Chapter II

Background and Literature Review

Ethanol fermentation can be operated in batch, fed batch, and continuous mode. This is shown in a simplified diagram in Figure 2.1 [2]. In general, variations in medium composition, requirement of cells recycling, and unproductive steps, for example charge, discharge, and cleaning steps are drawbacks in batch process. Fed batch can be applied to control the medium composition. Duration of fermentation, ethanol yield, and fermentation efficiency can be improved with optimization of the fermentation process and implementation of new technologies. With proper operation, implementation of continuous instead of batch process will increase ethanol productivity, reduce production cost, and improve process efficiency. The process can be carried out by using free cells, flocculating cells, or immobilized cells [3].

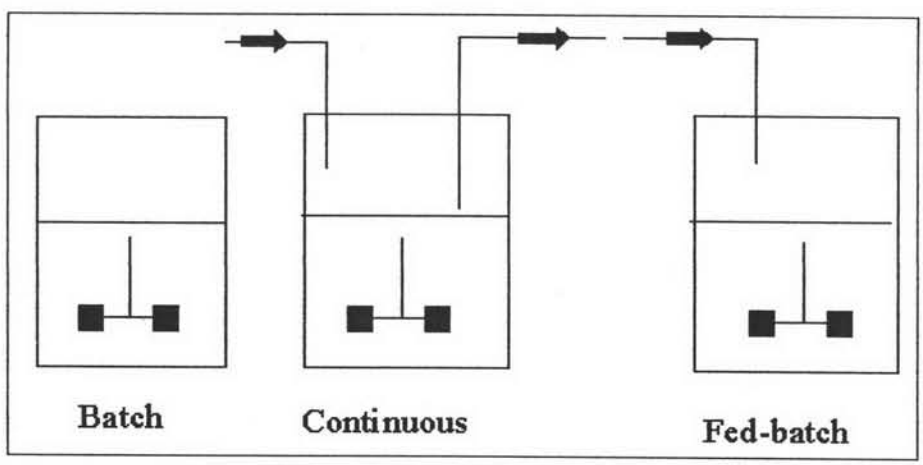

Figure 2.1 Diagram of batch, fed batch, and continuous process [2]

Alcoholic fermentation using freely suspended cells provides larger area for contact between cells and nutrients in the medium. The technology for suspended cell culture is more readily available as compared to immobilized cells counter part. However, there are disadvantages including high cost for microbial recycling, high contamination risks, susceptibility to environmental variations, and limited of dilution rate in fermentation due to washout. Figure 2.2 shows a schematic diagram of a suspended cells fermentation system equipped with cell/biomass recycle system [2].

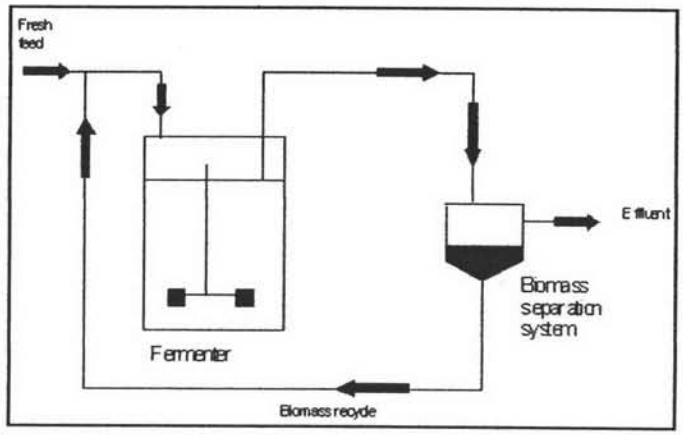

Figure 2.2 Suspended cells fermentation with cell recycle [2]

The use of flocculating instead of suspended cells as producers can improve downstream processing but there may be additional expenses related to equipment necessary for sedimentation and recycling of the cells. Moreover, the cells' aggregate (flocs) themselves possess additional diffusion limitation than suspended cells [4]. A simple description of flocculated cells fermenter is given in Figure 2.3 [2].

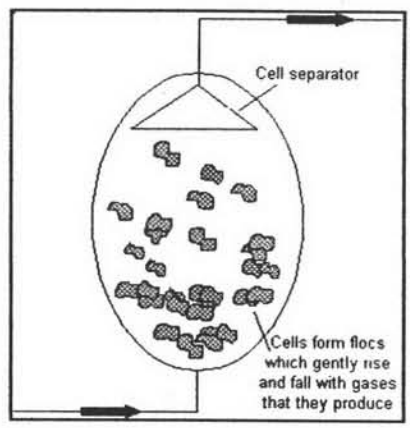

Figure 2.3 Flocculated cells fermenter [2]

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 [3,5]. Thus, immobilized cell technology is considered promising for ethanol production and chosen as the focus of this work. A representation of immobilized cell reactor is presented in Figure 2.4 [2].

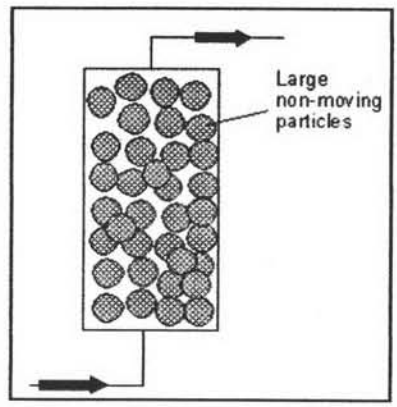

Figure 2.4 Immobilized cells bioreactor in fixed bed configuration [2]

2.1 Immobilized cell system

Immobilized cell technology has gained many interests since 1980s. Thousands of documents in various journals and papers are currently available via scientific search websites [5]. Immobilization of cells as biocatalysts is almost as common as enzyme immobilization. It can be defined as the restriction of cell mobility within a finite space [6]. Main application fields of IC consists of biosyntheses and bioconversions, environment, food processing, biosensors, and optical. Motivation for development of immobilized cell systems emerged from their potential advantages. Some potential advantageous characteristics of IC over suspension cultures are as following [2,4-6].

