Studies of penicillin acylase immobilization using membranes for the production of 6-aminopenicillanic acid (6- APA)

A

THESIS

SUBMITTED TO THE

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BY

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UNDER THE GUIDANCE OF

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DEDICATED TO

MY GUIDE & FAMILY

Contents

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Studies of penicillin acylase immobilization using membranes for the production of 6-aminopenicillanic acid (6- APA)"** submitted by Mr. D. M. Thakar was carried out by the candidate under my supervision/guidance at Chemical Engineering and Process Development Division, National Chemical Laboratory, Pune (India). Such materials as has been obtained from other sources, has been duly acknowledged in the thesis.

Dr. H. V. Adikane **December 2009**

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DECLARATION BY THE CANDIDATE

This is to certify that the work incorporated in the thesis entitled **"Studies of penicillin acylase immobilization using membranes for the production of 6-aminopenicillanic acid (6- APA)"** is my own work conducted under the supervision/guidance of Dr. H.V. Adikane, at Chemical Engineering and Process Development Division, National Chemical Laboratory, Pune (India). I further declare that to the best of my knowledge, this thesis does not contain any part of work, which has been submitted for the award of any degree either of this university or any other university without proper citation. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Signature of the candidate December 2009

Dnyaneshwar M. Thakar

Abstract:

Penicillin G acylase is an enzyme used commercially for the production of 6-aminopenicillanic acid (6-APA) an intermediate for the preparation of semisynthetic penicillins. The enzyme is also involved in synthesis of the anti-platelet agent and in enzymatic activation of pro-drugs in cancer therapy. Because of industrial importance, the search of suitable carrier for the enzyme immobilization always remains a major thrust area of research. However, the mass transfer limitations in the carrier remain a major barrier to further improvement of the enzyme performance at industrial level. The accumulation of product and generation of pH gradients are the major drawback in the conventional form of the carrier such as bead. However, most of the recent studies indicate the immobilization trend of using carrier with bead configuration. In comparison with the conventional sources, polymeric membranes appear to be suitable carrier for the enzyme immobilization. Because, substrate molecules can be convected through the membrane support, rather than having to diffuse to a bead or particle to reach the active site of an enzyme, this may enhance the mass transfer efficiency significantly. However, irrespective of advantages such as easy scale up, high throughput etc., the fouling of polymeric membrane remains a major problem which reduces productivity and increases maintenance and operating costs. Generally when the carries has to undergo different chemical reaction to immobilize the enzyme, it may results in the reduction of pore size and enhancement in fouling. Therefore, it is desirable that the carrier should have the high pore size, chemical and mechanical stability and biodegrability to overcome the after use disposal problem. In the present work, we have carried out an evaluation of the utility of biodegradable polymeric membrane in 6-APA production, as well as a search for new low cost

microbial growth substrate as to make the overall process economical. The thesis is divided into seven chapters.

Chapter 1: General Introduction

This part comprises a literature survey related to 1. Enzyme immobilization and methods of immobilization; 2. Enzyme reactor and properties of immobilized enzymes; 3. Penicillin acylase; 4. Immobilization of penicillin acylase; 5. Membrane technology.

Chapter 2: Use of black liquor a waste product of pulp and paper industry as a complex organic source for the production of *Escherichia coli* penicillin G acylase.

Penicillin G acylase is an important industrial enzyme involved in the syntheses of various semi-synthetic penicillins and reduction in its production cost is a major concern. In this work, we report the effect of different industrial waste material such as black liquor, molasses, and spent wash used as a complex organic source on the production of penicillin G acylase**.** In comparison with the cornsteep liquor a conventional complex organic source, 26% higher penicillin acylase activity was obtained with black liquor. However, black liquor showed 2.25 fold higher penicillin G acylase activity in comparison with the growth media without complex organic source. No change in conventional pH, temperature and growth period was observed for the production of penicillin G acylase using black liquor. The results indicate that the black liquor of any source can be used for the production of penicillin G acylase.

Chapter 3: Purification of penicillin G acylase using ultrafiltration polymeric membrane.

Different ultrafiltration membranes were used to understand their role in the purification of penicillin G acylase. The 4.2 fold purification with 31% recovery of penicillin G acylase was obtained using 300,000; 100,000 and 30,000 molecular weight cutoff membrane. The permeate of 300,000; 100,000 and retentate of 30,000 molecular weight cutoff membrane showed similar pattern on the SDS-PAGE. The results indicate the possibility of obtaining high purification and recovery of penicillin G acylase using 300,000 and 30,000 molecular weight cutoff membrane. It can be concluded that the adaptation of present purification method at industrial level will give significant economical advantage.

Chapter 4: Studies for the preparation of cellulose based porous membrane suitable for the biotech applications.

The different experiments were carried out to develop a novel membrane casting solution. The developed membrane casting solution comprises a mixture of cellulose based water insoluble polymer, water soluble polymer, amine and two water miscible solvent. The membrane casting solution was spread on the glass plate using glass rod and then membrane was recovered by immersing the plate in water. The prepared membrane showed high flux, good hydrophilicity, excellent film-forming ability, good mechanical properties, and high chemical reactivity. The involvement of heating, cooling and highly hazardous chemicals is almost negligible in the developed process of membrane preparation.

Chapter 5: Studies of penicillin G acylase immobilization using highly porous cellulose based polymeric membrane.

The different ionic molecules/compounds were used as a ligand for the immobilization of penicillin G acylase on the highly porous cellulose based polymeric membrane prepared as described in chapter 4. The immobilized enzyme activity around 250 U_{App} was obtained with the ligand such as proline, tryptophan, casein acid hydrolysate and brilliant green. Comparatively, proline showed less IMY% (percentage immobilization yield - 58) but higher RTA% (percentage of activity retention - 71) and specific activity (145 U_{App} g^{-1}). However, the crosslinked preparation of brilliant green obtained using glutaraldehyde showed 82 ± 2.7% immobilized enzyme activity after the completion of successive five cycles. In comparison with the free enzyme, the enzyme immobilized on the brilliant green coupled membrane showed around 2.4 fold increase in Km value (47.4 mM) as well as similar optimum pH (7.2) and temperature (40 ºC). The immobilized enzyme retained almost 50% activity after 107 days and 50 cycles of operation. Almost 50% decrease in buffer flux after enzyme immobilization was observed. At the end of the 30 cycles, flux pattern shows around 38% decrease in buffer flux however, after 16 cycles of operation flux moves closer towards the steady state. The scale up studies carried out using 250 mL operating volume indicates the suitability of developed enzyme immobilized membrane for industrial applications.

Chapter 6: Studies of whole cell immobilization using polymeric membranes for the production of 6-amino penicillanic acid.

In comparison to the free / soluble enzyme immobilization, whole cell immobilization has many advantages such as higher operational stability, and carries out multi-step

cofactor requiring bioconversion. Whole cell immobilization eliminates the costly process of enzyme purification and extraction, and it is relatively easy. However, it has the disadvantages of low activity, diffusional restriction and the leaching of cellular components into the reaction mixture. The different experiments were carried out to overcome these problems. The evaluation of different parameters were carried out using different form of whole cell: 1. whole cell without any modification; 2. whole cell immobilized using novel membrane developed by us; 3. crosslinked whole cell; 4. whole cell immobilized using gelatin + alginate beads; 5. crosslinked solvent treated whole cell; 6. solvent treated whole cell immobilized using gelatin + alginate beads.

Chapter 7: Conclusion.

This part discusses the overall results obtained in the above different studies and conclusion indicating the potentially suitable process for the production of 6-aminopenicillanic acid (6-APA) at industrial level. The last part comprises the output of the thesis work in the form of patent and publications.

Chapter: 1

General Introduction

Chapter 1:

1. General introduction of -

1. 1. Enzyme immobilization:

Enzymes are protein molecules and known as biocatalysts, which serve to accelerate the biochemical reactions by several orders of magnitude. Enzymes are not permanently modified by their participation in reactions and display great specificity. It is cost-effective to use them more than once as they are not changed during the reactions. However, if the enzymes are in solution with the reactants and / or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term "immobilized" means unable to move or stationary. Thus, the immobilized enzyme is an enzyme that is physically attached to a solid support, over which a substrate is passed and converted to product. The immobilized enzyme can provide increased resistance to changes in conditions such as pH or temperature. This also allows enzymes to be held in place throughout the reaction. Thus, the immobilized enzyme can be easily separated from the products and may be used again. Therefore the immobilized enzyme is widely used in industry for enzyme catalyzed reactions.

Nelson and Griffin discovered in 1916 that the invertase when absorbed on a solid (charcoal or aluminum hydroxide) exhibited the same activity when uniformly distributed throughout the solution at the bottom of the reaction vessel. This discovery was the first of various enzyme immobilization techniques currently available. However, the proper selection of the immobilization techniques to obtain active and stable immobilized enzyme mostly depends on physical and chemical properties of an enzyme and support. This needs a careful investigation of different parameters to evolve most suitable techniques of immobilization for the particular enzyme.

Immobilized enzymes are very important for commercial uses as they possess many benefits which include:

1. Economical: The immobilized enzyme can be easily removed from the reaction thus making it easy to recycle, multiple or repetitive use.

2. Stability: Immobilized enzymes typically have greater thermal and operational stability than the soluble form of the enzyme and product is not contaminated with the enzyme.

3. Convenience: The ability to stop/start the reaction rapidly by removing/adding the enzyme from the reaction solution, long half life, predictable decay rates, elimination of reagent preparation, etc.

