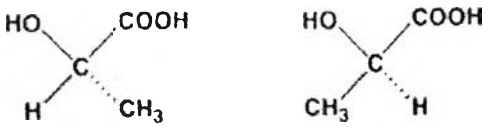
CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Theory

2.1.1 Lactic acid

Lactic acid is one of the most important organic acids produced by lactic acid bacteria, discovered by Swedish scientist C. W. Scheele in 1780 from sour milk [1, 2] but it was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881 [2]. Lactic acid is a three carbon organic acid which one terminal carbon atom is part of an acid or carboxylic group and the other is part of a methyl or hydrocarbon group. Lactic acid exists in two optically active stereo-isomers, the L(+)lactic acid and D(–)lactic acid.



L(+)lactic acid D(–)lactic acid

Figure 2.1 Lactic acid isomers [2]

Lactic acid is soluble in water and water miscible organic solvents but insoluble in other organic solvents. It exhibits low volatility. Other physical properties are tabulated in Table 2.1 [2, 28].

Lactic acid has a wide range of beneficial uses in the sectors relating to food preservation, flavour enhancement, cosmetics, etc. Since elevated levels of D(–)lactic acid is harmful to human metabolism and can resulted in acidosis and decalcification [3], L(+)lactic acid is the preferred isomer in food and pharmaceutical industries as humans have only L-lactate dehydrogenase that metabolizes L(+)lactic acid [1]. It also finds applications in the preparation of biodegradable polymers for medical uses such as surgical sutures, prostheses and controlled drug delivery systems [1, 3] for the reason that their properties approach those of petroleum derived plastics [2].

Technical-grade lactic acid extensively used in leather tanning industries as an acidulant for deliming hides and in vegetable tanning. Lactic acid also used as descaling agent, solvent, cleaning agent, slow acid-releasing agent and humectants in a variety of technical processes [1]. Finally, the potential applications of lactic acid are summarized in Figure 2.2 [3].

Table 2.1 Physical properties of lactic acid

Molecular weight	90.08
Melting point	16.8°C
Boiling point	82°C at 0.5 mm Hg 122°C at 14 mm Hg
Dissociation constant, K_a at 25°C	1.37×10^{-4}
Heat of combustion, ΔH_c	1361 KJ/mole
Specific Heat, C_p at 20°C	190 J/mole/°C

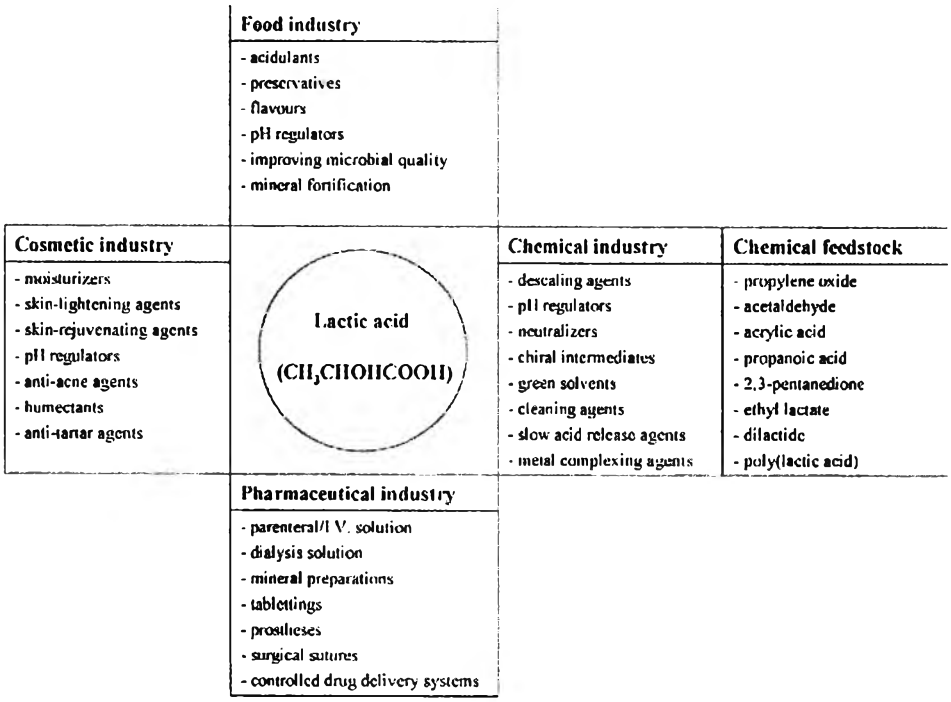


Figure 2.2 Diagram of the commercial uses and applications of lactic acid [3].

Currently, lactic acid is used in a wide variety of specialized industrial applications where the functional specialty of the molecule is desirable. The demand for lactic acid grows continually at 5-8% annual clip. Its use as a new material for synthesis of biodegradable plastics was identified in late 1940s and early 1950s. Demand for lactic acid is expected to increase as rated by different surveys due to its use in biodegradable plastics and other large-scale industrial products. By the end of year 2011, lactic acid demand is expected to shoot up to 200,000 MT world wide. The current global production of lactic acid is about 120,000 tons per year. New applications of L(+)lactic acid, such as a monomer in biodegradable plastics or as an intermediate in the synthesis of high-volume oxygenated chemicals, have the potential to greatly expand the market for it. Therefore, the biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative to environmental pollution caused by the petrochemical industry and the supply limitation of petrochemical resources.

2.1.2 Lactic acid fermentation

Lactic acid can be manufactured either by chemical synthesis or by microbial fermentations. Chemical synthesis results in racemic DL-lactic acid whereas stereospecific, L(+), D(–), and DL mixture, form is produced by fermentation using specific microbial strain [1, 4].

Lactic acid bacteria ferment sugars via different pathways resulting in homo-, hetero-, or mixed acid fermentation. Homofermentation gives only lactic acid as the end product of glucose metabolism, and the Embden-Meyerhof-Parnas pathway as shown in Figure 2.3A is used. In heterofermenation equimolar amounts of lactic acid, carbon dioxide and ethanol or acetate are formed from glucose via the phosphoketolase pathway, presented in Figure 2.3B. The ratio of ethanol and acetate formed is dependent on the redox potential of the system. This pathway is used by facultative heterofermenters, such as *L. casei*, for the fermentation of pentoses, and for the fermentation of hexoses and pentoses by obligate heterofermenters, organisms such as *Leuconostoc*.

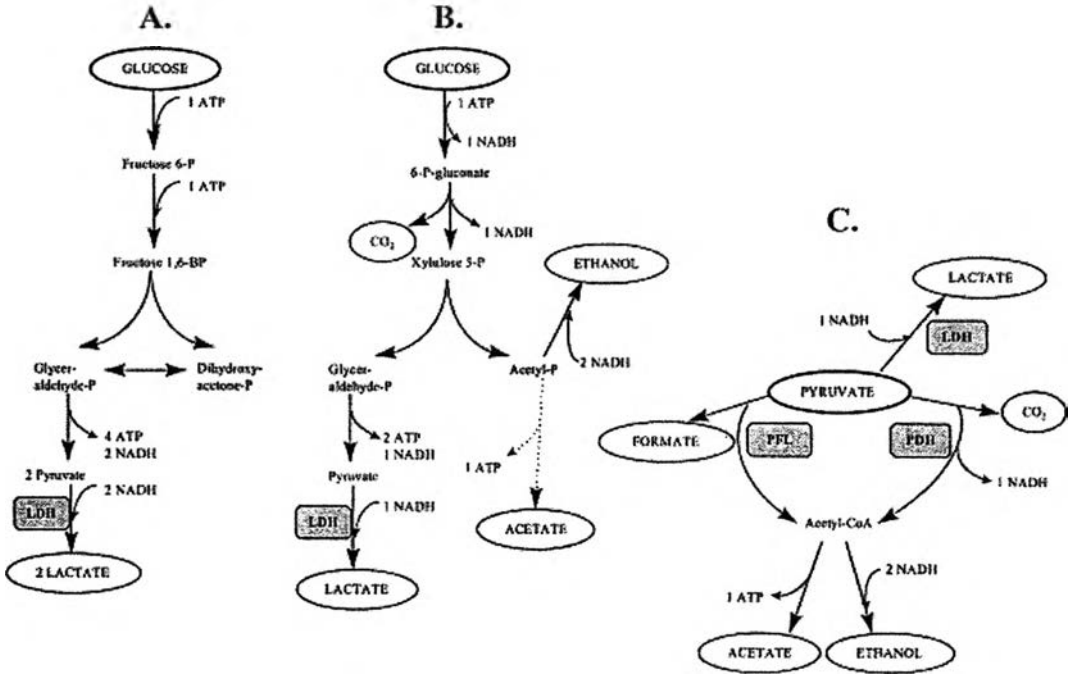


Figure 2.3 Catabolic pathways in lactic acid bacteria. Homofermentation (A), heterofermentation (B) and mixed acid fermentation (C). *P* = phosphate, BP = bisphosphate, LDH = lactate dehydrogenase, PFL = pyruvate formate lyase, and PDH = pyruvate dehydrogenase [4].