1. Higher cell concentration.
2. Higher possibility for biocatalyst regeneration in hostile condition.
3. Elimination washout problem which in turn enables the fermentation to be carried out at higher dilution rate.
4. Easier downstream processing of the product.
5. Possibility of avoiding costly cells recovery and recycle by reuse of cells.
6. Improving cells genetic stability in some cases.
7. Protection of cells from shear force (especially for shear sensitive cells).
8. More favorable microenviromental conditions.
9. Maintenance of cell activity by protection from toxins and inhibitors.
10. Higher production rates and yields.
11. Smaller fermenter requirements.
12. Capital and energy cost saving.

In a continuous fermentation, productivity in general can be improved by increasing the flow rate of the system which is usually represented as dilution rate.

Dilution rate is the ratio between flow rate and volume of reactor. The relationship between productivity and dilution rate is shown in Figure 2.5 [2].

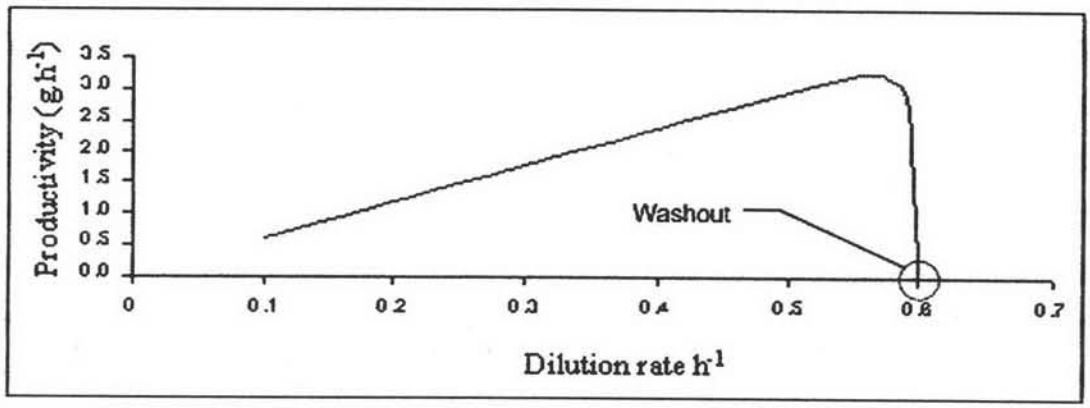

Figure 2.5 Productivity versus dilution rate curve [2]

After reaching an optimum value, the productivity will decrease drastically until it reaches nearly zero. In some cases, the optimum bioreactor volume is set by the critical dilution rate which corresponds to the dilution rate at which washout occurs. Figure 2.6 shows a relationship between substrate, product, and biomass concentration with critical dilution rate [2].

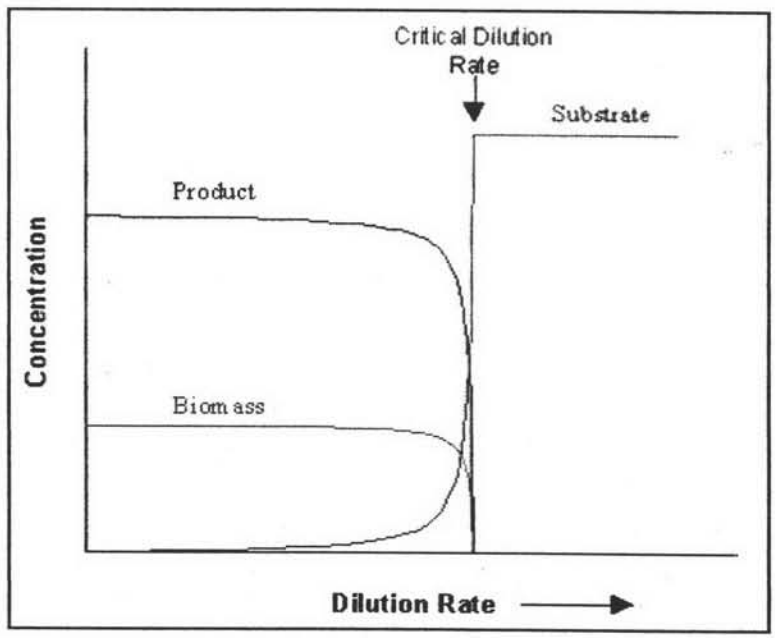

Figure 2.6 Concentration profile with variable dilution rate [2]

By immobilizing the cells inside the bioreactor, high cells concentration can be achieved even though the dilution rate has exceeded its critical value. With the combination between high dilution rate and high cell densities inside the reactor,

immobilized cells fermenter can maintain higher productivity as compared to conventional suspended cells culture.

Unfortunately, immobilization of cells also has some limitations and drawbacks. It should only be applied when the desired product is excreted by the cells. Furthermore, it often leads to systems for which diffusional restriction are important. In many cases, the control of microenviroment in immobilized biosystems is difficult due to the high degree of heterogeneity within such systems [6].

Immobilized systems can be classified into natural and artificial occurring ones [5]. In nature, some microorganisms can form biofilm by attaching to one another or even to surfaces. This attachment is facilitated by secretion of adhesive substance called glycocalyx by the cells [5,7,8]. In artificial immobilized cell system, cells are immobilized by using carriers/supports. Proper selection of carrier is extremely important for immobilized cell application because it will affect greatly on the performance of the system [7]. As every organism exhibits different interaction with different carriers, evaluation of carrier performance for an individual organism should be done in case by case basis.

2.2 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 [6]. In addition, cheap, non toxic, highly available, and environmentally friendly materials are often more preferred for cell immobilization purposes [8,9]. Selection of supporting materials will depend upon many factors including the resistance to microbial degradation, mechanical strength, type of fluid, surface characteristics, and the cost of materials. Carriers can be classified into organic, inorganic, porous, and charged carrier based on the nature of the supporting material [8].

2.2.1 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 [8] but less severe environmental pollution [9].

Some organic material from plants such as loofa sponge [1,4,9-11], sugar cane stalk [3], quince [12], apple cut [13], corn grit [14], and wood blocks [15] 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 [9]. Some examples of organic materials are shown in Figure 2.7 [16,17]. Naturally derived polymers such as alginate and chitosan have been studied intensively to evaluate their compatibility for cell immobilization.

Figure 2.7 Examples of organic materials for cell immobilization [16,17]

2.2.2 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 [5,8]. Grafting procedure is generally incompatible with cell viability [5]. Examples of this type of carrier are metal oxides, stainless steel mesh, polyacrylamide, and polystyrene [6]. Figure 2.8 shows some inorganic materials which can be used as cells carriers [18-20].