1.1.1. Methods of immobilization:

It is essential to choose a method for immobilizing an enzyme to a surface which will prevent loss of enzyme activity. There are mainly three methods available for immobilizing enzymes (Figure 1):

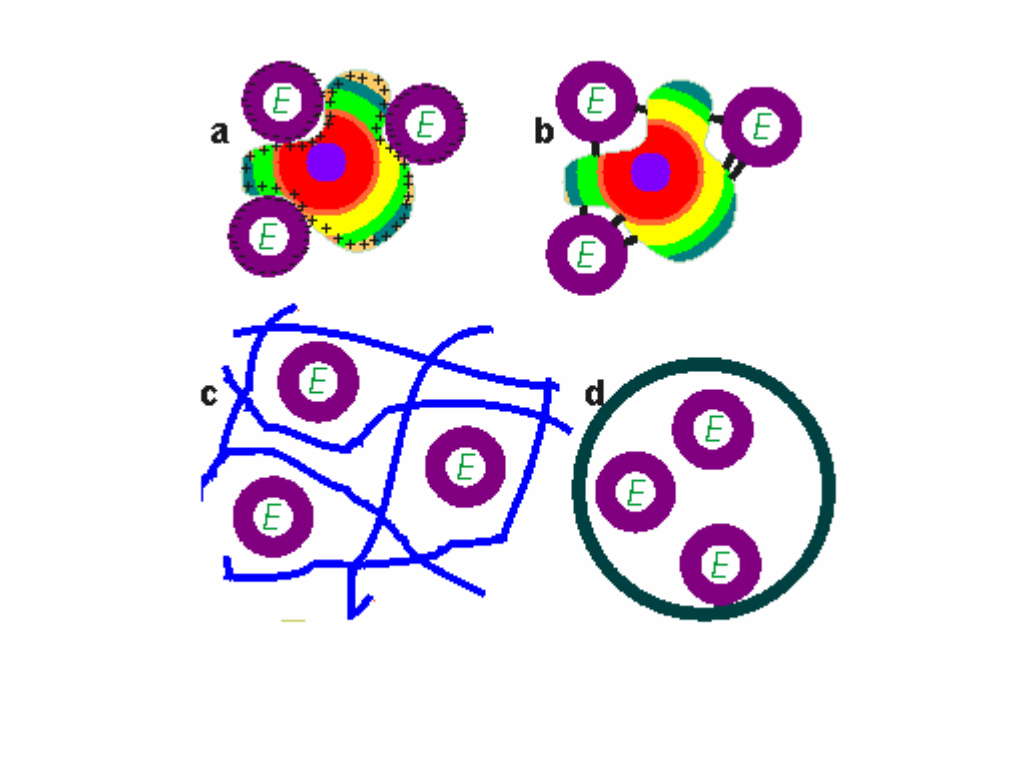
1. Adsorption: the binding of enzymes by physical / ionic interaction to water-insoluble carriers.

2. Covalent Binding: the binding of enzymes by covalent coupling to water-insoluble carriers.

3. Entrapping: incorporating enzymes into the lattices of a semi-permeable gel or enclosing the enzymes in a semi-permeable polymer membrane.

Figure 1: Methods available for immobilizing enzymes: (a) enzyme (E) non-covalently adsorbed to an insoluble particle; (b) enzyme (E) covalently attached to an insoluble particle; (c) enzyme (E) entrapped within an insoluble particle (d) enzyme (E) entrapped within a semi-permeable membrane.

(Source: http://www.lsbu.ac.uk/biology/enztech/immethod.html):



1.1.1.1. Adsorption:

Adsorption is a very simple method of an enzyme immobilization which has wide applicability. The immobilized enzyme in a directly usable form can be obtained by simply mixing an enzyme with a suitable adsorbent under appropriate conditions of pH, ionic strength, incubation period and washing off unbound enzyme. It is possible to achieve high enzyme loading using adsorption. The binding between adsorbent and the enzyme is mostly due to a combination of hydrophobic effects and the formation

of several salt links per enzyme molecule. The physical links between the enzyme molecules and the support generally weakened during use by inappropriate changes in pH or ionic strength. Therefore the choice of adsorbent is most important to avoid enzyme leakage during use. Although there are different choice of adsorbent mentioned in the literature such as ion-exchange matrices, porous carbon, clays, hydrous metal oxides, glasses and polymeric aromatic resins etc. But ion-exchange matrices can be used repeatedly to achieve desired economy by just washing off the used enzyme with concentrated salt solutions and reloading the active enzyme.

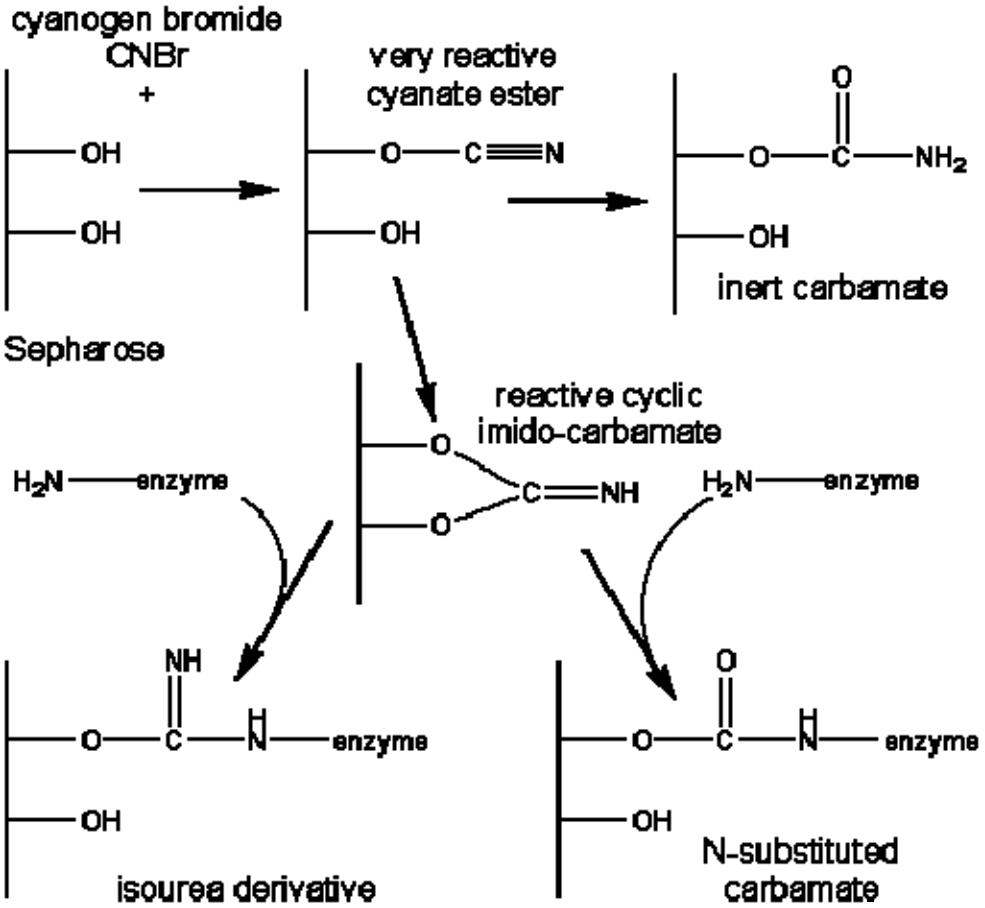
1.1.1.2. Covalent Binding:

In comparison to other methods, the covalent binding method is most effective and widely studied. The ultimate aim of the covalent binding is to avoid leaching of an enzyme into the surrounding solution. However, binding reaction has to be performed under conditions that should protect the catalytic activity of the enzyme and active site of the enzyme must be unaffected by the reagents used. In general, the functional groups of proteins suitable for covalent binding and which do not cause loss of the catalytic activity of the enzyme include (a) the alpha amino groups of chain and epsilon amino groups of lysine and arginine, (b) the alpha carboxyl group of chain end and beta and gamma carboxyl groups of aspartic and glutamic acids, (c) the phenol ring of tyrosine, (d) the thiol group of cysteine, (e) the hydroxyl groups of serine and threonine, (f) the imidazile group of histidine, and (g) the indole group of tryptophan. In some cases, it is possible to increase the number of reactive residues of an enzyme in order to increase the yield of immobilized enzyme and to provide alternative reaction sites to those essential for catalytic activity of the enzyme. The covalent binding method has become mostly applicable method of immobilization

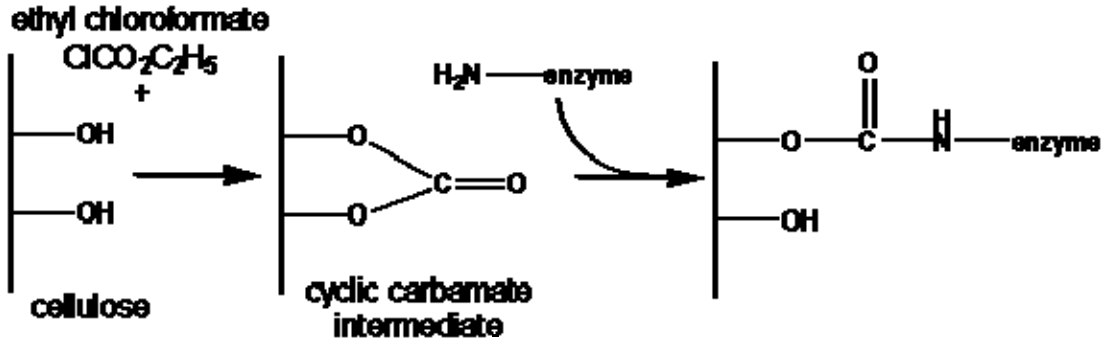
because of the wide variety of binding reactions, capabilities of covalent binding of functional groups with insoluble carriers and possibilities of activation to generate such groups. Figure 2 shows some of the most commonly used methods for the covalent immobilization of enzymes. The activation of sepharose by cyanogen bromide is a simple, mild and often successful method of wide applicability. Sepharose is a commercially available beaded polymer which is highly hydrophilic and generally inert to microbiological attack. The cyanogen bromide combines with hydroxyl groups of sepharose to give the reactive cyclic imido-carbonate, which reacts with primary amino groups on the enzyme under mildly basic conditions (Figure 2a). However, the high toxicity of cyanogen bromide has led to the investigation of alternative methods involving chloroformates to produce similar intermediates (Figure 2b). The bifunctional reagents such as carbodiimides are very useful as they allow the coupling of amines to carboxylic acids (Figure 2c). The mostly used bifunctional reagent to link enzymes to the insoluble carriers is glutaraldehyde (Figure 2d). Glutaraldehyde is particularly useful for producing immobilized enzyme membranes which is generally used in the preparation of biosensors. The enzymes can be coupled to even inert materials such as glass with the help of trialkoxysilanes (Figure 2e). There are numerous other methods available for the covalent attachment of enzymes. However, selection of proper methods to achieve highly stable and active immobilized is a most critical factor which varies from enzyme to enzyme.