2.1.3 Lactic acid bacteria

Today lactic acid bacteria consist of the gram-positive genera: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella.* Lactic acid bacteria are cocci, with the exception of Lactobacilli and Carnobacteria which are rods, unable to synthesize ATP by respiration, and that have lactic acid as the major end product from energy-conserving fermentation of sugars. Most lactic acid bacteria are facultatively anaerobic, catalase negative, nonmotile and nonspore forming [5, 29]. They have high acid tolerance and survive at pH 5 and lower. Their acid tolerance gives them a competitive advantage over other bacteria. The optimal temperature for growth varies between the genera from 20 to 45°C. Most of them are considered GRAS (Generally Regarded As Safe), but some strains of e.g. *Streptococci* are pathogenic.

Strains of lactobacilli were compared with regard to the fermentation of various sugars. Among the glucose users, *Lactobacillus salivarius* spp. *salivarius* ATCC 11741 emerges as the most efficient strain [4, 6].

L. salivarius is a gram-positive, non-spore forming bacillus bacterium, and also a facultative anaerobe. It is a homofermentative organism that is found occurring naturally in the human oral cavities, intestines, and vagina [5]. It is considered to be non-pathogenic and is sometimes used to produce lactic acid in fermented foods and is used as a probiotic to help prevent infections by other microorganisms [5, 29].

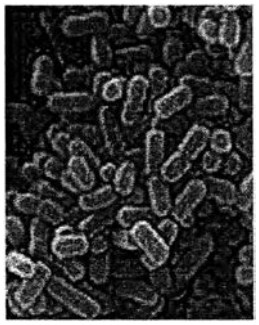


Figure 2.4 *L. salivarius*, rod-shaped bacteria [29].

Siebold et al. (1995) compared the production of lactic acid by three different Lactobacilli namely *Lactobacillus delbreuckii* DSM 20072, *Lactobacillus casei* DSM 20021 and *Lactobacillus salivarius* DSM 20492. The experiment datum showed that *L. salivarius* exhibited the highest specific growth rate and the highest specific productivity. Substrate inhibition occurred at 30 g L^{-1} glucose for *L. delbrueckii*, 26 g L^{-1} glucose for *L. casei*, and no serious substrate limitation was observed up to 100 g L^{-1} glucose for *L. salivarius*. 60 g L^{-1} lactate caused only slight product inhibition for *L. salivarius* [6].

Lactic acid bacteria have complex nutrient requirements, due to their limited ability to synthesize B-vitamins and amino acids. Therefore they are naturally found in nutrient-rich environments such as in plants, milk, and inside the human and animal bodies [4]. The addition of nutrients, for instance yeast extract, peptone, or corn steep liquor, and higher nutrient concentrations generally had a positive effect on the lactic acid production.

2.1.4 Cell immobilization

In the past two decades the immobilization of microorganisms, cells and parts of cells has gradually been introduced into microbiology and biotechnology [8]. Immobilized cell techniques are currently being exploited for various fermentation processes. This is mainly due to the numerous advantages that cell immobilization offers including enhanced fermentation productivity, feasibility of continuous processing, cell stability, maintain high cell density, and lower costs of recovery and recycling and downstream processing [10]. Cell immobilization may also protect cells against shear force. On the other hand, immobilization of cells also has some limitations and drawbacks. The cell may contain many catalytically active enzymes, which may catalyze unwanted side reactions. Also, the cell membrane itself may serve as a diffusion barrier, and may reduce productivity. Furthermore, the matrix may sharply reduce productivity if the microorganism is sensitive to product inhibition [7, 8, 10]. The main advantages and disadvantages of immobilized cell system are summarized in Table 2.2 [7 – 9, 11, 13].

Whole cell immobilization is defined as the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity. If whole microbial cells can be immobilized directly, procedures for extraction and purification can be omitted and the loss of intracellular enzyme activity can be kept to a minimum [8].

Immobilization systems can be classified into natural and artificial occurring one. In nature, a number of microorganisms may produce extracellular polymers, which are usually lumped together and form biofilm by attaching to one another or even to surfaces. In artificial immobilization, cells are immobilized by using carriers or supports. Proper selection of carrier is utmost important for immobilized cell application because it will have an effect on the system performance. Since every microorganism exhibits different interaction with different carries, evaluation of carrier performance for an individual microorganism should be done based on case by case.

Table 2.2 Advantages and disadvantages of immobilized cell system.

Advantages	Disadvantages
1. Higher reaction rates due to increased cell density	1. Mass transfer limitation due to diffusional barrier
2. Fermentation productivity enhancement	2. Complexity in preparation of the system before start up
3. Ability to conduct continuous operations at high dilution rate without washout problem	3. Sharply reduce productivity if the microorganism is sensitive to product inhibition
4. Ease of downstream processing leads to low costs of recovery and recycling	4. Limitation of mechanical stability
5. Possibilities for regenerating the biocatalytic activity of immobilized cell structures	
6. Protect cells against shear rate	
7. Reduction of risk of microbial contamination due to high cell density and fermentation activity	

2.1.4.1 Type of immobilized cell carriers

Various materials have been tested as cells carriers. In general, an ideal cell carrier should be rigid and chemically inert, should bind cells firmly, and should have high cell loading capacity [10]. In addition, cheap, non-toxic, highly available and environmental friendly materials are often more preferred for cell immobilization process [11]. The selection of an immobilized material will depend upon many factors including the resistance to microbial degradation, mechanical strength, type of fluid, surface characteristics, the cost and availability of materials, simple and effective immobilization process [30]. These materials can be classified into organic material, inorganic material, charged material, and porous material based on the origin of the support material [11].

A. Organic material

Organic material has a higher adsorbtivity compared to inorganic material owing to the larger variety of reactive functional groups, for example carboxyl, amino, hydroxyl, on the organic material surface. In addition, organic materials usually contain a certain amount of nutrients, which help the microorganisms to adhere and grow. Higher frequency replacement of organic material in immobilization system is needed as a result of its biodegradability. Some organic materials from plants such as loofa sponge [24 – 26, 31 – 36], sugarcane bagasse [37], and saw dust [38] have been reported for whole cell immobilization. Naturally derived polymers such as alginate and chitosan have been studied intensively to evaluate their compatibility for cell immobilization [10, 11].

B. Inorganic material

Inorganic material is usually considered to be resistant to microbial attack, to exhibit high thermal stability and to have good flow properties. To improve the adsorbability of the inorganic material, grafting by organic material which contains a large variety of reactive groups was performed. Examples of this type of carrier are metal oxides, glass, ceramics, stainless steel mesh, polyurethane, and polystyrene [10, 11].

C. Charged material

Since the surface charge of microorganisms in negative in general, positively charged material will be favourable for attachment of cell especially at the early stage of immobilization. The use of charged material could become a problem if substrate, product, and/or residual contaminants are charged and interact with the support material. This will hinder the cell growth for example by removing essential minerals from medium. The inhibiting effect will increase at higher charged material. Ion-exchange resins, gelatin, stainless sphere wire, and porous cellulose are example of charged materials [11].

D. Porous material

For macroporous carrier such as porous glass, cell will attach at the surface as well as the pore of the material. This will be leaded to higher cell loading compared with microporous carrier, zeolite. Cell can not enter the pore of microporous 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. More examples of porous material include agar, alginate, κ-carrageenan, polyacrylamide, chitosan, gelatin, cellulose, collagen, porous metal screen, polyurethane, silica gel, polystyrene, and cellulose triacetate [10, 11].

2.1.4.2 Immobilization techniques

Numerous biotechnological processes are advantaged by immobilization techniques and therefore several such techniques and support materials have been proposed. These techniques can be categorized into four major groups based on the physical mechanism employed for the immobilization of whole microbial cells: (a) immobilization on solid carrier surfaces or carrier binding, (b) entrapment within a porous matrix, (c) cell flocculation or aggregation, and (d) mechanical containment behind a barrier [7].

A. Immobilization on solid carrier surfaces or carrier binding

Cell immobilization on a solid carrier is carried out by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier. Whole cell is bound to solid support, fixed in the form of an active layer. When the substrate passes over the surface, enzymatic reactions change the substrate to the desired product. Immobilized cells may perform differently to an equivalent mass of freely suspended cells. As there are no barriers between the cells and the solution, cell detachment and relocation is possible with potential establishment of equilibrium between adsorbed and freely suspended cells. Systems using immobilized cells on a surface are popular because of the relative ease of performing this type of immobilization. Selection of suitable carriers for cell adsorption is usually based on adsorption capacity and strength of binding. For example, the adsorption capacity may vary from 2 mg/g for porous silica to 250 mg/g for wood chips. Thus, careful

selection of such support material for each particular case is needed to obtain the expected enhancement of immobilized cell system performance. Examples of materials used in this type of immobilization are cellulosic materials; DEAE-cellulose, wood, sawdust, delignified cellulose, and loofa sponge; inorganic materials; polygorskite, montmorilonite, hydromica, porous porcelain, and porous glass; etc. Solid materials like glass or cellulose can also be treated with polycations, chitosan or other chemicals to enhance their adsorption ability [7, 10, 11].

