γ –อะลูมินาในเจลอัลจิเนทสำหรับการตรึงเซลล์ในกระบวนการหมัก

นางสาว จีรานันท์ พูลศิริสมบัติ

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γ – ALUMINA DOPED ALGINATE GEL FOR CELL IMMOBILIZATION IN FERMENTATION PROCESSES

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จีรานันท์ พูลศิริสมบัติ : γ-อะลูมินาในเจลอัลจิเนทสำหรับการตรึงเซลล์ในกระบวนการหมัก (γ-ALUMINA DOPED ALGINATE GEL FOR CELL IMMOBILIZATION IN FERMENTATION PROCESSES) อ. ที่ปรึกษา: ผศ.ดร. เหมือนเดือน พิศาลพงศ์, อ. ที่ปรึกษาร่วม: รศ.ดร. สีรุ้ง ปรีชานนท์, 97 หน้า.

งานวิจัยนี้ได้มีการพัฒนาตัวพยุงแบบใหม่ คือ แกมมา-อะลูมินาล้อมด้วยอัลจิเนท (AEC) โดยนำมาตรึงเซลล์ยีสต์ตกตะกอน *Saccharomyces cerevisiae* M 30 สำหรับผลิตเอทานอลจากกากน้ำตาลและตรึงเซลล์แบคทีเรีย *Clostridium butyricum* DSM 5431 สำหรับการผลิต 1,3-โพรเพนไดออลจากกลีเซอรอล จากนั้นทำการประเมินความเหมาะสม และ เปรียบเทียบผลกับการใช้เซลล์แขวนลอย (SC) และการใช้แกมมา-อะลูมินา (AC) เป็นตัวพยุง ทำการหมักแบบครั้งคราวที่ทำซ้ำ (repeated batch) เพื่อประเมินความเป็นไปได้ในการนำกลับมาใช้ใหม่ ในการศึกษาการหมักเอทานอลที่ทำเป็นชนิดครั้งคราว (batch) พบว่า ปริมาณเอทานอลที่ผลิตได้ในระบบ SC, AC และ AEC มีค่าเท่ากับ 82.4, 77.1 และ 74.6 กรัมต่อลิตร ตามลำดับ และ เมื่อใช้ AEC ในการหมักแบบครั้งคราวที่ทำซ้ำ พบว่า AEC มีศักยภาพในการนำกลับมาใช้ใหม่และให้ผลของการหมักในเทอมของผลได้และอัตราการผลิตที่ดีสำหรับการนำกลับมาใช้ในรอบที่ 1, 2 และ 3 โดยมีค่าเทียบเท่ากับระบบ SC และ AC อีกทั้งยังมีอัตราการผลิตเอทานอลที่เสถียรกว่าระบบ SC จากการวิเคราะห์ผลในเทอมของผลได้ของปริมาณยีสต์ตรึงรูปในตัวพยุงแบบใหม่ จะมีค่าประมาณ 85.6 % และนอกจากนี้ AC และ AEC ยังมีประสิทธิภาพในการตรึงเซลล์แบคทีเรีย *C. butyricum* โดยในเทอมของผลได้ของปริมาณแบคทีเรียตรึงรูปในตัวพยุงแบบใหม่ มีค่าเท่ากับ 79.6% และ 83.2% ตามลำดับ อย่างไรก็ตาม พบว่า เกิดผลยับยั้งในการผลิต 1,3-โพรเพนไดออลจากการตรึงเซลล์บนแกมมา-อะลูมินา โดยในการหมักแบบครั้งคราว ถึงแม้ว่าปริมาณเซลล์สุดท้ายในระบบ AC และ AEC จะมากกว่าระบบ SC เล็กน้อย แต่พบว่าความเข้มข้น 1,3-โพรเพนไดออลสุดท้ายและสัดส่วนผลได้ของผลิตภัณฑ์มีค่าลดลงอย่างเด่นชัด โดยหลังจากทำการหมักเป็นเวลา 33 ชั่วโมง ปริมาณ 1,3-โพรเพนไดออลที่ผลิตได้ในระบบ SC, AC และ AEC มีค่าเท่ากับ 41.4, 20.6 และ 15.5 กรัมต่อลิตรและผลได้ของผลิตภัณฑ์มีค่าเท่ากับ 0.75, 0.58 และ 0.48 ตามลำดับ นอกจากนี้ เมื่อทำการหมักแบบครั้งคราวที่ทำซ้ำ ผลที่ได้ยืนยันการยับยั้งการผลิต 1,3-โพรเพนไดออลของแกมมา-อะลูมินาโดยเฉพาะในระบบ AEC ยิ่งไปกว่านั้นพบว่าเซลล์จากการหมักในระบบ SC และ AEC ไม่มีเสถียรภาพในการนำกลับมาใช้ใหม่ โดยคาดว่าจากการมีประจุเป็นบวกของแกมมา-อะลูมินา จะส่งผลไปขัดขวางการทำงานของเยื่อหุ้มเซลล์ ซึ่งเป็นสาเหตุให้เกิดผลยับยั้งกิจกรรมของเซลล์แบคทีเรีย *C. butyricum* ในการผลิต 1,3-โพรเพนไดออล

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต จีรานันท์ พูลศิริสมบัติ
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JIRANAN PULLSIRISOMBAT: γ – ALUMINA DOPED ALGINATE GEL FOR CELL IMMOBILIZATION IN FERMENTATION PROCESSES. THESIS ADVISOR: ASSISTANT PROFESSOR MUENDUEN PHISALAPHONG, Ph.D., THESIS COADVISOR: ASSOCIATE PROFESSOR SEEROONG PRICHANINT, Ph.D., 97 pp.

γ-Alumina (γ-Al_2O_3) doped alginate gel (AEC) was developed as a cell carrier in this study. The immobilization system of *Saccharomyces cerevisiae* M.30 for ethanol production from molasses and *Clostridium butylicum* DSM 5431 for 1,3-propanediol production from glycerol were used to evaluate the performance of the new carrier. Its feasibility for cell immobilization was examined and compared with suspended cell (SC) culture and immobilized cell on γ-Al_2O_3 (AC). Reusability of the carrier was evaluated by repeated batch mode. In a single batch system of ethanol fermentation, the final ethanol concentration of suspended cell, AC and AEC cultures were 82.4, 77.1 and 74.6 g/l, respectively. In repeated batch modes, AEC culture demonstrated a good potential of reusability. Its ethanol production and conversion yield of the 1st, 2nd and 3rd repeated batch were comparable to those of SC and AC cultures. In addition, its ethanol production was more stable than that of SC culture. The yeast cell immobilization yield of the new carrier was approximately 85.6 %. AEC and AC were also found to be effective for the cell immobilization of *C. butylicum* with the immobilization yield of 79.6% and 83.2%, respectively. However, the strong inhibition effect of cell-γ-Al_2O_3 immobilization on 1,3-propanediol production was observed. In a single batch system, although the final cell concentration of AC and AEC culture were slightly higher than that of the suspended culture, the final 1,3-propanediol concentration and the conversion yield were significantly decreased. After 33 hours of the cultivation, the final 1,3-propanediol concentration of suspended cell, AC and AEC culture were 41.4, 20.6 and 15.5 g/l, with the conversion yield of 0.75, 0.58 and 0.48, respectively. In the repeated batch fermentation, the strong inhibitory effect of γ-Al_2O_3, towards 1, 3-propanediol production, especially in the system of AEC culture was confirmed. Moreover, the instability of SC and AEC cultures for reuse was observed. Interfering of positive charge of γ-Al_2O_3 on the cell membrane was thought as the cause of inactivity of *C. butylicum* DSM 5431 on 1,3-propanediol production.

Department	Chemical Engineering	Student's signature.....
Field of study	Chemical Engineering	Advisor's signature.....
Academic year	2007	Co-advisor's signature.....

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CHAPTER I
INTRODUCTION

Fermentation processes are currently considered as a useful alternative for the industrial synthesis of bulk chemicals, solvents, pharmaceutical and agrochemical products. Their advantages over chemical catalysis are, for example, mild conditions for the operation, using low cost substrate materials from agricultural or industrial wastes including the minimization of chemical wastes. However, the common drawbacks associated with bioprocesses are the low concentration of products, low productivities, inhibition effects at high concentration of substrates or products and the instability of production under continuous process.

Fermentation processes can be conducted with batch, semi-continuous, or continuous mode. Free, flocculated, or immobilized cells can be used as the biocatalysts. Immobilized cell system offers many advantages, especially higher productivity and more stability for a long run. So far, many immobilization methods and carrier materials have been investigated. Various materials have been tested as cells carriers [1-7]. In general, an ideal cell carrier should be rigid and chemically inert, should bind cells firmly, and should have high loading capacity. Organic material has a higher adsorbtivity than inorganic material due to larger variety of reactive groups such as amino, carboxyl, and hydroxyl on the surface of the material. Entrapment, the physical restriction of cell within a confined space or network is one of most potential cell immobilization techniques and alginate is most frequently used as the biopolymer for the entrapment due to its mild gelling property and nontoxicity. However, practical applications of alginate entrapment method have been limited by the problems of physical and chemical stability towards microbial attacks and organic solvents. In contrast, an inorganic material such as alumina is known to be thermally and mechanically stable, non-toxic, and highly resistant against microbial attacks and organic solvents [1-7].

Therefore, in this study, γ – alumina doped alginate gel is developed as a carrier for fermentation processes. The immobilization system of *Saccharomyces cerevisiae* M.30 for ethanol production and *Clostridium butylicum* DSM 5431 for 1,3–propanediol production from glycerol are used to evaluate the performance of the new carrier. The activities of the immobilized cells are then compared to the systems

of free cells and immobilized cells adsorbed on γ– alumina. The studies are conducted in batch shaking flasks and the kinetics of the reactions is determined. Since this technology is still in the verge of development, information gained from experiments will be benefit for the further cell carrier development.

1.1. Objectives

1. To develop a new immobilized cell carrier for fermentation processes.
2. To investigate cell immobilization on γ-alumina entraped with alginate gel on fermentation systems of *Saccharomyces cerevisiae* M.30 for ethanol production and *Clostridium butylicum* DSM 5431 for 1,3–propanediol production.

1.2. Expected benefits

1. Invention of high performance cell immobilization carrier for fermentation processes.
2. Useful information for a better understanding of immobilized cell technology.

1.3. Working scopes

In the framework of the preliminary study of immobilized cell system by γ-alumina entrapment with alginate gel, the fermentations in this work are carried out in batch and repeated batch modes in 500 ml shaking flasks. The fermentation systems of *Saccharomyces cerevisiae* M.30 for ethanol production and *Clostridium butylicum* DSM 5431 for 1, 3–propanediol production are used for the performance evaluation of the carrier system of γ – alumina doped alginate gel in comparison with the systems of free cells and immobilized cells on γ-alumina. The comparison is included cell productivity, product concentration, production yield and stability.

1.3.1 Production of ethanol

The fermentation is carried out in aerobic system at shaking frequency of 150 rpm and temperature of 33°C. Molasses is used as a sole carbon source. The initial concentration of reducing sugar is controlled at 220 g/L and the initial pH is 5.0.

1.3.2 Production of 1,3–propanediol

The fermentation is carried out in anaerobic system at shaking frequency of 100 rpm and temperature of 32°C. Glycerol is used as a sole carbon source; the initial concentration of glycerol is varied from 40 - 130 g/L and the initial pH is 7.0.

CHAPTER II
BACKGROUND AND LITERATURE REVIEW

2.1. Immobilized cell system

Cell immobilization was defined as “the physical confinement or localization of viable microbial cells to a certain region of space with preservation of some desired catalytic activity” [8]. It is done by attaching cells to a bulk matrix or attaching cells to material surface to retain the cells in a certain area. It is being investigated for antibiotic, enzyme, alcohol, and acid production [9]. Many studies shown that cell immobilization could increase production rates by as much as two times [10]. Some potential advantageous characteristics of immobilized cell over suspension cultures are as following [11].

1. Prolong activity and stability of the biocatalyst. The immobilization support may act as a protective agent against physicochemical effects of pH, temperature, solvents or heavy metals.
2. Increase cell density per unit bioreactor volume, which leads to high volumetric productivity, shorter fermentation times and elimination of non-productive cell growth phases.
3. Increase substrate uptake and production yield.
4. Enhance feasibility of continuous processing.
5. Increase tolerance to high substrate concentration and/or end product Inhibition.
6. Simplify product recovery through reduction of separation and filtration of cell
7. Be able to regenerate and reuse of the biocatalyst for extended periods in batch operations, without removing it from the bioreactor.
8. Reduce the risk of microbial contamination due to high cell densities and fermentation activity.
9. Reduce the size of bioreactors with simplified process designs and therefore, lower capital costs.
10. Reduce maturation times for some products.

The above advantages become obvious through some of the most recent examples of research on immobilization techniques including the application in production of alcoholic beverages like wine, beer, distillates and potable alcohol production. The various supports used for cell immobilization are classified as organic, inorganic, natural supports and membrane systems. Natural supports are mainly of food grade purity and are used with minimum or no pre-treatment such as wood, sawdust, pieces of fruit etc. On the other hand organic materials are synthetically made (like plastic) or extracted from natural sources by more complex processes (like polymeric hydrogels).

2.1.1. Type of immobilized cell carriers

Various materials have been tested as cells carriers. They interact with the cell surface by physical or chemical bonds. A number of factors must be considered in the selection of a suitable carrier for large-scale use [11].

1. The carrier should have a large surface area, with functional groups for cells to adhere to.
2. The carrier must be easy to handle and regenerate.
3. Cell viability and operational stability of the immobilized biocatalyst must be high and retained for long times.
4. The biological activity of the immobilized cells should not be adversely affected by the immobilization process.
5. The porosity of the support should be uniform and controllable, allowing free exchange of substrates, products, cofactors and gases.
6. The carrier should retain good mechanical, chemical, thermal and biological stability and not be easily degraded by enzymes, solvents, pressure changes or shearing forces.
7. The carrier and immobilization technique should be easy, cost effective and amenable to scale-up.
8. The carrier should not affect product quality and readily accepted by consumers.

Carriers can be classified into organic, inorganic, porous, and charged carrier based on the nature of the supporting material [1].

2.1.1.1 Organic carriers

Organic material has a higher adsorbtivity than inorganic material due to larger variety of reactive groups such as amino, carboxyl, and hydroxyl on the surface of the material. Availability of a certain amount of nutrient in organic material will help attachment and growth of cell. Biodegradability of organic materials usually leads to higher replacement frequency [1] but less severe environmental pollution [2].

Some organic materials from plants such as loofa sponge [3], sugar cane stalk [4], and apple cut [5] have been tested for cell immobilization. They usually exhibit advantageous characteristics such as non-reactive, non-toxic, cheap, simple to use, and available in large quantities. Some examples of organic materials are shown in Figure 2.1. Naturally derived polymers such as alginate and chitosan have been studied intensively to evaluate their compatibility for cell immobilization

loofa sponge www.istockphoto.com

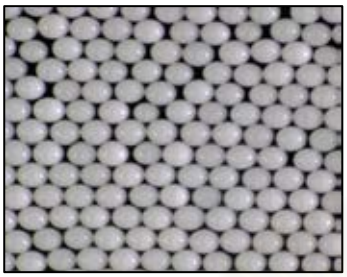
apple cut www.fiferorchards.com

Figure 2.1 Examples of organic materials for cell immobilization

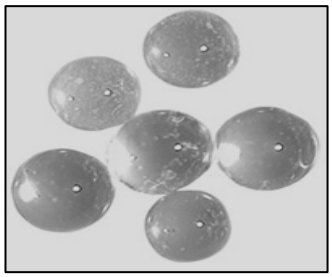
2.1.1.2 Inorganic carriers

Inorganic material usually has good resistant to microbial attack, high thermal stability, and good flow properties. To improve its absorbability, organic groups can be attached to the material by grafting with various coupling/cross-linking agents such as glutaraldehyde and carbodiimide [1]. Grafting procedure is generally incompatible with cell viability. Some inorganic carriers such as metal oxides, stainless steel mesh, alumina beads, and glass beads have been tested for cell immobilization. They usually exhibit advantageous characteristics such as suitable surface structure, high stability,

and cheap, Figure 2.2 shows some inorganic materials which can be used as cells carriers.



Alumina beads
www.taimei-chem.co.jp



Glass beads
www.optaminerals.com

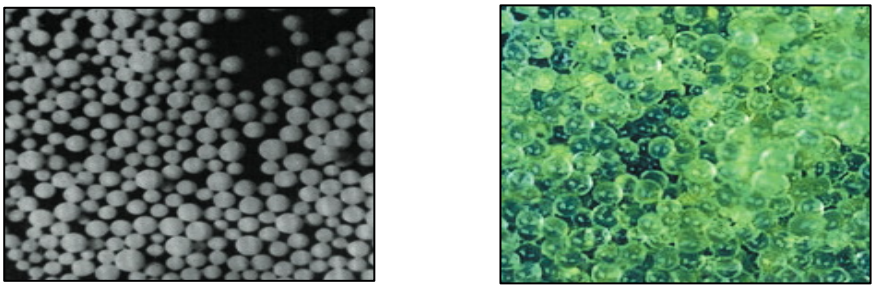
Figure 2.2 Inorganic materials for cells carriers

2.1.1.3 Charged carriers

Since surface charge of microorganisms is usually negative, positively charged material will favor attachment of the cell especially at the early stage of immobilization. However, the charged material could also interact with charged substrate, product, and/or residual contaminants [1]. Ion exchange resins, gelatin, stainless sphere wire, and porous cellulose are example of charged carriers [6].

2.1.1.4 Porous carriers

For macro porous carrier such as porous glass, cell will attach at the surface as well as the pore of the material. This will lead to higher cell loading compared to micro porous carrier such as zeolite. Cell can not enter the pore of micro porous material because the pore size is smaller than cell. Carrier bead which has small pore on the surface and large pore in the interior was found to be effective for cell immobilization [7]. More examples of porous carrier include agar, alginate, κ-carrageenan, polyacrylamide, chitosan, gelatin, cellulose, collagen, porous metal screen, polyurethane, silica gel, polystyrene, and cellulose triacetate. The pictures of some porous materials are shown in Figure 2.3.



Cellulose beads www.bio-world.com

alginate beads www.bio-world.com

Figure 2.3 Porous carriers for cell immobilization

2.2 Immobilization techniques

The immobilized cell systems based on the physical mechanism could be classified into four methods: attachment to a surface, entrapment within a porous matrix, containment behind a barrier and self-aggregation [12].

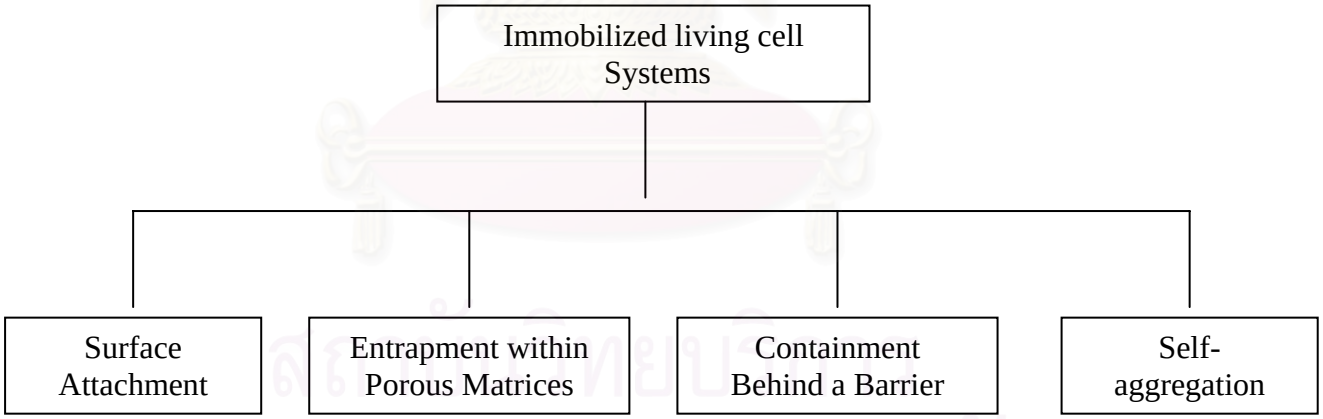


Figure 2.4 Classification of immobilized cell system [12]

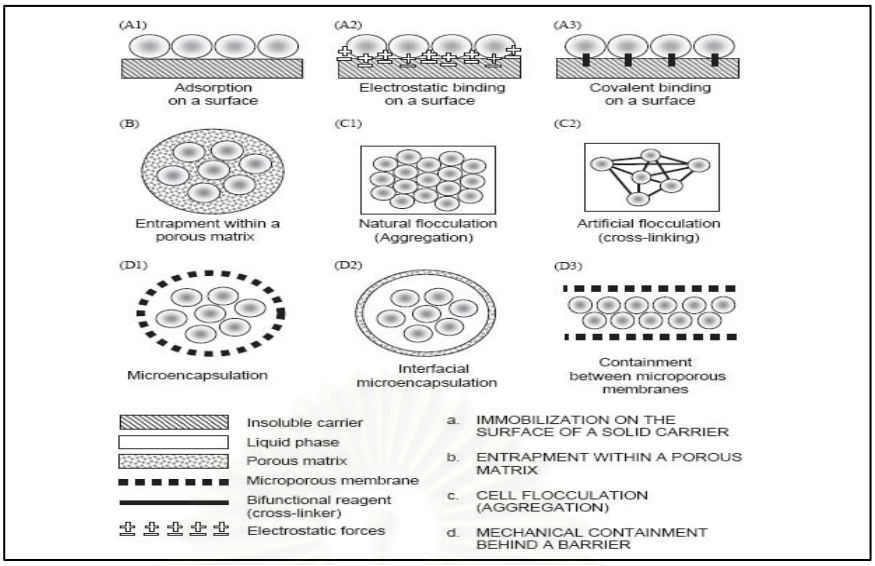


Figure 2.5 Basic methods of cell immobilization [12]

2.2.1 Attachment to a surface

Immobilization by adsorption is a popular immobilization method because it is a simple and fast technique. Microorganisms adsorb spontaneously on a wide variety of organic and inorganic supports (Figure 2.6). A suitable adsorbent should display a high affinity towards the biocatalyst and yet cause minimal denaturation. The binding of cells occurs through interactions such as van der Waals forces, ionic bonds, hydrogen bridges or covalent interactions.

Microbial cells exhibit a dipolar character and their charge depends on the pH of the solutions. Furthermore, the physiology of the cells has a strong influence on the adhesion. The charges on the cell wall depend also on cell type and environmental conditions. Plant ce1ls and some bacteria can attach to a surface due to the physicochemical effects and the production of extra-cellular polymers. Some types of animal cell have an absolute requirement for attachment to a surface before they can proliferate or produce bioproducts. Cell division is gradually inhibited by cell-cell contact as the culture reaches confluence. By the introduction of the microcarrier, animal cell cu1turing was revolutionized. Cells grow on the surface of microcarrier usually in the form of monolayer. As a result, internal mass transfer limitations are absent. On the contrary, biofilms on surfaces can be several millimeters thick, and mass transfer limitations even determine their long-term behaviors [12].

As there is no barrier between the cells and the solution, this method cannot be used where cell-free effluent is desired. The depth of a microbial biofilm often varies, especially with flow rate and so it is often difficult to accurately control biofilm processes. The industrial use of surface-attached cell systems has been extensively examined in the field of waste water treatment (biofilms) and only recently in mammalian cell culture (microcarriers). Bead-to-bead contact and abrasion of the surfaces can be a problem and the non-porous nature of the carriers limits the available surface area. Besides natural adsorption, cel1s can also be chemically bound to a surface by chelation to metal oxides or covalent bonding with coupling agents such as glutaraldehyde, aminosilane, etc. (Figure 2.6).

