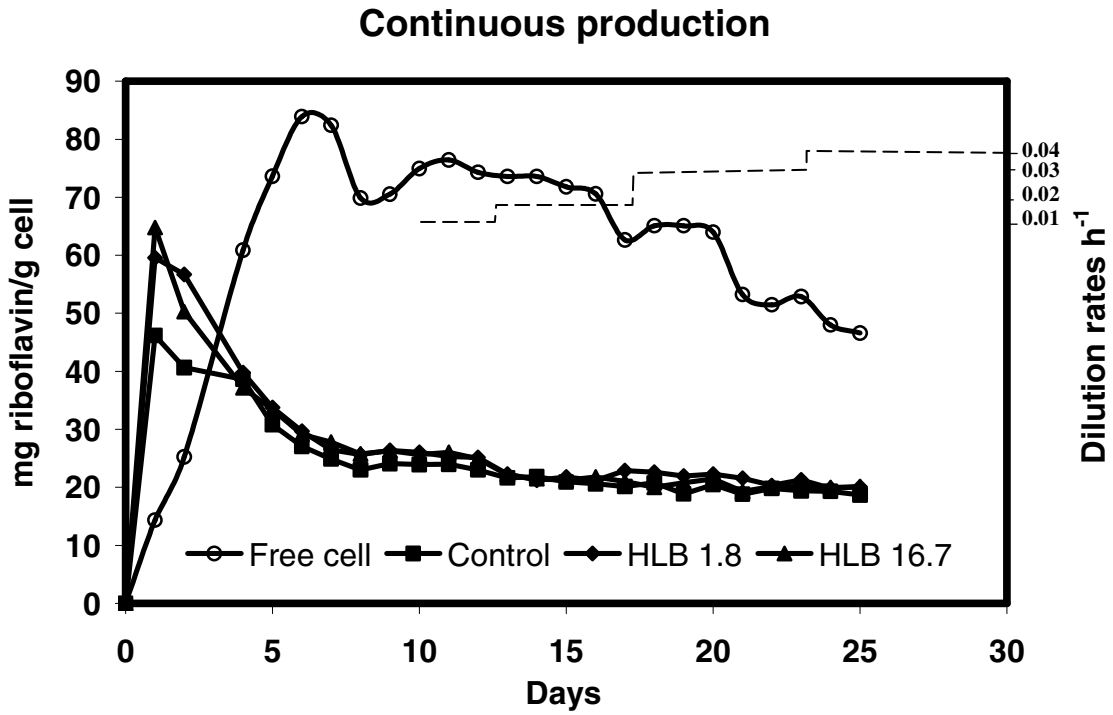

Figure 4.8 Normalized continuous riboflavin production (mg riboflavin / g cell) for a) a free (suspended) cell reactor, b) a reactor containing capsules made without addition of surfactant, c) a reactor containing capsules made with surfactant having HLB 1.8, and d) a reactor containing capsules made with surfactant having HLB 16.7. The first 5 days were batch mode.

This reduction by free cells at higher dilution rates was probably due to cell washout contributing to a loss of substrate utilization capability. Another explanation could be the possibility of increased concentration of several components showing antiflavinogenic activity (i.e. iron) in culture broth as a result of the increased dilution rate, similar to the observation mentioned by Buzzini and Rossi (1997).