Figure 2: Commonly used methods for the covalent immobilization of enzymes: (a) Activation of sepharose by cyanogen bromide. (b) Chloroformates may be used to produce similar intermediates to those produced by cyanogen bromide but without its inherent toxicity. (c) Carbodiimides may be used to attach amino groups on the enzyme to carboxylate groups on the support or carboxylate groups on the enzyme to amino groups on the support. (d) Glutaraldehyde is used to link enzymes to supports. (e) The use of trialkoxysilane to derivatise glass. (Source: http://www.lsbu.ac.uk/biology/enztech/immethod.html):

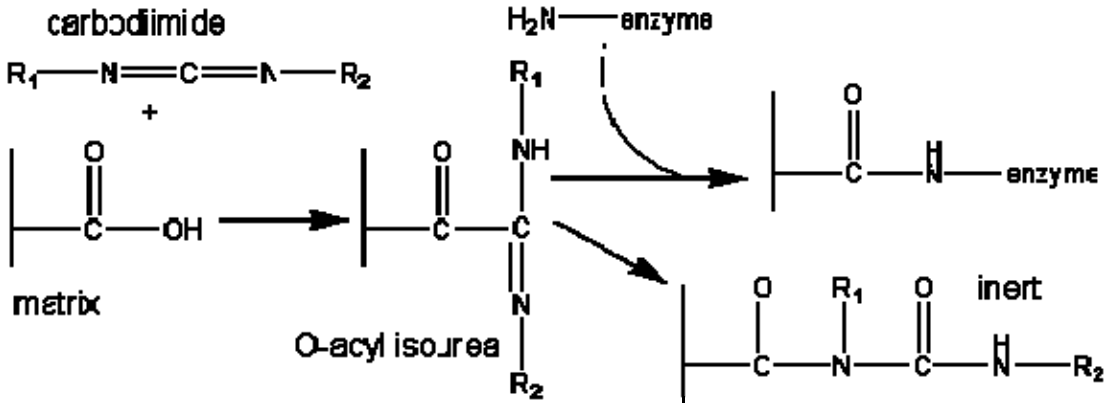
(a) Cyanogen bromide:



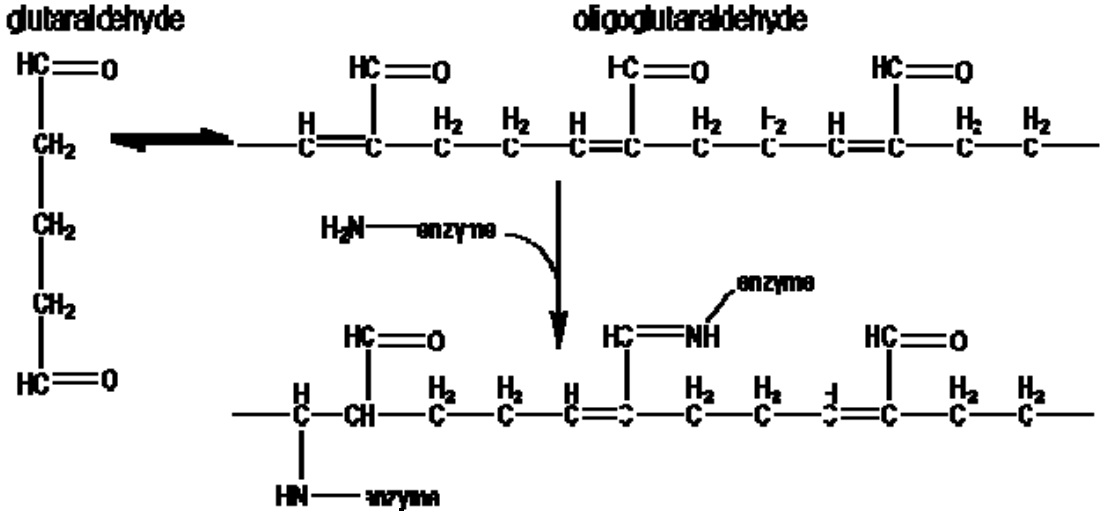
(b) Ethyl chloroformate:



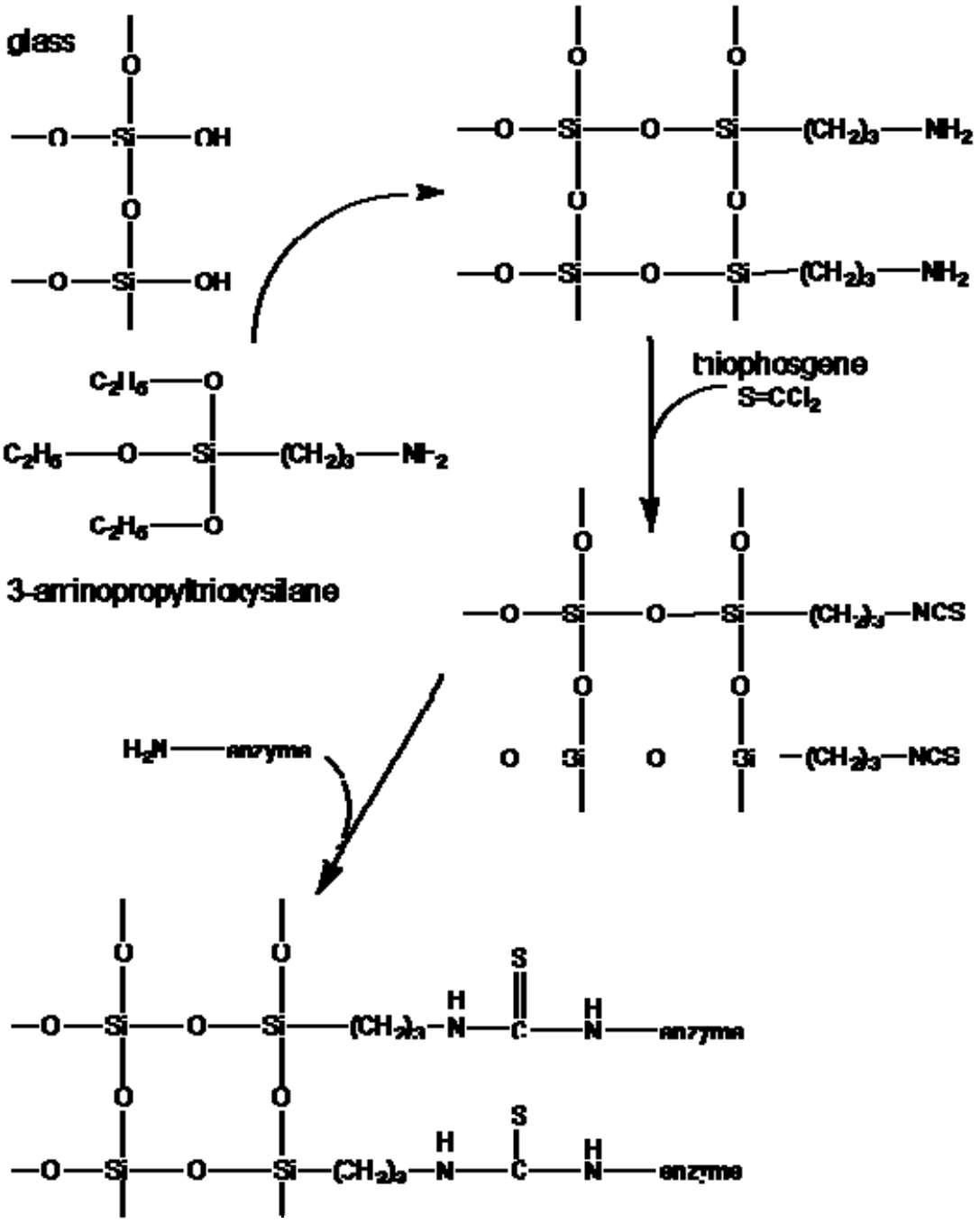
(c) Carbodiimide:



(d) Glutaraldehyde:



(e) 3-Aminopropyltriethoxysilane:



1.1.1.3. Entrapping:

In general, entrapment is a useful method of enzyme immobilization using gels or fibers where substrates and products are low molecular weight. A purely physical caging or covalent binding of an enzyme can be achieved using entrapment method.