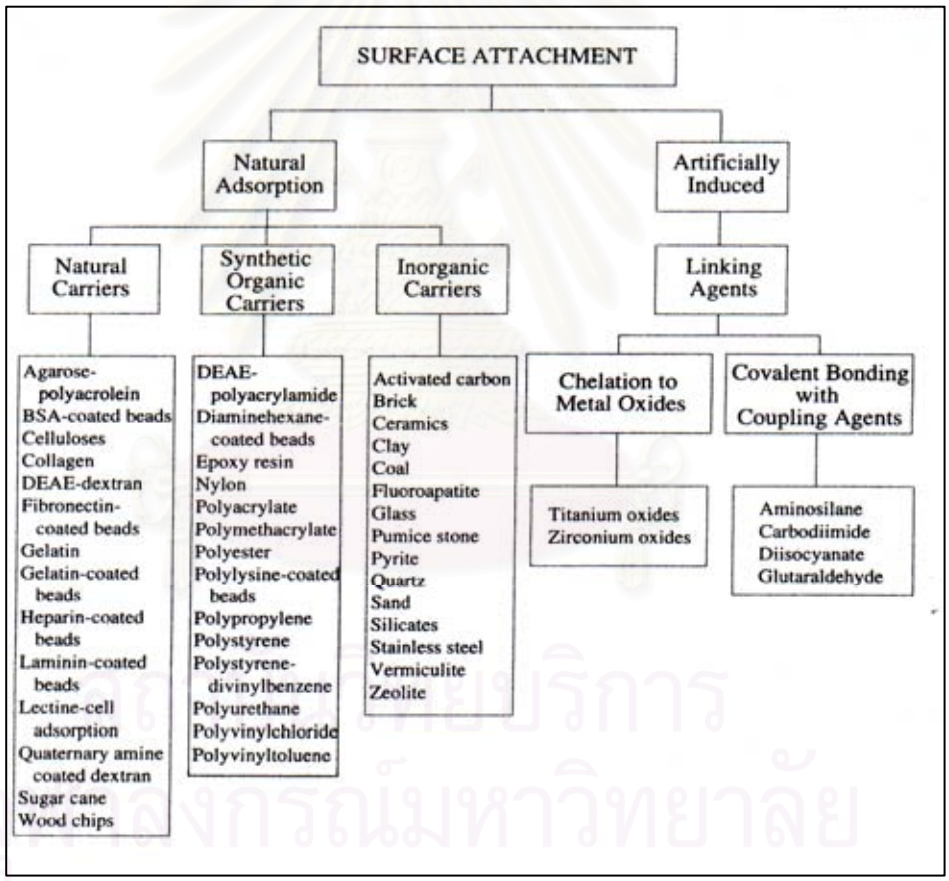


Figure 2.6 Immobilization by surface attachment [12]

2.2.2 Entrapment within Porous Matrices

Two categories of methods can be distinguished: (i) the porous matrix is synthesized in situ around the cells to be immobilized, i.e. gel entrapment; and (ii) the cells are allowed to move into the preformed porous matrix (Figure 2.7). Entrapped cells can reach high densities in the matrix and-compared to surface immobilization cells are well protected from the fluid shear. This dense packing may, however, again lead to transport limitations.

1. Gel entrapment. Mainly for its simplicity excellent cell containment most of the research in the domain of immobilized cells has used gel entrapment. A wide variety of natural (polysaccharides and proteins) and synthetic polymers can be gelled into hydrophilic matrices under mild condition allow cell entrapment with minimal loss of viability. Very high biomass loadings can be achieved. The polymer cell mixture can be formed in different shapes and sizes. The most common forms are small beads about 1 to 5 mm in diameter, Even though natural polymers dominate, synthetic polymers have recently been developed and applied for the immobilization of living cells. The synthetic polymers can be easily and artificially designed for adequate properties. The porosity of the gel as well as the ionic and hydrophobic or hydrophilic properties can be adjusted. Additionally, the mechanical strength and longevity of the gels formed from synthetic polymers are generally superior to those from natural polymers.

Gel-entrapment has the disadvantage of limited mechanical stability. It has been frequently observed that the gel structure is easily destroyed by cell growth in the gel matrix and carbon dioxide production. However, the gel structure can usual1y be reinforced, e.g. alginate gel was made stronger by the reaction of alginate with other moleclues like polyethyleneimine, glutaraldehyde cross-linking, silica, genipin, polyvinylalcohol or by partial drying of the ge1. Another important disadvantage compared to other immobilized cell systems is that the mass transfer limitations are more severe because mass transfer is by molecular diffusion only (and not by convection). The diffusion in the matrix itself is fast, and mainly oxygen transport can be limiting. Mass transfer of substrate and products is usually not limiting in the matrix but rather in the closely cell packed cell aggregates that are formed.

2. Preformed support. The cells are entrapped in a matrix which protects them from the shear field outside the particles. This is of particular importance for fragile cells

such as mammalian cells. Unlike gel immobilization systems, porous supports can be inoculated directly from the bulk medium. As with the adsorption method, cells are not completely separated from the effluent in these systems. Mass transport of substrates and products can be achieved by molecular diffusion as well as convection, by proper particle design and organization of external flow. Consequently mass transport limitations are less severe under optimal conditions. When, ideally, the colonized porous particles retain some free space for flow, immobilization occurs, partly by attachment to the internal surface, self-aggregation and retention in dead-end pockets within the material. In this case, cell adhesion is not very strong and the application of high external now rates can reversibly remove cells from the matrix. When high cell densities are obtained (e.g. animal or plant cells) convection is no longer possible and the particles behave as dense cell agglomerates with strong diffusion limitations. In this case the matrix only stabilizes and protects the cells from shear. A high degree of cell viability is retained in most entrapment method; the preformed support particles are more resistant to compression and disintegration than the gel particles [12].

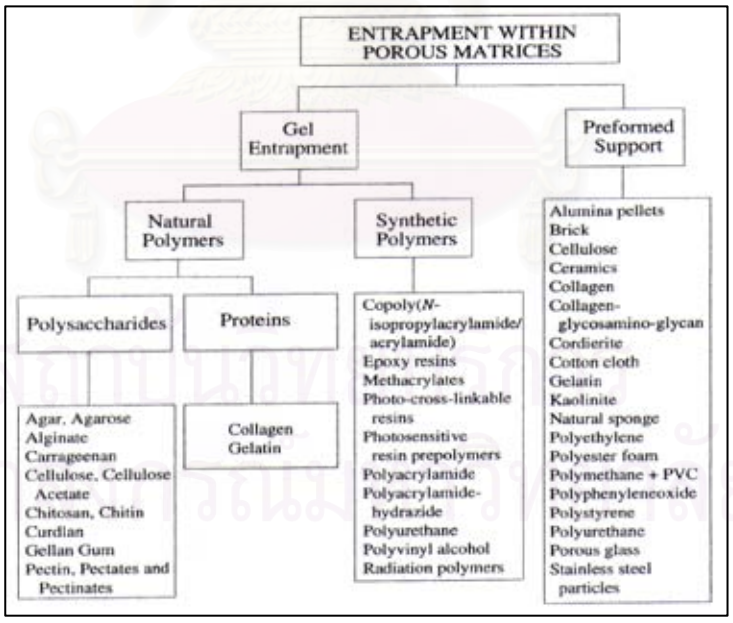


Figure 2.7 Immobilization by entrapment in porous matrices [12]

2.2.3 Containment behind a Barrier

Containment behind a barrier is ideal for several specialized systems, when total cell separation from the effluent is required, or when some high molecular weight or specific product (perm-selectivity) needs to be separated from the effluent, these systems are highly useful. The barrier can be either preformed (hollow fibred systems and flat membrane reactors) or formed around the cells to be immobilized (microcapsules and two-phase entrapment), as illustrated in Figure 2.8. The synthetic membranes are usually polymeric micro-filtration or ultra-filtration membranes, although other types of membranes have been used such as ceramic, silicone rubber or ion exchange membranes. Mass transfer through the membrane is not only dependent on the pore size and structure but also on the hydrophobicity /hydrophilicity and charge. Transport can be by diffusion and/of by flow induced by application of a pressure difference over the membrane. Various mild micro-encapsulation methods have been developed recently to entrap living cells, and some are combined with gel immobilization within the microcapsule. The barrier which immobilized cells can be as simple as the liquid/liquid phase interface between two immiscible fluids [12].

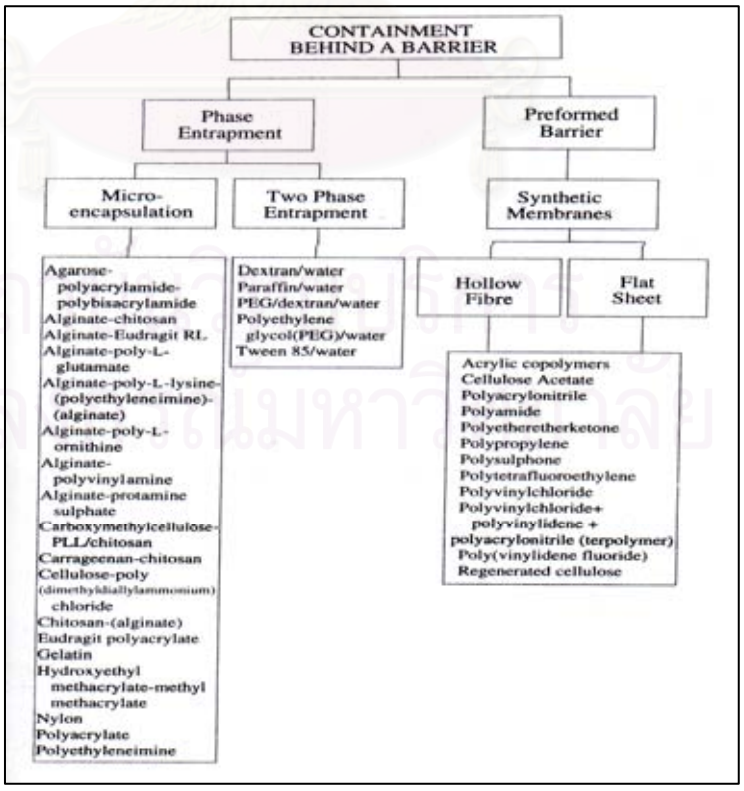


Figure 2.8 Immobilization by containment behind a barrier [12]

2.2.4 Self Aggregation of Cells

Cells that naturally aggregate, clump, form pellets or flocculate can also be considered as immobilized (Figure 2.9). Many industrially important products are produced during primary and secondary metabolism by fungal pellet, e.g. citric acid by *Aspergillus species*, antibiotics using *Penicillium* species, enzymes for the biodegradation of lignin, and decolonization of paper mill bleach plant effluents using *Phanerochaete chrysosporium*, cellulolytic enzymes by *Trichoderma reesei*. Microbial aggregates in anaerobic waste water treatment are used to speed up the digestion rate during methanogenesis and have been successfully applied in many full-scale installations, especially for sugar beet, potato, pulp and paper mill waste waters. Microbial aggregates can also be encountered in wine-making and brewing where yeast cells flocculate at the end of fermentation. The culturing of algae, plant cells, and animal cells can also result in aggregation phenomena.

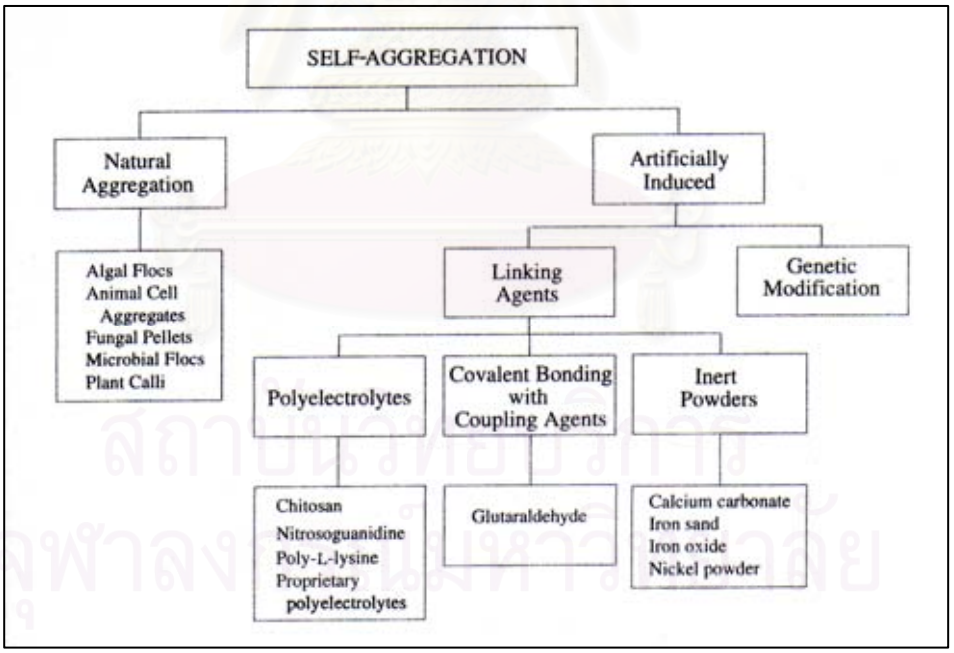


Figure 2.9 Immobilization by self- aggregation [12]

2.3 Ethanol Production

Ethanol or ethyl alcohol, CH_3CH_2OH, has been described as one of the most exotic synthetic oxygen-containing organic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, an antifreeze, a fuel, a depressant, and especially because of its versatility as a chemical intermediate for other organic chemicals.

Today ethanol production is much more refines and upgraded, leading to improved efficiency. This increase in production has lead to more uses than just beverages. In fact, beverages make up an extremely small portion of the ethanol industry. Ethanol has proven itself highly useful in a wide variety of areas such as medical application for sterility and anesthesia. It is also as cleaning solvents, chemical agents and of course the biggest consumption, as a powerful fuel source.

Due to the growing demand for energy and diminishing supply of fossil fuels, increasing attention has been given to alternative sources of energy. Alternative fuels need to be renewable, sustainable, efficient, cost-effective and safe [13]. In addition these fuels should reduce harmful pollutants and exhaust emissions. Ethanol is one of the alternative fuels being considered to replace some of the oil based fuels and fuels additives. When ethanol is burned it produces carbon dioxide and water. The carbon dioxide produced from burning ethanol is much less harmful then other gasses produced from burning gasoline decreasing the damage to the environment.

Ethanol can be produced in two different ways: the petrochemical route from the hydration of ethylene and the biotechnological route from microbial fermentation of agriculture biomass [14]. Microorganism under anaerobic growth conditions has the ability to utilize glucose and produce ethanol using the Entner Doudoroff pathway as shown in Figure 2.10

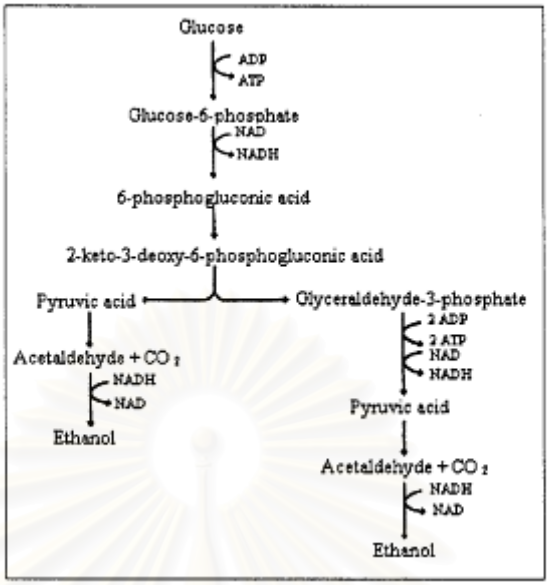


Figure 2.10 Entner Doudoroff pathways

Using the Entner Doudoroff pathway certain microorganism are able to produce two moles of ethanol and two moles of carbon-dioxide for every moles of glucose consumed. The theoretical yield of ethanol using this partway ($Y_{P/S}$) is 0.51 g of ethanol produced per gram of glucose consumed. The actual yield observed in industry is 90% of the theoretical yield. This decrease is due to cell maintenance and production of other by-product such as glycerol [15].

Much research has been done to determine which organism are the most efficient ethanol fermentors, and ways to modify these organisms to increase their efficiency. Common strains of organisms used in ethanol production include *Saccharomyces cerevisiae, Zymomonas mobilis* and *Candida tropicalis* [16]. All three of those strains exhibit high production rates and efficiency. However, *Saccharomyces cerevisiae* has shown the ability to maintain high production rates of ethanol in concentrations as high as 6 % it becomes inhibitory [17].

Industrial fermentation process use microorganism that are suspended in the fermentation media. These systems are usually run in batch and semi-batch. As a result, there are many limitations such as low cell densities, nutritional limitations and long down times needed for clean up and preparing the fermentation reactor. In order to keep cell densities high it is necessary to separate and recycle cells after each the

fermentation. Figure 2.11 shows a schematic diagram of suspension cells fermentation with cell recycle system

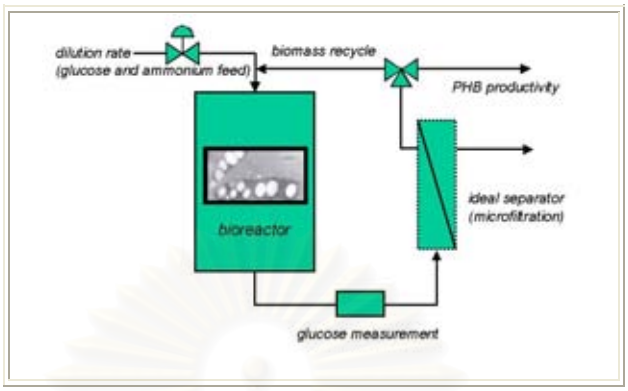


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Figure 2.11 Suspension cells fermentation with cell recycle system

The need of cell recycling can be avoided by using immobilized cell system because the cells in this system are bound to large non-moving particles/carriers. This fact in combination with other potential advantages including high concentration of active cells, gives immobilized cells technology superiority in achieving higher ethanol productivity than conventional methods. Thus, immobilized cell technology is considered promising for ethanol production and chosen as the focus of this work.

2.3.1 **Review of ethanol fermentation by immobilized carriers**

There have been many studies regarding ethanol fermentation by immobilized cells. Review on some of those studies is summarized in Table 2.1. For inorganic supports for cell immobilization Isono et al. (1994) found that *Saccharomyces cerevisiae* was immobilized on γ-alumina particles with binder polymers using a spray-dryer. The optimum pH for the immobilized yeast was found to be 4. The pre-soaking of γ-alumina particles in resin solution before immobilized with a spray-dryer improved cell immobilization and achieved high sucrose conversion to ethanol [18]. Continuous ethanol fermentation was successfully developed using a bioreactor with immobilized cell on the external and internal surfaces of inorganic, channeled, porous alumina beads [19]. Maximum productivity was achieved using fermentation broth (containing 16.7% glucose) with residence time of 4 hours [19].

Organic materials usually proposed as supports of immobilization in ethanol production are mainly polymeric materials, such as polysaccharides and are widely found in nature as constituents of cell walls of plants, crustaceans or insects, etc. The most commonly used polysaccharides for cell immobilization are alginates, cellulose, carrageenan, agar, pectic acid and chitosan. Jamai et al. (2001) reported the use of Ca-alginate as immobilization support to immobilized *Candida tropicalis* and *Saccharomyces cerevisiae* for glucose fermentation. They found that the productions of CO_2 by Ca-alginate immobilized *Candida tropicalis* as well as the lower supply of oxygen to the cells are the major factors that reduce ethanol production [20]. Fermentation of sugar by *Saccharomyces cerevisiae* for production of ethanol in an immobilized cell reactor (ICR) was successfully carried out to improve the performance of the fermentation process. The cell was entrapped in calcium alginate. By using ICR the yield was improved approximately 27%. Productivities of the ICR were 1.3, 2.3, and 2.8 g/l h for 25, 35, 50 g/l of glucose concentration [21].

Table 2.1 Studies on ethanol production by immobilized cells.

Author	Carrier		Shape and Dimension	Remark
	Type	Material		
Koutinas et al. (1988) [22]	Inorganic	Alumina pellets	Cylindrical Length = 5mm ∅ = 2.5 mm	1. *Zymomonas mobilis* cells were immobilized on pellets of alumina (Al_2O_3) based on opposite electrostatic forces 2. Rate of growth, ethanol production and glucose utilization increased when Al_2O_3 was added in the growth medium. 3. Many of cells escaped in the supernatant medium. Therefore, the separation of cells from the fermentation medium before further processing was required.
Kanellaki et al. (1988) [23]	Inorganic	Gamma-Alumina	Cylindrical Length = 5mm ∅ = 2.5 mm	1. Gamma-Alumina increased the final cell concentration, biomass yield and productivity. 2. The rate of ethanol production was markedly increased. Therefore, the low cost Gamma-Alumina seemed to be an extremely attractive porous solid that might be used for immobilizing *Saccharomyces cerevisiae*.

Table 2.1 Studies on ethanol production by immobilized cells.

Author	Carrier		Shape and Dimension	Remark
	Type	Material		
Hamdy et al. (1989) [19]	Inorganic	Alumina Beads	Sphere ∅ = 3.8 mm	1. Continuous ethanol fermentation was successfully achieved using a bioreactor with cells immobilized on to alumina beads. 2. The immobilized cell system was stored for a maximum of one year without loss of bioreactor activity. 3. The near-horizontal column was superior for continuous ethanol production as compared to the vertical column. 4. The total surface area available for attachment of cells and pore size were important factors for immobilization.
Isono et al. (1995) [18]	Inorganic	Gamma-Alumina	Sphere ∅ = 3 mm	1. Presoaking of alumina particles in resin solution increased immobilization yield and sucrose conversion to ethanol. 2. Addition of yeast extract increased ethanol production. 3. The optimum pH of fermentation shifted to acidic (pH 4). This restricted contamination and facilitated electrostatic attraction between alumina and cells.

Table 2.1 Studies on ethanol production by immobilized cells.

Author	Carrier		Shape and Dimension	Remark
	Type	Material		
Bekers et al. (1999) [6]	Inorganic	Stainless steel	Sphere ∅ = 6 mm	1. Carrier reuse was successful. 2. Modified wire sphere was used. 3. Average ethanol volumetric productivity was 0.92-1.25 g/l h.
Najafpour et al. (2004) [21]	organic	Alginate	Sphere ∅ = 5 mm	1. The ICR system exhibited a higher yield of ethanol production (38%) compared to the batch system. *2.* High glucose concentration (150 g/l) was used in continuous fermentation using immobilized *S. cerevisiae.*
Santos et al. (2005) [1]	Inorganic	Porous glass Zeolite beads	Sphere Porous glass ∅ =2.53 mm Zeolite beads ∅ =0.3-2x10^{-3} μm	1. Porous glass was more preferred than zeolite because of higher immobilization yield. 2. Cell concentration was higher in the immobilized system than the free cell system. 3. Productivity was 30 % lower for the immobilized system compared to the free cell system.

Table 2.1 Studies on ethanol production by immobilized cells.

Author	Carrier		Shape and Dimension	Remark
	Type	Material		
Kourkoutas et al. (2005) [24]	Inorganic and organic	Kissiris Alumina pellets Apple pieces	-	1. Immobilization cells were stored and remained active for longer time period than the free cells. 2. Immobilized cells were found to be more stable on storage than free cells. 3. Higher storage time periods resulted in contaminate of the wines when kissiris, apple-supported biocatalysts and free cells were used, while immobilized cells on γ-alumina pellets were unable to ferment after longer storage.

2.4 1,3- Propandiol Production

1,3-Propanediol, trimethylene glycol ($HOCH_2CH_2CH_2OH$) is produced on a much smaller scale than its isomer, propylene glycol. In spite of some interesting areas of application, the total production remains relatively small. The difficulties of manufacturing the product resulted in a poorly competitive price structure relative to other diols. 1,3- PD can be produced in two difference way: the petrochemical route from the oxidation of ethylene and the biotechnological route from bacterial fermentation.

1,3-PD is a typical product of glycerol fermentation and has not been found in anaerobic conversions of other organic substrates. Only very few organisms, all of them bacteria, are able to form it. They include enterobacteria of the general *Klebsiella* (*K. pneumoniae), Enterobacter (E. agglomerans)* and *Citrobacter (C. freundii*), lactoba-cilli *(L. brevis and L. buchneri)* and clostridia of *C. butyricum* and *C. pasteurianum* group [25-30]. All of them produces 1,3-propanediol as the primary product. Different organism produces different levels of product and by-products, as well as having different nutrient requirements. Many researches have been conducted looking for organism capable of producing higher product concentration, with faster production rates.