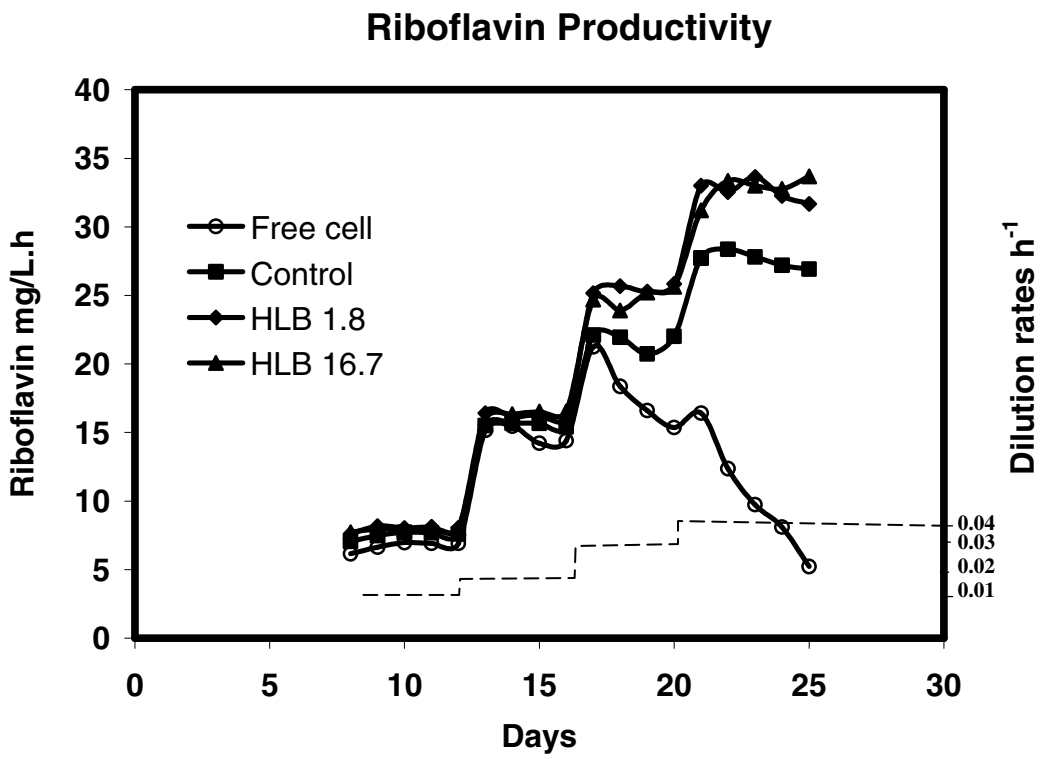

Figure 4.9 Variation of riboflavin productivity with time for a free cell reactor, a reactor containing capsules made without addition of surfactant, a reactor containing capsules made with surfactant having HLB 1.8, and a reactor containing capsules made with surfactant having HLB 16.7.

Interestingly, utilization efficiencies of the immobilized systems containing surfactants were similar to that of the free cell system at low dilution rates. However, at higher dilution rates, the systems with surfactants did not lose any efficiency. In fact, the HLB 1.8 system seemed to have higher efficiency at a dilution rate of 0.03 h^{-1}, while efficiency of the HLB 16.7 system decreased slightly at that rate. This indicates that the immobilized systems containing surfactants were much more efficient at high dilution rates than the free cell and control systems.