For instance, the lysine residues available on the surface of the enzyme can be derivatised by reaction with acryloyl chloride (CH_2=CH-CO-Cl) to give the acryloyl amides and then it may be copolymerised and cross-linked with acrylamide (CH_2=CH-CO-NH_2) and bisacrylamide (H_2N-CO-CH=CH-CH=CH-CO-NH_2) to form a gel. The enzyme can also be entrapped in cellulose acetate fibers by making up an emulsion of the enzyme plus cellulose acetate in methylene chloride and extrusion of this emulsion through a spinneret into a solution of an aqueous precipitant. Generally, calcium alginate has been widely used for the entrapment of microbial, animal and plant cells.

Another method of entrapment is the confinement of enzymes using membranes however, semipermeable nature of the membrane plays a critical role. The entrapment using membranes should ensure confinement of the enzyme and free passage for the reaction products. The entrapment of an enzymes using membrane can be achieved by simply placing the enzyme on one side of the semipermeable membrane whereas the reactant and product on the other side. Hollow fibers are the best example of entrapment of an enzyme using membranes. They are available commercially with large surface areas relative to their contained volumes (> 20 m^2 l^{-1}) and permeable only to substances of molecular weight substantially less than the enzymes. Hollow fibers are very easy to use for a wide variety of enzymes (including regenerating coenzyme systems) and the cost effect may be minimized by avoiding the cost of additional research and development associated with other immobilization methods. Another method is the encapsulation of an enzyme within small membrane bound droplets or liposomes. For instance, the enzyme is dissolved in an aqueous solution of 1, 6-diaminohexane and then dispersed in a solution of hexanedioic acid in the immiscible solvent, chloroform. The resultant reaction forms a thin polymeric shell

around the aqueous droplets which traps the enzyme. In general, liposomes are formed by the addition of phospholipid to enzyme solutions. They form concentric spheres of lipid membranes surrounding the soluble enzyme. After washing of the non-confined enzyme, the micro-capsules and liposomes can be transferred back to aqueous solution before use.

1.2. Enzyme reactor:

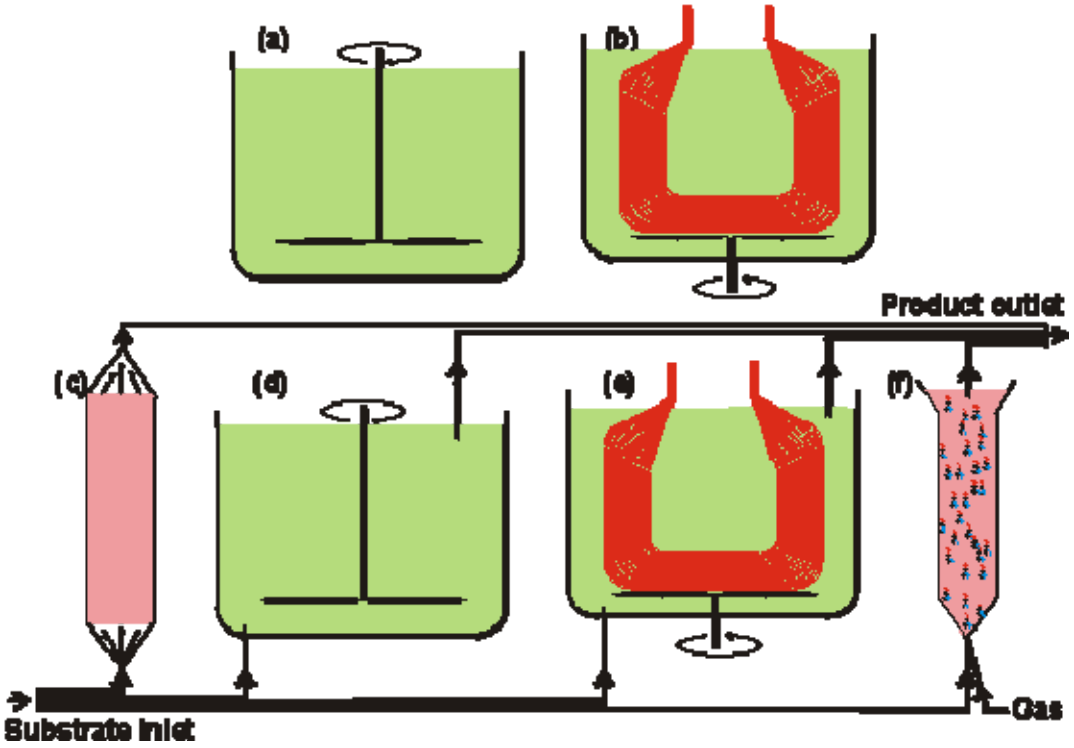
It is a vessel or series of vessels used to perform a desired enzymatic reaction. Figure 3 shows the different type of reactors being used to perform a desired enzymatic reaction. There are several important factors that determine the choice of reactor for a particular process which includes costs of substrate, downstream processing, labor, depreciation, overheads, process development and costs of building and running the enzyme reactor. The other factors such as form of the enzyme (free or immobilized), the chemical, physical and kinetics properties of an immobilized enzyme and the properties of the support of immobilization including whether it is particulate, membranous or fibrous, and its density, compressibility, robustness, particle size and regenerability also plays an important role. The scale of operation, the possible need for pH and temperature control, the supply and removal of gases and the stability of the enzyme, substrate and product are critical factors that determine the choice of reactor.

Generally, batch reactors consist of a tank containing a stirrer (stirred tank reactor, STR), which is normally fitted with fixed baffles to improve the stirring efficiency. After the reaction, rapidly removal of the entire product is practically possible using

batch reactors. This indicates that the enzyme and substrate molecules have identical residence times within the reactor. However, in some cases there may be a need for further additions of enzyme and/or substrate (fed -batch operation). In comparison to continuous processes, the operating costs of batch reactors are higher due to the regularly necessity for the reactors to be emptied and refilled. The major advantage of batch reactors is their simplicity in use and in process development. Therefore, they are preferred for small-scale production of highly priced products, especially where the same equipment is to be used for a number of different conversions. Batch reactors also offer a closely controllable environment that is useful for slow reactions, where the composition may be accurately monitored, and conditions (temperature, pH, coenzyme concentrations) varied throughout the reaction. They are also of use when continuous operation of a process proves to be difficult due to the viscous or intractable nature of the reaction mix. However, there are some drawbacks such as batch-to-batch variations, as the reaction conditions change with time and difficult to scale-up due to the changing power requirements for efficient mixing.

Figure 3: Diagrams of different enzyme reactor: a – Stirred tank batch reactor (STR); b – batch membrane reactor (MR); c - packed bed reactor (PBR), also called plug -flow reactor (PFR); d - continuous flow stirred tank reactor (CSTR); e - continuous flow membrane reactor (CMR); f - fluidized bed reactor (FBR):

(Source:http://www.lsbu.ac.uk/biology/enztech/reactors.html)



There are other reactors such as packed beds with downward flow, suspended particles in a fluid bed with upward flow of substrate, tubular reactors, membrane reactors, and many others. The physical nature of the surface becomes a major problem in some of these reactors. The support changes its behavior when coated with an enzyme. For instance, the supports that form excellent packed beds fail to do so when coated with enzyme. The particles which ideally self-suspend in a fluid bed then aggregate during use. This results in requirement of more power to pump through

substrate and lowered enzymatic activity due to decreased surface area. In case of porous glass supports many problems were encountered such as the glass itself could dissolve and loss of enzyme activity which may be overcome by the zirconium treatment of the glass surface. Generally, the non-biodegradable supports such as glass, silica, celite, bentonite, alumina, or titanium oxide etc., are being used in industrial reactor. However, the ultimate aim of the enzyme reactor is to achieve the highest specific activity, in terms of weight of enzyme and support employed and the simultaneously separation of product with reaction to avoid unfavorable equilibria. To achieve this aim lot of efforts are going on unabated in search of new supports and methods of enzyme immobilization.

1.3. Properties of immobilized enzymes:

The study of physical and chemical properties of the immobilized enzyme is essential in the selection of most suitable method for the enzyme immobilization. The understanding of the nature of enzyme and support also helps in obtaining highly active and stable immobilized enzyme. It is obvious that the changes in stability and kinetic properties of an enzyme may occur due to change in microenvironment after immobilization. However, it is desirable to achieve microenvironment as close to as soluble enzyme to obtain highly active and stable immobilized enzyme.

1.3.1. Stability

In general, the stability of the enzymes might be expected to either increase or decrease on immobilization. Particularly, the stabilization or denaturation of enzymic proteins depends on the microenvironment provided by the carrier. The enzyme immobilization may also help to overcome inactivation due to autodigestion of

proteolytic enzymes. It was observed that the enzymes coupled to inorganic carriers were generally more stable than those attached to organic polymers when stored at 4 or 23 °C. Immobilization may also provide the stability to denaturing agents.

1.3.2. Kinetic properties

Generally, a decrease in specific activity of an enzyme upon immobilization observed due to the denaturation of the enzymic protein caused by the coupling process. However, the changes occur in enzyme activity due to the actual process of immobilization have not been studied very much. The change in enzymic activity after immobilization may be attributed to the factor of microenvironment which may be drastically different from that existing in free solution. The change in microenvironment is a result of the physical and chemical character of the support matrix as well as the interactions of the matrix with substrates or products involved in the enzymatic reaction.

The enzyme and substrate interactions mostly depend on the chemical and physical properties of the support of immobilization, which influence the rate of reaction. The decrease in Michaelis constant (Km) by more than one order of magnitude has been found when substrate of opposite charge to the carrier matrix was used. This also happened at low ionic strengths and when neutral substrates were used. The rate of the enzyme reaction also depends on the diffusion of substrate from the bulk solution to the micro-environment of an immobilized enzyme. The rate at which substrate passes over the immobilized enzyme particle affects the thickness of the diffusion film, which in turn determines the concentration of substrate in the vicinity of the

enzyme and hence the rate of reaction. The effect of the molecular weight of the substrate can also be significant as diffusion of large molecules will obviously be limited by steric interactions with the matrix. Generally it was observed that the relative activity of immobilized enzymes towards high molecular weight substrates remains lower than low molecular weight substrates. However, in some cases this may appears advantageous as the immobilized enzymes can be protected from attack by large inhibitor molecules.