The fermentation of glycerol into 1,3-propanediol requires an anaerobic biological reactor. The classical reactor used for most fermentation studies is a batch reactor. Numerous studies have been conducted using simple closed vessels, tubes or flasks. The stirred tank, and airlift reactor [31] are two types of reactor that have been used in glycerol fermentation studies. It was found that specific productivities of 2.3-2.9 $gl^{-1}h^{-1}$ and final 1,3-propanediol concentrations of 50-58 g/l could be achieved in these reactors. 1,3-propanediol fermentation in batch, fed batch [32-33] and continuous processes have been investigated. Continuous fermentations of *Citrobacter freundii* in stirred tank reactor with a productivity of 1.38 $gl^{-1}h^{-1}$ was reported. However, the improved performance was achieved by continuous culture of *Klebsiella pneumoniae* in a stirred tank reactor in which productivities of 4.9-8.8 $gl^{-1}h^{-1}$ with final 1,3-propanediol concentrations of 35.2-48.5 g/l were obtained [34] .

Many organism are capable of anaerobic metabolism using glycerol as a sole substrate, all of which reduce the glycerol to 1,3-propanediol. Reductive glycerol dissimilation consists of two enzymatic steps, 1) a dehydration step, followed by 2) a

hydrogenation step. Oxidation glycerol dissimilation follows the glycolytic pathway to pyruvate, which typically results in the formation of acetate and butyrate. However, a number of other compounds can be formed from the glycolytic pathway intermediates. Enzymatic pathway is shown in Figure 2.12 [47].

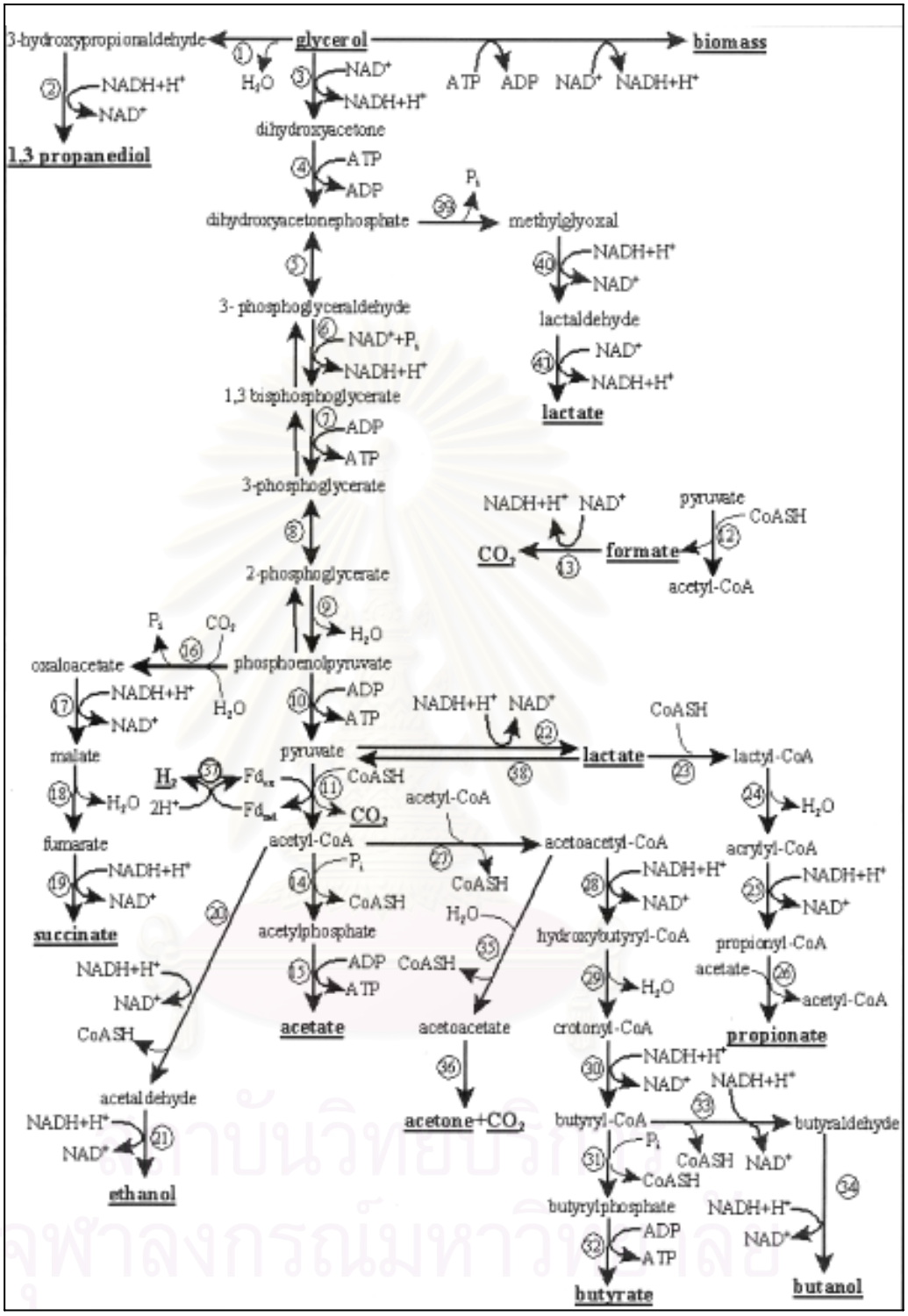


Figure 2.12 Enzymatic Fermentation Pathways Potentially Present in Glycerol Fermentation [47]

The importance of knowing all potential metabolic pathways is to determine the optimal pathway to use to maximize desirable products. In genetic engineering, multiple metabolic steps can be created in a host organism to produce an optimum amount of desirable products

2.4.1. **Review of 1,3-propanediol fermentation**

The organisms for 1,3-propanediol formation include species of *Klebsiella, Citrobacter, Enterobacter,* and *Clostridia.* Table 2.3 summarizes the organisms, which are capable of converting glycerol to 1,3-propanediol.

Clostidia

Two species appear capable of forming 1,3-propanediol are *Clostridium pasteurianum* and *Clostridium butyricum*

Clostridium pasteurianum is known as a classical acid producer and usually ferments carbohydrates to butyrate, acetate, carbon dioxide and hydrogen. However, it could form 1,3-propanediol when grown on glycerol as the sole carbon and energy source. Dabrock et al. 1992 [29] determined effect of pH, phosphate and iron limitations on 1,3-propanediol formation by *Clostridium pasteurianum*. *Clostridium pasteurianum* was grown on glycerol at pH 7 in batch mode; 27.6 g/l glycerol were consumed, and 1,3-propanediol, butanol and ethanol were obtained in concentrations of 10,3.3 and 1.4 g/l, respectively.

Heyndrickx et al. 1991 [33] observed the simlar situation in cultivation of *Clostridium butyricum* in which ethanol and butanol pathways were absent or at least very weak. *Clostridium pasteurianum* and *Clostridium butyricum* have the advantages that they are nonpathogenic, and 1,3-propanediol yield of *Clostridium butyricum* is close to the maximum.

Gunzel et al. 1991 [31] studied the conversion of glycerol to 1,3-propanediol by *Clostridium butyricum* DSM 5431 in fed batch culture up to scale of 2 m^3. Growth and product formation were optimal at pH 7 and 35 °C. Final concentration of 50 to 58 g/l of 1,3-propanediol (yield of 0.52-0.71 mol/mol and productivities ranging from 2.3-2.9 g/lh was obtained).

Remann et al. 1996 [34] studied the effect of methyl viologen addition and phosphate and iron limitation on production distribution during glycerol fermentation of *Clostridium butyricum* DSM 5431 in continuous culture. The acetate/butyrate ratio during glycerol fermentation was essentially influenced by the availability of iron. Therefore iron limitation proved to be a suitable means to achieve high 1,3-propanediol yield and to reduce butyrate formation.

Colin et al. 2000 [36] studied on inhibition during glycerol fermentation to 1,3-propanediol by *Clostridium butyricum* CNCM 1211. The initial concentration of the 1,3-propanediol affected the grown and fermentation of the bacterium more than the glycerol concentration. For initial glycerol at 20 g/l, the growth and fermentation were completely stopped at an initial 1,3-propanediol concentration of 65 g/l. However, for an initial 1,3-propanediol concentration of 50 g/l and glycerol at 70 g/l, the final concentration of 1,3-propanediol reached 83.7 g/l (1.1 M) with complete consumption of the glycerol. Therefore, during the fermentation, the strain tolerated a 1,3-propanediol concentration higher than the initial inhibitory concentration (65 g/l).

Himmi et al. 1999 [37] studied on essential nutrient requirements for glycerol fermentation into 1,3-propanediol by *C. butyricum.* Batches fermentation using low-nutrient medium (LNM) based on biotin as the sole growth factor were performed with glycerol as a carbon source. It was shown that only 4 μg/l of biotin was sufficient to convert 129 g/1 of glycerol into 67 g/1 of 1,3-propanediol (production yield = 0.63 mol/mol glycerol used).

Biebl et al. 1991 [28] studied with respect to growth inhibition by the accumulating products. During glycerol fermentation of *C. butyricum* growth was inhibition by end product at concentrations of 60 g/l for 1,3-propanediol, 27 g/1 for acetic acid and 19 g/1 for butyric acid at pH 6.5. Appreciable inhibition by glycerol was found only above a concentration of 80 g/l.

Klebsiella

Homann et al. 1990 [27] found *K. oxytoca* strains and *K. pneumoniae* were capable of forming 1,3-propanediol.

Zeng et al. 1993 [38] used the method of metabolic pathway stoichiometry to analyze the fermentation of glycerol to1,3-propanediol by *Klebsiella pneumoniae* DSM 2026.

Grown on glycerol in excess, the yield of 1,3-propanediol was 0.68-0.73 mol/mol of glycerol consumed.

Solomon et al. 1994 [39] studied on the product formation during anaerobic degradation of glycerol by *Klebsiella pneumoniae* DSM 2026, under glycerol limitation and glycerol excess in continuous cultures at 37 °C, pH 7 , dilution rates from 0.08 to 0.38 h^{-1} and feed glycerol concentrations of 15, 40, and 74 g/l. The major products of this fermentation were considered to be 1,3-propnaediol, ethanol, and acetate; the minor products were formate, lactate and succinate while acetoin and 2,3-butanediol were the by products of the anaerobic glycerol metabolism.

Chen et al. 2003 [40] studied the microbial production of 1,3-propanediol (1,3-PD) by *Klebsiella pneumoniae* under micro-aerobic conditions. The experimental results of batch fermentation showed that the microbial growth in fed-batch fermentation by *K. pneumoniae* DSM 2026 was faster under micro-aerobic than anaerobic conditions. The concentration, molar yield, and productivity of 1,3-PD in fed-batch fermentation under micro-aerobic conditions were 59.50 g/l, 51.75%, and 1.57 g l^{-1} h^{-1}, respectively.

Citrobacter

Homann et al. 1990 [27] studied anaerobic in batch cultures of *K. pneumoniae, K. oxytoca*, and *C.freundii*. They studied using purified strains isolated from sediment and sewage sludge. Two isolates were identified as *C. freundii* and given strain name of *Zu* and *K2*. For fermentation with the initial glycerol of 50 g/l at pH 7, the strain *Zu* and *K2* were formed in concentration 30 and 34 g/l of 1,3-propanediol with productivity of 1.33 and 1.37 g/l*h. *C. freundii* produces 1,3-propanediol with higher yield (0.64 mol/mol) than either *K. pneumoniae* (0.53 mol/mol) and *K. oxytoca* (0.42 mol/mol) because *C. freundii* produced less lactic and less ethanol than the other two strains.

Boenigk et al. 1993 [35] studied the conversion of glycerol to 1,3-propanediol by *Citrobacter freundii* DSM 30040 in single-stage and two-stage continuous. Glycerol concentration of 80 g/l were consumed, with the final of 1,3-propanediol concentration of 41 g/l and the overall productivity of 1.38 g l^{-1} h^{-1}.

Enterobacter

Enterobacter agglomerans was recently identified as a 1,3-propanediol producer by Barbirato et al. 1997 [30]. They studied the continuous culture of *Enterobacter agglomerans* CNCM 1210 under regulated pH condition (pH 7) with glycerol and/or glucose (20 g/l) as carbon source. Cultures grown on glucose produced mainly acetate, ethanol and formate. In contrast 1,3-propanediol was the main product form glycerol and the yield of 1,3-propanediol was 0.52 mol/mol

2.4.2. Review of 1,3-propanediol fermentation by immobilized carriers

Immobilized cells are typically immobilized in or onto an inert support material. Important properties of a material are its inert nature, size, shape, density, surface properties, and price. When cells are immobilized into a matrix, like the alginate entrapment method, these properties can be manipulated to some extent. However, when cells are immobilized onto a solid material, like adsorption to vermiculite, the properties of the support matrix can be selected to provide optimal physical characteristics.

Pflugmacher and Gottschalk 1994 [41] immobilized *Citrobacter freundii* on polyurethane particles for the fermentation of glycerol to l,3- propanediol. The reactor was a fixed bed loop reactor with a total volume of 500 ml. The temperature and pH were controlled at 36 °C and 6.9 respectively. They observed a maximum specific productivity of 8.2 g /lh with 400 mM glycerol feed and a substrate efficiency of 57 mole of 1,3-propanediol /100 mole of glycerol.

Table 2.2 1,3-Propanediol production by different strains of bacteria

Organism	Initial glycerol (g/l)	1,3-PD (g/l)	Yield (mol/mol)	References
K. pneumoniae DSM2026	125	56	0.54	Deckwer 1995 [42]
K .pneumoniae DSM2026 *C. freundii* Zu	50 53	22.30 28.15	0.53 0.64	Homann 1990 [27]
K .pneumoniae DSM2026	40	18	0.54	Chen 2003 [40]
K .pneumoniae DSM2026	60	31	0.62	Biebl 1998 [43]
C. freundii DSM 30040	34.5	19	0.57	Pflugmacher 1994 [41]
C. butyricum DSM 5431	55	26.6	0.58	Remainn 1998 [44]
C. butyricum DSM 5431	110	56	0.61	Deckwer 1995 [42]
C. butyricum DSM 5431	22	13	0.69	Biebl 1995 [45]
C. butyricum DSM 5431	97	58	0.71	Gunzel 1991 [31]
C. butyricum CNCM 1211	120	63.7	0.64	Colin 2000 [36]
C. butyricum CNCM 1211	70	37	0.64	Barbirato 1998 [46]
C. butyricum CNCM 1211	129	67	0.63	Himmi 1999 [47]

CHAPTER III
MATERIALS AND METHODS

Most of the materials and methods used in this work are of common practice in cell cultivation, cell immobilization, and fermentation technologies. A flocculating yeast strain, *Saccharomyces cereviceae* M30 was chosen based on its high ethanol productivity. The microorganism employed to produce 1,3-propanediol in this work was *Clostridium butyricum*, DSM 5431. Methods for cell carrier preparation were constructed based on simple sensible aseptic procedures that can be readily applied on bench scale fermentation experiments.

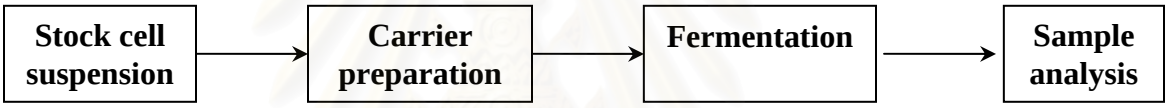


Figure 3.1 General flow diagram of the experiment work in this study

3.1 Microorganism and Stock cell suspension

Stock cell suspension preparation steps are outlined by Figure 3.2. *Saccharomyces cereviceae* M30 strain was kindly provided by Assoc. Prof. Dr Savitree Limtong, from Department of Microbiology, Kasetsart University, Bangkok. Stock culture was stored in PDA agar slant. Each starter culture was obtained by transferring cells from an agar slant into 500 ml Erlenmeyer flask containing 100 ml sterilized cultivation medium. The cultivation medium was composed of 5% w/v sugar from palm sugar, 0.05% w/v ammonium sulfate at pH 5. The medium was sterilized in autoclave for 20 minutes at 121°C before used. Cell cultivation was comducted in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 20 hours before harvesting. The cells were concentrated by decantation to obtain stock cell suspension. All procedures were done in aseptic condition.

A strain of *Clostridium butyricum*, DSM 5431 was obtained from American Type Culture Collection. This strain is identical to the American Type Culture Collection (ATCC) BAA-557^{TM}. Stock culture was stored at 4 °C in Reinforced Clostridial Medium (RCM), which is a rich media used for the general maintenance, and cultivation of wide varity of Clostridial species. For inoculum preparation, spores were transferred to RCM medium, heat shocked at 80°C for 10 min and incubated at 35°C under anaerobic conditions in tubes. The preculture medium contained 100 ml and following components (l^{-1} distilled water): glycerol 20 g; K_2HPO_4 1 g; KH_2PO_4 0.5 g; $(NH_4)_2SO_4$ 2 g; $MgSO_4.7H_2O$ 0.2 g; $CaCl_2.2H_2O$ 15 mg; $FeSO_4.7H_2O$ 5 mg; $CaCO_3$ 2 g; yeast extract 1 g; trace element solution SL7 2 ml. Preculture medium of 100 ml in 500 ml flask was sealed under nitrogen before sterilized in autoclave for 20 minutes at 121°C before used. Cell cultivation was carried out in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 100 rpm, 33°C for 20 hours and then was concentrated by decantation to obtain stock cell suspension.

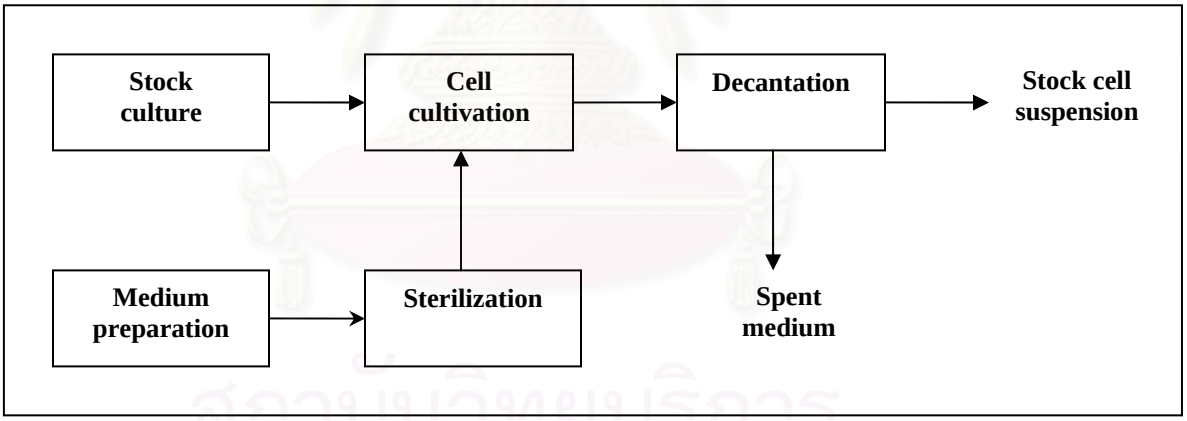


Figure 3.2 Methods of stock cell suspension preparation

3.2 Preparation of carriers for immobilization

Immobilization of cells in batch fermentation was investigated using two general methods. The first was the adsorption of cells onto the surface of γ-alumina powder. The second was entrapment of alumina-cells in calcium alginate matrix.

3.2.1 γ-Alumina carrier preparation

γ - Alumina powder was used as a solid support material with abundant surface area for cell adsorption. γ - Alumina powder was put on an aluminium plate and sterilized in autoclave for 15 minutes at 121°C before use.

3.3.2 Alginate-alumina carrier preparation

Preparation steps of entrapment alginate-alumina carriers are shown in Figure 3.3 Alumina powder was immersed in stock cell suspension for 20 hours to induce natural cells adhesion. All procedures were done in aseptic condition to minimize contamination. After that supernatant were removed. Alumina-cell mixture was added to the alginate solution to form an alginate-alumina-cell mixture with volumetric ratio of 1:1. The mixture was used to construct gel carriers. The formation was initiated by adding the alginate-alumina-cell mixture drop wisely into 0.12M $CaCl_2$ using a syringe with internal diameter of needle of 1.2 mm. Gel carriers with the diameter (∅) 3 mm were left to harden in $CaCl_2$ solution for 15 minutes to ensure that precipitation reaction reached completion. The carriers were then rinsed 3 times with NaCl 0.9% w/v.

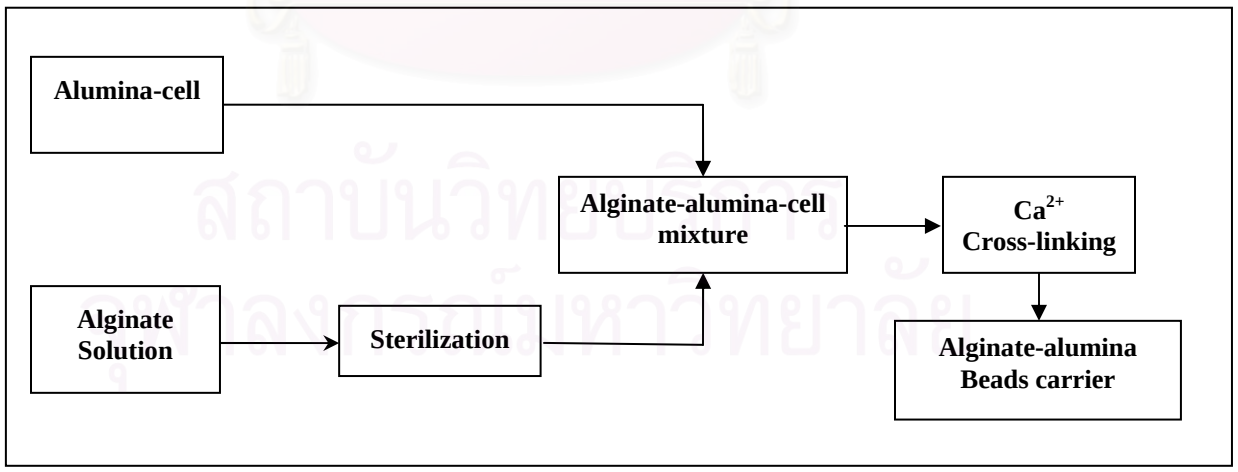


Figure 3.3 Preparation of alginate-alumina carrier

3.3 Fermentation in the shake flask

For ethanol fermentation, molasses based medium of 22% w/v reducing sugar with 0.05% w/v $(NH_4)_2SO_4$ at the initial pH of 5.0 was used as culture medium. Suspended cell (SC) culture was prepared by inoculating stock cell suspension (10%, v/v) into 200 ml fermentation medium in 500 ml Erlenmeyer flask. Batch fermentation in shake flasks was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C.

For 1,3-propanediol fermentation, glycerol was used as the sole carbon source. The medium composition for fermentation was similar to culture medium only without $CaCO_3$ and the initial concentration of glycerol was varied from 40-130 g/l. The volume of medium was adjusted to 100 ml in 500 ml Erlenmeyer flask and the system was sealed under nitrogen in order to promote anaerobic condition which is favorable to 1,3-propanediol fermentation by bacterial. Suspended cell (SC) culture was prepared by inoculating stock cell suspension (10%, v/v) into 100 ml fermentation medium. Batch fermentation was carried out in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 100 rpm, 32 °C maintained all the time at the initial point.

Immobilized cell cultures were inoculated aseptically with a known mass of their respective carriers. The samples were frozen before analysis of sugar, ethanol, 1,3-peopanediol and cell concentration in order to enable all samples to be analyzed at the same time. In repeated batch mode, decanted cells for SC or immobilized cells were transferred aseptically to fresh medium and new run was carried in the same configuration as the main run.

3.4 Fermentation in the bioreactor

For 1,3-propanediol fermentation, the medium composition for fermentation was similar to culture medium only without $CaCO_3$ and the initial concentration of glycerol was 80 g/l. Batch culture was conducted in a 1-l reactor filled with 560 ml of medium and inoculated 140 ml of preculture. During the fermentation, nitrogen gas (at a rate of 0.1 vvm) was infused into the culture medium. The agitation speed was 100 rev./min and the

pH was adjusted to 7 by automatic addition of 4 N NaOH. The incubation temperature was 32°C.