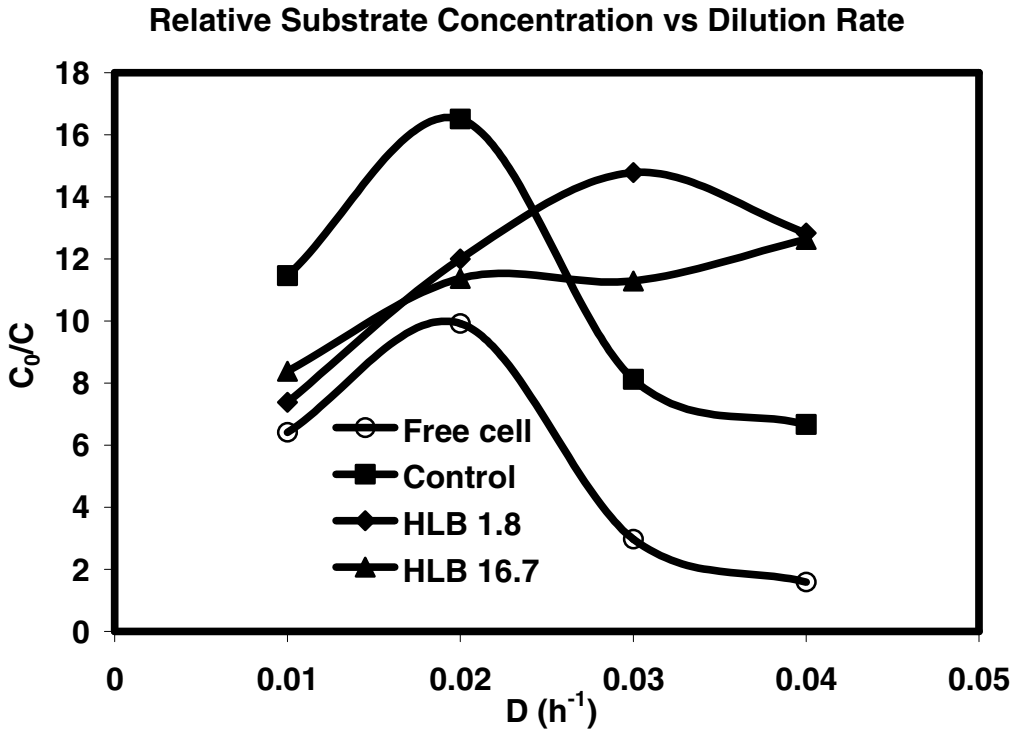

Figure 4.10 Variation of relative substrate concentration (C_0/C) with dilution rate (D) for a free cell reactor, a reactor containing capsules made without addition of surfactant, a reactor containing capsules made with surfactant having HLB 1.8, and a reactor containing capsules made with surfactant having HLB 16.7. C_0= influent glucose concentration (30 g/L), C= effluent glucose concentration (g/L).

4.5 Conclusion

The application of immobilized continuous riboflavin production techniques allowed good overall production efficiency. Dilution rates higher than 0.02 h^{-1} reduced riboflavin production in free cell and control systems.

In contrast, dilution rates higher than 0.02 h^{-1} did not reduce riboflavin production in the two immobilized systems containing surfactants. This could be explained in terms of diffusivity. The two immobilized systems containing surfactants contained capsules made with nonionic surfactants which, according to our previous work, increased both diffusion of substrate (glucose) and product (riboflavin) through the capsules' membrane.

Results suggest that the immobilized systems containing surfactant-modified capsules are superior to the free cell and control systems at higher dilution rates. They exhibit higher productivity and less fluctuation at higher dilution rates, while the free cell and control systems suffered significant reduction of substrate utilization capabilities at higher dilution rates. Results show great promise for the continuous immobilized cell reactor for riboflavin production using capsules made with nonionic surfactants.

4.6 Literature Cited

Buzzini P, Rossi J. 1997. Riboflavin production by yeasts as an alternative approach for the utilization of agro-industrial residues. Agro-Food-Ind Hi-Tech 8(5): 30-32.

Cunha MAA, Rodrigues RCB, Santos JC, Converti A, da Silva SS. 2007. Repeated-Batch Xylitol Bioproduction Using Yeast Cells Entrapped in Polyvinyl Alcohol-Hydrogel. Curr Microbiol 54(2): 91-96.

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CHAPTER V
SUMMARY AND FUTURE RECOMMENDATIONS

5.1 Summary

Cell immobilization techniques and their applications have been of interest for many decades. In this work, calcium alginate capsules were used for entrapment of the yeast *Candida flareri* (ATCC # 20850) for riboflavin production. There were two goals for this study. The first goal was to evaluate the effects of nonionic surfactants having various hydrophile-lipophile balances (HLB) on the diffusion coefficient of glucose and riboflavin through the hollow capsules. The second goal was to evaluate the performance of three immobilized yeast bioreactors producing riboflavin using the hollow calcium alginate capsules developed in the previous part.

The following conclusions can be drawn:

- The addition of surfactants that are more water soluble (high HLB) during capsule preparation increased the diffusion coefficient of riboflavin away from capsules.
- The addition of surfactants that are more oil soluble (low HLB) increased the diffusion coefficient of glucose into the capsules.
- The addition of surfactants in general increased the average thickness and average diameter of capsule membranes.
- The addition of surfactants resulted in capsules that have an irregular porous surface and less uniform membrane structure.