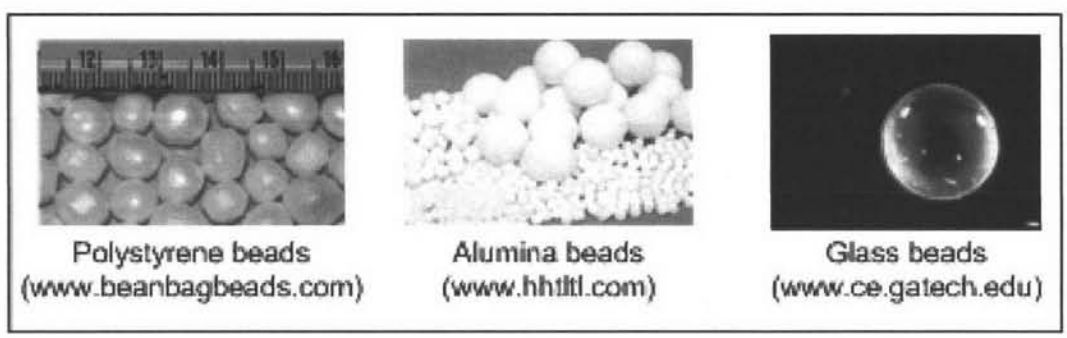

Figure 2.8 Some inorganic materials for cells carriers [18-20]

2.2.3 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. The charged material could also interact with charged substrate, product, and/or residual contaminants [8,21]. This will hinder cell growth for example by removing essential minerals for growth from medium. The inhibiting effect will increased at higher charged material [21]. Ion exchange resins, gelatin, stainless sphere wire, and porous cellulose are example of charged carriers [6,21-23].

2.2.4 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 [7]. Carrier bead which has small pore on the surface and large pore in the interior was found to be effective for cell immobilization [22].

Material with large pores such as loofa sponge can effectively be used for immobilization of segregating cell for instance flocculating yeast [1]. High porous material allows the cell to attach under low shear condition while the fluid outside the material can be moving at high speed [8]. 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 [6,21-24]. The pictures of some porous materials are shown in Figure 2.9 [25-28].

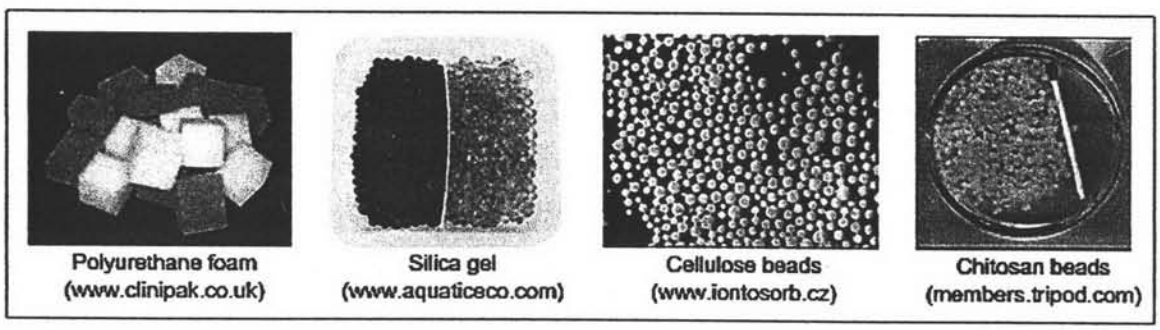

Figure 2.9 Porous carriers for cell immobilization [25-28]

2.3 Immobilization techniques

Immobilization is usually done by entrapment or binding of cells using physical or chemical forces [6]. There are several methods for immobilizing cells to supporting carrier: attachment or adsorption to carrier, covalent bonding or cross-linking, microencapsulation, membrane reactor, and entrapment within porous matrices [6,8,9]. Figure 2.10 provides physical descriptions of several techniques which have been applied for cells immobilization [29].

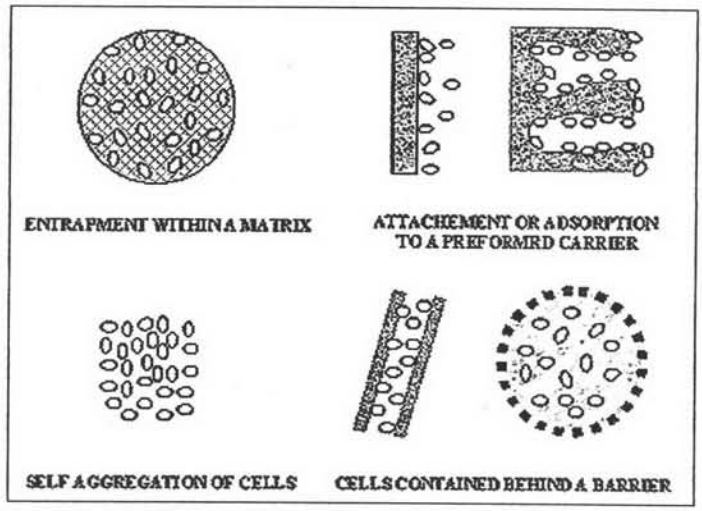

Figure 2.10 Description of some immobilization methods [29]

2.3.1 Attachment/adsorption to carrier

In natural ecosystems, microorganisms tend to concentrate at a surface. This fact is believed to be one of the reasons for benefit of attached growth system, leading to an efficient process in a more compact configuration [8]. Direct contact between nutrient and supporting carriers is also a major advantage of this system. High cell loading for adsorption system can be obtained by using microporous support materials. However, this may lead to intraparticle pore diffusion limitation at high cell densities. In addition, the control of microenvironmental conditions is found to be difficult for porous supports [6]. A ratio of pore to cell diameter of 4 to 5 has been recommended for this type of immobilization [6,7]. At small pore sizes, accessibility of the nutrient into inner surface of pores may become the limiting factor while for large pore sizes low specific surface area per unit of volume of the carrier will become a concern [6]. Therefore, there can be optimum pore sizes corresponding to maximum growth and/or bioconversion rate [6,24].