3.5 Analytical methods

Sugar concentration was determined using a modified 3,5-dinitrosalicylic acid (DNS) reagent method through a corresponding standard curve. Briefly, 0.2 ml of sample was hydrolyzed with HCl 37% w/v. After the hydrolysis was stopped, the sample was neutralized using NaOH 30% w/v. Centrifugation was performed and the supernatant was reacted with DNS reagent before the color intensity was measured by spectrophotometer at wavelength of 520 nm.

Ethanol assay was measured by gas chromatography (Shimadzu Model GC $7A_G$) equipped with Flame Ionization Detector (FID). A column with length of 2 m, outer diameter of 3.3 mm, and packed with Porapak Q 80-100 mesh was used in collaboration with N_2 as carrier gas. Flow rate of N_2 is 50 ml/min. The oven and detector temperature were 190°C and 240°C respectively. The samples were injected with volume of 1μL and injection temperature of 240°C.

1,3-propanediol assay was measured by HPLC equipped with refractive index Detector (RI). A column (Lichrocart C18) with length of 250 mm, outer diameters of 4 mm. Operating condition was: 20 mM H_3PO_4 as a mobile phase, flow rate 1.2 ml/min, column temperature at room temperature.

Free and immobilized cell concentrations were measured as cell dry weight. Samples of fermentation broth were centrifuged (2000 rpm, 15 min) and the cells were resuspended in water for free cell determination. A known mass of cell carriers was dissolved in sodium citrate 0.05 M. The suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. Drying of all samples was performed at 60°C for 3 days.

3.6 Scanning Electron Microscopy (SEM)

Carriers from the end of fermentation run were sputter-coated by gold and examined using JSM 6700-F (JEOL, Japan) scanning electron microscope.

3.7 Calculation of fermentation parameters

The yield of sugar consumption (Y_S, %) was calculated as the ratio of sugar consumption at the end of fermentation ($S_0 - S_F$, g/l) to initial sugar level (S_0, g/l). Immobilization yield (Y_I, %) was defined as the ratio of immobilized cell concentration (X_I, g/l) to total cell concentration (X_T, g/l) at the end of fermentation. The ethanol yield factor ($Y_{P/S}$, g ethanol/g sugar) was considered as the ratio of final ethanol concentration (P_F, g/l) to ($S_0 - S_F$). The 1,3-propanediol yield factor ($Y_{P/S}$, g 1,3-propanediol/g glycerol) was considered as the ratio of final 1,3-propanediol concentration (P_F, g/l) to ($G_0 - G_F$).

Chapter IV

Results and Discussion

4.1. Immobilization of *Saccharomyces cerevisiae* M30 for Ethanol Fermentation

Batch experiments were carried out to compare the amount of sugar consumption and ethanol production in system of suspension and that of immobilized cells. In fermentations of molasses medium contained the initial sugar concentrations of 220 g/l in shake flask culture either with free cells or immobilized cells of *Saccharomyces cerevisiae*. Ethanol fermentation in this experiment was carried out for 60 hours. Three cultures were evaluated in this study. Labels of each culture were given in Table 4.1.

Table 4.1 List of sample and labels for ethanol fermentation

Sample's Name	Label
Suspension cells culture	SC
Adsorption γ-Al_2O_3 culture	AC
Adsorption γ-Al_2O_3-Entrapment alginate culture	AEC

The results of the fermentations are summarized in Table 4.2. At the end of the fermentation, the total cell concentrations of immobilized cell cultures (IC) were higher than that of the SC culture. The final immobilized cell concentration of the system with AEC carriers was 4.12 g/l, which was slightly lower than that of the system with AC carriers (4.3 g/l). Increase of cell concentration in AC and AEC carriers was owing to cell growth inside the carriers (γ-Al_2O_3) during the fermentation. Similar result for AC was reported by Koutinas et al., 1988 studying on the fermentation of *Z.mobilis*. Kanellaki et al., 1988 reported that the immobilization

of AC led to positive effect to the growth of *Saccharomyces cerevisiae* since the surface of AC did not inhibit the growth of culture. The adsorption of cells on the surface of γ-Al_2O_3 should be due to high positive charge density on the surface of the carrier and negative charged on the yeast cell wall. Such attachment may depend not only on electrostatic forces, but also on chemical bonds between carboxyl groups, amino groups and hydroxyl groups of the cell wall and Al^{3+} ions [23]. Table 4.2 summarizes the final ethanol and cell concentration and yields of batch fermentation for 60 hours using SC, Ac and AEC cultures. Complete definitions of the parameters were previously described in section 3.7.

Table 4.2 Yield and end products of batch ethanol fermentation for 60 h using the cultures of suspended cells, AC-immobilized cells and AEC-immobilized cell

System	P_F (g/l)	X_T (g/l)		Y_I (%)	$Y_{P/S}$ (mol/mol)
		X_E	X_I		
SC	82.4	3.9	-	-	0.42
AC	77.1	0.46	4.3	89.3	0.40
AEC	74.6	0.59	4.12	85.6	0.39

The sugar concentration profile of this study was given in Figure 4.1. The concentration of sugar of all systems was decreased with increasing biomass and ethanol concentrations. The consumption of sugar on SC culture was higher than that of immobilized cell cultures. After 60 h, the sugar concentration of SC system was 27 g/l, whereas the final sugar concentrations of immobilized cell (IC) in AC and AEC carriers were 33 and 40 g/l, respectively. For all systems, trend of sugar concentration and ethanol production profile corresponded to the trend of the normal microbial growth.

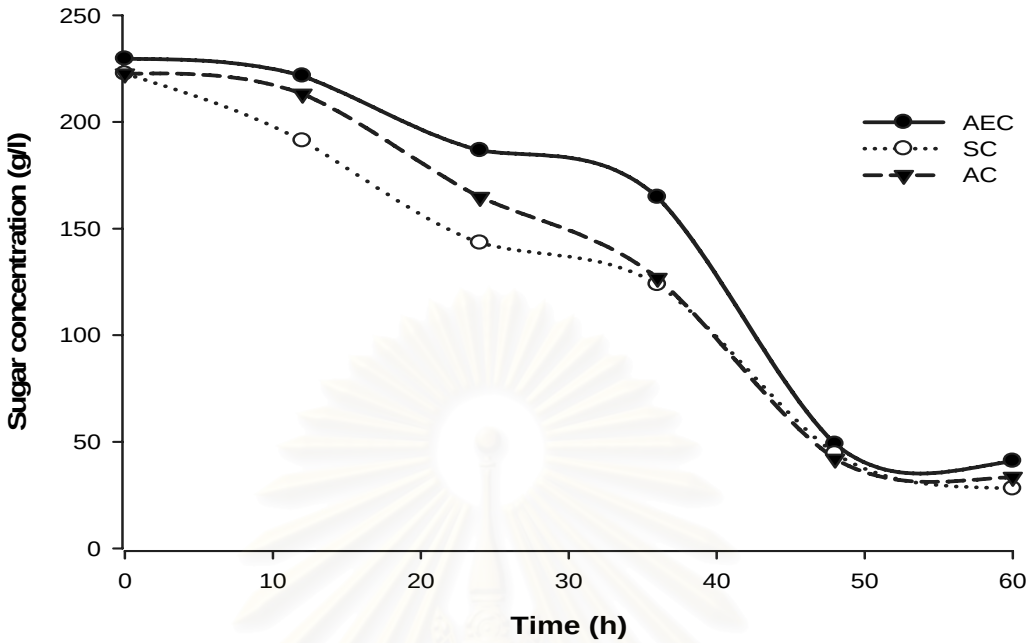


Figure 4.1 Sugar concentration profile of fermentation

Figure 4.2 shows a comparison of performance in terms of ethanol production. It was found that the sugars were consumed duration 60 h with the final ethanol concentration were 82, 77 and 74 g/l for SC, AC-immobilized cell and AEC-immobilized cell cultures, respectively. In a range of fermentation time from 12 to 36 hours, it was shown that the ethanol concentration of AEC carriers was lower than that of the system with AC carriers. This can be indicated that mass transfer resistance did affect cell growth and product formation. However, the final ethanol concentration of AEC increased until it reached to similar level with AC at the end of fermentation (60 hours). From these results, it can be summarized that the ethanol concentration of immobilized cell cultures (IC) were slightly lower than that of the SC culture. With reference to immobilized yeast growth on γ-Al_2O_3, Kanellaki et al., 1988 reported that the ethanol concentration and production yield of immobilized cell cultures (IC) were equal or lower than that of the SC culture. It is evident the presence of γ-Al_2O_3 causes slight changes in the final ethanol concentration and production yield.

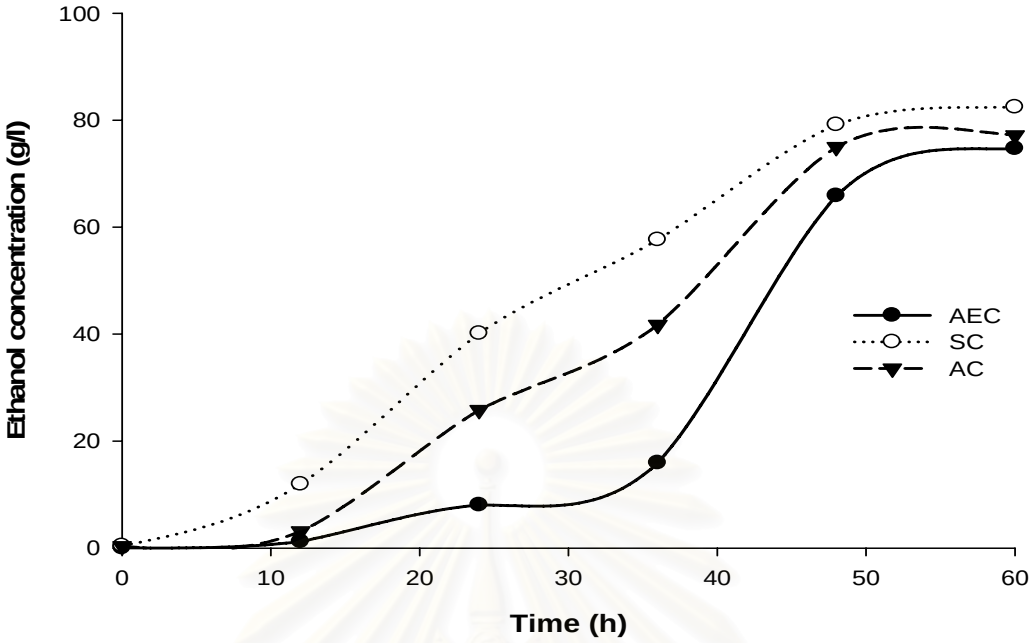


Figure 4.2 Ethanol concentration profile of fermentation

From the immobilization yield (Y_I) data (Table 4.2), it can be concluded that the new carriers (AC and AEC) were efficient in immobilizing yeast. Structural differences among the carriers were proposed as the main separating factor and will be further analyzed by comparing the SEM images. Y_I of AC (89.3 %) was high thus this carrier was considered effective for yeast immobilization.

In these view, the new carriers can be used for ethanol production by yeast. To improve productivity and yield, AEC can be used as carrier matrix in a packed column, and AC carrier in cell recycling for continuous fermentation. Beneficial properties of immobilized cell systems, such as protection from inhibition and promotion of cell activity had been reported elsewhere [48, 49, 51, 52]. Junter et al., 2004 proposed that the ability of cells to grow in the immobilized state makes it possible for the regeneration of the culture in hostile condition such as high ethanol concentration.

A series of scanning electron micrographs were taken for immobilized cell carriers, showing the appearance of outer surface and cross section of the carriers at the end of fermentation. Figure 4.3 shows an overall view of AEC. Yeast cells in AEC

were protected by film of alginate However, some cells may escape from the carrier if there was a leak on the surface (Figure 4.4).

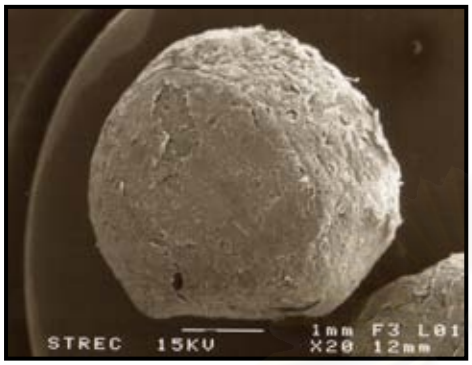


Figure 4.3 Overall view of AEC after batch fermentation for 60 hours

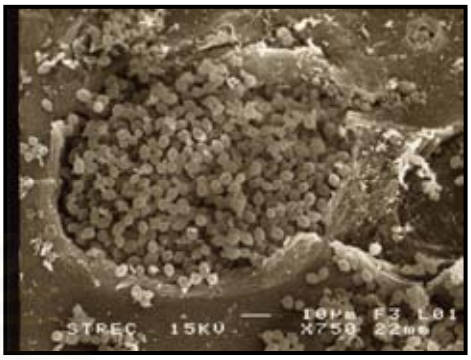


Figure 4.4 Leak on the surface of AEC after batch fermentation for 60 hours

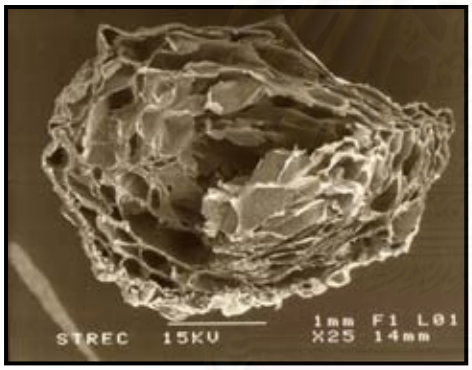


Figure 4.5 Cross section of AEC after batch fermentation for 60 hours

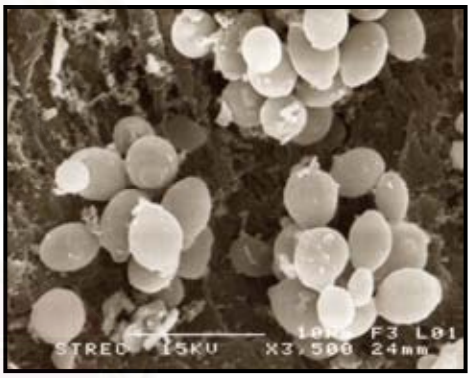


Figure 4.6 Closer look of the of AEC after batch fermentation for 60 hours

The cross sectional view of AEC was provided in Figure 4.5. Yeast can grow well inside the gel structure of AEC. The yeast was healthy as confirmed by the image in Figure 4.6. Figure 4.7 shows an overall view of AC. As can be seen from the picture there were many cells attached on γ-Al_2O_3 surface (Figure 4.8). Cell growth increased sufficiently during fermentation. In addition, considering the good cell attachment, it was assumed that most of the cells were strongly fixed on γ-Al_2O_3 surface.

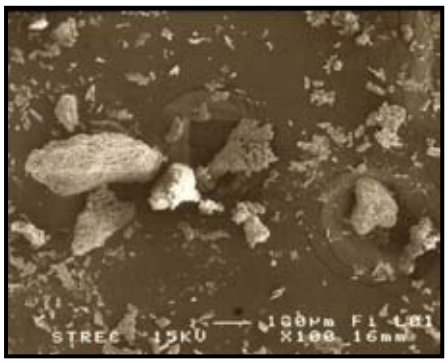


Figure 4.7 Overall view of AC
after batch fermentation for 60 hours

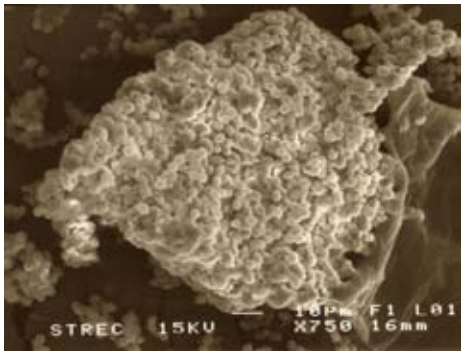


Figure 4.8 Surface of AC
after batch fermentation for 60 hours

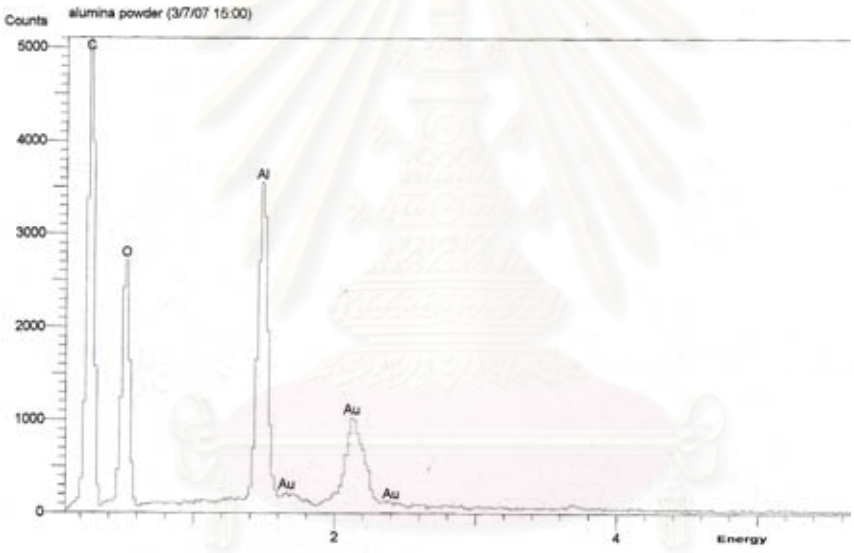
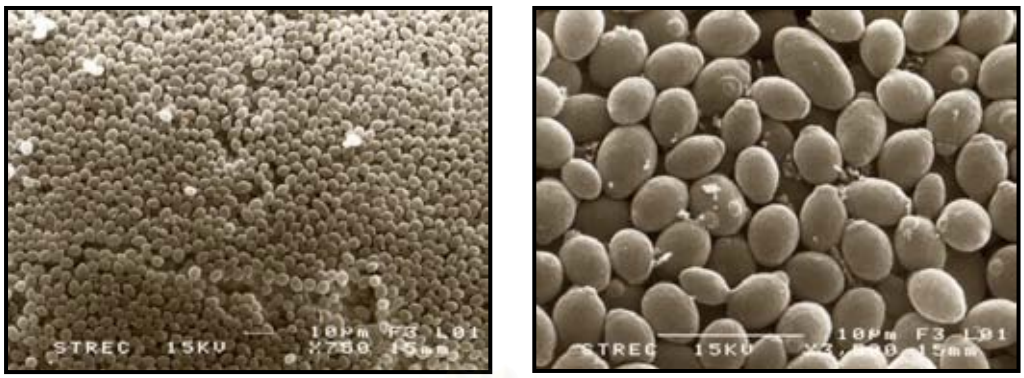


Figure 4.9 Spectra of superficial elemental composition of surface performed by energy dispersive X-ray spectroscopy after biomass immobilization

Figure 4.9 shows γ-Al_2O_3 peak in the elemental composition after cell immobilization. Figure 4.10 (A, B) shows an image of yeast living in suspended cell culture (SC). The cells appeared healthy and retained their normal oval shape



(A) (B)

Figure 4.10 Yeast from SC broth after batch fermentation for 60 hours

4.2. Repeated batch Fermentation of *Saccharomyces cerevisiae* M30

Ethanol production using γ-Al_2O_3 as a carrier for *S cerevisiae* M 30 was examined by a 4-cycle repeated batch fermentation using cane molasses as the carbon source. The duration of each batch was 48 h. There were three cultures in this study: Suspended cells (SC), γ-Al_2O_3 immobilized cells (AC) and γ-Al_2O_3 doped alginate immobilized cells (AEC). The results of fermentations are shown in Figure 4.11-4.12. For the first batch, after 48 h ethanol concentration of the SC system was 77.06 g/l, whereas the final ethanol concentrations of immobilized cells (IC) in AC and AEC carriers were 67.25 g/l and 69.73 g/l, respectively.

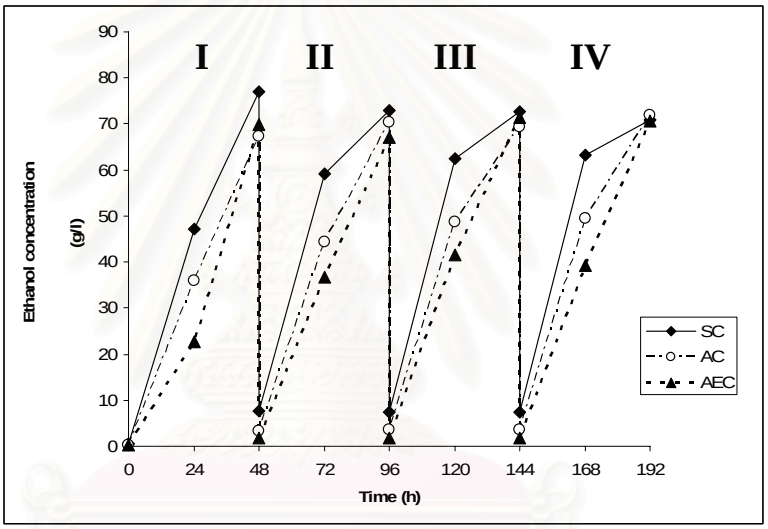


Figure 4.11 Ethanol concentrations in repeated batches of SC, AC and AEC

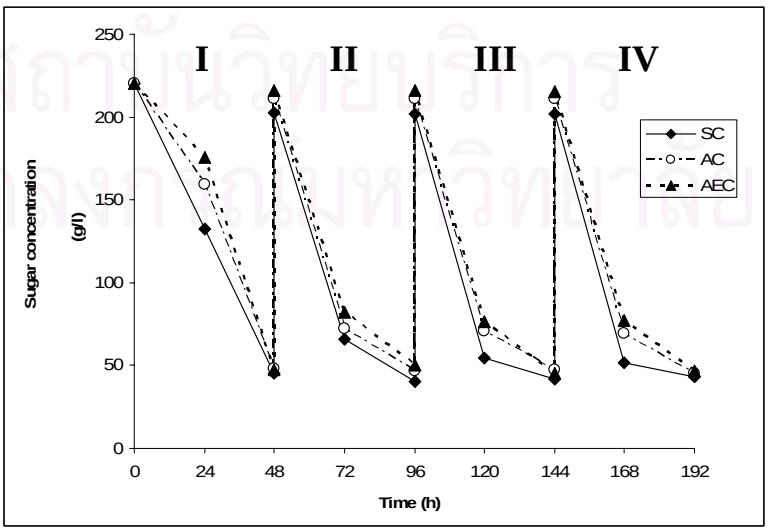


Figure 4.12 Sugar concentrations in repeated batches of SC, AC and AEC

In the second and the third batch, all system exhibited high ethanol productions without any occurrence of the lag phase. The ethanol concentration profile followed the trend of sugar concentration curve. This demonstrated that the yeast used in this study was highly active, which resulted in high sugar consumption and high level of ethanol. In the fourth batch, the majority of sugar was consumed within 48 h with the final ethanol concentrations being 70.87, 71.80 and 70.74 g/l for SC, AC-immobilized cells, and AEC-immobilized cells, respectively. At the end of the fourth batch, the total cell concentrations of IC cultures were higher than that of the SC culture. Increases in cell concentrations in AC and AEC carriers were due to immobilized cells in the carriers during the fermentation. The immobilized cell concentration of the system with AEC carriers was 4.96 g/l, which was slightly lower than that of the system with AC carriers (5.25 g/l) however; the ethanol production was still in a high level (Table 4.3).