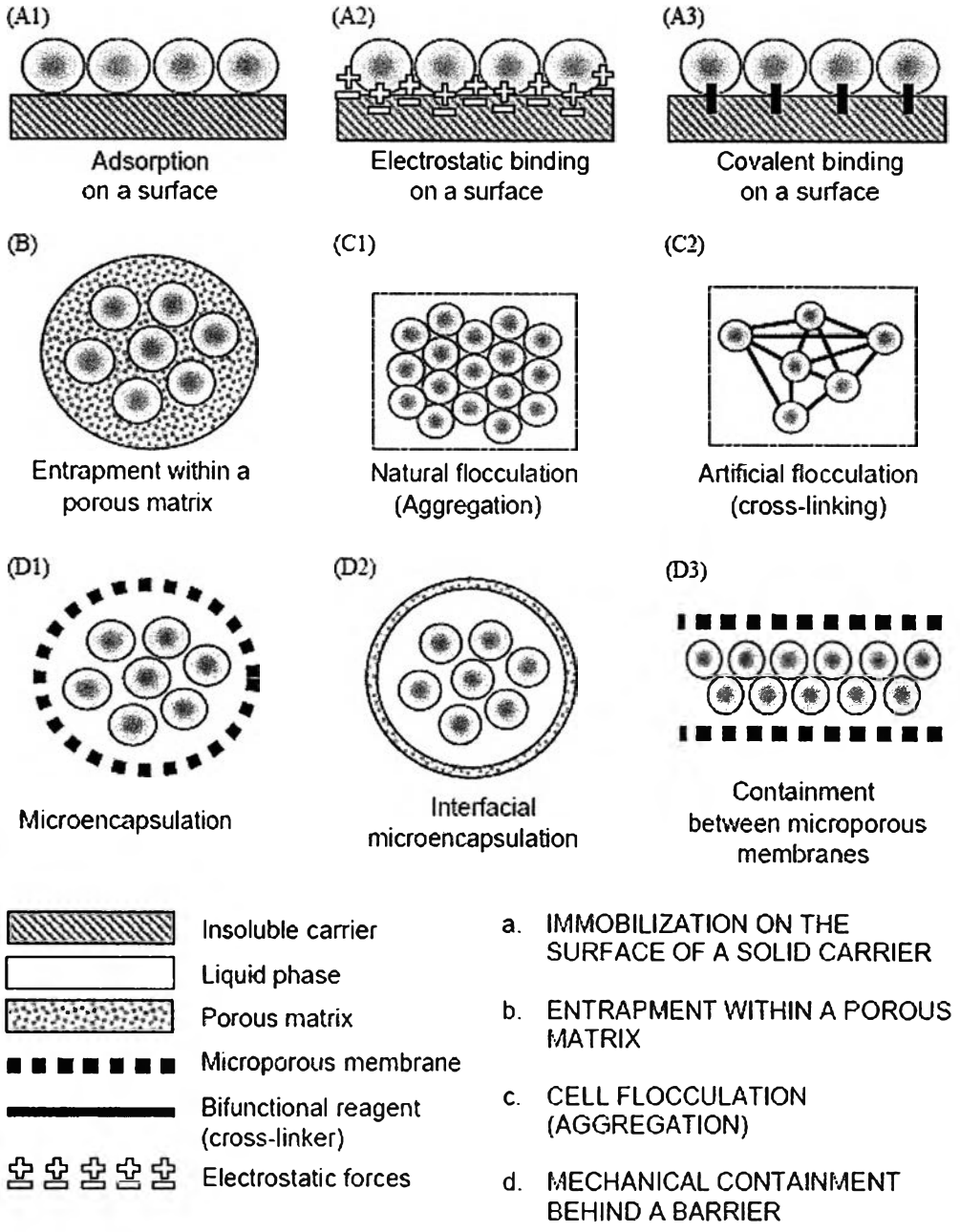


Figure 2.5 Basic methods of cell immobilization [7].

B. Entrapment within a porous matrix

Physical entrapment within porous matrix is the most widely used technique for cell immobilization. In applying entrapment, microbial cells are included within a rigid network to prevent cells from diffusing into the surrounding medium, while still allowing mass transfer of nutrients and metabolites. Various materials for instance collagen, gelatin, agar, alginate, carrageenan, chitosan, polygalacturonic acid, cellulose triacetate, polyacrylamide, polyvinyl alcohol, and polystyrene are used extensively as cells carriers. The advantage of this method is that cell leakage may act as a diffusion barrier. Furthermore, several drawbacks such as restrictive diffusion of nutrients, metabolites, oxygen, and product occurred due to the gel matrix and the high cell density in the gel bead, the chemical and physical instability of the bead and the non-regenerability of the gel bead should be concerned. However, production of large amount of gel beads needed for its commercial application is expensive and also required specialized equipment. [7, 8, 10, 12]

C. Cell flocculation or aggregation

Cell flocculation is an aggregation of cells to form a larger unit or the property of cells in suspensions to adhere in clumps and sediment rapidly. Flocculation can be considered as an immobilization technique as the large size of the aggregates makes their potential use in reactors included packed-bed, fluidized-bed, and continuous stirred-tank possible. The ability of microorganism to form aggregates is mainly observed in moulds, fungi and plant cells. However, artificial flocculating agents or cross-linking agents, e.g. chitosan, can be used to enhance aggregation in cell cultures that do not naturally flocculate [7, 8].

D. Mechanical containment behind a barrier

Containment of cells behind a barrier can be attained either by use of microporous membrane filters or by entrapment of cells in a microcapsule or by cell immobilization on to an interaction surface of two immiscible liquids. This type of immobilization is ideal when cell free product and minimum transfer of compounds are required. Membrane bioreactor technology is widely used in cell recycling and continuous processes. The major disadvantages of cells immobilization between

microporous membranes are mass transfer limitations and possible membrane biofouling caused by cell growth [7].

2.1.5 Chitosan

Chitosan (poly[1→4)-β-linked 2-amino-2-deoxy-D-glucose] is the principal derivative of chitin, a natural polymer. It is prepared from chitin by N-deacetylation at a varying extent that is characterized by the degree of deacetylation. Therefore, it is the copolymer of N-acetyl-D-glucosamine and D-glucosamine. When the degree of deacetylation of chitin is up to 50%, it becomes soluble in aqueous acidic media and is called chitosan. For better understanding the difference between chitin and chitosan, the both structures are shown below.

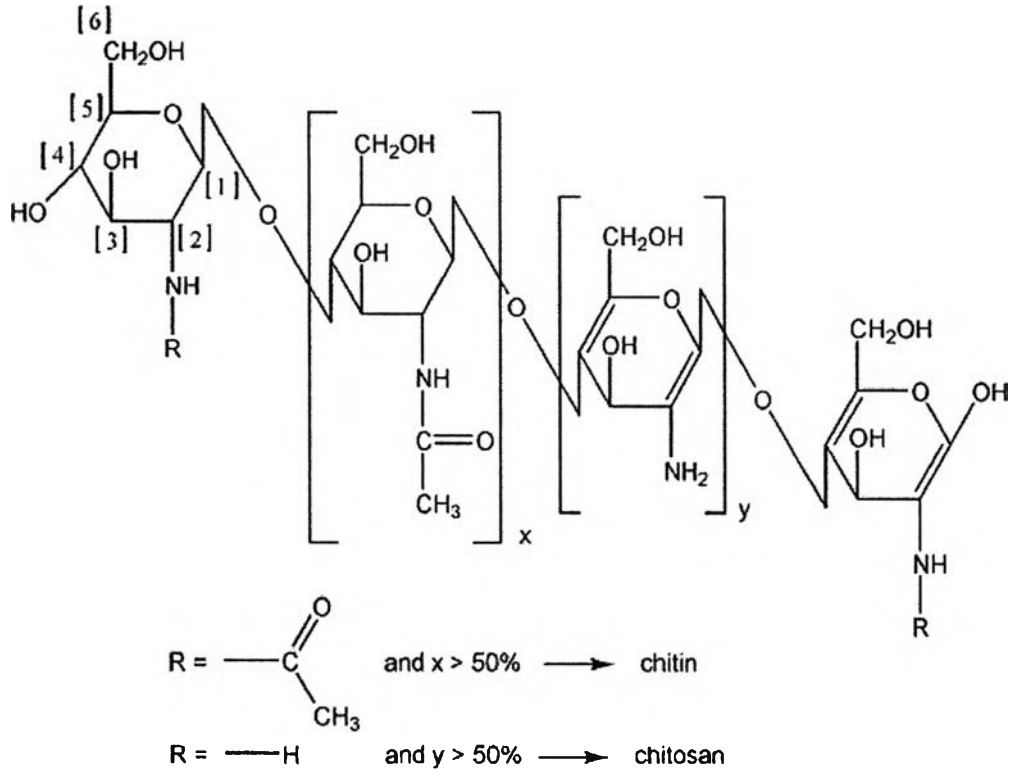


Figure 2.6 Structure of chitin and chitosan [39].

The main parameters influencing the characteristics and properties of chitosan are molecular weight (MW), degree of deacetylation (DD) which represents the molar

fraction of deacetylated units, crystallinity, and the purity of the product. These parameters are determined by the conditions set during preparation [39, 40].

Chitosan is a semicrystalline polymer in the solid state. It is readily soluble in dilute acidic solutions below pH 6.0. This is because chitosan can be considered a strong base as it possesses primary amino groups with a pKa value of 6.3. The presence of the amino groups indicates that pH substantially alters the charged state and properties of chitosan. At low pH, these amines get protonated and become positively charged and that makes chitosan a water-soluble cationic polyelectrolyte. On the other hand, as the pH increases above 6, amines in chitosan become deprotonated and the polymer loses its charge and becomes insoluble. The soluble-insoluble transition occurs at its pKa value around pH between 6 and 6.5. As the pKa value is highly dependent on the degree of N-acetylation, the solubility of chitosan is dependent on the DD and the method of deacetylation used including isolation and drying conditions. Furthermore, the solubility of chitosan also relates to ionic concentration, pH, nature of the acid used and the distribution of acetyl groups along the chain [39].