In many cases, a higher metabolic activity was observed by using attached growth system when compared with suspended (free cells) counterparts. Possible causes for higher metabolism within attached growth system include higher amount of active biomass concentration in this system and concentration of nutrient around attached biofilm due to its slimy nature. A physiological difference between attached and suspended microorganisms is also been proposed as a distinction factor. It has been suggested that with attachment, the activation of different genes occurs, which responds in a faster growth rate, increased metabolic activity, and greater resistance to toxicity or contamination. Increased resistance to toxicity has also been attributed to the occurrence of protective extracellular matrix surrounding the biofilm. The diffusion barrier, together with ion exchange role of this matrix, reduces the concentration of toxic compounds around attached biofilm [8].

Selection of suitable materials for cell adsorption is usually based on adsorption capacity and strength of binding. Adsorption capacity for example may vary from 2 mg/g for porous silica to 250 mg/g for wood chips. Porous glass carriers have loading capacities of 10^8 to 10^9 cells/g that are less or comparable to 10^9 to 10^{11} cells/ml capacities obtained in gel entrapment system [6].

A microbial structure called glycocalyx (capsule) which consists of extracellular polysaccharides or protein is believed to play an important role in microbial attachment to solid surfaces. Apart from this structure, there are several forces that also have been attributed for governing microbial attachment: electrostatic interactions, covalent bond formation, hydrophobic interactions, and partial covalent bond formation between microorganisms and hydroxyl groups on the surfaces. None of these forces could be considered as the dominant one. The strength of the attachment and the composition of the forces which govern it vary with different environmental conditions, different microbial species, different surface properties, and with different fluid properties [6,8]. As surface properties such as surface charge of both the cell and carrier play an important role in cells attachment, some efforts have been directed for modification of these properties either physically [23] or by chemical treatment for example with proteins, ions, and cationic polymers [4,21,22]. However, some treatments have been reported to have detrimental effects on cell growth and productivity. Thus, careful selection and application of such treatments for each particular case are often needed to obtain the expected enhancement of immobilized cells system performance [10].

Electrostatic forces involved in microbial attachment to solid surfaces are usually manifested in the form of ionic and hydrogen bond. These forces are considered to be weaker than covalent bond when compared individually. Nevertheless, they are capable of producing a relatively firm binding if the number of bonds is sufficiently large. The contribution of electrostatic interactions is significantly high in the initial stages of adsorption. Since surface charge of almost all microorganisms is usually negative and in many cases, the immobilization carrier is also negatively charged, an electrostatic repulsive force can prevent adsorption to progress [8]. In practice, negatively charged cells do attach to surfaces that possess net negative charge because there are other stronger forces that overcome the electrostatically originated repulsive force such as the formation of covalent and hydrogen bonding. In systems where positively charged supports for instance ion exchange resins and gelatin are used, electrostatic can be the dominant governing force for microbial attachment [6,8].

For neutral surfaces, adsorption of cells may be facilitated by chemical bonding such as covalent and hydrogen bonding or van der Waals forces [6]. The outer surfaces of microbial cells and cell walls contain large quantities of various reactive groups. Covalent bonds are usually formed between those ligands and organic groups that are available on the surface of support material [8].

Since in many cases attachment of microorganisms to supporting materials takes place in a liquid system, hydrophobic interactions between the support and microorganisms become very important. Hydrophobic groups on the microbial surface can interact with the ones on the carrier while removing water molecules which separate the microorganisms from the carrier surface. In addition, there are facts proving that some microorganisms prefer to grow on hydrophobic surfaces than hydrophilic ones [8].

In microbial immobilization process, hydroxides are usually formed on the surface of the support as results of the reaction between the support and water from the immobilization solution. Suitable amino or carboxyl groups on the cell surfaces can replace those hydroxyl groups resulting in the formation of partial covalent bonds between support and cells [8].

Adsorption in general is a simple and inexpensive method of cell immobilization [6, 4]. Although a higher diffusivity can be obtained by this method when compared to entrapment method [24], limited cell loading capacity and rather weak binding forces reduce the attractiveness of this method [6]. Most of the time, hydrodynamic shear stress around adsorbed cells should be mild enough to prevent cells detachment from support

surfaces [6]. Examples of materials used for cells adsorption include loofa sponge [1,4, 9-11], sugar cane stalks [6], porous glass [6-7], porous silica, alumina, ceramics, zeolite [7], quince [12], apple cut [13], corn grit [14], wood block [15], porous cellulose [6,21,22], gelatin, chitosan [6,24], activated carbon, wood chips, and polypropylene ion-exchange resins [6].

2.3.2 Covalent bonding/cross-linking

Covalent bonding is the most widely used method for enzyme immobilization but it is not as widely applied for cell immobilization [6]. Functional groups on cells and support material surfaces are usually not compatible to each other for covalent bonding so that coupling agents such as glutaraldehyde and carbodiimide are often needed to activate ligands on the microbial and carrier surface. This activation step will facilitate the formation of covalent bonds between the microorganisms and carrier. On the other hand, this process will expose the microorganisms to potent reactive groups which possess toxic effects [6,8]. Some inorganic carriers such as titanium and zirconium oxide have been designed to provide satisfactory functional groups required for covalent bonding in order to perform well even without the use of coupling agents [6].

Covalent bonding forces are stronger than adsorption forces, resulting in more stable binding [6]. However, with growing cells, cells leakage as a result of cell division becomes a problem when using covalent bonding as a method for cell immobilization [6,8]. Because of cell leakage, covalent bonding method is often associated with low cell loading when compared to other immobilization methods [8]. Furthermore, suitable materials with desired functional groups are rather limited. Among of such materials are carboxy methyl cellulose (CMC) plus carbodiimide; carriers with aldehyde, amine, epoxy, or halocarbonyl groups; Zr(IV) oxide; Ti(IV) oxide; and cellulose plus cyanuric chloride. Carriers with –OH groups are treated with cyanogen bromide (CNBr), materials with $-NH_2$ groups are treated with glutaraldehyde, and supports with COOH groups are treated with carbodiimide for forming covalent bonds with protein groups on cell surfaces [6].