1.4. Enzyme penicillin acylase:

The four industrial research groups independently reported the discovery of penicillin G acylase in 1960 [1– 4]. Since then, penicillin G acylase (penicillin amidohydrolase: E.C. 3.5.1.11) is at the forefront of interest of enzyme science and technology because of its considerable importance in the pharmaceutical industry. This newly discovered enzyme was initially employed as an industrial catalyst for the manufacture of 6-APA from penicillin G long before the use of biocatalysis in the fine chemicals industry. It is being used commercially for the hydrolysis of penicillin G (Pen G) and cephalosporin G (Cep G) to 6-aminopenicillanic acid (6-APA) and 7-amino-3-deacetoxycephalosporanic acid (7-ADCA), intermediates for the production of semisynthetic penicillins and cephalosporins (Figure 4). 6-APA and 7-ADCA are the building blocks of semi-synthetic penicillins (ampicillin, amoxicillin, cloxacillin, salbactum) and cephalosporins (cephadroxil, cefalexins, etc.). Figure 5 shows some examples [5, 6]. Penicillin G acylase is a heterodimeric protein consisting of a small β-subunit and a large subunit, which are formed by the processing of a single polypeptide precursor (Figure 6). Penicillin G acylase belongs to N-terminal nucleophile hydrolases that share a common fold around the active site bearing a

catalytic serine, cysteine or threonine at the N-terminal position [7, 8]. Penicillin G acylase is a rare example of successful application of enzymes for the large-scale production of valuable pharmaceuticals [9, 10]. Penicillin G acylase current industrial application refers to the production of 6-APA from either penicillin G or V [5]. The annual consumption of PGA is estimated to be in the range of 10–30 million tons [11].

Figure 4: Enzymatic conversion of penicillin G and cephalosporin G into 6-APA and 7-ADCA leaving phenyl acetic acid as common side product [12].

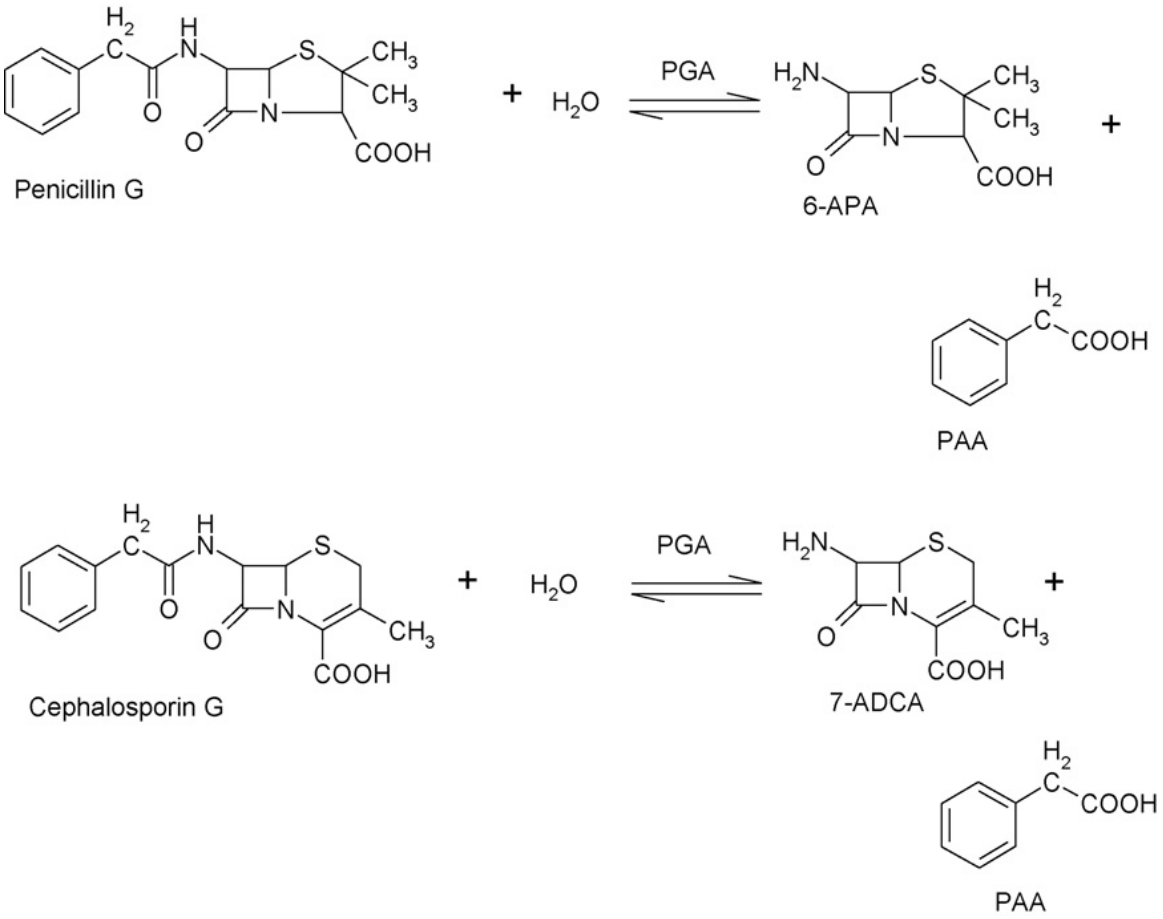


Figure 5: Penicillin G acylase catalyzed synthesis of some important β-lactam antibiotics [5].

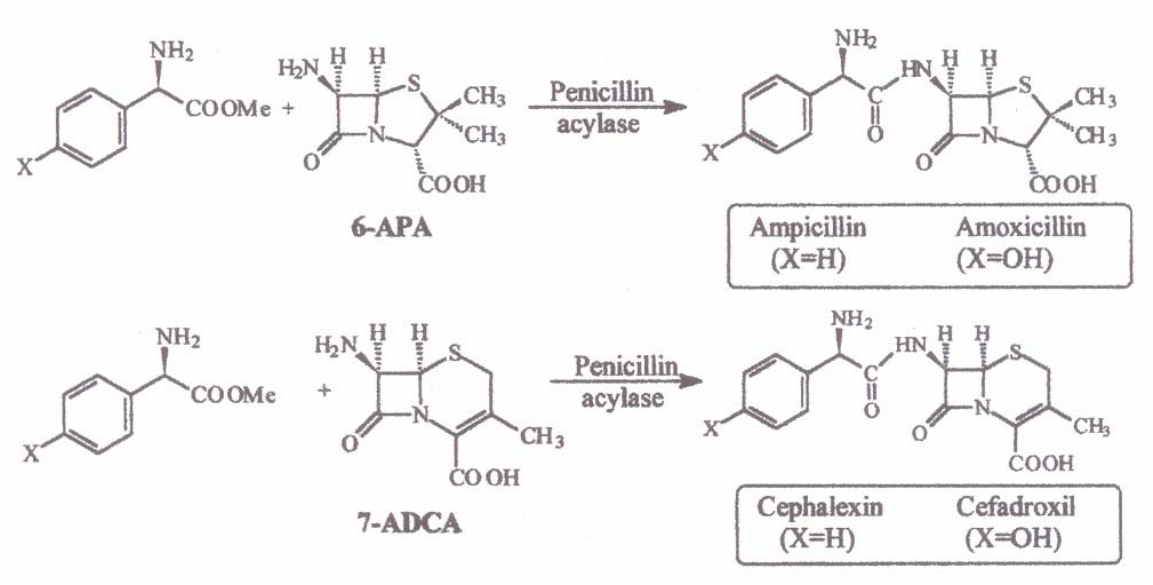
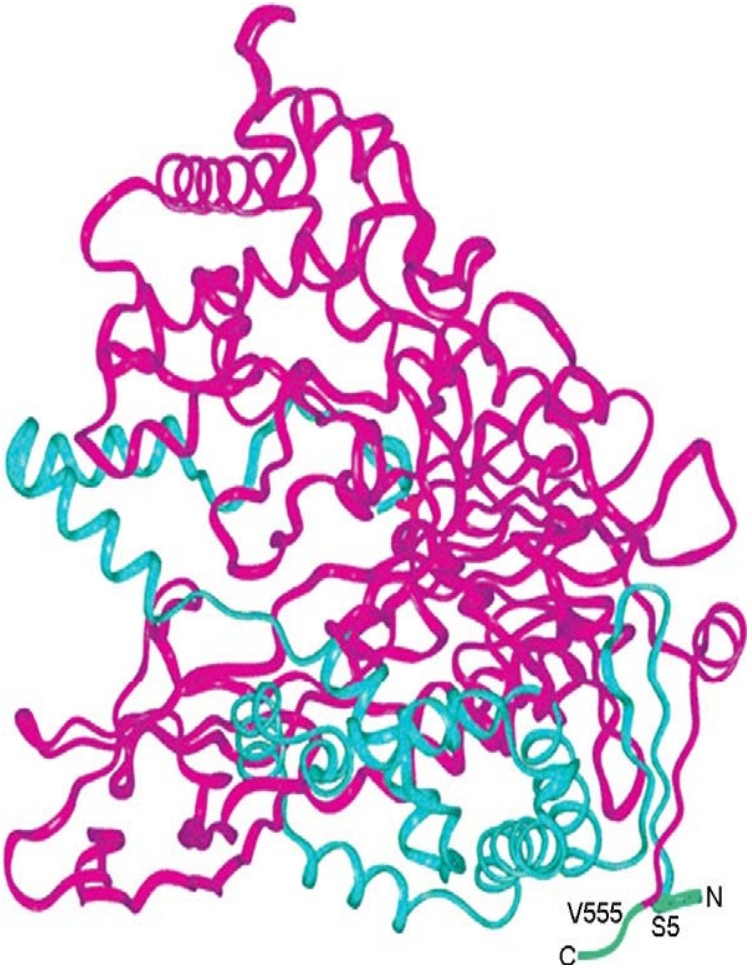


Figure 6: Penicillin G acylase dimeric structure showing beta-subunit - magenta, A-subunit — blue ribbon. The polypeptide regions trimmed from the N terminus of the A-subunit and from the C terminus of the beta-subunit are indicated in green. The amino acid residues to be connected with the four amino acids linker are labeled. In red, at the center of the molecule, the catalytic serine residue is indicated [12].