Chitosan is considered to be a cationic polyelectrolyte because it has free primary amino groups distributed regularly in its molecular chain. When it dissolves, it possesses high positive charge on $-NH_3^+$ groups, allowing it to adhere to negatively charged surfaces and able to aggregate with polyanionic compounds [41, 42].

Chitosan has unique biological properties: biodegradability, nontoxicity, physiological inertness, and remarkable affinity to proteins. Its properties also make the most exciting use in biotechnology as cell and enzyme immobilization supports [42].

2.1.6 Fermentation [10]

Microorganisms grow in a suitable nutrient medium and convert them into biological products. Nutrients are used for energy production, biosynthesis and product formation. When consuming nutrients, biomass increases with time. The growth rate directly relates to cell concentration. Microbial growth rate is characterized by the specific growth rate (μ), defined as follows;

$$\mu \equiv \frac{1}{X}\frac{dX}{dt} \tag{2.1}$$

Where X is cell mass concentration (g/l), t is time (h), μ is specific growth rate (h^{-1}).

Batch fermentation

Batch fermentation is a simple form of cultivation. It is widely used in laboratory and industry. It refers to cell cultivation in a vessel with initial medium without nutrient addition or removal.

Batch growth patterns and Batch growth kinetics.

When seed culture is inoculated into liquid medium, the organisms utilize the dissolved nutrients from the medium and convert them to biomass. The growth of organisms normally follow typical growth curve which includes 1) lag phase 2) logarithmic or exponential growth phase 3) deceleration phase 4) death phase.

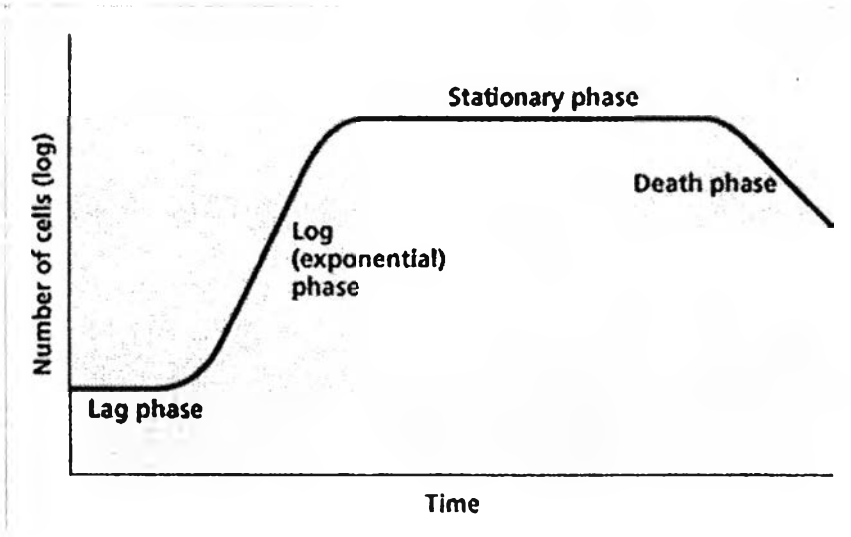


Figure 2.7 Typical growth curve for bacteria [43].

Lag phase is a period of cell adaptation to new medium. It occurs immediately after inoculation. New molecular constituents are reorganized and new enzymes are synthesized, depending on new medium compositions. Cell mass may increase only a

little without cell number density increment. In the case of small amount of inoculation and low viable cell fraction, pseudo-lag phase may occur. It is because of small inoculum size or poor inoculum condition, not because of adaptation. Long lag phase may happen when nutrient and growth factor concentrations are low.

Logarithmic growth phase or exponential growth phase occurs after cell adjustment to new environment. Cell multiplication is rapid causing exponential increase of cell mass and cell number density with time. All components of a cell can grow with the same rate which is called balanced growth. The average composition of a single cell remains constant. The specific growth rate can also be determined from either cell number or cell mass. The growth rate is independent of nutrient concentration because the nutrient concentration is still large. The exponential growth rate is first order

$$\frac{dX}{dt} = \mu X \tag{2.2}$$

$$X = X_0 \quad \text{at } t = t_0$$

Its integration yields

$$\ln \frac{X}{X_0} = \mu t \quad \text{or} \quad X = X_0 e^{\mu t} \tag{2.3}$$

Where X and X_0 are cell concentration at time t and t=0.

Deceleration growth phase comes after the exponential phase. Depletion of one or more nutrients or toxic by-product accumulation can cause deceleration of growth. It occurs for a short time period. Unbalanced growth occurs because of the rapid change of environment. Restructuring of cells happens due to nutrient depletion or waste accumulation so that the cells can survive in hostile environment.

Stationary phase starts when deceleration phase stops. The net growth rate is zero or no cell division or the growth rate equals to the death rate. Cells are still active and produce secondary metabolites, non-growth-related products. There may be one or more of these phenomena taking place;

1. Constant total cell mass concentration but decreased viable cells.
2. Cell lysis and the drop of viable cell mass.
3. No cell growth but secondary metabolite production from active metabolism of cells.

When inhibitory product is accumulated in the medium, it slows down cell growth rate and finally stops at a certain level of inhibitor concentration.

Death phase or decline phase begins at the end of stationary phase. Dead cells lyse and release intracellular nutrients into the medium.

Death rate is as follows;

$$\frac{dN}{dt} = -k_d^{'} X \quad \text{or} \quad N = N_s e^{-k_d^{'} t} \tag{2.4}$$

Where N_s is cell concentration at the end of stationary phase, $k_d^{'}$ is first-order death-rate constant.

Some stoichiometric parameters related to growth kinetics are used to describe growth kinetics. Yield coefficients are based on the material consumed.

$$\text{Growth yield is } Y_{X/S} \equiv -\frac{\Delta X}{\Delta S} \ . \tag{2.5}$$

Yield coefficient based on product formation is as follows;

$$\text{Product yield is } Y_{P/S} = -\frac{\Delta P}{\Delta S} \ . \tag{2.6}$$

Microbial growth, product formation and substrate utilization can be expressed in the specific rate forms, which are normalized by X. The specific rates have the benefit of using to compare fermentation effectiveness.

$$\text{Specific growth rate } (\mu) \ \mu \equiv \frac{1}{X}\frac{dX}{dt} \ .$$

$$\text{Specific rate of product formation } q_p = \frac{1}{X}\frac{dP}{dt} \ . \tag{2.7}$$

$$\text{Specific rate of substrate utilization } q_s = -\frac{1}{X}\frac{dS}{dt} \ . \tag{2.8}$$

Three major categories of products from microbes are classified as following;

1. Growth-associated product formation occurs at the same time as microbial growth. The specific rate of product formation is proportional to the specific growth rate.
2. Nongrowth-associated products are produced during the stationary phase of zero growth rate. The specific rate of product formation is constant.

3. Mixed-growth-associated products are produced during the slow growth and stationary phase. The specific rate of product formation follows linear equation as $q_p = \alpha\mu + \beta$. (2.9)

Lactic acid fermentation is an example of mixed-growth-associated products.

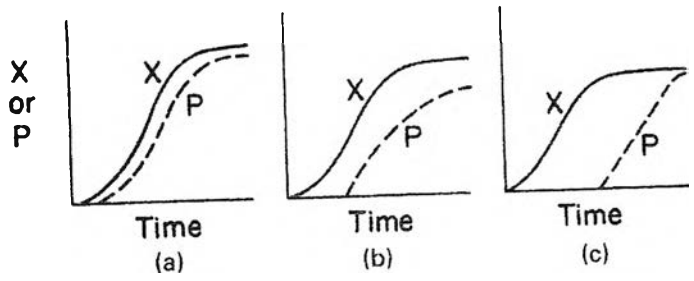


Figure 2.8 Kinetic patterns of growth and product formation in batch fermentations: a) growth-associated, b) mixed-growth-associated, and c) nongrowth-associated [10]

Growth kinetics

Unstructured and nonsegregated models are simple and applicable to some situation if interest. They assume fixed cell composition, equivalent to assuming balanced growth. The assumption is valid in exponential phase of batch culture but fails during transient condition.

Monod equation

Monod equation is similar to Michaelis-Menten kinetics for enzyme reactions. A single chemical species, S, is assumed. It is growth rate limiting. Increase of S can influence growth rate while other nutrient concentration changes have no effect. The specific growth rate is related to substrate concentration as follows;

$$\mu = \frac{\mu_m S}{K_S + S} \tag{2.10}$$

Where μ_m is the maximum specific growth rate when $S >> K_s$, Ks is saturation constant, equals to the concentration of the rate-limiting substrate at the value of one-

half of the maximum specific growth rate. That is K_s =S when $\mu = \frac{1}{2} \mu_m$. The Monod equation can be well applied with slow growth and low population density.

Adsorption of cells on inert support surfaces [10]

The major advantage is direct contact between nutrient and support materials. Using microporous support materials can obtain high cell loadings. There are two factors affecting the selection of a suitable support material: adsorption capacity and strength of binding. The binding forces between cell and support surface depend on support surface properties and type of cells. Electrostatic forces are dominant when support surfaces are positively charged. For negatively charged support surfaces, cells adhesion is by covalent binding or H bonding. Some specific chelating agents can be used to enhance cell-surface interactions.

The immobilization method of adsorption is simple and inexpensive. However, its disadvantages are limited cell loadings and rather weak binding forces. The hydrodynamic shear used should be very mild to avoid cell removal from support surfaces.