Cross-linking refers to the direct linking of microorganisms to each other to form a large insoluble three dimensional network/aggregate of cells [6,8]. Some cells may even be cross-linked after adsorption onto support surfaces. The links may be formed by physical or chemical cross-linking. Physical cross-linking can be done by polyelectrolytes, polymers such as chitosan, and salts (for example $CaCl_2$, $Al(OH)_3$, and $FeCl_3$) that act as cells aggregation agents (flocculent). Chemical cross-linking finds expression in covalent

bonds formation. Covalent bonds are constructed by using chemical agents such as glutaraldehyde [6]. Because the process is similar to the one used in aforementioned covalent bonding method, chemical cross-linking also suffers from the same problems that are related to covalent bond method. Generally speaking, cross-linking like covalent bonding is not widely practiced for cell immobilization because of their corresponding disadvantages [6,8].

2.3.3 Microencapsulation

The microencapsulation method consists of wrapping droplets containing microorganisms with a thin membrane to form a microcapsule [8]. Microcapsules essentially are hollow and spherical particles bound by semi permeable membranes [6]. The entrapped microorganisms can freely move within the microcapsules and consume nutrients that penetrate through the membrane. Nylon, collodion, polystyrene, acrylate, polylysine-alginate hydrogel, cellulose acetate-ethyl cellulose, polyester, and cellulose nitrate are examples of membrane material used to construct microcapsules [6,8]. Membranes with different composition and molecular weight (MW) cutoff can be chosen for a certain field of application in order to retain some high molecular weight products inside while allowing passage of low molecular weight nutrients and products [6]. These microcapsules usually have a diameter of 10 to 100 μm. In general, good diffusive mass transfer across the membrane has become the major attraction of this technique but in practical, toxicity and instability of the membrane have hindered this method from reaching its full scale application [8]. Intraparticle diffusion limitations are less severe in microcapsules due to the presence of liquid cell suspension in the intracapsule space and more cells per unit of volume can be packed into such carriers [6]. Unfortunately, cell growth and metabolic gas production may cause mechanical rupture of the capsules [8].

2.3.4 Membrane reactor

In membrane reactors, microorganisms are isolated from bulk liquid by using sheets of membrane [6,8]. The membrane will allow selective permeation of essential nutrients from bulk liquid to reach the microorganism while at the same time immobilizing the microorganisms by physically isolating them from the bulk liquid. Both porous and dense membranes can be applied for this purpose [8].

Hollow fiber membrane module is the simplest manifestation of membrane reactor suitable for cell immobilization. This device is a mass transfer analog of the shell and tube

heat exchanger in which the tubes are made of semi permeable membranes. In typical configuration, cells are inoculated on the shell side and allowed to grow there while nutrient solution is pumped through the tube side. Nutrients diffuse through the membrane and reach the cells. In a reverse direction, metabolic products from cell will diffuse back into the flowing nutrient stream. Most of the time, conventional hollow fiber unit does not perform well with living cells due to diffusional limitations so that modification is often needed. Modifications involving multiple membrane types, each for a particular purpose (for example for gas exchange or extractive product removal) or changes of membranes configuration to improve convective flux within the cells layer have been proposed. Several commercial reactors of this type have been applied animal cells cultivation [6].

Fouling of the membrane is the main concern in the application of membrane reactor. This phenomenon finds expression in pore blockage for porous membrane and concentration polarization layer for dense membrane. Fouling problems can be solved in general membrane application by physical or chemical cleaning procedures. However, these cleaning procedures may imply harmful effects to the microorganisms so that most of the time they can not be used for solving fouling problem encountered in membrane used for cell immobilization purpose [8].

2.3.5 Entrapment within porous matrices

Physical entrapment within porous matrices is the most widely applied technique for cell immobilization [6]. Figure 2.11 shows a representative diagram of cells immobilization by entrapment in porous matrices [2]. Various matrices such as porous polymers (agar, alginate, κ-carrageenan, polyacrylamide, chitosan, gelatin, cellulose, and collagen), porous metal screen, polyurethane, silica gel, polystyrene, and cellulose triacetate have been evaluated as cells carriers [6,9,11,21-24]. Higher diffusion restriction becomes a main problem for this entrapment method while higher viable biomass concentration, higher resistance to contamination, and better genetic stability are proposed as advantages that can be obtained from this process when compared to other immobilization techniques [8].

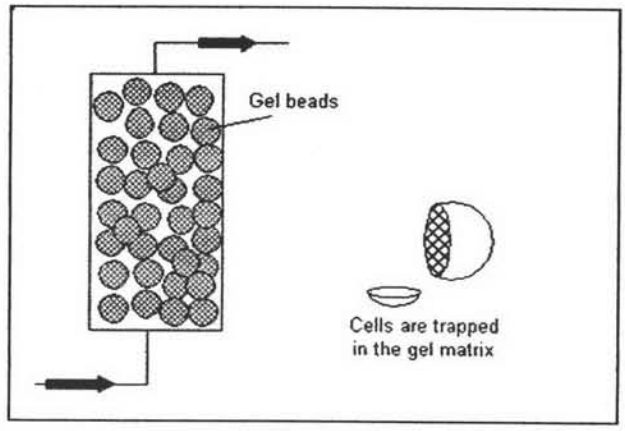

Figure 2.11 Cells entrapment in gel matrix [2]

In polymeric cells carriers, cells are entrapped inside the polymer matrix. The carriers are usually prepared in the form of beads. These beads should be porous enough to allow the transport of substrates and products in and out of the bead [6]. Small pores of the polymeric matrix retain the microorganisms but allow penetration of substrates towards the entrapped cells because of its hydrophilic nature [8].

First step of entrapping microorganisms inside polymer beads consists of suspending the microorganisms within a liquid solution which contains macromolecule monomers. The next step is gelation/polymerization of the solution to produce a polymeric gel containing viable biomass. Depending on the nature of monomers used, polymerization may be carried out by various chemical and physical processes. Such processes include lowering or raising the temperature, precipitation of polymers, ion-exchange/ionotropic gelation of monomers by multivalent cations, polycondensation, cross-linking, and photochemical reactions [6,8]. Gelatin and agar beads can be made by reducing the temperature of their templates so that solidification of the gel will occur [6].

Polymers such as polystyrene, cellulose triacetate, and collagen can be precipitated by changing the solvent or pH of the solution. The initial polymer solution has to be prepared with an organic solvent or a water-solvent mixture, for example ethanol and acetone. Inactivation or even cells death may result during precipitation as a consequence of direct contact between cells and solvents [6].