- Higher glucose consumption rates were observed for the immobilized systems than for the free cell system.
- Dilution rates higher than 0.02 h^{-1} reduced riboflavin production in the free cell system.
- Dilution rates higher than 0.02 h^{-1} did not reduce riboflavin production in the control system and the reactors having capsules made with Span 85 (HLB 1.8) and Tween 20 (HLB 16.7)
- All three reactors using cells immobilized in capsules exhibited better operational stability and less fluctuation at higher dilution rates, while the free cell system suffered reduced substrate utilization rates at higher dilution rates.

It appears that capsules made with the addition of nonionic surfactants exhibit superior performance in continuous systems at higher dilution rates. These results show great promise for the continuous immobilized cell reactor for riboflavin production using capsules made with nonionic surfactants.

5.2 Future Recommendations

Results showed that the addition of nonionic surfactant during the manufacturing of alginate capsules increased diffusion of glucose in a chemically defined media. However, no study has been conducted to test the use of a substrate of agro-industrial origin for a similar study. Buzzini and Rossi (1997) developed a continuous process to produce riboflavin by *Candida tropicalis* cells immobilized in calcium alginate using concentrated rectified grape must as the substrate. Their system was able to produce up to 600 mg/L riboflavin for more than 1 month. However, reduction of riboflavin production was observed in culture broth at higher dilution rates. They speculated that these higher

dilution rates were too high in comparison to the velocity of vitamin biosynthesis. Another explanation, which they did not address, is the possibility of diffusion limitation of substrate and other nutrients through the support matrix. Therefore, it is recommended that the effects of nonionic surfactants on the diffusional properties of alginate capsules using raw materials should be investigated.

In addition, results showed that the addition of surfactants resulted in capsules that have an irregular porous surface and a less uniform membrane. Their addition may have rendered the membrane more permeable by making the capsules more porous, thus increasing solute diffusion through it. Accordingly, Zhang et al. (2006) studied the effect of Span 85 and Tween 60 on the pore size of alginate capsules prepared by the emulsification method. Their work aimed at experimentally controlling the pore sizes of alginate capsules. Their work showed that the optimum HLB value which gave the narrowest pore size distribution was 12.3. However no work exists on the effects of HLB of surfactants on pore size distribution of calcium alginate capsules made by the extrusion method. Therefore it is also recommended that the effect of nonionic surfactants on the porosity of calcium alginate capsules prepared by the extrusion method should be investigated.

5.3 Literature Cited

Buzzini P, Rossi J. 1997. Riboflavin production by yeasts as an alternative approach for the utilization of agro-industrial residues. Agro-Food-Ind Hi-Tech 8(5): 30-32.

Zhang FJ, Cheng GX, Gao Z, Li CP. 2006. Preparation of Porous Calcium Alginate Membranes/Microspheres via an Emulsion Templating Method. Macromol Mat and Eng. 291(5): 485-492.

APPENDICES

A.1 Example of computer code (Mathematica 5.2) used to solve Crank's diffusion model

```
<< Statistics`NonlinearFit`
data = {{0, 1.0076}, {1, 0.8505}, {2.5, 0.8082}, {5, 0.7636}, {7.5, 0.7387}, {10,
0.7251}, {15, 0.7091}, {20, 0.7035}, {30, 0.7022}, {40, 0.6953}, {50, 0.6949}, {60,
0.6944}};

NonlinearRegress[data, (2.13 / 3.13) (1 + 0.22725 Exp[-476.41 delta x] + 0.08603 Exp[-
1662.89 delta x] + 0.04244 Exp[-3624.00 delta x] + 0.02484 Exp[-6366.58 delta x] +
0.01620 Exp[-9891.70 delta x] + 0.01137 Exp[-14199.93 delta x] + 0.00846 Exp[-
19164.33 delta x] + 0.00652 Exp[-24941.91 delta x] + 0.00518 Exp[-31479.59 delta x] +
0.00421 Exp[-38777.36 delta x]), {x}, {delta,0.0000001},

RegressionReport →{BestFitParameters, StartingParameters, ParameterCITable,
EstimatedVariance, ANOVATable, AsymptoticCorrelationMatrix, FitCurvatureTable},
ShowProgress →True]
```