Passive Immobilization: Biological films

Biological films are the growth of cells in multilayer on solid support surfaces. Its formation can occur commonly in natural and in industrial fermentation systems. The binding forces between cell and support material may be very complicated.

Nutrient diffuses into the stagnant biofilm and products diffuse out into liquid medium. The biofilm thickness is important and affects its performance. Thin biofilms have low conversion rates due to low biomass concentration. Thick biofilm may have limited growth caused by diffusion limitations. Within thick biofilm, nutrient-depleted regions may be developed. Optimum biofilm thickness results in maximum bioconversion rates.

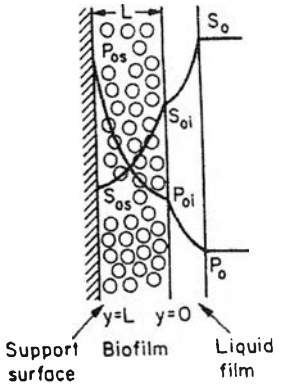


Figure 2.9 Schematic representation of a biofilm [10]

Diffusional limitations in immobilized cell systems

Extra diffusional limitations may occur in immobilization of cells comparing to suspension cell cultures. The diffusional limitations depend on the relative rate of bioconversion and diffusion. It can be described using Damkohler number.

$$Da = \frac{\max.rate\,of\,bioconversion}{\max.rate\,of\,diffusion} = \frac{r_{max}}{(D_e/\delta)S_0} \qquad (2.11)$$

Where r_{max} is the maximum rate of bioconversion (mg substrate / l.h), D_e is the effective diffusivity of the rate-limiting substrate, δ is the thickness of diffusion path or liquid film, and S_0 is the bulk substrate concentration in liquid phase. D_e/δ is the mass transfer coefficient $k_{L,}$ when film theory is applied.

If Da >> 1, the rate is diffusion limited. For Da << 1, the rate is limited by the rate of bioconversion. For Da $\approx$ 1, the diffusion and bioreaction rates are comparable. Da < 1 is desirable to reduce diffusion limitations when the productivity does not improve upon immobilization.

Diffusional limitations may be external, which is between fluid and cell surface in adsorption and covalent binding. If the limitation is because of external mass transfer, liquid-phase turbulence may be increased to increase the reaction rate.

2.2 Literature review

2.2.1 Lactic acid bacteria selection

Several lactic acid bacteria information was tabulated in Table 2.3, according to operating conditions and kinetic parameters. It was found that *Lactobaciluus salivarius ssp. salivarius* was selected as L(+)lactic acid producer in this research because it is one of the high yield producers which glucose as substrate.

2.2.2 Lactic acid production by suspended cell

Anatkatat [44] produced lactic acid in a batch fermentor using lactic acid bacteria *Lactobacillus salivarius subsp. salivarius* ATCC 11741.Glucose from cassava starch hydrolysate (CSH) and brewer's yeast autolysate (BYA) were used as carbon and nitrogen source for the producing lactic acid. The effect of pH, commercial grade glucose (CG) concentration, glucose from cassava starch hydrolysate (CSH) concentration, yeast extract concentration (YE) and brewer's yeast autolysate (BYA) concentration on cell growth and lactic acid production were investigated. In addition, the comparison between CG and CSH, YE and BYA were studied. Moreover, we studied the effect of the bitterness of BYA on lactic acid production. We found that the CG and YE had more good effect for lactic acid production than CSH and BYA. Whereas, a higher lactic acid yields were investigated from using CSH and BYA as a carbon and nitrogen source, so it recommends that the blending of CG with CSH and YE with BYA for lactic acid production is the one choice for reduce the cost of carbon and nitrogen source. The optimum pH, commercial grade glucose (CG) concentration, cassava starch hydrolysate (CSH) concentration and brewer's yeast autolysate (BYA) concentration for lactic acid production were found to be 5.5, 70 g L^{-1}, 70 g L^{-1} and 48 ml L^{-1}, respectively. Moreover, the result shows that the bitterness of BYA didn't effect the lactic acid production but the debittering of the bitterness was good for cell growth.

Table 2.3 The lactic acid production [44]

Organism	FM	Substrate	Nutrient	Operating Conditions	X ($g L^{-1}$)	S ($g L^{-1}$)	LA ($g L^{-1}$)	μ_{max} (h^{-1})	Y_{XS} (g/g)	Y_{PS} (g/g)
L.casei KH-1	Batch	Glucose	YE 0.697% – CSL 1.708%	37°C, pH 5.7, 24 h	5.38	22.15	-	0.49	0.31	0.81
L.casei ssp. casei DSM 20011	Cont	Glucose	YE	36°C, pH 6.4, 16 h	-	40.00	-	0.47	0.11	0.96
L.bulgaricus PTCC 1332	Batch	Glucose	YE	37°C, pH 5.5, 48 h	9.00	6.00	24.00	-	0.15	0.40
L.plantarum PTCC 1058	Batch	Glucose	YE	37°C, pH 5.5, 48 h	9.12	6.00	29.00	-	0.15	0.83
L.delbruekii ssp. delbruekii PTCC 1333	Batch	Glucose	YE	37°C, pH 5.5, 48 h	8.76	6.00	15.00	-	0.14	0.25
L.lichmannii PTCC 1057	Batch	Glucose	YE	37°C, pH 5.5, 48 h	7.86	6.00	1.68	-	0.13	0.02
L.casei PTCC 1055	Batch	Glucose	YE	37°C, pH 5.5, 48 h	8.22	6.00	6.18	-	0.13	0.10
L.casei ssp. casei PTCC 1608	Batch	Glucose	YE	37°C, pH 5.5, 48 h	8.40	6.00	19.00	-	0.14	0.31

Table 2.3 The lactic acid production (Cont.) [44]

Organism	FM	Substrate	Nutrient	Operating Conditions	X ($g L^{-1}$)	S ($g L^{-1}$)	LA ($g L^{-1}$)	μ_{max} (h^{-1})	Y_{XS} (g/g)	Y_{PS} (g/g)
L.lactis ssp. lactis PTCC 1403	Batch	Glucose	YE	37°C, pH 5.5, 48 h	7.80	6.00	2.91	-	0.13	0.04
L.amylophilus GV6	Batch	Corn starch	Red lentil 2% + Baker's yeast 1%	37°C, pH 6.5	-	-	12.20	-	-	0.92
L.delbrueckii ssp. delbrueckii ATCC9649	Batch	Wheat flour Hydr	YE	37°C, pH 6.0, 168 h	-	119.00	109.00	-	-	0.91
L.delbrueckii ssp. bulgaricus DSM 20081	Batch	Wheat flour Hydr	YE	45°C, pH 6.0, 168 h	-	144.00	26.00	-	-	0.18
L.casei ssp. rhamnosus	Batch	Glucose	YE	45°C, pH 6.0	3.17	30.00	27.80	0.68	-	0.90
L.salivarius ssp. salivarius	Batch	Glucose	YE	45°C, pH 6.0	3.05	30.00	27.50	1.41	-	0.99
L.helveticus	Cont, Recirc	Whey perm Powder	YE	42°C, pH 5.5, 138 h	-	-	28.29	-	-	0.88
L.casei NRRL B441	Batch	Barley starch	YE	37°C, 48 h	-	130.00	-	-	-	0.98
L.casei LA 041	Batch	CSL + glucose	YE	42°C, pH 6.25, 84 h	2.55	-	112.5	-	-	0.87

Table 2.3 The lactic acid production (Cont.) [44]

Organism	FM	Substrate	Nutrient	Operating Conditions	X ($g L^{-1}$)	S ($g L^{-1}$)	LA ($g L^{-1}$)	μ_{max} (h^{-1})	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)
L.plantarum ATCC 21028	batch	Lactose	YE	37°C, pH 6.0	-	-	-	0.36	0.39	1.02
L.rhamnosus NBRC3863	batch	Glucose	Spent cells – YE	42°C, pH 6.0	-	-	-	-	-	0.99
L.casei ATCC 10863	batch	RHH – glucose	YE	38°C, pH 6.0, 18 h	6.70	21.00	-	-	-	0.44
L.delbrueckii NCIM 2365	batch	Cane sugar	YE	42°C, pH 6.0, 24 h	3.90	80.00	66.00	-	-	0.97
L.delbrueckii NRRL B445	batch	Whey – glucose	YE	pH 6.5, 48 h	-	-	24.50	-	-	0.82
L.delbrueckii NRRL B445	batch	Whey – glucose	Soya flour	pH 6.5, 48 h	-	-	23.00	-	-	0.77
L.delbrueckii NRRL B445	batch	Whey – glucose	$(NH_4)_2SO_4$	pH 6.5, 60 h	-	-	23.50	-	-	0.79

Abbreviations: FM = fermentation mode; LA = lactic acid; Yp/s = yield of g LA per g substrate provides; Ref = reference; YE = yeast extract; CSL = corn steep liquor; hydr = hydrolysate; perm = permeate; RHH = ram horn hydrolysate; cont = continuous culture; recirc = recirculation of cells

2.2.3 Production of lactic acid by immobilized cell system

Cell immobilization is an effective method of improving the efficiency of substrate utilization and productivities of various fermentation processes and its practical application has been reported in both the food and wastewater-treatment industries. However, in order to use this technology in industrial production, the immobilization carrier must be very cheap and cell immobilization should be achieved with minimal additional cost. Renewable and biodegradable materials are the interesting alternative for immobilized cell carriers because of their simple and economical immobilization process. Immobilization of viable cells in agricultural residues such as loofa sponge, sugarcane bagasse, wood chips/shavings, saw dust, rice husk and straw have been investigated.