Table 4.3 Yields and end products of repeated batch ethanol fermentation for 48 h for each batch using of SC, AC and AEC

Batch	P_F (g/l)	X_T (g/l)		$Y_{P/S}$ (g/g)	Y_S (g/g)
		X_E	X_I		
I					
SC	77.06	-	-	0.43	0.79
AC	67.25	-	-	0.39	0.78
AEC	69.73	-	-	0.40	0.78
II					
SC	72.88	-	-	0.40	0.80
AC	70.27	-	-	0.40	0.78
AEC	67.17	-	-	0.39	0.76
III					
SC	72.75	-	-	0.40	0.79
AC	69.46	-	-	0.40	0.77
AEC	71.80	-	-	0.40	0.79
IV					
SC	70.87	4.5	5.25	0.39	0.78
AC	71.80	-	4.96	0.41	0.78
AEC	70.74	-	-	0.40	0.78

Yield (Y) is an important parameter in a technological point of view and Table 4.3 demonstrates this parameter. Sugar consumption (Y_S) of AC was also similar to that of AEC and SC, suggesting that the new AC and AEC carriers were reliable in terms of substrate utilization. Instability of the SC culture in the repeated batch fermentation was observed from the comparison of its final ethanol concentration and $Y_{P/S}$ from batch to batch. The ethanol concentration in SC culture dropped from 77.06 g/l in the 1st batch to 71-73 g/l in the 2nd-4th batch, which maybe attributable to the negative effect high ethanol concentration on cell activity and viability. The ethanol production of IC cultures in AC and AEC carriers were relatively stable, which can be attributed to ethanol yield factor ($Y_{P/S}$) of AC and AEC carriers were quite stable in all batches. Koutinas and Kanellaki (1988) reported that specific ethanol productivity of immobilized cells on γ-Al_2O_3 was slightly higher than free cell. The repeated batch study also revealed protective ability of the immobilization matrix. In the medium of same composition, more cells were grown in the carriers than in the liquid broth. The matrix of the carriers may protect the yeast by fortification from toxins and inhibitor [3,5-7].

During the first batch, the yeast in immobilized systems was still acclimatizing with the new microenvironment thus its ethanol yield factor was consequently lower than SC. In repeated batch 4, the cells in the carrier were better protected from their environment than free cell; they could utilize more fraction of sugar for ethanol production as indicated by relatively higher $Y_{P/S}$ for AC and AEC than SC. It is interesting to note that the level of $Y_{P/S}$ of immobilized systems was more comparable to SC in repeated batch 4 than the first batch. This result is proposed as an indication that the immobilized yeast had been well acclimatized to environmental condition in repeated batch 4.

In conclusion, AC and AEC were successfully developed and applied in repeated batch ethanol fermentation. In this study, the AC and AEC carriers have many advantages including reusability and stability. To improve productivity and yield, AEC can be used as carrier matrix in a packed column, and AC carrier in cell recycling for continuous fermentation.

4.3 1,3-Propanediol Fermentation (*C. butyricum* DSM 5431)

4.3.1 RCM Tube Cultures and Pre-Culture Media

Clostridium butyricum DSM 5431 is a Gram-positive, anaerobic spore-forming rod. The spores are heat-resistant. For culture preparation, a 1 ml of *Clostridium butyricum* DSM 5431 stored in liquid medium at 4 °C in a glass tube is aseptically transferred to 15 ml of the sterilized medium in 20 ml glass tube. The medium is kept anaerobic by bubbling pure nitrogen for 5 min and is then kept at 35° C for 20 hour growth. A large amount of bubbles from the fermented gas are observed during 12- 20 hours of the cultivation. Figure 4.13 demonstrates the characteristics of the culture at 20 hours in the glass tube.

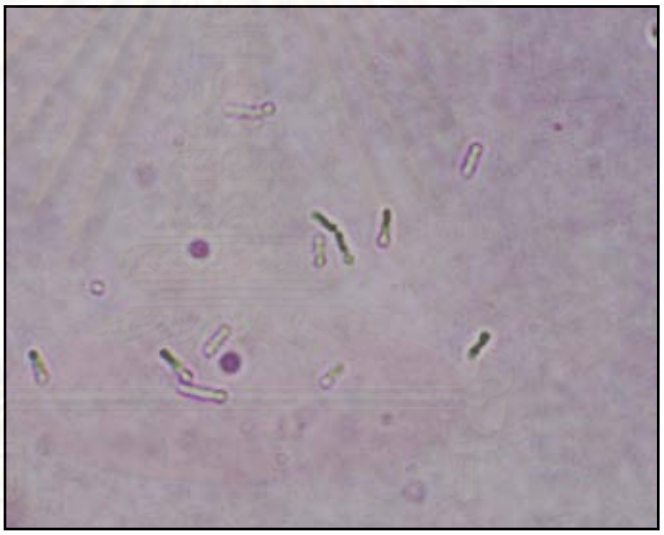


Figure 4.13 *Clostridium butyricum* DSM 5431 in RCM medium at 20 hours of the cultivation in the glass tube

4.3.2 Fermentation Media Cultures

The 15 ml of the prepared culture was aseptically transferred to 150 ml of the sterilized medium in the 500 ml flask. The medium was also kept anaerobic by bubbling pure nitrogen for 15 min. The agitation speed was maintained at 100 rpm and the temperature was controlled at 32° C. The culture tube and the culture flask are demonstrated in Figure 4.14. It was found that *Clostridium butyricum* DSM 5431 could grow well and produce 1,3- PDO during the cultivation. Every 4 hours of the operation, 3 ml of fermentation broth was aseptically taken from the shaking flask for the determination of products, cells and substrates (glycerol).

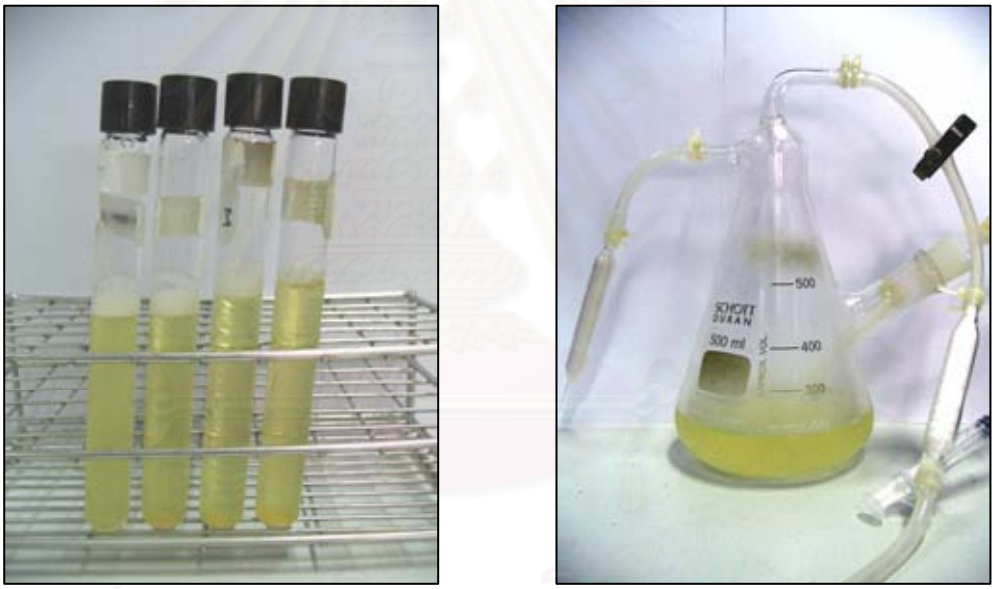


Figure 4.14 Show the culture tube and the culture flask

Figure 4.15 Batch fermentation in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA)

Figure 4.16 demonstrates the characteristics of the culture at 30 hours in the shaking flask. C*lostridium butyricum* DSM 5431 in the shaking flask is in rod-shape connected together as a long tail. In comparison with the culture in the 10 ml tube (Figure 4.13), it is shown that cell morphology changed when agitation condition was changed. Besides the mass transfer effect on cells, the mixing of broth is also a crucial character for transporting substance into cells.

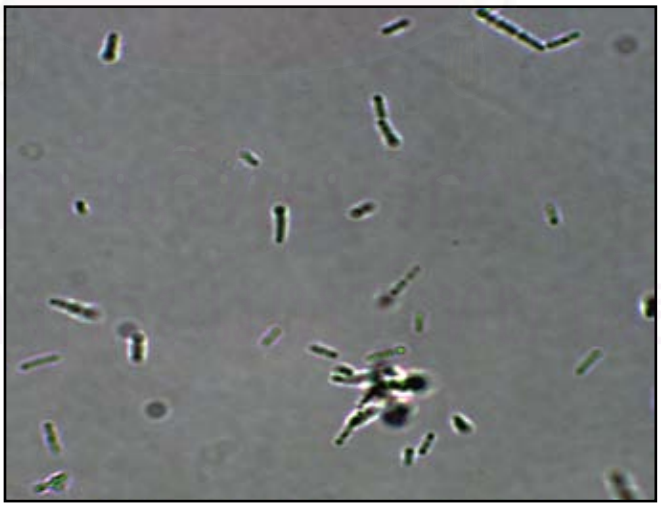


Figure 4.16 *Clostridium butyricum* DSM 5431 in the shaking flask at 30 hours

4.3.3 Shake Flask Culture 1

In this study, the effect of substrate inhibition on biomass growth and 1,3-propanediol production were investigated. Batch cultures in shake flask were carried out at different glycerol concentrations. The initial glycerol concentrations were varied from 40 – 130 g/L. The results of this experiment were shown in Figure 4.17-4.18.

The concentration of glycerol slightly decreased while the cell density and 1,3-propanediol concentration to some extent increased during 30 hours of cultivation (Figure 4.17-4.18). The maximum cell density of *Clostridium butyricum* DSM 5431 in batch fermentation was 0.369 g/l. The optimal glycerol concentration was 60-100 g/l.

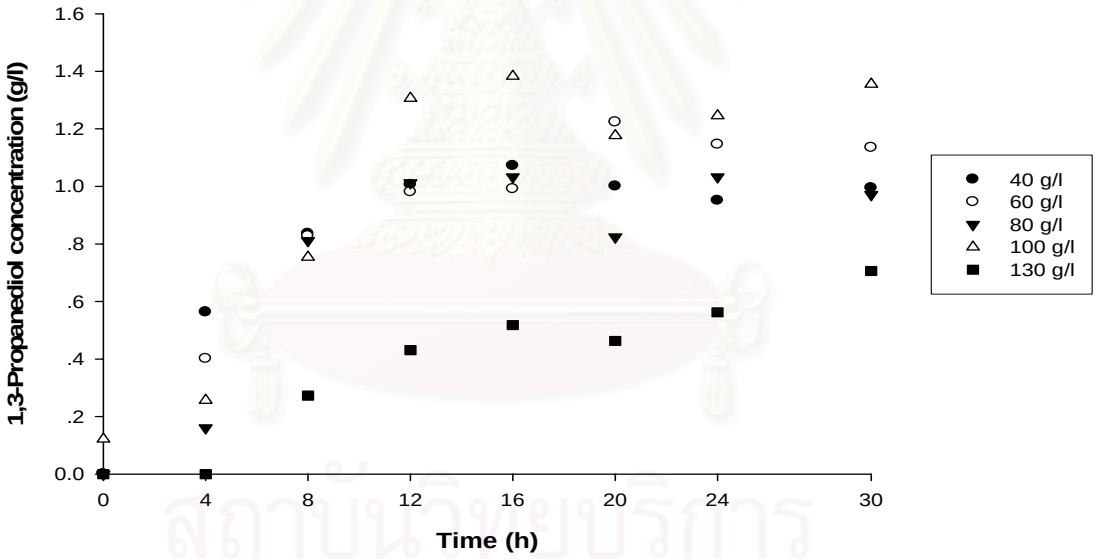


Figure 4.17 1,3-PD concentrations in batch fermentation with initial concentrations of 40-130 g/l glycerol versus time, 500 ml flask

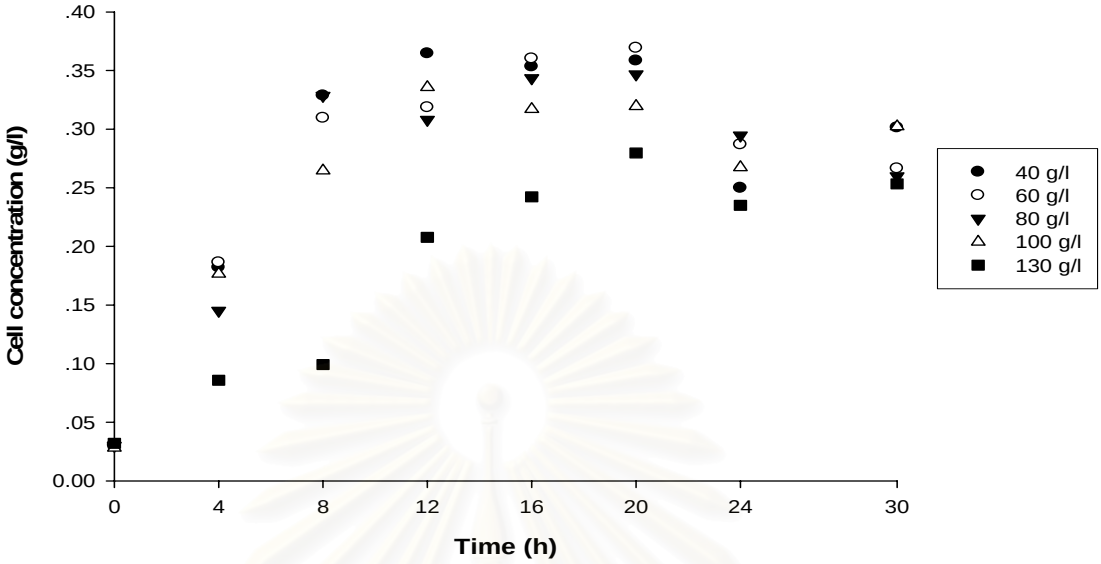


Figure 4.18 Cells density in batch fermentation with initial concentrations of 40-130 g/l glycerol versus time, 500 ml flask

Overall, the concentration profile (1,3-propanediol) was low compared to other reports [26-40]. The possible causes of low 1,3-propanediol production could be according to anaerobic condition in the system. According to literature [26-40], production of 1,3-propanediol using glycerol as the sole carbon source was always done under controlled anaerobic conditions. Usually it was required to deliver N_2 into the shake flask or fermentor throughout the whole processes. Therefore, the improved methodology to maintain anaerobic condition was carried on. Batch cultures were carried out in 500 ml flasks, at 32 °C, with an initial glycerol concentration of 60-100 g/l. The controlled anaerobic condition was improved by increase N_2 gassing time from 5 min to 15 min. The results of this experiment were shown in Table 4.4.

Table 4.4 Final glycerol and 1,3-propanediol concentrations and production rate in batch fermentation with initial concentration of 60-100 g/l glycerol 500 ml shaking flask

Glycerol (g/l)	1,3-PD concentration (g/l)	Production rate (g/l*h)
60	1.53	0.063
80	1.74	0.072
100	1.47	0.061

From Table 4.4 and Figure 4.17-4.19, it was found that the optimal glycerol concentration was 80 g/l, whereas the substrate inhibition was apparent from 100 to 130 g/l glycerol. It was noticed that high concentration of glycerol would delay the lag phase during fermentation.

Under strictly anaerobic condition of this experiment, 1,3-propanediol concentration and production rate were higher than those of previous experiment. It could be concluded that the production of 1,3-propanediol from glycerol by *Clostridium butyricum* DSM 5431 required strictly anaerobic conditions.

1,3-Propanediol concentration was increased during the course of fermentation however, the concentration profile (1,3-propanediol) was low compared to other reports [26-40]. The possible causes of low 1,3-propanediol production could be according to low cell concentration in the system. Therefore, further studies on the effect of cell loading were carried on.

4.3.4 Shake Flask Culture 2

In this study, we investigated the effect of cell loading by increase % inoculums from 10% (v/v) to 20 % (v/v). The fermentation was carried out with the initial glycerol concentration of 80 g/l in shake flask culture for 72 hours. The results are shown in Figure 4.19-4.20.

Figure 4.19 shows the fermentation performance in terms of glycerol and 1,3-propanediol concentration. From this relation, it is observed that the bacterium was more active in the way that it consumed more glycerol from fermentation medium. The maximum 1,3-propanediol concentration was increased to 4.6 g/l (Figure 4.20).

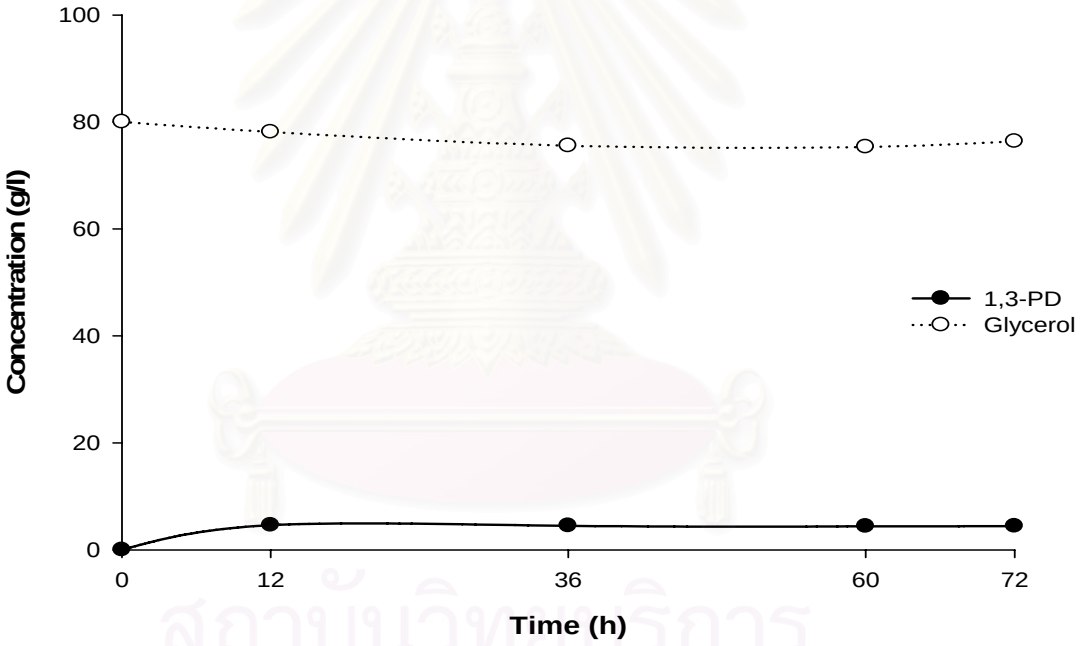


Figure 4.19 Glycerol and 1,3-PD concentrations in batch fermentation with initial concentration of 80 g/l glycerol versus time, 500 ml flask

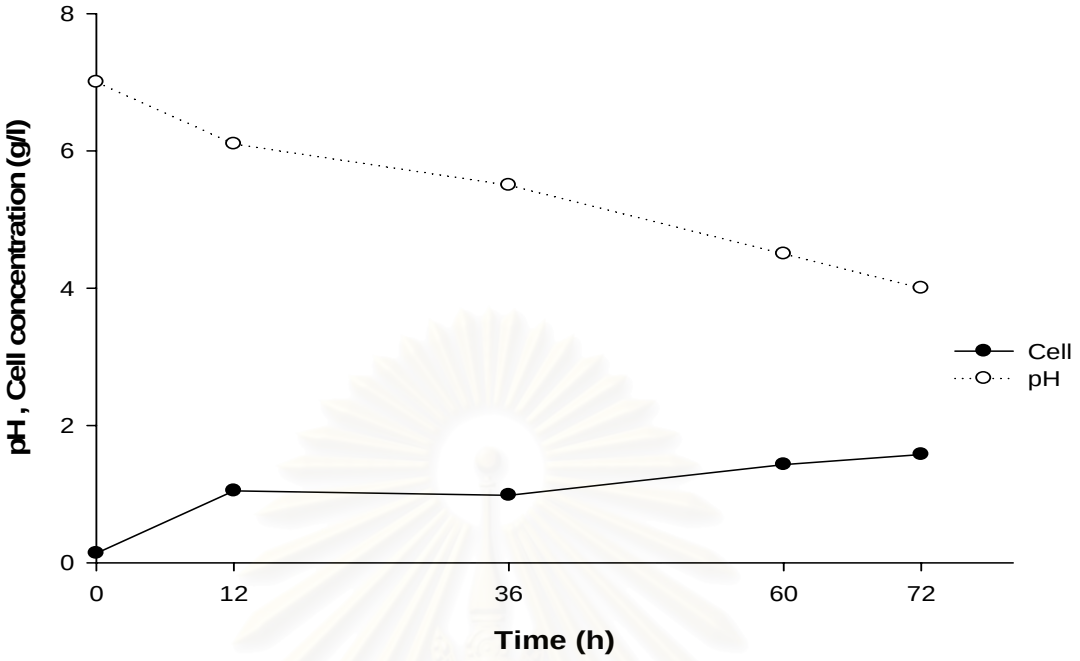


Figure 4.20 1,3-propanediol concentrations and pH-change in batch fermentation with initial concentration of 80 g/l glycerol versus time, 500 ml flask

After 12 hours of the cultivation in shake flasks, an apparent pH-drop to 6 was observed as shown in Figure 4.20. The 1,3-propanediol concentration was almost constant after this stage but the cell density profile remained gradually increased. The pH dropped from 7 to 4 after 72 hours of fermentation. This observation demonstrates that medium pH is very important to 1,3-propanediol fermentation process. Growth rates of bacteria are optimal at pH around 7 and fall quickly at a lower pH value. In general, the pH of the broth influences the maximum growth rate, inhibitory potentials of substances, and the synthesis of most metabolites [55].

1,3-propanediol in this experiment was higher than the previous experiment (Section 4.3.3) however, the concentration of 1,3-propanediol profile was low compared to other reports [26-40]. The possible causes of low 1,3-propanediol production could be according to pH-drop phenomenon. The information regarding the extent of pH drop led to the decision that pH control was necessary.

4.3.5 Batch culture in the 1 liter fermentor

Batch culture was carried out in the 1 liter fermentor with 80 g/l initial glycerol concentration. The fermentor was equipped with pH regulation and regulated by automatically adding sterilized NaOH solution. The fermentation were maintained in an optimal range (T= 33 °C, pH= 7) under anaerobic conditions (N_2 gassing).

1,3-Propanediol fermentation in this experiment was carried out for 33 hours. Glycerol was used up in 33 hour and converted into 1,3-propanediol at maximum concentration of 41.37 g/l, a yield of 0.76 mol PDO /mol glycerol and productivity of 1.3 g/l.h . The biomass of 3.11 g/l was obtained as shown in Figure 4.22. The concentration of 1,3-propanediol from the operation in the fermentor was about 9 times higher than that of the shake flask (Section 4.3.4). Similar results have also been previously reported (Gunzel et al., 1991). The *C. butyricum* strain was able to produce 1,3-propanediol of approximately 18-23 g/l and productivities of 0.7-2.2 g/l.h in batch fermentation of 50 g/l initial glycerol.