Ion-exchange gelation takes place when a water soluble polyelectrolyte is mixed and reacted with salt solution to form a solid gel. The most popular example of this type of gelation is the formation of Ca-alginate gel by mixing Na-alginate solution with $CaCl_2$ solution. Others examples of polymers obtained by ionotropic gelation are Al-alginate, Ca-Al carboxymethyl cellulose, Mg pectinate, κ-carrageenan, and chitosan polyphosphate.

Among these polymers, alginate and κ-carrageenan are the most widely used ones for cell immobilization purposes [6,23]. Further stabilization of these ionic gels can be done by covalent cross-linking [6].

Polymers produced by polycondensation will have high chemical and mechanical stability because of the formation of covalent networks. Usually, liquid precursors are cured with a component containing multifunctional reactive ligands such as hydroxyl, amino, epoxy, and isocyanate groups. Examples of polymers prepared by polycondensation include epoxy, polyurethane, silica gel, gelatin-glutaraldehyde, albumin-glutaraldehyde, and collagen-glutaraldehyde. Harsh reaction conditions including high temperature, low or high pH values, and toxicity from functional groups may adversely reducing the activity and viability of cells [6].

Cross-linking is usually initiated by copolymerization of the base monomers with vinyl containing monomers, such as methylene bis-acrylamide. Acrylamide, methacrylamide, and 2-hydroxyethyl methacrylate are the most widely used base monomers for producing cross-linked polymers. One popular example of such polymers is polyacrylamide which is obtained from copolymerization of acrylamide and bis-acrylamide. After a polymeric block is formed, the block is then pressed through a sieve plate to obtain regular shaped particles. Methods other than cross-linking for instance suspension and emulsion polymerization technique can also be performed to produce polymeric beads for cells entrapment [6].

In some cases, the monomers solution is added dropwise for example by syringe into the gelating solution in order to form spherical beads containing entrapped microorganisms. After formation, the polymeric gel beads can be utilized directly or placed in nutrient medium to encourage subsequent cell growth inside the beads prior to usage. Furthermore, the beads can be dried and then stored until use [8].

Based on the origin of the material, there can be natural and synthetic polymers. Natural polymers such as alginate and carrageenan are mainly isolated from algae. Another example of natural polymer is chitosan which is derived from the exoskeleton of marine animals such as shrimps and crab. Gelation of these materials can be done by either cooling and/or contacting with ionic solutions such as sodium hydroxides (NaOH) and calcium chloride ($CaCl_2$) solution. On the other hand, numerous chemicals or photochemical reactions can be applied for polymerization of synthetic polymers for example polyacrylamide, polyvinyl alcohol (PVA), and polyethylene glycol (PEG) [8].

Generally speaking, natural polymers are considered to be less mechanically stable than the synthetic ones. In practical, physical strength of polymer matrix can be enhanced by increasing monomers and crosslinking agent concentration but at the same time this procedure will decrease the pore size of polymer beads. Because of their organic origin, natural polymers are also more vulnerable to biodegradation when compared to synthetic counterparts. On the other hand, higher diffusivity and milder preparation condition than that of synthetic polymers have become main consideration for the use of these natural derived materials [8].

Diffusional properties of a certain polymer are affected by the pore size, actual size, and microbial loading within the polymers beads. An increase in diffusivity was observed when polymer with big pore size and high water content was used. This fact suggested that diffusional limitation is less severe when porous polymers are used for cell immobilization. Diameter of 0.2-1.2 mm is claimed as the most effective bead size [30]. Insufficient supply of nutrients can happen if microbial loading in the beads is too high because in this case, diffusion rate of nutrients is slower than their consumption rate by microorganisms [8].

In some area of application such as packed bed reactor, disruption of the polymeric beads caused by inadequate mechanical strength has become a major concern. This can happen because in general, gel beads are soft and mechanically fragile [6,23]. Several hardening materials such as propylene glycol ester, ethyleneimine, colloidal silica, glass or plastic core, and gelatin shell have been tested in attempt to increase the resistance of gel beads to disruption [6,8]. When an inner core system is used, such core is often inactive because of diffusional limitations [6]. Another alternative for enhancing mechanical and structural strength of polymeric matrix is by using freeze-drying method [8].

In many cases, change of physiological properties of microorganisms such as activity, generation time, optimum temperature, and production rate have been detected in immobilized biosystem. This indicates that immobilized microorganisms will behave differently than their suspending counterparts as the result of their adaptation to the new environment. Unfortunately, the precise physiological change within the microorganisms due to the entrapment process can not be known before hand because it varies irregularly from one species to the other [8].

Entrapment method has also found to be favorable for the use of genetically engineered organisms (GNO) which have found wider application range nowadays. In suspended cells system, GNO cells are usually overrun by their native counterparts. This can be avoided for GNO cells entrapped in polymeric beads because the polymer matrix

provides isolated environment for the cells so that they are protected from competition with more dominant native microorganisms [8].

Other key issue related to entrapment method is production cost. This is especially true when entrapment method is used in production of low value bulky product such as ethanol. Simple, efficient, and economic large scale production process is therefore needed for commercial use of this method. At present, several reported methods in literature have become available for this purpose for example capillary jet breaking method and resonance nozzle technique [8].

2.4 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. Entrapment method, in particular the one which involves the use of alginate and carrageenan as building material is widely investigated for ethanol fermentation. Alginate is an anionic linear copolymer derived from algae. It is well known as a frequently used polymer because of its mild gelling properties and non-toxicity [31]. Gelation of alginate is usually done by contacting alginate with Ca^{2+} salt solution. The Ca^{2+} cation acts as a crosslinking agent for alginate monomers to form their polymeric network.

Other natural polymers such as cellulose and chitosan have also been subjects of great interests. Cellulose carriers offer advantages by being cheap, low toxic, and environmental friendly. Cellulose carriers can be prepared as macroporous beads with controlled porosity by adjusting preparation condition to conform to requirement of each particular field of application [21,22].

Chitosan is a cationic polysaccharide which is biodegradable and has high tensile strength compared to other biopolymers [32]. It is produced from chitin containing materials such as crab and shrimp shells by N-deacetylation process [33]. Due to its positive surface charge and biocompatibility, chitosan is considered to be effective for supporting human and mammalian cell functions, proliferation, and differentiation without additives [32].