Goncalves et al. [12] researched on the immobilization of *Lactobacillus delbrueckii* NRRL B445 on inert supports with the intention of lactic acid production. All experiments were done in the continuously recycled packed reactors at pH 6.3 and 42°C. Four inert adsorbent supports were used for immobilization: raschig rings of sintered glass, beads of sinter glass, beads of porous glass, and irregular ceramic particles. The best support was found to be the beads of sinter glass, yielding the highest volumetric lactic acid productivity. In addition, the effects of pH and substrate concentration under immobilization were evaluated. The results indicated that pHs above or below the optimum for suspended cell systems can be used in the immobilized reactor while maintain lactic acid productivity. To simplify downstream processing by keeping the glucose concentration close to zero in the effluent, the glucose concentration in the feed has to be chosen in conjunction with the dilution rate.

Yoo et al. [45] encapsulated the *Lactobacillus casei* subsp. *rhamnosus* ATCC10863 in liquid-core alginate capsules for lactic acid production. The immobilization method of *L. casei* cells was investigated using alginate capsules that possess an interphasic membrane and a liquid core. The capsules were found to offer more space for cellular growth than gel-core beads, which resulted in 1.5 fold higher cell concentration than in the latter. However, the Ca-alginate structure was unstable during repeated batch fermentations for lactic acid production. Ba-alginate capsules were chemically and physically more stable than the Ca-alginate capsules in phosphate and lactate solutions. Attempts were also made to use various hardening

agents to stabilize the structure of the Ba-alginate capsules. It was found that the treatment with a mixture of chitosan and $BaCl_2$ solution gave the best results for hardening. Finally, stable lactic acid production was possible with a productivity of more than 2.7 g L^{-1} h^{-1} by *L. casei* cells immobilized in chitosan-coated Ba-alginate capsules. The cell leakage from the capsules was maintained relatively low during repeated batch fermentations.

Senthuran et al. [21] experimented on lactic acid production by recycle batch fermentation using immobilized cells of *Lactobacillus casei* subsp. *rhamnosus* DSM 20021. The fermentation set-up comprised of a column packed with polyethyleneimine-coated foam glass particles, Pora-bact A, and connected with recirculation to a stirred tank reactor vessel for pH control. The immobilization of *L. casei* was simply performed circulating the culture medium inoculated with the microorganism over the beads. At this stage, a long lag period preceded the cell growth and lactic acid production. Subsequently, for recycle batch fermentations using the immobilized cells, the reducing sugar concentration of the medium was increased to 100 g L^{-1} by addition of glucose. The lactic acid production started immediately after onset of fermentation and the average reactor productivity during repeated cycles was about 4.3 to 4.6 g L^{-1} h^{-1}, with complete substrate utilization and more than 90% product yield. Sugar consumption and lactate yield were maintained at the same level with increase in medium volume up to at least 10 times that of the immobilized biocatalyst. The liberation of significant amounts of cells into the medium limited the number of fermentation cycles possible in a recycle batch mode. Use of lower yeast extract concentration reduced the amount of suspended biomass without significant change in productivity, thereby also increasing the number of fermentation cycles, and even maintained the D-lactate amount at low levels. The product was recovered from the clarified and decolorized broth by ion-exchange adsorption.

Senthuran et al. [22] optimized the lactic acid production by immobilized *Lactobacillus casei* subsp. *rhamnosus* DSM 20021 in recycle batch reactor. Different nutritional and process parameters influencing lactic acid production, adsorbed to Poraver beads in a recycle batch reactor system, were studied in an attempt to set up a system having a long operational lifetime and permitting use of high substrate concentrations for maximal conversion to the product. The presence of lactose, even as a minor fraction of the total sugar amount, was necessary for complete utilization

by the organism for growth and conversion to lactate. Hydrolyzed whey protein constituted a richer source of nitrogen compared to yeast extract. Addition of lactate to the medium at the start of the process resulted in severe inhibition compared with the normal process. For a homofermentative process, pH 6.0 was found to be optimal. The overall productivity of the recycle system was higher under all conditions studied in comparison with the batch process using free cells. Enhancement in productivity in the recycle batch reactor was also accompanied by an increase in density of suspended cells. However, the contribution of the suspended cells to the overall reactor productivity was not noticeable. The bead size of the matrix was found to be important for operational stability of the reactor.

Cotton et al. [46] developed a unique plastic composite support (PCS) that stimulates biofilm formation in order to perform the continuous lactic acid fermentation by *Lactobacillus casei* subsp. *rhamnosus* ATCC 11443. The optimized PCS blend for *L. casei* contains 50% wt/wt of agricultural product, 35% wt/wt ground soy hulls, 5% wt/wt soy flour, 5% wt/wt yeast extract, 5% wt/wt dried bovine albumin, and mineral, and 50% wt/wt of polypropylene (PP) produced by high-temperature extrusion. The PCS tubes have a wall thickness of 3.5 mm, outer diameter of 10.5 mm, and were cut into 10 cm lengths. Six PCS tubes, three rows of two parallel tubes, were bound in a grid fashion to the agitator shaft of a 1.2 litres vessel fermentor. It was founded that PCS stimulated biofilm formation, supplied nutrients to attached and suspended cells, and enhanced lactic acid production. Biofilm thickness on the PCS tubes was controlled by the agitation speed. The PCS biofilm reactor and PP control reactor achieved optimal average production rates of 9.0 and 5.8 g L^{-1} h^{-1}, respectively, at 0.4 h^{-1} dilution rate and 125 rpm agitation with yields of approximately 70%.

Elezi et al. [38] immobilized cells of *Lactobacillus brevis* on dilignified cellulosic (DC) material in the purpose of producing lactic acid for food additive. DC material used as the support for immobilization was produced from wood sawdust after treatment with NaOH solution and heating for 3 hours at the boiling point for removal of lignin. For the immobilization of cells, *L. brevis* cells were inoculated to synthetic medium with DC material then the flask was incubated at 30°C and allowed to ferment overnight. When the immobilization was completed, the fermented liquid was decanted and the supported biocatalyst was washed with fresh medium and ready

to use for lactic acid production. The results indicated that yields of 80 and 100% conversion using glucose were obtained at 30°C in 1 day of fermentation time. Lactic acid fermentation using whey as substrate was obtained at 30°C in 1-1.5 days, resulting in 70% yield, whereas the remaining lactose in whey was converted to alcohol byproduct, leading to 90% lactose exploitation and 100% conversion. Cell immobilization of *L. brevis* on DC material was proved by its reuses in repeated batch fermentations and through electron microscopy. A series of 10 repeated batch fermentations without any loss in cell activity showed a tendency for high operational stability. The presence of DC material resulted in a drastic drop of the fermentation time from 48 to 13 hours.

Roble et al. [32] produced L(+)lactic acid from raw cassava starch in a circulating loop bioreactor with cells immobilized in loofa sponge. Microorganisms used in this experiment are *Aspergillus awamori* IAM 2389 and *Lactococcus lactis* spp. lactis IFO 12007. *A. awamori* functioned on the saccharification process and *L. lactis*, an aerotolerant anaerobe, was employed for lactic acid fermentation. *A. awamori* was immobilized directly in cylindrical loofa sponge while the *L. lactis* was immobilized in sliced loofa sponge with alginate as the polymer support. In the loofa sponge alginate gel cube, the sponge serves as skeletal support for the gel with the cells. The alginate gel formed a hard outer layer covering the soft porous gel inside. By controlling the rate and frequency of broth circulation between the riser and downcomer columns, the riser could be maintained under aerobic condition while the downcomer was under anaerobic condition. Repeated fed-batch L(+)lactic acid production was performed for more than 400 hours and the average lactic acid yield and productivity from raw cassava starch were 0.76 g lactic acid/g starch and 1.6 g lactic acid L^{-1} h^{-1}, respectively.

Shahbazi et al. [47] performed the immobilization of *Bifidobacterium longum* NCFB 2259 and *Lactobacillus helveticus* ATCC 15009 in sodium alginate beads and on a spiral-sheet bioreactor for the production of lactic acid from cheese whey. *B. longum* immobilized in sodium alginate beads showed better performance in lactose utilization and lactic acid yield than *L. helveticus*. In the spiral-sheet bioreactor, a lactose conversion ratio of 79% and lactic acid yield of 0.84 g of lactic acid/g of lactose utilized were obtained during the first run with the immobilized *L. helveticus*. A lactose conversion ratio of 69% and lactic acid yield of 0.51 g of lactic acid/g of

lactose utilized were obtained during the first run with immobilized *B. longum* in the spiral-sheet bioreactor. In producing lactic acid *L. helveticus* exhibited better when using the spiral-sheet bioreactor and *B. longum* showed better performance in gel bead immobilization system.