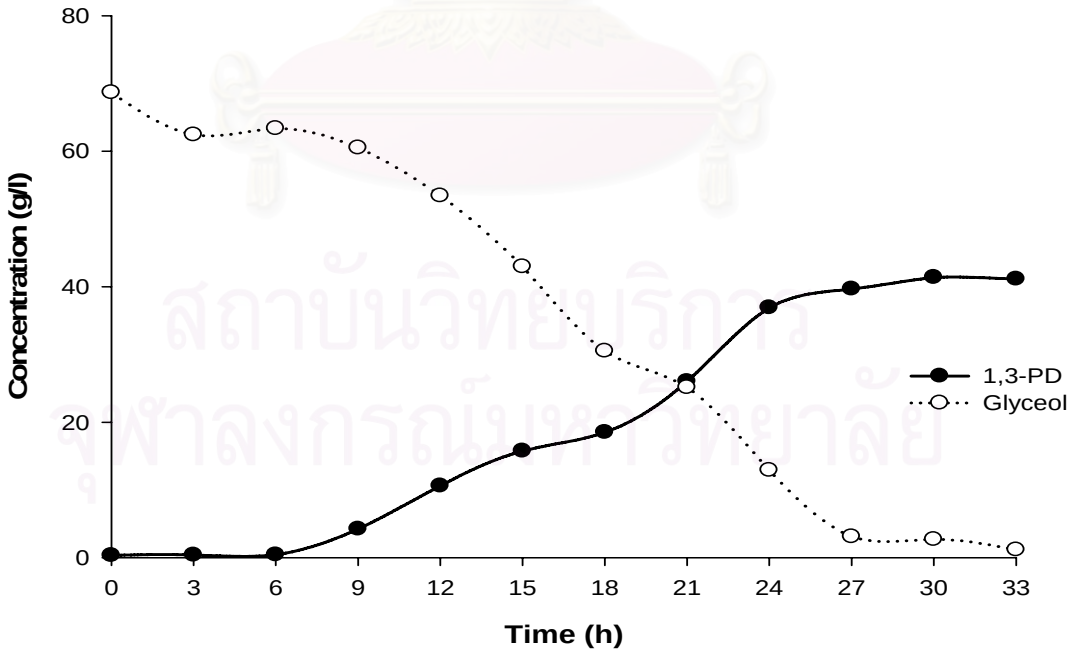


Figure 4.21 Glycerol and 1,3-PD concentration in 1 liter fermentor

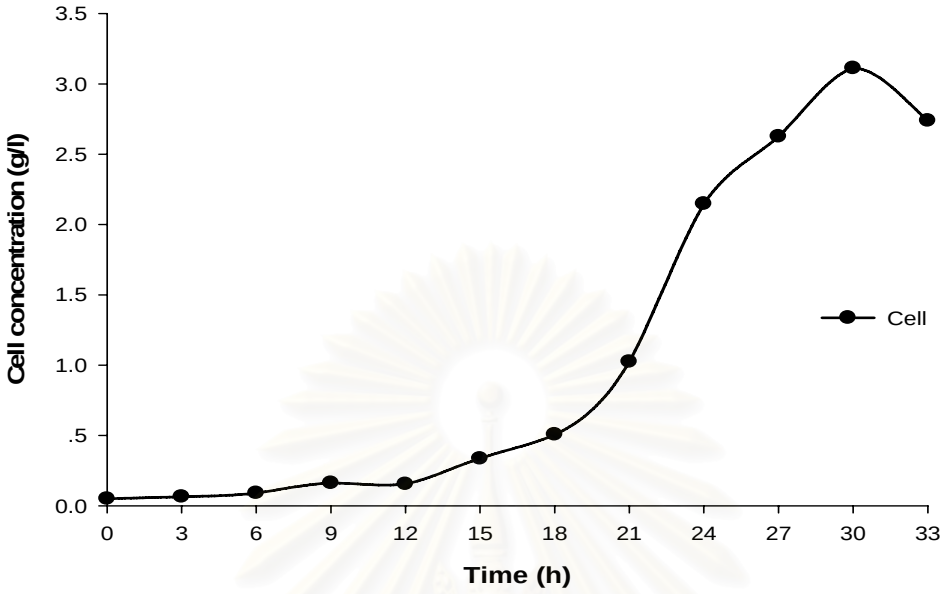


Figure 4.22 Cell concentrations in 1 liter fermentor

It was clear that the biomass and 1,3-propanediol formation were greatly influenced by pH level. Low pH inhibited the cell growth and 1,3-propanediol formation. The controlled pH was beneficial to the consumption of glycerol and enhancement of the yield of 1,3-propanediol. The maximum concentration of 1,3-propanediol was obtained in pH 7. Hanno biebl et al., 1992 proposed that *C. butyricum* DSM 5431 grew well in a pH range between 6.5 and 7.5, with an optimum at pH 7, and was stimulated by sparging with N_2.

SEM observation shows the image of *C. butyricum* DSM 5431 in suspended cell culture. The cells appeared normal rod-shape (Figure 4.23). The bacteria were healthy as confirmed by the image in Figure 4.24. In addition, closer look to the cells, the formation of filaments (polysaccharides and proteins) on cell surface was observed (Figure 4.24).

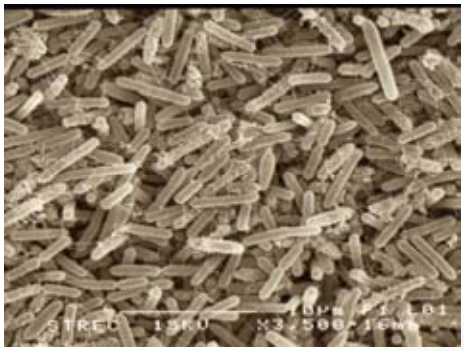


Figure 4.23 *C. butyricum* DSM 5431 from suspension cell

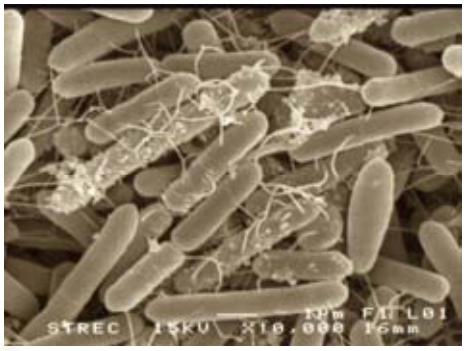


Figure 4.24 The formation of filaments in cells

The 1,3-propanediol productivity (41.37 g/l in 30 hours) in this work was comparable and the 1,3-propanediol yield (0.76) was slightly higher than that in the previous reports. In recent years, 1,3-propamediol fermentations were conducted with *C. butyricum* strains DSM 5431, B593, VPI 3266 (Gunzel et al., 1991; Biebl et al., 1992; Saint-Amans et al., 1994). It previously proposed that 1,3-propanediol was a growth-associated product and the conversion was 0.5-0.6 g/g [31,45,44].Generally, the anaerobic fermentation of glycerol using clostridia presents a higher conversion yield than that obtained during the culture conducted by the enterobacteria. The presently best enterobacteria strain, *K. pneumoniae* DSM 2026 achieved the conversion yield of 0.45-0.53 g/g (Biebl et al., 1992; Menzel et al., 1997; Ya Nan Zhao et al., 2006).

Large efforts have been made in order to increase the conversion yield and productivity of 1,3-propanediol. In literature, the optimizations of natural glycerol converting route include batch and fed batch cultures [47], continuous culture [35], microaerobic culture [40], co-substrate fermentation [45] and two-stage continuous fermentation [53]. Generally, maximum concentration of 1,3-propanediol (55-60 g/l) was obtained in batch and fed batch culture [47]. Continuous culture has the advantage of relatively high productivities (e.g. 4.9-8.8 g/L h with 1,3-propanediol concentrations of 35.2-48.5 g/l at low dilution rates).

Immobilization technique has displayed excellent propertied in the realm of bioconversion [40]. Griffiths and Bosley [54] employed POLYHIPE^{TM} polymers to

immobilized *C. freundii* for 1,3-propanediol production. Pflugmacher and Gottschalk [41] immobilized *C. freundii* on modified polyurethane particles and obtained a high productivity of 1,3-propanediol (81 g/l.h).

In next study, we investigate the potential use of γ-Al_2O_3 in immobilizing C. *butyricum* for production of 1,3-propanediol. The concentration and yield of 1,3-propanediol from glycerol medium under anaerobic operation was reported and compared with the results of free cell culture.

4.4 Immobilization of *C.butyricum* DSM 5431 for 1,3-Propanediol Fermentation

Glycerol consumption and 1,3-propanediol production by batch fermentation using immobilized cell cultures were compared with that using suspended cells. Batch fermentation with 80 g/l initial glycerol concentration in 700 ml fermentor either with free cells or immobilized cells of *C. butyricum* was carried out for 33 hours. Three cultures were evaluated in this study. Labels of each culture were given in Table 4.5.

Table 4.5 List of sample and labels for 1,3-propanediol fermentation

Sample's Name	Label
Suspension cells culture	SC
Adsorption γ-Al_2O_3 culture	AC
Adsorption γ-Al_2O_3-Entrapment alginate culture	AEC

The results of the fermentations are summarized in Table 4.6. At the end of the fermentation, the total cell concentrations of immobilized cell cultures (IC) were higher than that of the SC culture. The final immobilized cell concentration of the system with AEC carriers was 2.97 g/l, which was slightly lower than that of the system with AC carriers (3.48 g/l) However, the concentration profile of 1,3-propanediol of immobilized cell cultures (IC) were low when compared to that of suspension cell culture (SC) in the previous experiment (Section 4.3). Glycerol was consumed during 33 h of cultivation with the final 1,3-propanediol concentration of 20.6 g/l and 15.4 g/l for AC-immobilized cell and AEC-immobilized cell cultures, respectively (Figure 4.25-4.26).

Table 4.6 Yield and end products of batch 1,3-propanediol fermentation for 33 h using the cultures of suspended cells, AC-immobilized cells and AEC-immobilized cells

System	P_F (g/l)	X_T (g/l)		Y_I (%)	$Y_{P/S}$ (mol/mol)
		X_E	X_I		
SC	41.37	3.11	-	-	0.75
AC	20.61	0.89	3.48	79.63	0.58
AEC	15.47	0.60	2.97	83.19	0.48

The 1,3-propanediol yield ($Y_{P/S}$) of the immobilized cells (IC) in AC and AEC carriers were lower than that of SC culture. Low $Y_{P/S}$ and Y_S in IC cultures is strong indication of the occurrence of inhibition, caused by γ-Al_2O_3 based carrier.

The low glycerol consumption of the system with AC and AEC carriers indicated that the bacteria in the systems were less active than the suspended culture. From 1,3-propanediol profiles, it could be considered that γ-Al_2O_3 had negative effects on 1,3-propanediol production. High positive charge density on the surface of the carrier (γ-Al_2O_3) was considered negative interfering in the activities of the cells. The adverse effects on cell membrane could affect its enzyme activities. This assumption reasonably well fitted with the previously observation of the inhibition of cell activities by decreasing of pH (Section 4.3.5).

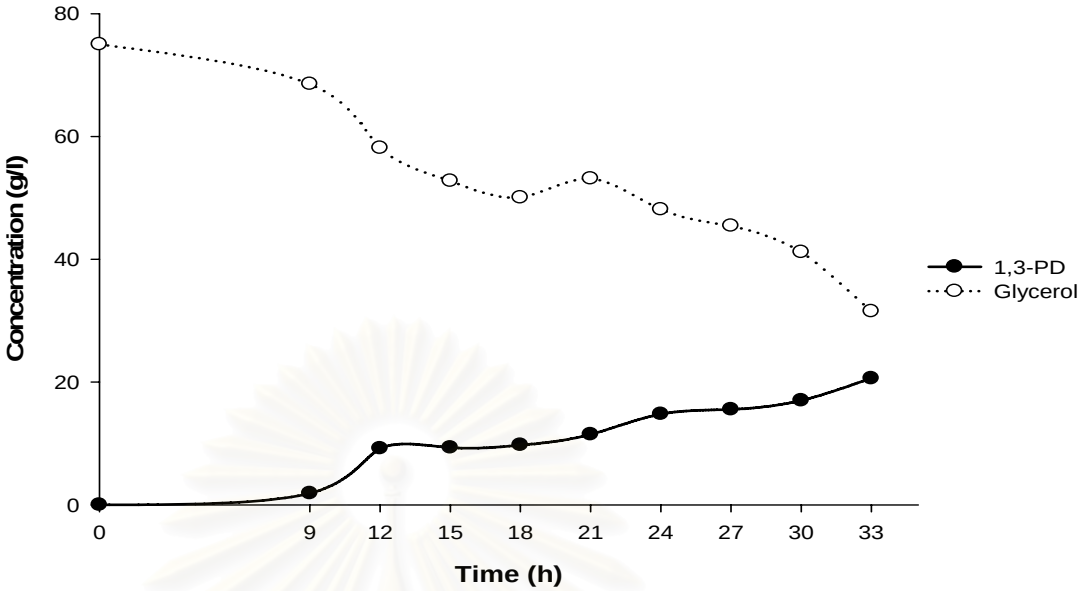


Figure 4.25 Glycerol and 1,3-PD concentration in AC culture

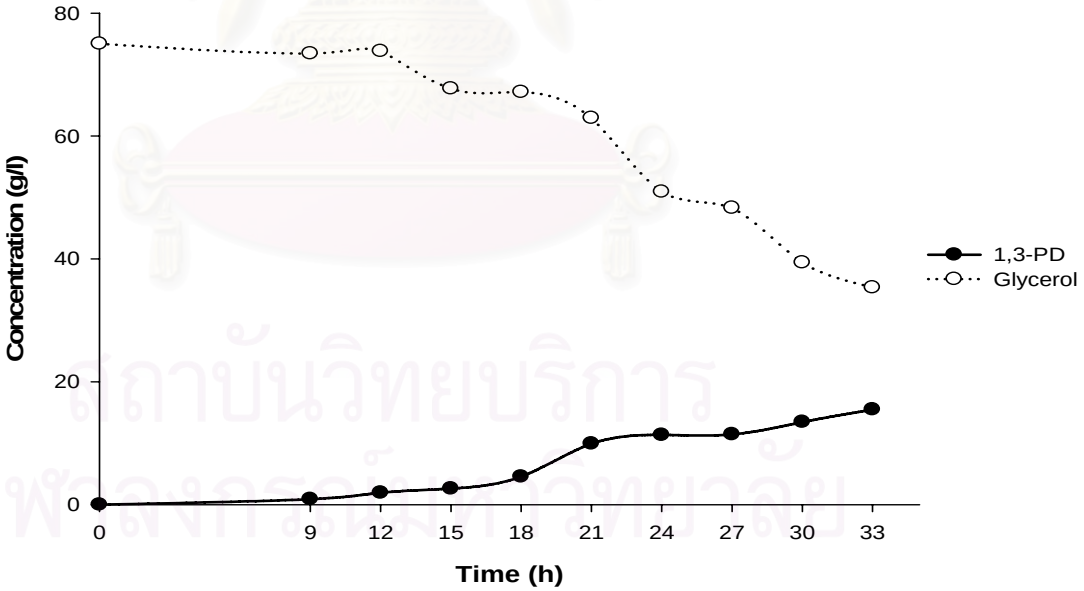


Figure 4.26 Glycerol and 1,3-PD concentration in AEC culture

Figure 4.27 showed the cells immobilized on AC surface after 33 hours of the fermentation. The surfaces of AC in almost all positions were covered with a layer of immobilized cells. It appears that strong binding between *C. butyricum* DSM 5431 cells and γ-Al_2O_3 was obtained, which resulted in immobilization yield factor (Y_I) of AC and AEC carriers were 79.6% and 83.1 % respectively. Such attachment could depend on electrostatic forces and chemical bonds between carboxyl, amino and hydroxyl groups of cell wall and Al^{3+} ions [23]. Koutinas et al., 1988 studying on the fermentation of *Z.mobilis*. They found that the growth of *Z.mobilis* was not inhibited in the presence of γ-Al_2O_3 in batch culture. *Z.mobilis* strain grew well when γ-Al_2O_3 was added to the medium. In this case, the production yield and the productivity were higher when compared to free cells.

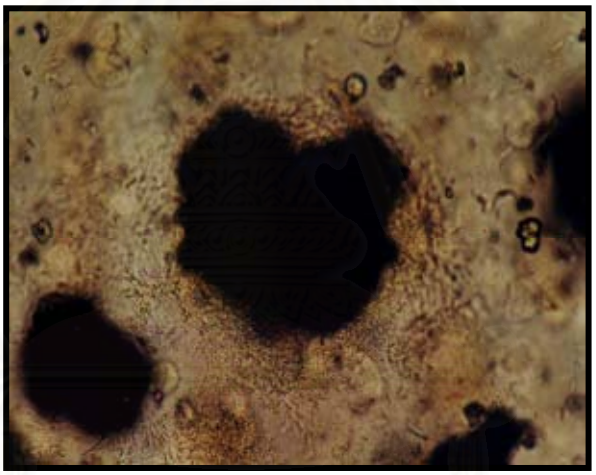


Figure 4.27 Cells immobilized on γ-Al_2O_3 after batch fermentation

In next study, we examined the carrier reusability in 1,3-propanediol fermentation. The concentration and yield of 1,3-propanediol from glycerol medium under anaerobic operation was reported and compared with the results of free cell cultures.

4.5 Repeated batch Fermentation of *C. butyricum* DSM 5431

A 4-cycle repeated batch fermentations was carried out for 1,3-propanediol production by *C. butyricum* DSM 5431 with glycerol as the carbon source in 700 ml fermentor. The duration of each batch was 24 h. There were three cultures in this study: Suspended cells (SC), γ-Al_2O_3 immobilized cells (AC) and γ-Al_2O_3 doped alginate immobilized cells (AEC). The results of the fermentations are shows in Figure 4.28-4.29. For the first batch, after 24 h the 1,3-propanediol concentration of the SC system was 36.9 g/l, whereas the final 1,3-propanediol concentrations of immobilized cells (IC) in AB and AEB carriers were 14.78 g/l and 11.35g/l, respectively.

Table 4.7 Yields and end products of repeated batch 1,3-propanediol fermentation for 24 h for each batch using of SC, AC and AEC

Batch	Time (h)	P_F (g/l)			$Y_{P/S}$ (mol/mol)			X (g/l)		
		SC	AC	AEC	SC	AC	AEC	SC	AC	AEC
I	12	10.6	9.2	1.9	-	-	-			
	24	36.9	14.8	11.4	0.79	0.67	0.57	-	-	-
II	12	17.7	9.3	3.5	-	-	-			
	24	32.7	13.9	6.0	0.72	0.52	0.45	-	-	-
III	12	16.6	12.5	3.5	-	-	-			
	24	33.5	11.6	4.5	0.75	0.40	0.36	-	-	-
IV	12	10.1	12.3	1.6	-	-	-			
	24	26.4	12.5	3.3	0.48	0.46	0.19	4.38	4.56	3.91

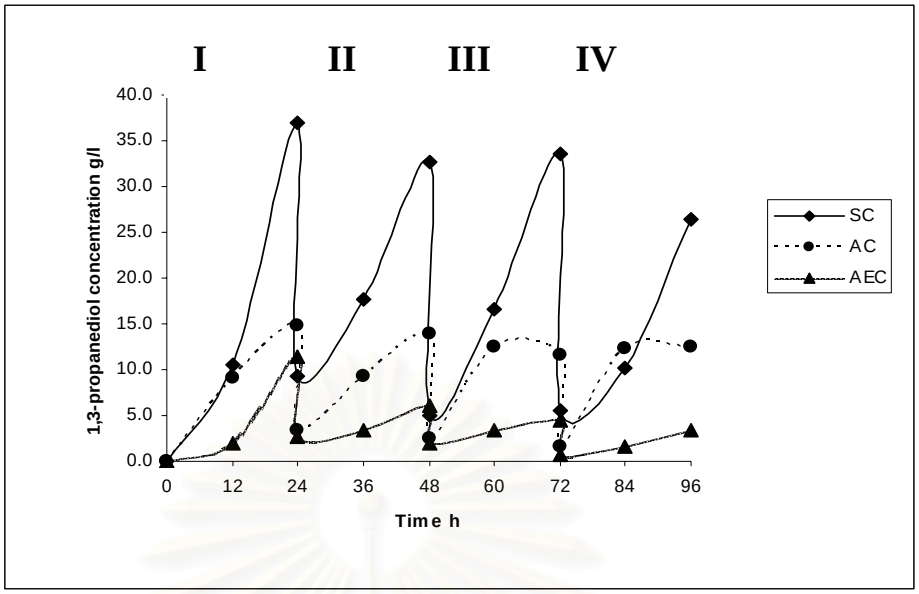


Figure 4.28 The 1,3-propanediol concentration profiles in 4-cycle repeated batches using SC, AC and AEC cultures

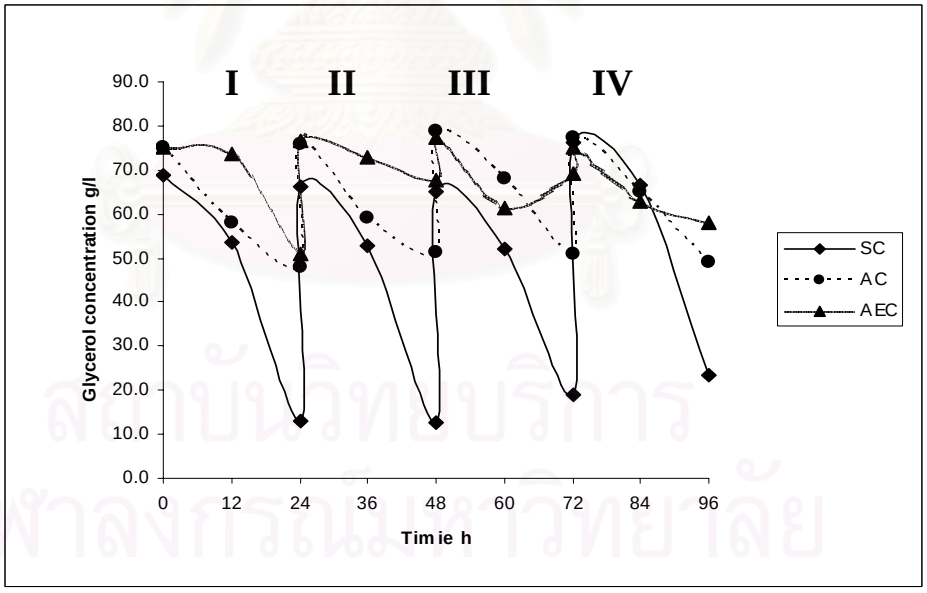


Figure 4.29 The Glycerol concentration profiles in 4-cycle repeated batches using SC, AC and AEC cultures

The 1,3-propanediol concentration of all systems decreased from the first to the fourth batch. At the end of the fourth batch, The final 1,3-propanediol concentration of the system with AB and AEB carriers were 12.46 g/l and 3.32 g/l respectively, which was significantly lower than that of the system with SC culture (26.41 g/l).

Instability of the SC culture was observed from the comparison of $Y_{P/S}$ in the first and the fourth batches as shown in Table 4.7. From the results, Instability of the SC culture in the repeated batch fermentation can be attributable to the negative effect of high 1,3-propanediol concentration on cell activity and viability. While the stability of the AC culture was relatively higher than that of SC culture, the stability and the productivities of AEC-immobilized cell cultures were significantly decreased. The negative effect of γ-Al_2O_3 on the cell activity together with the decrease in diffusivity from alginate entrapment was though to be the main cause of this low performance.

In conclusion, although in term of ethanol fermentation, the application of AC and AEC carriers with *S. cerevisiae* were promising, AC and AEC based carrier was not suitable for immobilizing *C. butyricum* DSM 5431 for 1,3-propanediol production. The application of other carrier for 1,3-propanediol fermentation is considered in the further. The comparison of ethanol fermentation and 1,3-propanediol fermentation using SC, AC and AEC cultures was summarized in Table 4.8.