In 6-APA production, penicillin G is a predominant substrate although penicillin V is also used at an industrial scale. The side chain molecules, phenylacetic acid and phenoxy-acetic acid produced by the deacylation of penicillin G and penicillin V respectively, are usually recovered and recycled as precursors to the fermentation. The industrial production of 6-APA has undergone a remarkable transformation due to the fact that penicillin acylase-catalyzed processes have replaced traditional chemical conversions. The traditional chemical synthesis of 6-APA began around 1970 at Gist-Brocades and consisted of a one-pot deacylation of the fermentation product penicillin G using a procedure requiring hazardous chemicals and solvents. This approach remained in use for 15–20 years until it was largely replaced by the penicillin G catalyzed hydrolysis of penicillin G, which affords 6-APA in good yield. An annual worldwide production of 9,000 tons 6-APA is produced enzymatically from penicillin G and V [13].

1.4.1. Immobilization of penicillin acylase:

Enzymatic production of 6-APA is only economically viable when immobilized biocatalysts are used. Significant cost savings result from the advantages of immobilized penicillin acylase over soluble enzyme, e.g., conventional handling of the biocatalyst, easy separation of the enzyme from the product, re-use of the enzyme and enhanced stability. Penicillin acylase from E. coli, *Bacillus megaterium* or *Alcaligenes faecalis* have been immobilized following different methods that range from binding to prefabricated carrier materials to packaging in enzyme crystals or powders [14 - 17]. The resulting immobilized biocatalysts are currently available from several companies, e.g., Gist-Brocades, Recordati, Rhm or Roche. All these immobilization techniques aim to reduce manufacturing costs and mass transfer

limitations in order to increase their competitiveness for industrial applications [18]. Research in this field is now focused on the preparation of very robust biocatalysts that may improve enzyme stability over a broad range of temperature and pH, and that may tolerate organic solvents. The major breakthrough in this field was the development of cross-linked enzyme crystals (CLECs), which are prepared by crystallization of the enzyme followed by crosslinking with glutaraldehyde [19]. Cross-linked enzyme crystals of penicillin G acylase from *E. coli* (SynthaCLEC-PA; Altus Biologics) combine the features of essentially pure enzyme and high tolerance to organic solvents, and have proved to work equally well for hydrolysis and synthesis of β-lactam antibiotics. Recently, cross-linked enzyme aggregates (CLEAs) of penicillin G acylase from *E. coli* have been prepared by physical aggregation of the enzyme under non-denaturing conditions, followed by cross-linking with glutaraldehyde [20, 21]. Such CLEAs were more efficient than CLECs of the same enzyme in the kinetically controlled synthesis of ampicillin in aqueous media as well as in a broad range of polar and apolar organic solvents [22]. On the other hand, covalent binding to commercial epoxy-activated acrylic beads (Eupergit C) has also resulted in a relative improvement of the operational stability of penicillin G acylase for industrial application [23]. Following the same approach, penicillin V acylase from *S. lavendulae* has recently been immobilized on Eupergit C [24, 25], showing favorable results for the production of 6-APA [26], and enhanced pH and thermal stability [27]. In spite of the stabilization due to enzyme attachment to the support, immobilized penicillin G acylase on Eupergit C exhibited lower turnover rates in penicillin G hydrolysis than the enzyme in solution. This fact has been attributed to diffusion limitations of substrate and product inhibition [28]. A new epoxy activated support called Sepabeads-EP has emerged for the immobilization of industrial

enzymes [29], with excellent results. In fact, penicillin G acylase immobilized on epoxy-Sepabeads is more stable than the immobilized derivative on Eupergit C following the same immobilization protocol [30]. Another recent approach for efficient immobilization of penicillin G acylase is based on the formation of an enzyme-fatty lipid biocomposite film into which the enzyme is incorporated [31]. Finally, whole-cell penicillin G acylase immobilized derivatives have proved to compete well with biocatalysts prepared with the isolated enzyme. In fact, whole cells of several strains of E. coli and its mutants containing penicillin G acylase activity have been entrapped within polymethacrylamide beads [32], gluten matrix [33], and open pore gelatin matrix [34], yielding immobilized preparations that were effective in the hydrolysis of penicillin G. Addition of cosolvents to the reaction medium has recently been reported as a novel approach for the improvement of penicillin acylase-catalyzed hydrolysis of penicillins for 6-APA production, as well as for the stabilization of the biocatalyst. As a matter of fact, the hydrolysis rate of penicillin V catalyzed by penicillin acylase from *S. lavendulae* was increased by using organic cosolvents in monophasic systems. Further investigations led to the conclusion that the enhanced hydrolytic activity was attributed to a nonessential activation of the enzyme in the presence of water-soluble short chain alcohols [25, 35]. Finally, selection of glycerol and glycols leads to both enzyme activation and stabilization [36, 37], which could be very interesting for industrial manufacture of 6-APA.

Currently, penicillin G acylase has emerged as a serious alternative to traditional chemical procedures for the manufacture of β-lactam antibiotics, small peptides and pure isomers from racemic mixtures. However, in spite of their environmental and economic benefits, few successful examples of penicillin G acylase catalyzed processes have actually replaced the industrial production. There is lot of to be done

with the help of modern developments in enzyme technology such as immobilization, non-aqueous biocatalysis and site-directed mutagenesis to make penicillin G acylase a successful example in biotechnological applications of an enzyme at industrial level.

1.5. Membrane technology:

A membrane technology has been employed with increasing frequency to replace or complement conventional industrial methods for separation, concentration, and purification. This trend can be attributed to the fact that membranes exhibit an intrinsically high efficiency of energy utilization [38]. In the last decade, several alternative applications for membranes have been investigated. These alternative applications do not rely on the abilities of these membranes to selectively reject some solutes while allowing solvent molecules and other solute species to pass through the membrane. Instead, they take advantage of such other membrane properties as high effective diffusivity, high chemical stability, and high membrane surface area/volume ratios [39]. One such application involves the use of membranes as supports for immobilization of enzymes via either physical or chemical binding procedures.

The recent entry of membranes in the form of “membrane bioreactor” in biodegradation and biotransformation reaction encourages their major involvement in future commercial biotech processes. Membrane bioreactor is an integration of membrane with bioreactor which simultaneously performs biocatalysis and membrane separation. Currently, membrane bioreactor is attracting more and more study and industry interests as the most promising reactive separation bioprocess. Membrane bioreactor can be operated easily and reduced energy consumption as well as it helps in obtaining relatively higher yield and conversion than the conventional bioreactors. They are being successfully used in the food, chemical, medicinal and biological

industry, as well as in the environmental treatment [40]. In near future, with the progress of related technologies and research fields such as novel membrane material, high-active biocatalysts, biocatalyst immobilization approaches and process design optimization and so on, it may efficiently combined the selective permeation of membrane with a bioreactor to obtain more economic and environmental benefits.

The major advantages of the membrane bioreactor can be summarized as follows:
1. Selectivity of biocatalysts is usually much higher than that of the conventional chemical catalysts. Moreover, the bioreaction condition in membrane reactor is very mild, and the products can be selectively permeated through the membrane. As an environmental friendly technology, byproducts can be suppressed.

2. Biocatalysts-loaded membrane in membrane reactor has a similar effect to a biofilm in nature.

3. As the products are continuously extracted from the reaction zone, the bioreaction thermodynamic equilibrium limit will be breached. The bioreaction conversion and/or yield will be correspondingly increased.

4. In membrane reactor, it is easy to realize that the free diffusion can be replaced by the convective mass transport, which will largely enhance both mass transport rate and reaction rate.

5. A continuous process with automatic control can be effectively realized in the membrane reactor.

6. Processes based on membrane bioreactive separation may largely predigest those downstream purification processes, save labor and energy, integration of membrane

and bioreactor can also shortage operation cycle. Furthermore, either free biocatalyst with a suitable biocatalyst cut-off membrane or immobilization biocatalyst in membrane reactor will reduce biocatalyst loss and save dosage, which will decrease the total cost of facilities and operation.

The membrane reactor can be classified on the basis of biocatalyst immobilization and membrane function. Generally, the membrane reactor is classified into two types (Figure 7):

(a). Biocatalysts are suspended in solution and compartmentalized by membrane in a reaction vessel, where the membrane only serves as a separation function.

(b). Biocatalysts are immobilized within membrane, and the membrane acts as a support for the biocatalysts as well as a separation unit.

Figure 8 shows the detailed classification of membrane reactor and enzyme immobilization approach.