A.2 Statistical Analysis, Student *t* comparison

Statistical comparison was performed using *Student's t-test* for the comparison of two means. It is the test of the null hypothesis that the means of two normally distributed populations are equal. If two data sets are given and each set its mean, standard deviation, and number of data points. The test can be used to determine whether the means are distinct according to the following equations:

$t = \dfrac{\overline{M}_d}{s_{\overline{M}_d}}$ Where $\overline{M}_d$ is the difference between samples means (μ_1 - μ_2) and $s_{\overline{M}_d}$ is the estimated standard error of the difference between means ($s_{\overline{M}_d} = \sqrt{\dfrac{s_1^2 + s_2^2}{n}}$).

Where s_1^2 and s_2^2 are the standard error for samples 1 and 2 and *n* is sample size. If probability *p* of *t* is less than the significance level then samples 1 and 2 and statistically distinct. *P* can be obtained from any published *t*-table

Table A.1 Student's *t* comparison of capsules thicknesses. All data are compared to control

		HLB			
	control	**1.8**	**6**	**11**	**16.7**
Thickness	96.33	148.56	129.62	129.60	116.73
	101.76	153.79	131.93	133.78	114.61
	99.81	146.36	139.91	128.16	109.92
Mean	99.30	149.57	133.82	130.51	113.75
M_d	0.00	50.27	34.52	31.21	14.45
Std. Error, s	2.75	3.82	5.40	2.92	3.48
s_{Md}		2.72	3.50	2.32	2.56
t		18.51	9.87	13.48	5.64
dF = n_1+n_2-2		4.00	4.00	4.00	4.00
p		>5E-4	15E-4	>5E-4	48E-4

Table A.2 Student's *t* comparison of combined diffusion of glucose. All data are compared to control

		HLB			
	control	**1.8**	**5.94**	**11**	**16.7**
D_m	1.29E-04	2.35E-04	2.02E-04	3.28E-04	3.85E-04
	1.31E-04	2.31E-04	1.92E-04	3.34E-04	3.65E-04
	1.26E-04	2.19E-04	1.97E-04	3.45E-04	3.78E-04
Mean	1.28E-04	2.29E-04	1.97E-04	3.35E-04	3.76E-04
M_d	0.00	1.0E-04	6.9E-05	2.1E-04	2.5E-04
Std. Error, s	2.52E-06	8.33E-06	5.00E-06	9.07E-06	1.02E-05
s_{Md}		5.02E-06	3.23E-06	5.43E-06	6.08E-06
t		19.97	21.30	38.15	40.73
dF = n_1+n_2-2		4.00	4.00	4.00	4.00
p		> 0.0005	> 0.0005	> 0.0005	> 0.0005

Table A.3 Student's *t* comparison of combined diffusion of riboflavin. All data are compared to control

		HLB			
	control	**1.8**	**5.94**	**11**	**16.7**
D_m	1.25E-04	3.71E-04	2.27E-04	2.24E-04	6.54E-05
	1.19E-04	3.31E-04	2.17E-04	2.69E-04	6.16E-05
	1.18E-04	3.52E-04	2.53E-04	2.11E-04	6.97E-05
Mean	1.21E-04	3.51E-04	2.32E-04	2.35E-04	6.56E-05
M_d	0.00	2.3E-04	1.1E-04	1.1E-04	5.5E-05
Std. Error, s	3.82E-06	2.00E-05	1.82E-05	3.05E-05	4.05E-06
s_{Md}		1.18E-05	1.07E-05	1.77E-05	3.22E-06
t		19.57	10.38	6.43	17.19
dF = n_1+n_2-2		4.00	4.00	4.00	4.00
p		> 0.0005	0.0003	0.0035	>0.0005

A.3 Calculation of maximum specific growth rate μ_{max}

Table A.4 shows free cell growth data. To calculate the maximum specific growth rate, Ln[X] is plotted versus t. The slope of the linear relationship is μ_{max}

Table A.4 Free cell growth data obtained from batch runs.