Table 2.1 Studies on ethanol production by immobilized cells

Ref. No.	Author	Carrier			Shape and Dimension	Remark
		Type	Method	Material		
[34]	Lee et al. (1983)	Organic	Entrapment	Alginate	Sphere ∅=1.8 mm	1. Stirred tank was less preferable than packed bed due to gel damage by stirrer. 2. Cell deactivation was observed after 20 days continuous operation. 3. Intermittent cell regeneration was performed.
[35]	Chien and Sofer (1985)	Organic	Entrapment	Alginate	Sphere ∅=3.3, 4.5, and 5.65 mm	1. Specific ethanol productivity of immobilized yeast in a batch was two-thirds than free cell. 2. Small bead and high flow rate were beneficial for ethanol productivity.
[36]	Elisabetta et al. (1992)	Organic	Entrapment	Gelatin	Sphere ∅=3 mm	1. Hardened gelatin had higher mechanical strength and immobilization capacity than alginate.
[24]	Shinonaga et al. (1992)	Organic	Entrapment	Chitosan	Sphere ∅=2 mm	1. Cross-linked bead was used as carrier. 2. Immobilization capacity was comparable with Ca-alginate and higher than inorganic glass.
[37]	Arasaratnam et al. (1994)	Organic	Entrapment	Alginate	Sphere	1. Ethanol production decreased with increasing alginate concentration.

Ref. No.	Author	Carrier			Shape and Dimension	Remark
		Type	Method	Material		
[11]	Ogbonna et al. (1994)	Organic	Adhesion	Loofa sponge	Cylinder	1. The sponge was stable under pH and autoclaving. 2. Compared to other adsorption carrier, the sponge had low density and highly porous. 3. Immobilization by simply inserting the sponge in flocculating yeast suspension. 4. Repeated batch and continuous fermentation was performed.
[4]	Ogbonna et al. (1996)	Organic	Adhesion	Loofa sponge and fiber	Cylinder and fiber	1. Chitosan was used as flocculant for non-flocculating cells. 2. Excess chitosan inhibited ethanol synthesis. 3. Fiber was less effective than sponge for cell immobilization.
[10]	Ogbonna et al. (1997)	Organic	Adhesion	Loofa sponge	Cylinder and small cube	1. Flocculating cell was preferred for immobilization. 2. Dense cube was better than whole sponge.
[23]	Bekers et al. (1999)	Inorganic	Adsorption	Stainless steel	Sphere ∅=6 mm	1. Modified wire sphere was used. 2. Carrier reuse was successful.

Ref. No.	Author	Carrier			Shape and Dimension	Remark
		Type	Method	Material		
[38]	Nigam (2000)	Organic	Entrapment	Carra-geenan	Sphere ∅=0.8 mm	1. Continuous packed bed fermentation demonstrated higher ethanol production than free cell.
[22]	Sakurai et al. (2000)	Organic	Entrapment	Cellulose	Sphere ∅=3.5 mm	1. Repeated batch for 20 days was successful. 2. Physical structure was significant for immobilization.
[44]	Kiran Sree et al. (2000)	Organic	Entrapment	Alginate	Sphere ∅=5 mm	1. In repeated batch, alginate bead had better productivity than free cell system. 2. Alginate preserved viability of yeast cell.
[1]	Ogbonna et al. (2001)	Organic	Adhesion	Loofa sponge	Cylinder ∅=9 cm x 12 cm	1. Fermentation in 50 liters bubble column was demonstrated. 2. External loop column was used for immobilization.
[39]	Najafpour et al. (2004)	Organic	Entrapment	Alginate	Sphere ∅=5 mm	1. Fixed bed continuous fermentation with productivity higher than batch was performed. 2. High level of sugar (150 g/L) was used.
[40]	Kobayashi and Nakamura (2004)	Organic	Entrapment	Alginate	Sphere ∅=4 mm	1. Productivity higher than free cell was observed due to higher dilution rate.

On contrary, it is also found to be toxic to a broad spectrum of bacteria and fungi as some chitosan products are reported to be contaminated by large amount of endotoxin. Shinonaga et al. (1992) have demonstrated that cross-linked chitosan beads (CCB) can be used as yeast cells carrier for ethanol production. It was also shown that CCB as a carrier for yeast had a higher ethanol production capacities than calcium alginate beads. The scanning electron micrograph of chitosan bead is shown in Figure 2.12 [24].

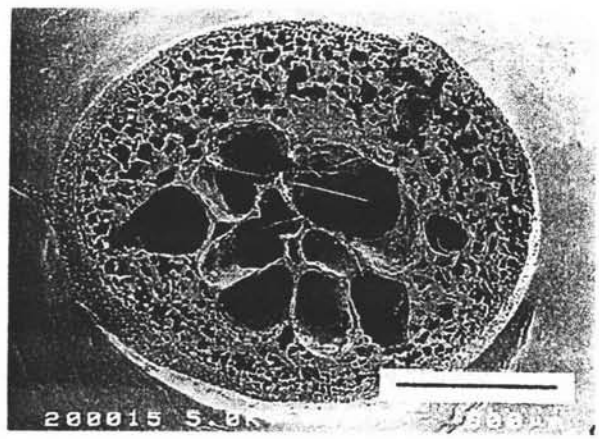

Figure 2.12 Electron micrograph of chitosan bead [24]

However, practical application of polymeric entrapment method has been limited by the problems of physical and chemical stability of gel as well as mass transfer limitation within the gel beads [6,8,11,23]. Furthermore, large scale production of these beads requires complex and sophisticated equipment which in the end will increase production cost of the resulting ethanol [11].

From mass transfer point of view and ease of immobilization procedure, passive adsorption to solid surfaces (attachment method) is preferred to entrapment method. However, cell adsorption alone is rarely satisfactory since the governing forces are usually not strong enough to prevent cell leakage and detachment from the carriers. Thus, very low cell loading is often observed with consequent low productivity. In view of this, biomass support particles (BSPs) such as polyurethane foams, ceramic beads, and stainless steel mesh which combines the advantages of both entrapment and attachment method has been extensively investigated [11].

Although high cell loading can be obtained, mass transfer restriction still persists in the usage of BSPs. In addition, since most of them are made from non-biodegradable materials, there are concerns about the after use impact of these materials towards the

natural ecosystems. Additional problems also arise if there are potentially toxic materials involved in the preparation of BSPs [11].