Figure 7: Types of membrane reactors

(Source:http://www.chemistrymag.org/cji/2004/061005ne.htm;Jan.3, 2004 Vol.6 No.1 P.5)

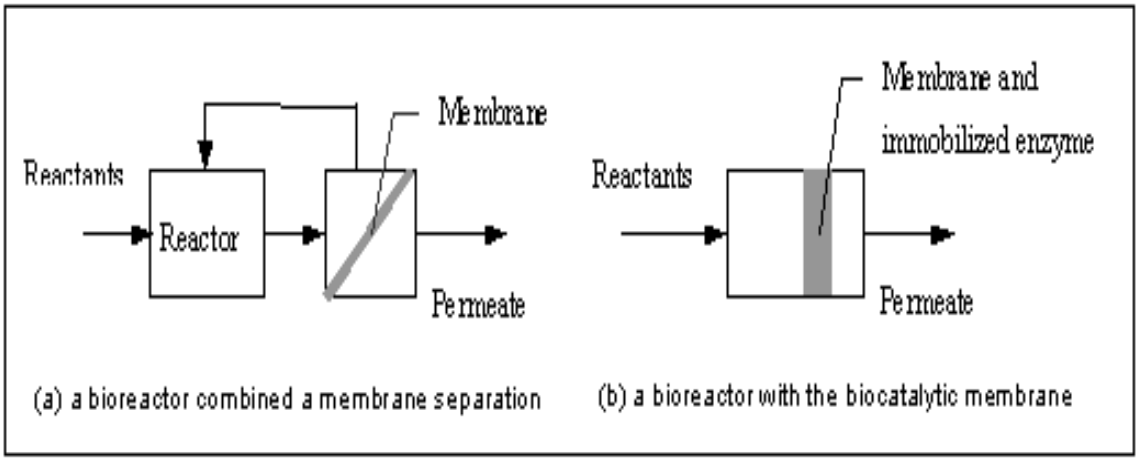
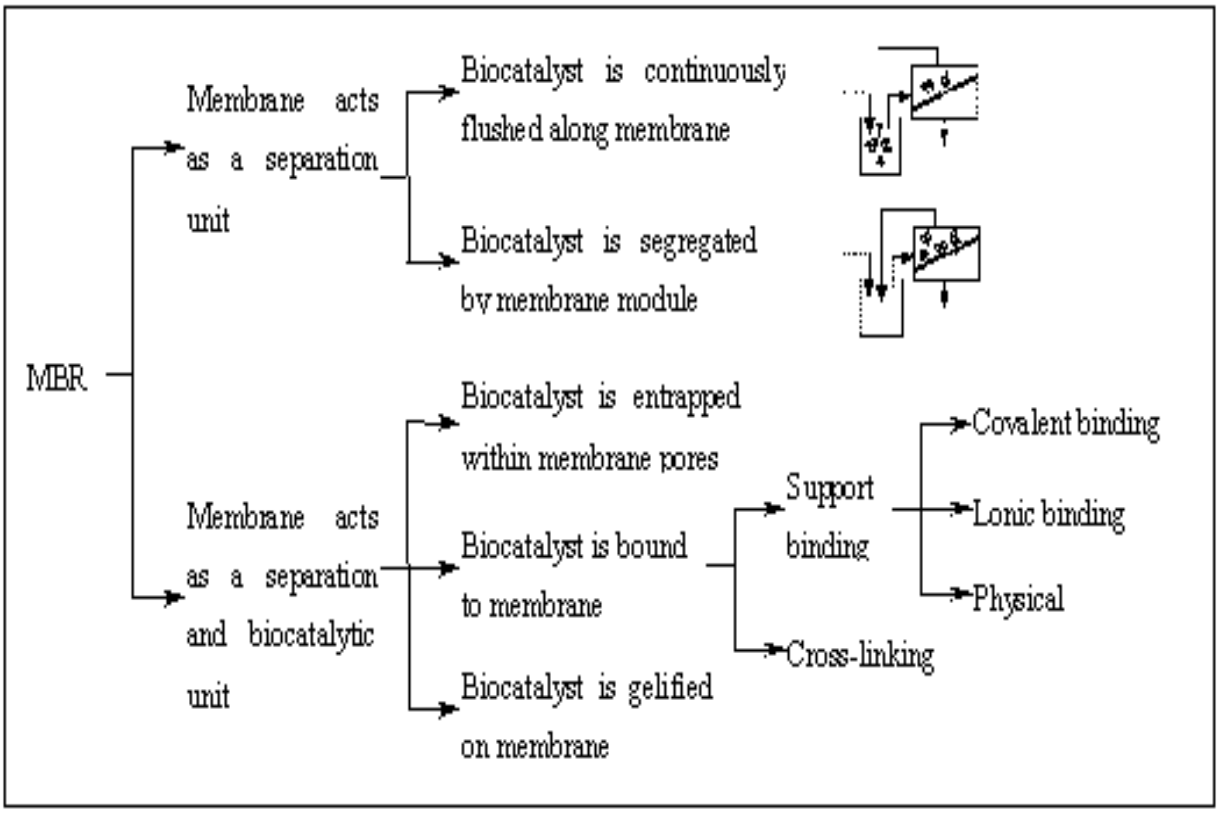


Figure 8: Examples of biocatalytic membrane reactors (MBR) with enzymes immobilized using different approaches.

(Source: http://www.chemistrymag.org/cji/2004/061005ne.htm; Jan.3, 2004 Vol.6 No.1 P.5)



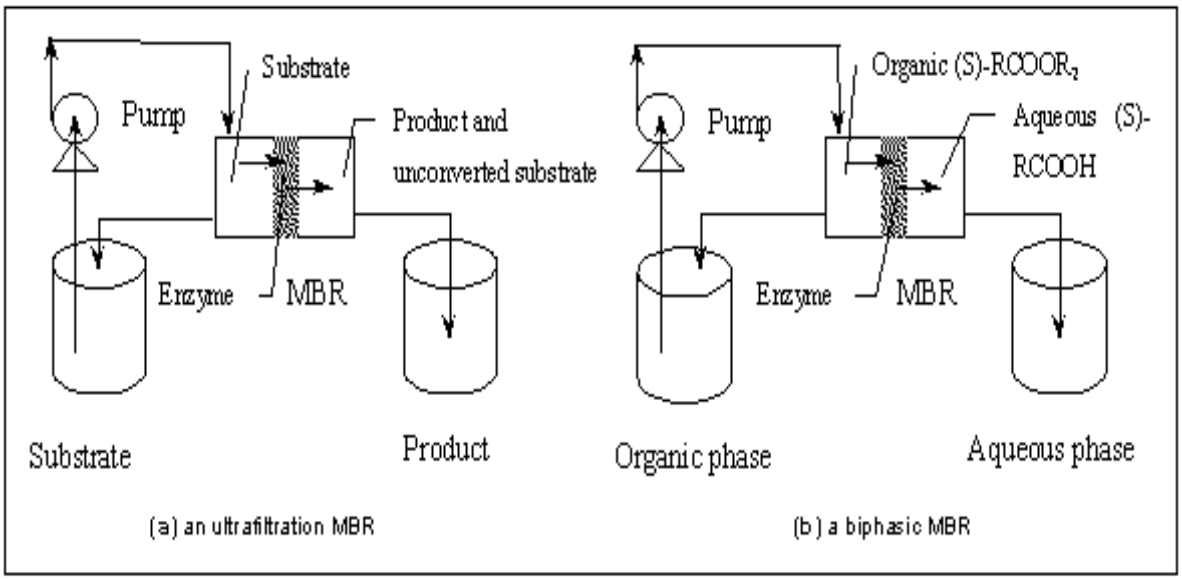
Further, the membrane reactor can be classified on the basis of different shapes of membrane and membrane modules as well as by the types of membrane materials such as organic, polymeric, inorganic, etc. However, membrane reactors are generally classified on the basis of their operation modes, such as ultrafiltration membrane (UF) bioreactor, biphasic (organic and aqueous) membrane bioreactor and so on. In a UF membrane reactor, substrates and products are homogeneous, as shown in Figure 9a, if substrate has a higher molecular weight, it can not pass through the UF membrane;

while the product easily do so and can be recovered from the other side of the membrane. If substrates and products have different solubilities (e.g. an ester and its hydrolysis products), as Figure 9b shown, a biphasic membrane reactor may be used. In such a bioreactor, the biocatalyst-loaded membrane is located between the organic and aqueous phases.

Figure 9: Examples of biocatalytic membrane reactors with two different operation approaches.

(Source: http://www.chemistrymag.org/cji/2004/061005ne.htm; Jan.3,

2004 Vol.6 No.1 P.5)



1.5.1. Application of membrane:

1.5.1.1. In food industry:

Membrane bioreactors have been widely used in alcohol fermentation [41], starch hydrolysis [42, 43], juice clarification [44] and so on. The use of membrane coated

with active materials such as diatomite, active carbon, hemp powder in beer and alcohol production is also reported [41], which results in increase by 30-40% beer yield as well as it save energy. The effect of enzyme concentration and space time on the performance of the membrane reactor was studied in one step starch hydrolysis to produce high dextrose equivalent sugar syrups using continuous recycle membrane reactor [42]. The experimental results of this study showed that as the enzyme concentration and space time increase, the conversion is enhanced, but capacity and productivity are decreased. In another study, a hollow-fiber membrane bioreactor for a continuous production of isomaltooligosaccharide following a two-step procedure was used [43]. During the first step, starch was solubilized in water with pH5.3-5.8 and partially hydrolyzed with an α-amylase and isoamylase. In the second step, saccharifying enzymes (β-glucosidase, epiphyte amylase) converted liquefied starch into final product isomaltooligosaccharide. This helps in saving enzyme dosage and production period as well as in obtaining a stable high quality product. A comparative study was carried out between integrated membrane process and the conventional methods for the clarification of apple juice. It showed 8% decrease in total manufacturing costs using an initial enzyme percentage of 0.5% (v/v), as an optimal enzyme concentration with better permeation flux [44]. This indicates the significant role of membrane reactor in acceleration of the food industry development.