Time (d)	Cell concentration (g/L)	Ln[X]
0	1.10	0.10
12	1.43	0.36
24	2.34	0.85
36	2.77	1.02
48	5.05	1.62
60	6.75	1.91
72	8.08	2.09
96	10.80	2.38
120	12.55	2.53
144	14.01	2.64

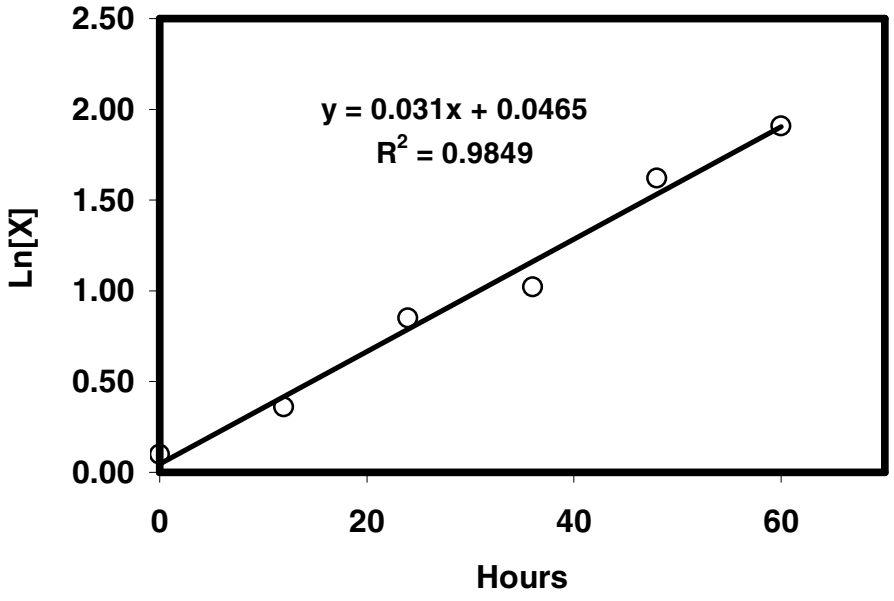

Figure A1. Plot of Ln[X] versus time, the plot yielded a slope of 0.031 h^{-1}

A.4 Calcium alginate beads.

One of the objectives of this study was to compare diffusion coefficients in capsules vs. solid beads. However, two problems occurred while working with the beads. The two problems were low riboflavin yields, possible due to significant diffusion limitations, and cell leak into the broth. Cell leak can be reduced by adding a secondary calcium alginate layer around the beads. This, however, will introduce more diffusion limitations. Figures A.2 and A.3 show cell leak into the broth and riboflavin yields respectively.