4.6 Comparison of ethanol fermentation and 1,3-propanediol fermentation using AC and AEC carriers

Table 4.8 Fermentation parameters of ethanol fermentation and 1,3propanediol fermentation using AC and AEC carriers

Parameters	Ethanol fermentation	1,3-propanediol fermentation
Substrate	Molasses	Glycerol
Strain	Yeast (*S. cerevisiae* M30)	Bacteria (*C. butyricum* DSM 5431)
Culture conditions	• Batch and repeated batch fermentation (shake flask) • Aerobic system Optimal conditions • pH 4-5 • 150 rpm • 33 °C pH control is not necessary	• Batch and repeated fermentation (shake flask & fermentor) • Anaerobic system Optimal conditions • pH 7 • 100 rpm • 32 °C pH control is necessary
Analytical method	• Biomass concentration by UV-spectrophotometer & Dry weight • Sugar concentration by UV-spectrophotometer • Ethanol concentration by gas chromatography (GC)	• Biomass concentration by UV-spectrophotometer & Dry weight • Glycerol and 1,3-PD concentration by high performance liquid chromatography (HPLC)

Parameters	Ethanol fermentation	1,3-propanediol fermentation
Single batch	• Final ethanol concentration (P_F) SC = 82.4 g/l AC = 77.1 g/l AEC = 74.6 g/l • $Y_{P/S}$ SC = 0.42 AC = 0.40 AEC = 0.39 • Final cell concentration (X_T) SC = 3.9 g/l AC = 4.76 g/l AEC = 4.71 g/l • Time for fermentation = 60 hours • AC and AEC carriers can be used for ethanol production by *S. cerevisiae* M 30	• Final 1,3-propanediol concentration (P_F) SC = 41.37 g/l AC = 20.61 g/l AEC = 15.47 g/l • $Y_{P/S}$ SC = 0.75 AC = 0.58 AEC = 0.48 • Final cell concentration (X_T) SC = 3.11 g/l AC = 4.37 g/l AEC = 3.57 g/l • Time for fermentation = 33 hours • AC and AEC carrier was not suitable for immobilizing *C. butyricum* DSM 5431 for 1,3-PD production
Repeated batch	• Time for fermentation of each batch = 48 hours 1st batch • Final ethanol concentration (P_F) and $Y_{P/S}$ SC = 77.06 g/l, $Y_{P/S}$ = 0.43 AC = 67.25 g/l, $Y_{P/S}$ = 0.43 AEC = 69.73 g/l, $Y_{P/S}$ = 0.43	• Time for fermentation of each batch = 24 hours 1st batch • Final 1,3-propanediol concentration (P_F) and $Y_{P/S}$ SC = 36.9 g/l, $Y_{P/S}$ = 0.79 AC = 14.8 g/l, $Y_{P/S}$ = 0.67 AEC = 11.74 g/l, $Y_{P/S}$ = 0.57

Parameters	Ethanol fermentation	1,3-propanediol fermentation
Repeated batch	2^{nd} batch • Final ethanol concentration (P_F) and $Y_{P/S}$ SC = 72.88 g/l, $Y_{P/S}$ = 0.40 AC = 70.27 g/l, $Y_{P/S}$ = 0.40 AEC = 67.17 g/l, $Y_{P/S}$ = 0.39 3^{rd} batch • Final ethanol concentration (P_F) and $Y_{P/S}$ SC = 72.75 g/l, $Y_{P/S}$ = 0.40 AC = 69.46 g/l, $Y_{P/S}$ = 0.40 AEC = 71.80 g/l, $Y_{P/S}$ = 0.40 4^{th} batch • Final ethanol concentration (P_F) and $Y_{P/S}$ SC = 70.87 g/l, $Y_{P/S}$ = 0.39 AC = 71.80 g/l, $Y_{P/S}$ = 0.41 AEC = 70.74 g/l, $Y_{P/S}$ = 0.40 • AC and AEC carriers were successfully developed and applied in repeated batch ethanol fermentation by *S. cerevisiae* M 30	2^{nd} batch • Final 1,3-propanediol concentration (P_F) and $Y_{P/S}$ SC = 32.7 g/l, $Y_{P/S}$ = 0.72 AC = 13.9 g/l, $Y_{P/S}$ = 0.52 AEC = 6 g/l, $Y_{P/S}$ = 0.45 3^{rd} batch • Final 1,3-propanediol concentration (P_F) and $Y_{P/S}$ SC = 33.5 g/l, $Y_{P/S}$ = 0.75 AC = 11.6 g/l, $Y_{P/S}$ = 0.40 AEC = 4.5 g/l, $Y_{P/S}$ = 0.36 4^{th} batch • Final 1,3-propanediol concentration (P_F) and $Y_{P/S}$ SC = 26.4 g/l, $Y_{P/S}$ = 0.48 AC = 12.5 g/l, $Y_{P/S}$ = 0.46 AEC = 3.3 g/l, $Y_{P/S}$ = 0.19 • AC and AEC carrier was not suitable for immobilizing *C. butyricum* DSM 5431 for 1,3-PD production, according to the negative effect of γ-Al_2O_3 on the cell activity

CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study, we investigated the potential use of γ-Al_2O_3 in immobilizing *S. cerevisiae* for production of ethanol and immobilizing C. *butyricum* for production of 1,3-propanediol

5.1.1 Ethanol production by *S. cerevisiae* from molasses

- The production of ethanol by immobilized *S. cerevisiae* in the AC and AEC carriers demonstrated desirable characteristics, including repeated cultivations with high density of biomass, stable cell activity.
- In terms of ethanol fermentation performance, the AC and AEC carriers were very promising. In a single batch system, the final ethanol concentrations of suspended cell (SC), AC-immobilized cells and AEC-immobilized cells were 82.4, 77.1 and 74.6 g/l respectively. Our first results clearly showed that AC and AEC were not provoked significantly undesirable effects concerning the fermentation of molasses by *S. cerevisiae*.
- In repeated batch fermentation, AC and AEC showed a good potential of reusability. It has been successfully reused in 3 subsequent batch fermentation cycles. The ethanol production of IC cultures in the AC and AEC carriers were more stable than suspended cell culture (SC). In main batch, ethanol yields of the AC and AEC systems ($P_{F/AC}$ = 67.2 g/l and $P_{F/AEC}$ = 69.7 g/l) were lower than that of suspended culture ($P_{F/SC}$ = 77.06 g/l). However, after passing through acclimatization period, ethanol yields of AC and AEC were equivalent with that of the SC culture. Overall, the ethanol conversion yield factor ($Y_{P/S}$) of all the systems remained constant at 0.4 (g ethanol/ g sugar).

5.1.2 1,3-propanediol production by C. *butyricum* from glycerol

- The AC and AEC based carriers were found unfavorable for *C. butyricum* DSM 5431 immobilization.
- After 33 hours of the cultivation in the fermentor although the cell concentration in all systems were comparable, the productions of 1,3-propanediol in the immobilized systems were significantly decreased. The final 1,3-propanediol concentration of SC system was 41.3 g/l, whereas that of the systems with AC and AEC carriers were 20.61 g/l and 15.47 g/l, respectively. This observation elucidated the inhibitory effect of γ-Al_2O_3 on cell activity. The 4-cycle repeated batch fermentation, especially of the SC and AEC cultures revealed the instability of the cell cultures. The result demonstrated that AC and AEC were inadequate for *C. butyricum* DSM 5431 immobilization for 1,3-propanediol production.

5.2 Recommendations

Based on this study, further improvements are recommended

1. The potential use of AC and AEC in other similar bio-systems should be evaluated in the future.
2. In term of ethanol fermentation, further work involving the application of AC and AEC carrier in a continuous fermentation in a scale larger than this work is considered necessary.
3. In term of 1,3-propanediol fermentation by *C. butyricum*, the application of other carriers for immobilizing bacteria is considered in the further work.

REFERENCES

1. J.C. Santos, S.I. Mussatto, G. Dragone, A. Converti, S.S. Silva, Evaluation of porous glass and zeolite as cells carriers for xylitol production from sugarcane bagasse hydrolysate, Biochem. Eng.J. 23 (2005): 1-9.
2. Y. Cohen, Biofiltration-the treatment of fluids by microorganism immobilized into the filter bedding material: a review, Bioresour. Technol. 77 (2001): 257-274.
3. J.C. Ogbonna, S. Tomiyama, H. Tanaka, Development of a method for immobilization of non-flocculation cells in (Luffa cylindrical) sponge, Process. Biochem. 31(1996): 737-744.
4. J.N. de Vasconcelos, C.E. Lopes, E.P. de Franca, Continuous ethanol production using yeast immobilized on sugar-cane stalks, Brazil. J. Chem. Eng. 21 (2004): 357-365.
5. Y. Kourkoutas, M. Douma, A.A. Koutinas, Continuous winemaking using quince-immobilized yeast at room and low temperatures, Process Biochem. 39 (2002): 143-148.
6. M. Bekers, E. Ventina, A. Karsakevich, Attachment of yeast to modified stainless steel wire spheres, growth of cells and ethanol production, Process Biochem. 35 (1999): 523-530.
7. A. Sakurai, Y. Nishida, H. Saito, M. Sakakibara, Ethanol production by repeated batch culture using yeast cells immobilized within porous cellulose carriers, J. Biochem. 35 (1999): 526-529.
8. S.F Karel, S.B. Libicki, The immobilization of whole cells engineering principles, Chem. Eng. Sci. 40(1985): 1321-1354.
9. J. McGhee, G. Julian, R. Detroy, Continuous and static fermentation of glucose to ethanol by immobilized *Saccharomyces cerevisiae* cells of different ages, Applied and Envirol. Microbio. 44 (1982): 19-22.
10. G. Galazzo, J. Bailey, In vivo nuclear magnetic resonance analysis of immobilization effects on glucose metabolism of yeast *Saccharomyces cerevisiae*, Biotechnol. Bioeng. 33 (1998): 1283-1289.

11. Y. Kourkoutas, A. Bekatorou, I.M. Banat, Immobilization technologies and support materials suitable in alcohol beverages production: review, Food Microbio. 21(2004): 377-397.
12. G. Ronnie, V. Gino, Immobilized living cell systems. John Wiley& Sons (1996).
13. L.H. Chum, R.P Overend, Biomass and renewable fuels, Bioprocess Technol. 17 (2001): 187-195.
14. S. Alfenore, D. Cameleyre, L. Benbadis, C Bideaux, Aeration strategy: a need for very high ethanol performance in *Saccharomyces cerevisiae* fed-batch process, Appl Microbiol Biotechnol. 63 (2004): 537-542.
15. K. Shuler, L. Michael, F. Kargi, Bioprocess engineering basic concepts, (2002) Prentice Hall. New York.
16. L. Jamai, K. Sendide, Physiological difference during ethanol fermentation between calcium alginate-immobilized *Saccharomyces cerevisiae* and *Candida tropicalis*, FEM. Microbiol Letters. 204 (2001): 375-379.
17. P. Dantigny, M. Gruber, Transition rate kinetics from ethanol oxidation to glucose utilization within a structured model of baker's yeast, Appl Microbiol Biotechnol. 45 (1996): 199-203.
18. Y. Isono, G.i. Araya, A. Hoshino, Immobilization of *Saccharomyces cerevisiae* for ethanol fermentation on γ-alumina particles using a spray- dryer, process Biochemistry. 30 (1995): 743-746.
19. M.K. Hamdy, K. Kim, C.A. Rudtke, Continuous ethanol production by yeast immobilized on to channeled alumina beads, Biomass. 21 (1990): 189-206.
20. L. Jamai, K. Sendide, K. Ettayebi, F. Errachidi, O.H. Alami, Physiological difference during ethanol fermentation between calcium alginate-immobilized *Candida tropicails* and *Saccharomyces cerevisiae*, FEMS Microbiology Letters. 204 (2001): 375-379.
21. G. Najafpour, H. Younesi, K.S.K. Ismail, Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cereviceae*, Bioresour. Technol. 92 (2004): 251-260.
22. A.A. Koutinas, M. Kanellaki, A. Lykourghiotis, M.A. Typas, C. Drainas, Ethanol production by *Zymomonas mobilis* entrapped in alumina pellets, Appl Microbiol Biotechnol. 28 (1988): 235-239.

23. M. Kanellaki, A.A. Koutinas, K. Kane, M. Nicolopoulou, Ethanol production by *Saccharomyces cerevisiae* promoted by γ-alumina, Biotechnol Bioeng. 34 (1989): 121-125.

24. Y. Kourkoutas, M. Kanellaki, A.A. Koutinas, Effect of storage of immobilized cells at ambient temperature on volatile by-product during wine-making, Food Microbio. 74(2006): 217-223.

25. H. SchuÈtz, F. Radler, Anaerobic reduction of glycerol to propanediol-1,3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. Syst Appl Microbiol. 5 (1984): 169-178.

26. W. Forsberg, Production of 1,3-Propanediol from Glycerol by *Clostridium acetobutylicum* and Other Clostridium Species, Applied and Environmental Microbiology. 53 (1987): 639-643.

27. T. Homann, C. Tag,H. Biebi, W. Deckwer, B. Schink, Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains, Appl Microbiol Biotechnol. 33 (1990): 121-126.

28. H. biebl, S. Marten,H. Hippe,W. Deckwer, Glycerol converion to 1,3-Propanediol by newly isolated clostridia, Appl Microbiol Biotechnol. 36 (1992): 592-597.

29. B. Dabrock, H. Bahl, G. Gottschalk, Parameters Affecting Solvent Production by *Clostridium pasteurianum*, Applied and Environmental Microbiology. (1992): 1233-1239.

30. F. Barbirato, C. Camarasa Claret, J.P. Grivet, A. Bories, Glycerol fermentation by a new 1,3-propanediol-producing microorganism; *Enterobacter agglomerans*, Appl Microbiol Biotechnol. 43 (1995): 786-793.

31. B. Gunzel, S. Yonsel, W. Deckwer, Fermentative production of 1,3-propanediol from glyerol by *Clostridium butyrium* up to a scale of 2 m^3, Appl Microbiol Biotechnol. 36 (1991): 289-294.

32. E. Petitdemane, C .Durr, S. Abbad Andaloussi, G. Raval, Fermentation of raw glycerol to 1,3- propanediol by new strains of *Clostridium butyricum*, Journal of Industrial Microbiology. 15 (1995): 498-502.

33. M. Heyndrickx, P. De Vos, The fermentation of glycerol by *Clostridium butyricum* LMG $1212t_2$ and $1213t_1$ and *C. Pasteurianum* LMG 3285 Appl Microbiol Biotechnol. 34 (1991): 637-642.

34. A. Reimann, H. Biebl, Production of 1,3-propanediol by *Clostridium butyricum* in continuous culture with cell recycling, Appl Microbiol Biotechnol. 49 (1998): 359-363.

35. R. Boenigk, S. Bowien, Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*, Appl Microbiol Biotechnol. 39 (1993): 453-457.

36. T. Colin, A. Bories, Inhibition of Clostridium butyricum by 1,3- propanediol and diols during glycerol fermentation, Appl Microbiol Biotechnol. 54 (2000): 201-205.

37. El. Himmi, A. Bories, Nutrient requirements for glycerol conversion to 1,3-propanediol by *Clostridium butyrium*, Bioresource Technology 67 (1999): 123-128.

38. A.P. Zeng, H. Biebl, Pathway analysis of glycerol fermentation by *Klebsiella pneumoniae*: Regulation of reducing equivalent balance and product formation, Enzyme Microb Technol. 15 (1993).

39. B. Ogbe solomon, A.P. Zeng, H. Biebl, Effects of substrate limitation on product distribution and H2/CO2 ratio in *Klebsiella pneumoniae* during anaerobic fermantation of glycerol, Appl Microbiol Biotechnol. 42 (1994): 222-226.

40. X. Chen, D. Zhang, W.T. Qi, Microbial fed-batch production of 1,3-propanediol by *Klebsiella pneumoniae* under micro-aerobic conditions, Appl Microbiol Biotechnol. 63 (2003): 143-146.

41. U. Pflugmacher, G. Gottschalk, Development of an immobilized cell reactor for the production 0f 1,3-propanediol by *Citrobacter freundii*, Appl Microbiol Biotechnol. 41 (1994): 313-316.

42. W. Dieter Drckwer, Microbial conversion of glycerol to 1,3-propanediol, FEMS Microbiology Review. 16 (1995): 143-149.

43. H. Biebl, A.P. Zeng, K. Menzel W.D. Deckwer, Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by *Klebsiella pneumoniae*, Appl Microbiol Biotechnol. 50 (1998): 24-29.

44. A. Reimann, S. Abbad-Andalouss, H. Biebl, H.Petitdemange, 1,3-propanediol formation with product-tolerant mutants of *Clostridium butyricum* DSM 5431 in continuous culture: Productivity carbon and electron flow, Applied Microbiology. 84 (1998): 1125-1130.

45. H. Biebl, S marten, Fermentation of glycerol to 1,3-propanediol: use of cosubstratrs, Appl Microbiol Biotechnol. 44 (1995): 15-19.

46. F. Barbirto, E.H. Himmi, T.Conte, A. Bories, 1,3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries, Industrial Crops and Products. 7 (1998): 281-289.

47. H. Biebl, A.P. Zeng, K. Menzel W.D. Deckwer, Microbial production of 1,3-propanediol, Appl Microbiol Biotechnol. 52 (1999): 289-297.

48. M.L. Shuler, F. Kargi, Bioprocess engineering, Prentice Hall, New Jersey, 2001.

49. J.C. Santos, S.I. Mussatto, G. Dragone, et al., Evaluation of porous glass and zeolite as cells carriers for xylitol production from sugarcane bagasse hydrolysate. Biochem. Eng. J. 23 (2005): 1-9.

50. G.A. Junter, T. Jouenne, Immobilized viable microbial cells: from the process to the proteome or the cart before the horse, Biotechnol. Adv. 22 (2004): 633-658.

51. N. Kiran Sree, M. Sridhar, K. Suresh, et al., High ethanol production by repeated batch using an immobilized osmotolerant *Saccharomyces cereviceae*, J. Ind. Microbiol. Biotechnol. 24 (2000): 222-226.

52. W. Carvalho, S.S. Silva, J.C. Santos, A. Converti, Xylitol production by Ca-alginate entrapped cells: comparison of different fermentation systems, Enzyme Microb. Technol. 32 (2003): 553-559.

53. S. Papanikolaou, P. Ruiz-Sanchez, B. Pariset, High production of 1,3-propanediol from industrial glycerol by a new *isolated C. butyricum* strain, Biotechnol. 77(2000): 191-208.

54. M.S. Griffiths, J.A. Bosley, Assessment of macroporous polystyrene-based polymers for the immobilization of *C. freundii*, Enzyme Micro. Tevhnol.15 (1993): 109-113.

55. Ya-Nan Zhao, Guo Chen, Shan-Jing Yao, Microbial production of 1,3-propanediol from glycerol by encapsulated *K.pneummoniae*. Biochem. Eng. J. 32 (2006): 93-99.

56. Saint-Amans, S Perlot, G Goma, G Soucaille, High production of 1,3-propanediol from glycerol by *C. butyricum* VPI 3266 in a simply controlled fed-batch system, Biotechnol. 16(1994): 831-836.