On the other hand, lignocellulosic materials such as sugar cane bagasse and stalk, wood chips, rice husk, and straw have also been extensively studied because they are considered to be cheap, biodegradable, and originated from renewable materials. In general, poor mass transfer and low immobilization yield are often attained due to their unsuitable and less controllable structure even though there are some reports about successful application of these materials. Furthermore, preparation of stable beds with such materials is very difficult since there is a cell concentration gradient along the bed while interparticulate cells often plug the beds resulting in channeling of nutrients [11].

More recently, one example of lignocellulosic material namely loofa sponge has been investigated extensively for ethanol production [1,4,10,11]. Loofa (*Luffa cylindrica*) is a plant which grows well in both tropical and subtropical climates. A physical description of the plant is presented in Figure 2.13 [41].

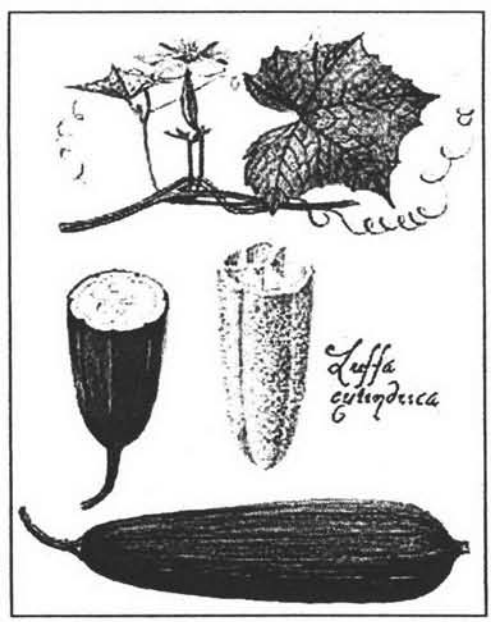

Figure 2.13 The picture of loofa plant [41]

The sponges obtained from dried loofa fruit are produced in large amount in most African and Asian countries where they are usually used for bath scrubber and dish washing. The sponges are light, cylindrical in shape, and made up of an interconnecting void within an open network of fibers. As a result of their random lattice of small cross section coupled with very high porosity, they are considered as very potential for cells immobilization [11]. Figure 2.14 shows a picture of loofa sponge [42].

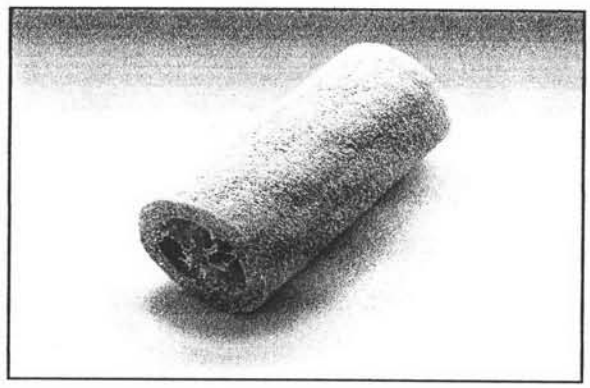

Figure 2.14 Loofa sponge [42]

Ogbonna et al. (1994) have demonstrated that loofa sponge is an excellent cells carrier. It could be used for more than 35 repeated batches without any loss of cell activity. Stable continuous ethanol fermentation using loofa immobilized cells in a jar fermenter was also successfully conducted for more than 500 hours [11]. This is particularly true for immobilization of flocculating yeast. For non-flocculating yeast, addition of flocculent such as chitosan is needed to obtain high degree of immobilization. Moreover, there is an optimum value of chitosan to be added into the system as reported by Ogbonna et al. (1996) because excess amount of chitosan will introduce an inhibitory effect over cell growth and ethanol production [4]. It was also found that loofa sponge was stable when tested against wide range of pH value and repetitive autoclaving. Immobilization was carried out using a simple method of passive adhesion. Loofa sponge was added directly to the cells suspension and then left for 20 min for self attachment of the cells into the loofa matrix. Surprisingly, the cells remained firmly attached to the sponge in a jar fermenter even at an aeration rate of 0.5 vvm and agitation speed of 300 rpm despite of the fact that the pore of loofa was much larger when compared to the size of the cells [11]. Scale up of fuel ethanol production from beet juice using loofa sponge had been successfully done in 50 liters immobilized bioreactor [1].

However, there are also doubtful results reported for the use of loofa sponge for cell immobilization. Liu et al. (1998) reported that aggregate with size above 500 μm was required to obtain high immobilization yield of plant cells on loofa sponge. This fact is contradictory with the result from Ogbonna et al. (1994) which claimed that high immobilization yield with loofa sponge had been achieved when flocculating yeast cells were used, considering that flocculating yeast cells aggregate (floc) had a size below 100 μm. Therefore, all reports regarding successful application of loofa sponge as cell carrier should be treated carefully.

It can be concluded that in general, most of the carriers tested for ethanol production nowadays still suffer from many drawbacks which limit their use in commercial scale. Inventing new type of carriers is one solution for this problem. In an attempt to do so, evaluation for the performance of newly designed supporting materials for cell immobilization namely loofa reinforced gel carriers was carried out in this work. Alginate and chitosan were chosen as the materials which were reinforced by loofa sponge based on many reports regarding their potential use as cell carriers. All materials chosen for the construction of these new carriers were biodegradable because of their natural origin. By doing so, environmental concern related to the use of these materials was eliminated. The methods for cell immobilization were diversified into adsorption/adhesion and entrapment method. By doing so, comparison between the two methods could be done simultaneously.

As mentioned before, instability of polymer gels including alginate and chitosan often becomes a problem for their application in immobilized biosystems. Reinforcement of these materials with strong fibers of loofa sponge is expected to alter their mechanical strength. When compared to previous reported attempts to enhance the strength of polymeric carriers such as the use of a hard core and a soft gelatin shell [6], the use of loofa sponge as reinforcement material is simpler and inexpensive. Furthermore, the occurrence of inactive zone inside the carrier as reported in the use of a hard core can be avoided in the new carriers because of the macroporous nature of loofa sponge [6]. The organic nature of loofa sponge was expected to have positive effects on cells attachment to the supports as organic materials were suggested to possess rich functional groups suitable for cells adsorption [8].