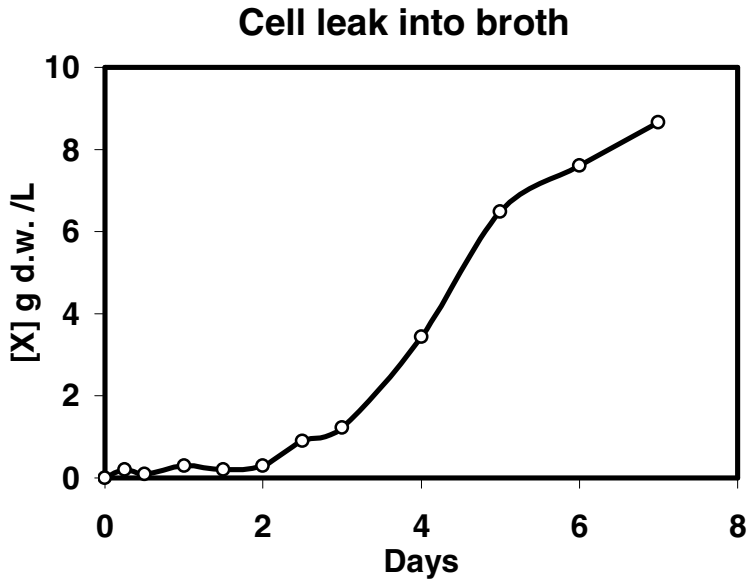

Figure A.2 Cell leak profile into the broth. After day two cell concentrations increased exponentially.

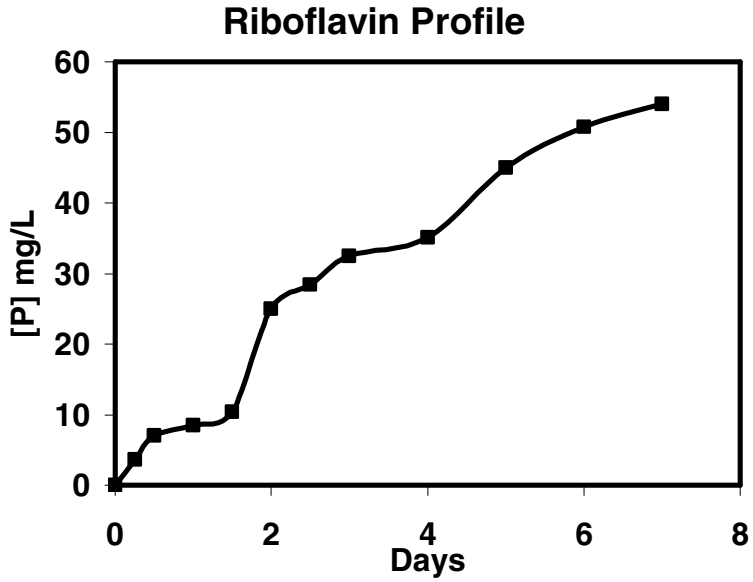

Figure A.3 Riboflavin production profile. Riboflavin concentration remained low even after cell leak became significant.

VITA

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Title of Study: RIBOFLAVIN PRODUCTION BY ENCAPSULATED *CANDIDA FLARERI*

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Major Field: Biosystems Engineering

Scope and Method of Study: Calcium alginate capsules were used for entrapment of the yeast *Candida flareri* for riboflavin production. The first goal was to evaluate the addition of nonionic surfactants to the capsule membrane. The diffusion coefficients of glucose and riboflavin through the hollow capsules were then evaluated by monitoring the change in solute concentration in the bulk solution. The second goal was to evaluate the performance of the immobilized yeast in continuous bioreactors producing riboflavin. Fermentation efficiencies of three immobilized systems and a suspended growth system were compared. The immobilized systems included a control (calcium alginate capsules made without surfactants), a system with capsules made with the addition of Span 85 (HLB 1.8), and a system with capsules made with the addition of Tween 20 (HLB 16.7).

Findings and Conclusions: Results indicate that the addition of surfactants that are more oil soluble (low hydrophile-lipophile balance, HLB) during capsule preparation increased the diffusion coefficient of riboflavin away from capsules, while the addition of surfactants that are more water soluble (high HLB) increased the diffusion coefficient of glucose into the capsules. Their addition may have rendered the membrane more permeable by making the capsules more porous, thus increasing solute diffusion.
The application of immobilized continuous riboflavin production techniques resulted in increased riboflavin productivity compared to the suspended growth system. Two of the immobilized systems contained capsules made with nonionic surfactants. Results showed that immobilized systems containing surfactant-modified capsules resulted in greater productivity than the control system (containing capsules made without surfactants). Results show great promise for the continuous immobilized cell reactor for riboflavin production using capsules made with nonionic surfactants.

ADVISER'S APPROVAL: _____