Shen and Xia [15] researched on the lactic acid production from cellulosic waste by cells of *Lactobacillus delbrueckii* ZU-S2 immobilized in calcium alginate gel beads. Cellulosic waste without pretreatment was hydrolyzed by cellulase and cellobiase in order to obtain the cellulosic hydrolysate. Cell immobilization was done by dripping the cell-sodium alginate solution in calcium chloride solution. After solidification, the immobilized gel beads will be obtained. The final concentration of lactic acid and the yield of lactic acid from glucose were 48.7 g L^{-1} and 95.2%, respectively, which were comparative to the results of pure glucose fermentation. The immobilized cells were quite stable and reusable and the average yield of lactic acid from glucose in the hydrolysate was 95.0% in 12 repeated batches of fermentation. The suitable dilution rate of continuous fermentation process was 0.13 hour, and the yield of lactic acid from glucose and the productivity were 92.4% and 5.746 g L^{-1} h^{-1}, respectively. The production of lactic acid by simultaneous saccharification and fermentation (SSF) process was carried out in a coupling bioreactor, the final lactic acid concentration was 55.6 g L^{-1}, the conversion efficiency of lactic acid from cellulose was 91.3% and the productivity was 0.927 g L^{-1} h^{-1}. By using fed-batch technique in the SSF process, the final concentration of lactic acid and the productivity increased to 107.6 g L^{-1} and 1.345 g L^{-1} h^{-1}, respectively.

Idris and Suzana [16] investigated the effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobilized *Lactobacillus delbrueckii* subsp. *delbrueckii* ATCC 9649. Batch fermentation of liquid pineapple waste to lactic acid was performed using immobilized *L. delbrueckii* under anaerobic condition for 72 hours. The results indicated that maximum concentration of lactic acid was produced after 56 hours of fermentation when 2.0% sodium alginate concentration was used. Maximum lactic acid production was also obtained with a 1.0 mm bead diameter at initial pH of 6.5 and temperature of 37°C. At last, it is feasible to use liquid pineapple waste to produce lactic acid using immobilized *L. delbrueckii* in calcium alginate bead.

Ganguly et al. [48] revealed the production of lactic acid by *Rhizopus oryzae* RBU2-10 immobilized in loofa sponge. First of all, pH and temperature stability of loofa sponge were assessed. The pH stability of the sponge was determined by soaking the sponge in the buffer solutions of varying pH, 1.1 – 14, for two weeks and then observing the change in shape, structure and texture of the sponge. The temperature stability of the sponge was observed by repeatedly autoclaving the sponge at 121°C for various lengths of time, 20 – 40 minutes. It was found that shape and texture of the loofa sponge remained intact after these treatments. Immobilization stage was performed by inoculated the loofa sponge with *R. oryaze* followed by incubation under shaking at 50 rpm and 30°C. After 24 hours, the immobilized mycelia were washed and the medium was replaced with fresh rice starch medium for further incubation. Subsequently, repeated batch fermentation for lactate production was carried out. At the end of each fermentation cycle, the immobilized matrices were retrieved by sieving, washed with sterile water and then transferred to the similar volume of fresh medium for the next cycle of cultivation. From the obtained datum, it can be concluded that the medium having four pieces of loofa sponge, 1.008 cm^3, per 100 ml medium and inoculated with 3 x 10^6 spores mL^{-1} resulted maximum production, 80.75 g L^{-1}, of lactic acid in 48 hours of fermentation. Focusing on repeated batch fermentation, lactic acid production could be carried out for 10 cycles. Remarkably higher levels of productivity, 1.66 – 1.84 g L^{-1} h^{-1}, was obtained during first five cycles of fermentation with a maximum productivity, 1.84 g L^{-1} h^{-1}, achieved during third cycle of fermentation.

John et al. [17] developed a process based on low cost production media for fermentative production of L(+)lactic acid. Various process parameters were optimized for the L(+)lactic acid production from cassava starch hydrolyzate-based medium by immobilized whole cells of *Lactobacillus delbrueckii* NCIM 2025 in alginate gel bead. The parameters considered for optimization were Na-alginate and $CaCl_2$ concentration, initial cell concentration, bead solidification time, bead size and incubation time of fermentation. Reusability of immobilized cells up to six batches was observed without any decline in lactate production. Lactate yield for *L. delbrueckii* was 0.93 g lactic acid/g reducing sugar with a production rate of 0.33 g L^{-1} h^{-1}. Furthermore, a column bioreactor packed with immobilized *L. delbrueckii* was run for three weeks in a continuous mode with a lactate yield of 0.75 – 0.95 g lactic

acid/g reducing sugar with an average production rate of 0.48 g L^{-1} h^{-1}. The study revealed that agro residues like cassava bagasse could be used as source material for developing a low cost technology for lactate production.

Rangawamy and Ramakrishna [49] demonstrated an integrated dual reactor system for continuous production of lactic acid by *Lactobacillus delbrueckii* NCIM 2365 using biofilms developed on reticulated polyurethane foam (PUF). In the biofilm development period, *L. delbrueckii* cells were inoculated to the PUF cube and incubated at 42°C for 12 hours on a rotary shaker, the PUF cubes were washed with sterile distilled water to remove the unadsorbed cells. Fresh MRS medium was added to the PUF cubes and incubated at 42°C for 24 hours to allow the cells to proliferate inside the PUF matrix. This procedure was repeated three times to ensure proper biofilm formation within the PUF matrix. The rate of lactic acid production was significantly high with a volumetric productivity of 5 g L^{-1} h^{-1} over extended period of time. When coupled to a bioreactor, the system could be operated as dual reactor for over 1000 hours continuously without augmentation of inoculum and no compromise on productivity.

2.2.4 Immobilization carriers in adsorption technique

Luffa cylindrical, loofa, grows well in both tropical and subtropical climates and the sponges are produced in large quantities in most African and Asian countries where they are currently used for bathing and dish washing [24, 25]. The sponge gourd, fruit of loofa, has a ligneous netting system in which the fibrous cords are disposed in a multidirectional array forming a natural mat. This fibrous vascular system is composed of fibrils glued together with natural resinous materials of plant tissue [27]. The loofa fibres composed of 60% cellulose, 30% hemicellulose and 10% lignin. As a result of their random lattice of small cross sections coupled with very high porosity, their potentiality as carriers for cell immobilization is very high [24, 25]. Furthermore, immobilization can be achieved by simply inoculating the cells into the reactor containing the loofa sponge bed.

Ogbonna et al. [24] developed the immobilization method for flocculating yeast cells, *Saccharomyces cerevisiae* IR-2, in loofa sponge and evaluated the suitability of loofa sponge as an immobilized cell carrier by ethanol production from both sucrose and molasses. Immobilization was performed by simply inserting loofa

sponge in a flocculating yeast cell suspension, the flocs were entrapped within the lattice structure and the immobilization was completed in less than 20 minutes. The immobilized cells grew inside the void volume, yielded a high cell concentration of over 4.4 g cells/g loofa sponge. In ethanol production from sucrose, more than 35 repeated batches were performed without any loss in the activity of the immobilized cells. Also with both sucrose and molasses media, more than 500 hours of stable continuous processing were possible. During the continuous process, the cells remained firmly immobilized to the loofa sponge and the free cell concentration in the effluent was less than 0.1 g L^{-1} throughout the fermentation period.

Ogbonna et al. [33] successfully designed the method for immobilization of non-flocculating cells in loofa sponge by adding chitosan as a flocculant. In this research, *Candida brassicae* was selected to use as the non-flocculating strain. In the reactor containing a loofa sponge bed, chitasan was introduced to a *C. brassicae* cell suspension to induced cell flocculation and the cells were efficiently immobilized. During ethanol production by the immobilized cells, the free cell concentration in the broth was controlled at the desired level by intermittent addition of chitosan to the reactor. The immobilized cell concentration increased but their specific ethanol productivity decreased with an increase in the chitosan concentration. The maximum ethanol productivity was obtained at a low chitosan concentration of 0.03 g L^{-1}. With this optimal concentration, the cell concentration, ethanol yield and productivity were, respectively, 2, 1.3 and 3 times higher than those of the suspension culture.

Ogbonna et al. [25] experimented on the immobilization of both flocculating and non-flocculating cells in loofa sponge for continuous ethanol production in column-type bioreactors. Immobilization of non-flocculating cells, *Candida brassicae*, was achieved by addition of chitosan, flocculating agent, to the reactor. However, the amount of immobilized non-flocculating cells per unit sponge was lower, and consequently the volumetric ethanol productivity was about 20% less than that of the flocculating cell. In comparison with a fixed bed made of a single cylindrical loofa sponge, the amount of sponge per unit reactor volume was 2 times higher when sliced loofa sponge was used to construct the fixed bed. Furthermore, the use of sliced loofa sponge resulted in 2- and 4-fold increases in the amount of immobilized flocculating and non-flocculation cells per unit sponge, respectively. Consequently, higher volumetric ethanol productivities were obtained when sliced loofa sponge was used for bed construction. In the case of flocculating cells,

Saccharomyces cerevisiae IR-2, the volumetric ethanol productivity was 66% higher than that obtained with a cylindrical loofa sponge.

Ogbonna et al. [34] effectively produced ethanol from sugar beet juice by using immobilized *Saccharomyces cerevisiae* IR-2 in cylindrical loofa sponge in a bubble column bioreactor. By using an external loop bioreactor, constructing the fixed bed with cylindrical loofa sponges, dividing the bed into upper, middle and lower sections with approximately 1 cm spaces between them and circulating the broth through the loop during the immobilization, uniform cell distribution within the bed was achieved. By means of this method, the system was scaled up to 50 litres and when compared with the 2 litres bubble column bioreactor, there was no significant differences ($P > 0.05$) in ethanol productivity and yield. By using external loop bioreactor to immobilize the cells uniformly on the loofa sponge beds, efficient large scale ethanol production systems can be constructed.