APPENDICES

APPENDIX A
STANDARD CURVE
AND
EXPERIMENTAL DATA

Standard curve for ethanol fermentation

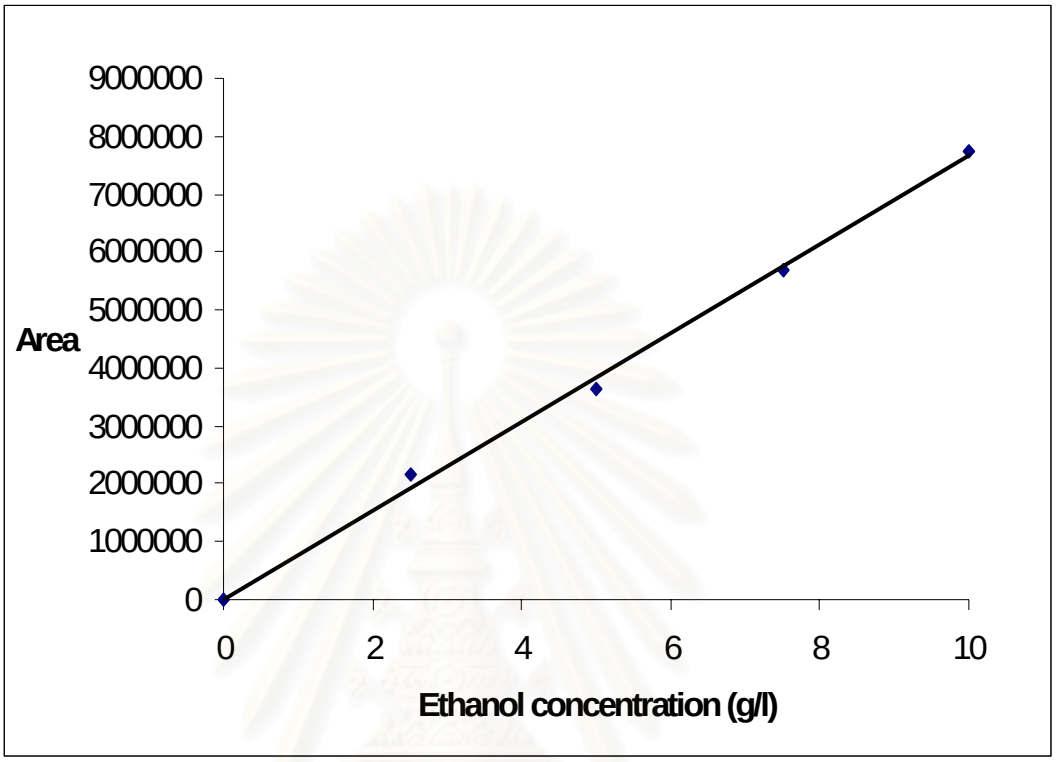


Figure A-1 Standard curve for ethanol concentration analysis

Curve area = 765983 * X

Standard curve for ethanol fermentation

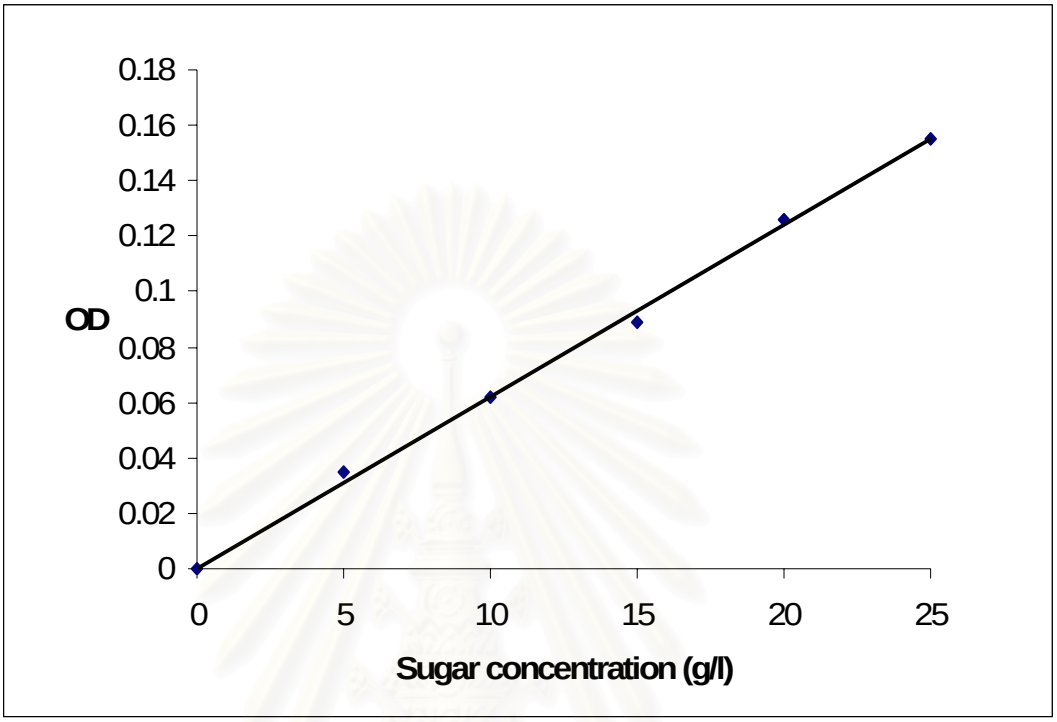


Figure A-2 Standard curve for sugar concentration analysis

$$OD = 0.0062 * X$$

Standard curve for ethanol fermentation

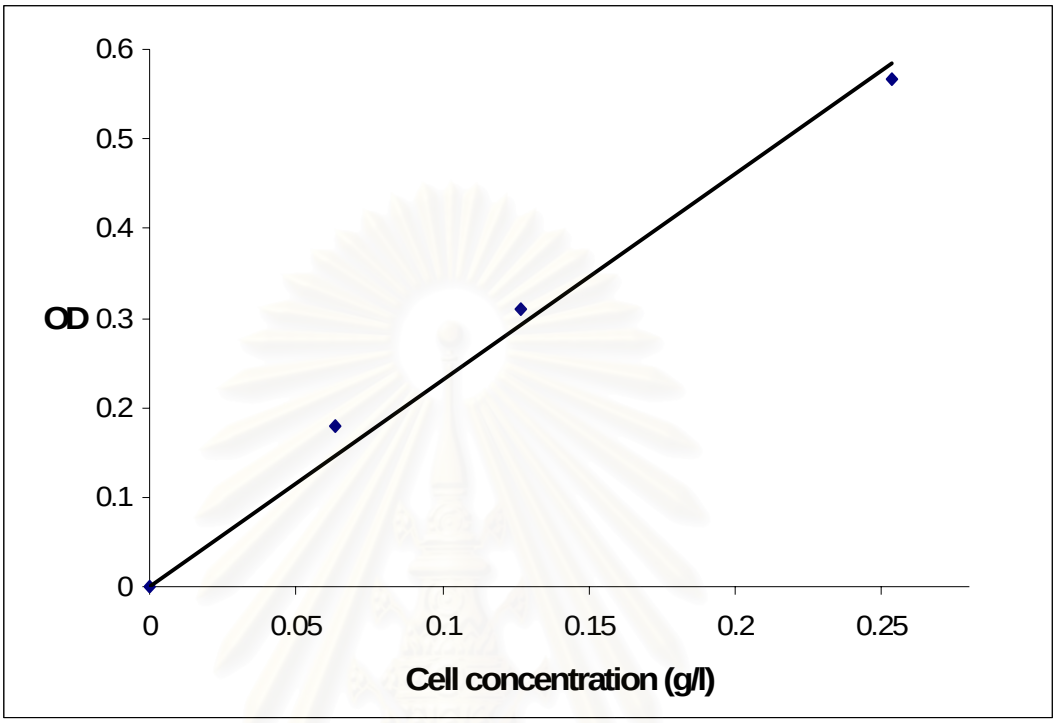


Figure A-3 Standard curve for Cell concentration analysis

$$OD = 2.303 * X$$

Standard curve for 1,3-propanediol fermentation

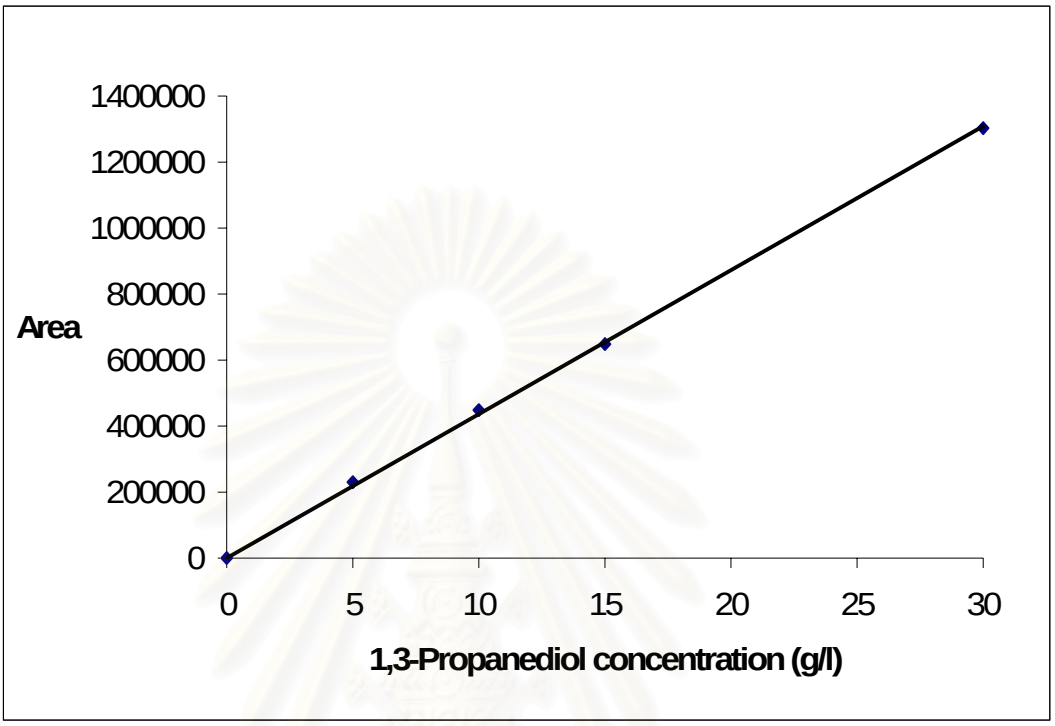


Figure A-4 Standard curve for 1,3-propanediol concentration analysis

Curve area = 43590 * X

Standard curve for 1,3-propanediol fermentation

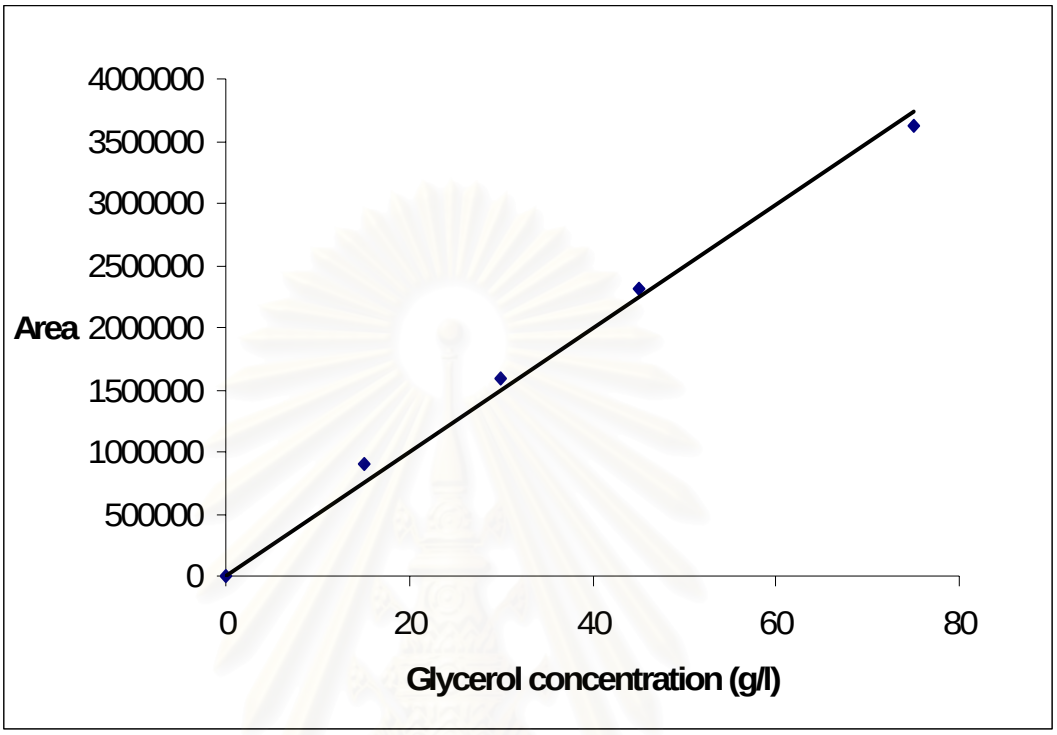


Figure A-5 Standard curve for glycerol concentration analysis

Curve area = 49760 * X

Standard curve for 1,3-propanediol fermentation

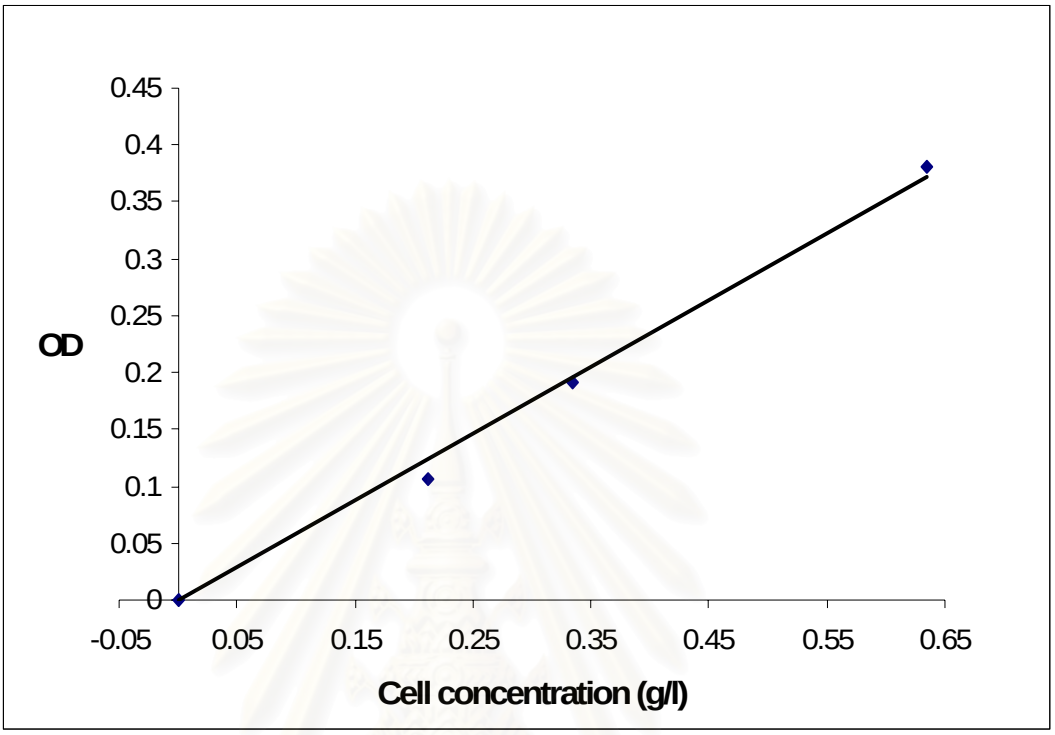


Figure A-6 Standard curve for cell concentration analysis

$$OD = 0.5943 * X$$

Table A-1 Experimental data with time, 1,3-PD and glycerol concentration of batch fermentation in shaking flasks with ranging from 40-130 g/l of initial glycerol at 32 °C

Time (h)	1,3-Propanediol concentration (g/l)					Glycerol concentration (g/l)				
	40	60	80	100	130	40	60	80	100	130
0	0.00	0.00	0.00	0.12	0.00	42.19	62.01	80.27	98.47	107.66
4	0.56	0.40	0.16	0.26	0.00	44.94	60.40	70.46	90.45	111.47
8	0.84	0.83	0.81	0.75	0.27	39.96	52.73	71.01	88.00	96.27
12	1.01	0.98	1.01	1.31	0.43	44.27	61.40	69.11	90.38	109.36
16	1.07	0.99	1.03	1.38	0.52	45.62	57.08	69.85	88.00	98.66
20	1.00	1.22	0.82	1.18	0.46	38.64	53.20	64.24	80.95	113.32
24	0.95	1.15	1.03	1.25	0.56	35.42	52.76	66.81	81.04	106.90
30	0.99	1.13	0.97	1.36	0.71	27.76	49.11	65.33	75.04	103.08

Table A-2 Experimental data with time and cell concentration of batch fermentation in shaking flasks with ranging from 40-130 g/l of initial glycerol at 32 °C

Time (h)	Cell concentration (g/l)				
	40	60	80	100	130
0	0.031	0.030	0.030	0.028	0.032
4	0.18	0.18	0.14	0.17	0.08
8	0.32	0.30	0.32	0.26	0.09
12	0.36	0.31	0.30	0.33	0.20
16	0.35	0.36	0.34	0.31	0.24
20	0.35	0.36	0.34	0.31	0.27
24	0.24	0.28	0.29	0.26	0.23
30	0.30	0.26	0.25	0.30	0.25

Table A-3 Experimental data with time, 1,3-PD, glycerol and cell concentration of batch fermentation in shaking flasks with 80 g/l of initial glycerol at 32 °C

Time (h)	1,3-PD concentration (g/l)	Glycerol concentration (g/l)	Cell concentration (g/l)
0	0.00	80.00	0.07
12	4.62	78.12	1.05
36	4.50	75.55	0.98
60	4.40	75.32	1.43
72	4.42	76.36	1.58

Table A-4 Experimental data with time, 1,3-PD, glycerol and cell concentration of batch fermentation in reactor with 80 g/l of initial glycerol at 32 °C

Time (h)	1,3-PD concentration (g/l)	Glycerol concentration (g/l)	Cell concentration (g/l)
0	0.34	68.66	0.05
3	0.39	62.43	0.06
6	0.44	63.36	0.09
9	4.23	60.49	0.16
12	10.59	53.40	0.15
15	15.74	42.96	0.34
18	18.51	30.49	0.51
21	26.03	25.11	1.02
24	36.91	12.89	2.15
27	39.65	3.12	2.63
30	41.37	2.70	3.11
33	41.14	1.16	2.74

Table A-5 Experimental data with time, 1,3-PD and glycerol concentration of cell immobilization in shaking flasks with 80 g/l of initial glycerol at 32 °C

Time h	1,3-Propanediol (g/l)			Glycerol (g/l)		
	SC	AC	AEC	SC	AC	AEC
0	0.00	0.00	0.00	80.00	80.00	80.00
12	4.62	3.64	3.01	78.12	80.57	68.39
36	4.50	3.51	3.14	75.55	77.32	66.85
60	4.40	3.44	3.02	75.32	75.32	66.35
72	4.42	3.73	3.06	76.36	75.71	67.64
84	4.50	3.54	3.09	75.59	75.20	67.39
96	4.35	3.41	3.19	75.67	73.88	66.56
108	4.43	3.53	2.90	73.37	73.01	65.75
120	4.40	3.43	3.12	73.61	72.42	62.80
132	4.36	3.34	2.86	72.91	72.29	62.95
144	4.64	3.16	2.87	69.04	70.35	62.70
156	4.83	3.51	3.00	73.04	73.05	67.10
168	4.50	3.46	3.00	71.73	74.57	66.82
180	4.63	3.52	3.03	73.05	73.33	68.27

Table A-6 Experimental data with time, 1,3-PD and glycerol concentration of cell immobilization in shaking flasks with 80 g/l of initial glycerol at 32 °C (added 1,3-propanediol)

Time h	1,3-Propanediol (g/l)			Glycerol (g/l)		
	SC	AC	AEC	SC	AC	AEC
0	0.00	0.00	0.00	80.00	80.00	80.00
12	31.51	25.30	27.03	64.75	65.78	71.08
36	33.97	27.01	24.16	62.27	62.03	70.87
60	32.11	23.73	24.88	62.87	62.85	69.09
72	34.30	24.16	25.24	61.46	58.65	66.74
84	34.36	27.21	27.92	60.15	59.28	67.75
96	33.36	24.23	25.40	58.54	56.42	64.30
108	33.45	25.88	24.29	59.96	58.84	65.75
120	32.13	23.96	24.88	59.56	58.11	65.81
132	31.23	25.05	25.42	58.00	58.93	65.50
144	32.84	22.86	23.11	60.05	57.72	63.46
156	30.65	24.66	25.90	58.90	58.99	67.51
168	32.90	24.34	24.18	58.30	58.94	66.59
180	34.52	24.07	25.24	58.71	59.15	67.51

Table A-7 Experimental data with time, 1,3-PD and glycerol concentration of cell immobilization in reactor with 80 g/l of initial glycerol at 32 °C

Time h	Concentration (g/l)			
	1,3-PD AC	1,3-PD AEC	Glycerol AC	Glycerol AEC
0	0.00	0.00	70.00	73
3	-	-	-	-
6	-	-	-	-
9	1.87	0.89	68.53	73.41
12	9.17	1.94	58.13	73.80
15	9.32	2.62	52.77	67.71
18	9.74	4.55	50.05	67.14
21	11.46	9.93	53.14	62.91
24	14.79	11.36	48.10	50.92
27	15.53	11.44	45.43	48.28
30	17.00	13.44	41.15	39.38
33	20.61	15.47	31.52	35.33

Table A-8 Experimental data with time, ethanol and sugar concentration of ethanol batch fermentation in shaking flask at 33 °C

Time h	Ethanol concentration (g/l)			Sugar concentration (g/l)		
	SC	AC	AEC	SC	AC	AEC
0	0.40	0.34	0.05	220.00	220.00	220.00
12	11.92	3.12	1.21	191.16	213.12	221.60
24	40.12	25.81	8.05	143.24	164.70	186.66
36	57.62	41.74	15.92	123.78	126.77	164.70
48	79.16	74.97	65.86	44.42	41.92	48.91
60	82.44	77.17	74.68	27.95	33.44	40.93

APPENDIX B
EXPERIMENTAL METHODS

B-1 Sugar analysis

Sugar (sucrose) concentration was determined using a modified DNS reagent method. All disaccharides in the samples and standard sucrose solutions were first hydrolyzed to their monomers by using acid solution at elevated temperature. The acid residue was then neutralized using a basic solution and the resulting precipitates were settled by centrifugation. After centrifugation, the supernatant was reacted with DNS reagent at high temperature resulting in the formation of brown colored solution. The solution was then diluted before being analyzed by using spectrophotometer. The absorbance of the sample was compared with standard sucrose solutions to obtain the corresponding sucrose concentration. Complete step by step procedures are provided in the following sections.

B-1.1 NaOH and HCl solution preparation

NaOH 30% w/v was prepared by dissolving 30 g of NaOH pellets in 100 mL of water. The reaction is highly exothermic so that the preparation should be done in water bath in order to avoid excess heat generation. Weighing time of NaOH pellets should be minimized because of the hygroscopic nature of NaOH. Solution of 37% w/v HCl was obtained by diluting concentrated HCl solution with DI water. Beware of the acid vapor and wear protective gloves when preparing the solutions. Commercially available HCl 37% can be also be used directly.

B-1.2 DNS reagent preparation

DNS powder is toxic and easy to airborne so that it should be handled with caution. This powder should be added slowly in the mixing process because it is not easy to dissolve. After preparation, the resulting yellow colored reagent is best used in fresh condition so that it is not suggested to keep unused for long time (more than 1 month). The reagent is usually kept in brown bottle to protect it from degradation originated from light for example sun light. The complete preparation steps are:

1. Dissolve 1.633 g NaOH 98% w/w in 20 mL of water. Mix the solution with magnetic stirrer.
2. Under stirring, slowly add 1 g of 3,5-dinitrosalicylic acid powder into the solution.
3. Dilute by adding 50 mL of water. Stir until it is homogeneous.
4. Add 30 g Na-K tartrate & mix it thoroughly.
5. Adjust the volume to 100 mL.
6. Keep the reagent for 3 days before use.

B-1.3 Standard sucrose solution preparation

Standard sucrose solutions were prepared first by making the source solution which was the solution with the highest sucrose concentration as the upper limit. The source solution was then diluted with water so that a set of standard solution with increasing sucrose concentration (for instance 0, 6.25, 12.5, 18.75, and 25% w/v) was obtained. The detailed procedures are as follows:

1. Dry ±2.6 g sucrose at 100-105°C in hot air oven for 2 hours.
2. Put the dried sucrose in desiccator for cooling.
3. Dissolve 2.5 g of the sucrose in 10 mL of water to obtain the source solution.
4. Prepare each 2 mL standard solution in small labeled bottle by serial dilution of suitable amount of source solution and diluting it with water as shown in detail in Table A-7. Use auto pipette for the transfer purpose.

Table B-1 Standard sucrose solution preparation

Source solution (mL)	Water (mL)	Sucrose concentration (% w/v)
0	2	0
0.5	1.5	6.25
1	1	12.5
1.5	0.5	18.75
2	0	25

B-1.4 Sample treatment I

In the first treatment, sample was hydrolyzed using HCl 37% in boiled water bath. After the hydrolysis reaction was stopped, NaOH was added into the solution. The sample was then centrifuged for removing suspended solids. Procedures of the first treatment are:

1. Mix 0.1 mL of sample with 0.9 mL DI water in 16 x 100 mm screw cap tube.
2. Blend the sample with 0.25 mL HCl 37%.
3. Put the tubes in boiling water bath for 10 minutes.
4. Stop the reaction by placing the tubes in ice bath.
5. Add 0.4 mL NaOH 30% w/v and then mix with vortex mixer.
6. Centrifuge the sample at 3000 rpm for 10 minutes.
7. Precautions and notes:
    a) Use vortex mixer for mixing the fluid in the tubes.
    b) Be cautious when handling the hot apparatus.
    c) The level of boiled water and ice bath must be sufficiently higher than the liquid level in the tubes to ensure good heating and cooling of the sample.

B-1.5 Sample treatment II

In treatment II, supernatant obtained from treatment I was reacted with DNS reagent in boiled water bath. In normal circumstances, the mixture of DNS reagent and the sample was readily homogenous. In some cases, yellow precipitates of DNS were formed as an indication of the insufficient basic condition. This can be solved by adding NaOH solution until homogenous solution is obtained.

The solution's color transformed from yellow to reddish brown in the course of reaction. The color intensity represents the corresponding sugar concentration. Solution with higher sugar content will have darker color. After the reaction was ended, the solution was diluted with sufficient amount of water until its absorbance spectrum obtained by spectrophotometer was well distributed along the range of concentration being considered (the absorbance measured was not more 0.7). Shimadzu UV-2450 UV-

Visible spectrophotometer was used for absorbance measurement. Sample containing only water (0% sugar) which had been treated in the same manner as the other samples was used as blank. At every absorbance measurement, fresh standard solution should be used. Complete procedures are described in the following paragraph.

1. Mix 0.1 mL of supernatant obtained from treatment I with 0.5 mL DNS reagent in 16 x 100 mm screw cap tube.
2. Boil the solution for 10 minutes using water bath.
3. Put the tubes in ice bath to stop the reaction.
4. Dilute with water and transfer to other tube until the desired color intensity is gained.
5. Measure the absorbance at 520 nm. Use sample with 0% sugar as blank.
6. Obtain the standard curve by plotting absorbance versus sucrose concentration of standard sucrose solution.
7. Use the standard curve to gain sugar concentration of the samples.

B-2 Determination of cell concentration

Cell concentration was determined by separation of cell from its carrier or medium followed by measurement by spectrophotometer. The cell concentration was obtained by comparing the absorbance of sample with its corresponding standard curve. The standard curve was made by measuring a set of samples of known cell concentration (with dry weight basis). The medium in this study could be classified in 2 forms: liquid and gel. The concentration of cells in a liquid medium was referred as free cell concentration. In the case of cells confined in or attached to gels, the gel was first dissolved before separated from cells by centrifugation.

B-2.1 Dry weight of cell

Dry weight of cell was determined by separating the cells from their suspending liquid medium by centrifugation. The cells were then dried and their weight was measured as the representative of their concentration in the initial suspension. The procedures are:

1. Centrifuge the cell containing medium at 2000 rpm for 15 minutes.
2. Remove the supernatant (discarded or to be used for other analysis).
3. Add HCl 0.1 N to the cell pellet and mix with vortex mixer.
4. Centrifuge the suspension at 2000 rpm for 15 minutes.
5. Discard the supernatant.
6. Disperse the cell pellet with DI water.
7. Repeat step 4-6.
8. Transfer the cell suspension to a pre-weighted aluminum dish.
9. Dry the cell in hot air oven at 100°C for 2 hours.
10. Measure the weight of the cells.
11. Precautions and notes:
    a) The cells cake is fragile. Pour out all of the supernatant in one cycle instead of several cycles.
    b) Dry and measure the weight of aluminum dishes before use.
    c) The dry weight of the cells is obtained as the difference between the weight of the aluminum dish which contains cells and the weight of empty dish.

B-2.2 Free cell concentration

A set of cell suspension with known cell concentration was used as standard. This solution was analyzed at the same time with samples of fermentation and used to generate standard curve of cell concentration. The complete procedures are:

1. Dilute sample with DI water in 16 x 100 mm rimless tube.
2. Centrifuge the cell suspension at 2000 rpm for 15 minutes.
3. Remove the supernatant.

4. Add HCl 0.1 N and mix with vortex mixer.
5. Centrifuge the suspension at 2000 rpm for 15 minutes.
6. Discard the supernatant.
7. Disperse the cell pellet with DI water.
8. Repeat step 5-8.
9. Measure the absorbance of sample at 660 nm.
10. Precautions and notes:

    a) Dilute the sample with DI water before optical density measurement if the cell concentration is too high (its absorbance value is too high).

    b) Mix every sample with vortex mixer before spectrophotometry to ensure homogeneity of the sample.

B-2.3 Immobilized cell concentration

Before the cell concentration could be measured, a measured amount of carrier should be dissolved to obtain cell suspension. The dissolution of the gel can be done by immersing the gel in several chemicals such as EDTA, sodium citrate, potassium citrate, and phosphate buffer. In this study, the dissolution of gel was carried out using sodium citrate 0.05 M solution. The complete procedures are as follows:

1. Dissolve appropriate amount of weighted gel carrier with 6 mL sodium citrate 0.05 M in 16 x 100 mm rimless tube.
2. Continue with same procedures as step 2-9 of Section B-2.2.
3. Precautions and notes:

    a) Refresh the sodium citrate solution if the gel hasn't been dissolved in a single cycle. Repeat this procedure until all gel has been dissolved.

    b) Intermittent mixing with vortex mixer is recommended to promote faster gel dissolution.

    c) To minimize measurement error caused by sample contamination, the dissolution process can be carried out in at temperature about 4°C.

VITA

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