Vignoli et al. [26] investigated the capacity of *Zymomonas mobilis* ATCC29191 bacteria immobilized in loofa sponge to produce sorbitol from fructose sources and the importance of difference factors in the fermentation process. The experiments were performed in times 12, 24, and 36 hours, containing 200, 250, and 300 g L^{-1} sucrose. The fermentative process was analyzed with free and immobilized cells. The results showed that *Z. mobilis* could be immobilized in loofa sponge after adding chitosan for cell flocculation. The sucrose concentration was not a relevant factor for the process but the fermentation time and the type of process were significant. The use of cells in the immobilized form and the 36 hours time period presented maximum sorbitol productions. The production with immobilization was practically twice that obtained with free cells, increasing of 25 g L^{-1} with free cells for 50 g L^{-1} with immobilized cells.

Phisalaphong et al. [35] developed an alginate-loofa matrix as a cell carrier for ethanol fermentation owing to its porous structure and strong fibrous nature. The matrix was effective for cell immobilization and had good mechanical strength and stability for long-term use. The immobilization yield of the new carrier was approximately 87%. In addition, after a storage period of 4 months, yeast cells remained firmly immobilized and still active.

Hideno et al. [50] evaluated the feasibility of using loofa sponge for immobilization of cellulase-producing microorganisms by acetylating loofa sponge. Acetylation was achieved by autoclaving process of loofa sponge immersed in acetic

anhydride at various temperatures for various times. The degree of acetylation, as inferred by the weight percentage gain (WPG), was enhanced by increasing both temperature and the acetylation duration. The acetylation of a piece of loofa sponge in an autoclave at 120°C for 20 minutes resulted in a WPG of about 8%, which was sufficient to protect the loofa sponge against cellulose degradation. The acetylated loofa sponge prepared under this condition was not decomposed by commercial cellulase and its structure was maintained for more than 720 hours during repeated-batch treatments with commercial cellulase. Flocculating yeast, *Saccharomyces cerevisiae* IR-2, and a fungus, *Trichoderma reesei* QM9414, was successfully immobilized in the acetylated loofa sponge. In each case, the percentage of immobilized cells was as high as that obtained using nonacetylated loofa sponge. Acetylation has no adverse effects on cell growth and immobilization of *T. reesei* QM9414, as well as on cell growth and ethanol production by *S. cerevisiae* IR-2. *T. reesei* QM9414 immobilized on an acetylated loofa sponge was effectively used for repeated-batch cellulase production from commercial cellulose powder.

Saudagar et al. [31] produced clavulanic acid by immobilize *Streptomyces clavuligerus* MTCC 1142 on loofa sponge discs. Best yield of 1125 μg mL^{-1} of clavulanic acid was reached with two discs of loofa sponge, each approximately 0.136 g dry weight, and 120 hours duration in the first cycle. Data obtained during four reusable cycles showed reduction in the initiation time of clavulanic acid production, resulting in higher levels of clavulanic acid in shorter time duration.

Meleigy and Khalaf [36] researched on the biosynthesis of gibberellic acid (GA) from milk permeate in repeated batch operation by a mutant *Fusarium moniliforme* cells immobilized on loofa sponge disc. GA production from milk permeate was studied by 28 mutants of *Fusarium moniliforme*, among which mutant γ-14 was selected as the best producer. Experiments were carried out in shake flasks and fermentative process was analyzed with free and immobilized cells. Immobilization of mutant γ-14 cells onto loofa sponge discs was studied with respect to the optimization of the incubation temperature, initial pH, inoculum size (number of discs), and its reusability for GA production. Best yield of GA, 2.40 g L^{-1}, was recorded by immobilized cells under optimized cultural conditions, 4 immobilized discs, 30°C, and pH 5, more than 2.8 times greater than previously reported by free cells. Data obtained during four reusable cycles showed hit stability of GA production

and reduction in the initiation time of acid production, resulting in higher levels of GA in shorter time duration. In conclusion, immobilization of mutant γ-14 cells onto loofa sponge discs permitted repeated reuse under the specified fermentation conditions for GA production from milk permeate.

Sugarcane baggase, which is a complex material, is the major by-product of the sugarcane industry. It is a fibrous residue of cane stalks left over after the crushing and extraction of the juice from the sugarcane. Sugarcane bagasse consists of approximately 50% cellulose, 25% hemicellulose, and 25% lignin [51].

Santos et al. (2005) revealed that sugarcane bagasse can be used not only as a source of xylose for the bioproduction of xylitol, but also as a carrier for immobilizing the cells in this case was *Candida guilliermondii* FTI 20037. The immobilization procedure is quite simple and can be easily integrated with the bioconversion itself. Activation of the raw bagasse with epichlorohydrin and hexamethylene diamine prior to cell immobilization improved the yield of immobilization and xylitol productivity. The increase in the amount of bagasse used to immobilize the cells led to substantial increases in both cell yield and immobilization yield. Under such conditions, xylitol yield was reduced owing to a higher concentration of suspended solids in the flasks, which favours oxygen dissolution into the medium. The average diameter of the bagasse used to immobilize the cells did not influence significantly any of the response variables evaluated: xylitol yield, cell yield, xylitol productivity, and yield of immobilization [37].

Mulinari and Silva [52] chemically modified the sugarcane bagasse to obtain the cellulose for using in the adsorption of sulphate ions in aqueous solution. To isolate the cellulose, the sugarcane bagasse was pretreated with sulphuric acid solution, followed by centrifugation with the purpose of separating the rich pentosanes solution. Extracted lignocellulosic fraction was delignificated with sodium hydroxide solution being obtained the crude pulp and bleached with sodium chloride. Finally, the bleached cellulose dried in a store at 50°C for 12 hours before used. The treated celluloses were immersed in the zirconium oxychloride in aqueous hydrochloric acid solution. The material was precipitate with ammonium solution. The obtained materials were characterized by thermogravimetry, scanning electron microscopy and surface area measurements. Sulphate ion was adsorbed on the Cell/ZrO_2.$n$$H_2O$ by immersing this solid in an aqueous solution of Na_2SO_4. After the

adsorption, the amount of sulphate ion concentration in the supernatant solution was determined using turbidimetric method by UV-vis absorption spectra, in the wavelength at 420 nm. The results were satisfactory showing that the Cell/ZrO_2.nH_2O hybrid material obtained presented good adsorption capacity.

Krishna et al. [23] discovered the chitosan-treated polypropylene (PP) matrix as immobilization support for lactic acid production using *Lactobacillus plantarum* NCIM 2084. Treatment of the matrix with chitosan was done by incubating the polymer beads in chitosan solution, 0.05, 0.5, 1.0, 2.0, and 5.0 g L^{-1}, for 30 minutes under sterile conditions. After that cell suspension was added to the treated matrices and agitated at 110 rpm at room temperature for 12 hours. The biocatalyst adsorbed on the 1.0 g L^{-1} chitosan treated PP matrix proved to be most effective in terms of conversion, yields and productivity. In addition, repeated batch fermentation experiments showed that the immobilized biocatalyst could be recycled effectively 11 times. Studies were also carried out in a packed bed reactor with media recirculation. A high productivity of 7.66 g L^{-1} h^{-1} could be obtained with a conversion of 94% and a yield of 97% at an average residence time of 30 hours.

Hsu et al. [53] enhanced the production of xanthan gum, an industrially important microbial polysaccharide, by using *Xanthomonas campestris* NRRL B-1459 cells adsorbed to cotton fibres. To further evaluate the role of xanthan polymers during cell adsorption, 2 types of hydrophilic fibrous matrices woven with looping namely cotton towel and viscose rayon towel were employed in this study. Aqueous solution of branched polyethylenimine (PEI), a cationic polyelctrolyte, was used as the surface modification agent for the matrices. From the experimental data, 2 distinct stages of *X. campestris* cells adsorption were proposed. During the initial stage (0 to 3 hours), the hydrophillicity of fibre, which surpasses electrostatic interactions between cells and fibre surface, is the determining factor for attracting cells to the fiber surface. Consequently in the xanthan producing stage (up to 28 hours), xanthan polymers enhance cell retention on fibre surface, whereas the overall cell load is dependent on the initial amount of cells adsorbed. It can be concluded that the immobilized *X. campestris* cells were capable of producing xanthan gum, with final xanthan concentration higher than those produced by suspending cells and the untreated cotton exhibited the highest immobilization efficiency and xanthan production.

Huang et al. [54] reported the technique of cell immobilization using porous biomass support particles (BSPs). In the present study, the immobilization of *Escherichia coli* K12 using BSPs was investigated in shake flask culture. The density of the cells immobilized within the BSPs was evaluated by measuring their intracellular lactate dehydrogenase (LDH) activity. Since *E. coli* cells were not successfully retained within reticulated polyvinyl formal (PVF) resin BSPs with matrices of relatively small pores, 60 μm, coating the surface of the BSPs with various polymers was examined as a way of promoting cell attachment. When positively charged polyamino acids such as poly-L-lysine, poly-L-arginine, poly-L-histidine, and poly-L-ornithine were adsorbed onto the particle surface, they were found to increase the immobilized cell density, while neutral and negatively charged poly-amino acids including poly-L-asparagine and poly-L-glutamic acid were not effective. These results indicate that *E. coli* cells can be efficiently immobilized in PVF resin BSPs by electrostatic interaction between the negatively charged ions of the cell surface and the positively charged polymers adsorbed onto the BSP surface. A significantly high immobilized cell density was also achieved by coating the surface of the BSPs with the synthetic polymeric amine polyethyleneimine. In particular, polyethyleneimine is an attractive coating polymer because of its relatively low cost.