1.5.1.2. In pharmaceutical and biomedical industry:

An application of membrane reactor in pharmaceutical industry has a unique potential that conventional processes will never attain. In the production of penicillin a very valuable antibiotic, different studies have been carried out using membrane technologies [45, 46]. The production of 6-amino-pencillanic acid using membrane

reactor with immobilized cell is also reported [45]. In this study, the whole penicillin acylase cell was entrapped in the microfiltration or ultrafiltration membrane with the capacity to cut off a suitable molecular weight using polysulfone hollow-fiber membrane or flat membrane. This helps to achieve higher yield and purity than conventional processes may be due to a large surface area-volume ratio. However, it shows some scale-up problems under backflushing mode. The use of ceramic membrane has been reported for the cultivation of *penicillin chrysogenum*, immobilized in the upper surface of a porous membrane [46]. There are reports regarding the use of membrane technology in lactic acid production, an important chemical and pharmaceutical additive and preservative agent [47, 48]. The eight times high productivity of lactic acid was obtained using membrane reactor in comparison with the batch reactor, whereas the biomass concentration (77g L^{-1}) in the membrane reactor was nineteen times that found in the batch culture [47]. The highest volumetric productivity 7.5 g L^{-1} h^{-1} and the specific productivity 3.54 h^{-1} of lactic acid from whey permeate in a semicontinuous production mode using bioreactor coupled with nanofiltration membranes was also reported [48]. There are some successful applications of membrane reactor in production of other medicines (cyclodextrins, microbial alginate, glycerol, monoglyceride, formaldehyde, amino acids and so on). In addition, many fine-chemicals and pharmaceuticals may be produced by resolution method in membrane reactor [49]. This indicates the significant role of membrane reactor in medicine production. The use of hollow-fiber bioreactor in the production of recombinant anti-carcinoembryonic antigen (anti-CEA) diabody and minibody for clinical applications showed important role of membrane technology in biomedical area [50].

1.5.1.3. In the management of environment:

The membrane reactor has been used in the treatment of sewage water, purification of underground, surface water and treatment of domestic and industrial wastewater [40]. The membrane reactor has been reported to be worked continuously during 130 days for the treatment of fermentation wastewater using rotary disk ultrafiltration membrane module coupled with an aerated bioreactor [51]. The treatment of waste gas streams using a bundle of hollow fiber hydrophobic porous membranes serving as a contactor between the gas and the oil phases was also reported [52]. Where, the pollutants were biodegraded in a membrane reactor, which contains a second bundle of porous hydrophobic membranes separating the oleic and aqueous phases, and serves as a support for the biofilm.

These all application of membrane reactor in different fields strongly suggests the utility of membrane technology in biotech industry. However, there are some technical challenges [40, 53] still to overcome, such as biocatalyst deactivation, when the biocatalyst is immobilized within the membrane or high mechanical stress to maintain a good transmembrane flux; biofouling and cleaning in MBR; bioprocess design for large-scale production; control of reaction and kinetic mechanisms; computer simulation and optimization of MBRs and so on. Therefore, in order to fully establish the industrial use of membrane reactor in fine chemical, pharmaceutical, and food industries, some indispensable studies on these challenges need to be continued in the coming years.

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Chapter: 2

Use of black liquor a waste product of pulp and paper industry as a complex organic source for the production of *Escherichia coli* penicillin G acylase

Chapter 2:

2.1. Summary

The aim of the present study was to search a new low cost microbial growth substrate for the production of as penicillin G acylase. As the penicillin G acylase is an important industrial enzyme involved in the syntheses of various semi-synthetic penicillins, the reduction in its overall production cost is a major concern. In this work, we report the effect of different industrial waste material such as black liquor, molasses, and spent wash used as a complex organic source on the production of penicillin G acylase**.** In comparison with the cornsteep liquor a conventional complex organic source, 26% higher penicillin acylase activity was obtained with black liquor. However, black liquor showed 2.25 fold higher penicillin G acylase activity in comparison with the growth media without complex organic source. No change in conventional pH, temperature and growth period was observed for the production of penicillin G acylase using black liquor. The results indicate that the black liquor of any source can be used for the production of penicillin G acylase.

2.2. Introduction

The growing demand from legal and social sources for the safe disposal of environmentally hazardous industrial waste material put an economical burden on the concerned industry. The conversion of these materials in to high value product may help to ease this burden significantly. Black liquor is a most polluted waste material of pulp and paper industry because of its toxicity and poor biodegradability [1]. Approximately, annual world production of black liquor is around 500 million tones [2]. Lots of efforts are going on to make black liquor suitable for safe disposal [3]. However, not much has been done to produce commercially viable products using black liquor. Recently, black liquor was used for the production of hydrogen [4]. In

the present study, we have used black liquor along with other industrial waste material for the production of high value product such as an enzyme penicillin G acylase.

Penicillin G acylase (EC 3.5.1.11) is being used in industrial syntheses of various semi-synthetic penicillins, it hydrolyzes penicillin G and other phenyl-acetic derivatives. It also involved in synthesis of the anti-platelet agent and in enzymatic activation of pro-drugs in cancer therapy [5]. Penicillin G acylase is an intracellular enzyme produced by several microorganisms [6]. In general, complex organic source (COS) such as yeast extract, malt extract, cornsteep liquor and hydrolyzed protein or amino acid mixture are being used as a major media component along with other nutrient and inducer for the cultivation of penicillin G acylase producing microorganisms [6]. Mostly, cornsteep liquor is reported to be used as a COS for the cultivation of widely used bacterial strains of *Escherichia coli* for the production of penicillin G acylase [7, 8]. Recently, the cheese whey a waste product of cheese manufacturing industries was used for penicillin G acylase [5]. The overall objective of the present study was to produce a high value product such as an enzyme penicillin G acylase using industrial waste material. The different studies were carried out to check the suitability of industrial waste material for PGA production. Our findings may help to reduce overall production cost of penicillin G acylase and a high value byproduct from waste material to the respective industry.

2.3. Materials and methods

2.3.1. Materials

The black liquor a waste of pulp and paper industry was obtained from the locally available sources. The black liquor termed as “black liquor I” has pH - 9.0; COD

(chemical oxygen demand) – 3135 ± 47 mg l^{-1}; total protein – 6.06 ± 0.25 mg ml^{-1}; total sugar – 0.056 ± 0.002 mg ml^{-1}. The black liquor termed as "black liquor II" has pH – 10.5; COD – 18359 ± 114 mg l^{-1}; total protein – 8.8 ± 0.18 mg ml^{-1}; total sugar – 0.118 ± 0.001 mg ml^{-1} and used after diluting it 10 times with water. The molasses a by-product of sugar industry and spent wash a waste of molasses based distillery were obtained from the locally available sources and used without pretreatment. Cornsteep liquor (CSL) was obtained from Anil Starch, Ahamdabad, India and was pretreated after preparation of 12% (w/v) solution in water. The CSL solution was adjusted to pH 8.0 with 1 N NaOH, steamed for 30 min, cooled and clarified by filtering it through the Whatman filter paper with pore size of 0.78 μm.

2.3. 2. Microorganism

PA producing microorganism *Escherichia coli* (NCIM 2400) was obtained from the National Collection of Industrial Microorganisms, Pune, India. The culture was maintained routinely on nutrient agar slants.

2.3. 3. Penicillin G acylase production

Penicillin G acylase production was carried out using growth medium containing (w/v): 4% COS; 0.3% peptone; 0.3% yeast extract; 0.35% NaCl; 0.3% K_2HPO_4; 0.03% KH_2PO_4; 0.1% phenylacetic acid and the pH was 7.2. The inoculum was developed by inoculating 250 ml conical flask containing 50 ml growth medium and incubated at 23 ºC for 24 h on a rotary shaker (300 rpm). The inoculum was transferred aseptically into a Gallenkamp fermenter (1l) with magnetic stirring containing 500 ml growth medium. The fermentation was carried out at 23 ºC under agitation at 300 rpm and 0.5 vvm aeration for 24 h. After fermentation, the cells were

collected by centrifugation (10,000 g). The harvested cells were suspended in 50 mM sodium phosphate buffer pH 7 and then disrupted in a Branson sonifier. The disrupted material was centrifuged at 10,000 g for 15 min and the supernatant was treated as an enzyme penicillin G acylase.

2.3. 4. Penicillin G acylase assay

Enzyme assay was carried out using 3.72% of penicillin G K^+ salt (Hindustan Antibiotics Ltd. Pimpri, India) as a substrate in 0.1 M phosphate buffer pH 7.0. To the mixture of 0.2 ml sample containing enzyme and 0.8 ml 0.1 M phosphate buffer pH 7.0, 1.0 ml substrate was added and then incubated at 40 ºC for 1 h. The reaction was terminated by pipetting 0.2 ml of the reaction mixture into 3.8 ml of citrate-phosphate buffer, pH 2.5. The enzymatic activity was estimated by determining the amount of 6-aminopenicillanic acid (6-APA) formed using p-dimethylaminobenzaldehyde reagent [9]. Specific activity of the enzyme was expressed as μmoles of 6-APA formed per minute per mg of protein at pH 7.0 and 40 ºC (U mg^{-1}).

2.3. 5. Analytical methods

Protein was determined using folin-ciocalteau's reagent [10]. Total protein was estimated using biuret reagent [11]. Prior to the estimation of total protein, equal volume of black liquor and 10% TCA (Trichloroacetic acid) was mixed together, centrifuged and the precipitate was dissolved in 0.1N NaOH. Total sugar was estimated using phenol and sulphuric acid reagent [12]. COD was estimated using conventional titration method [13]. All values given are averages of three determinations.

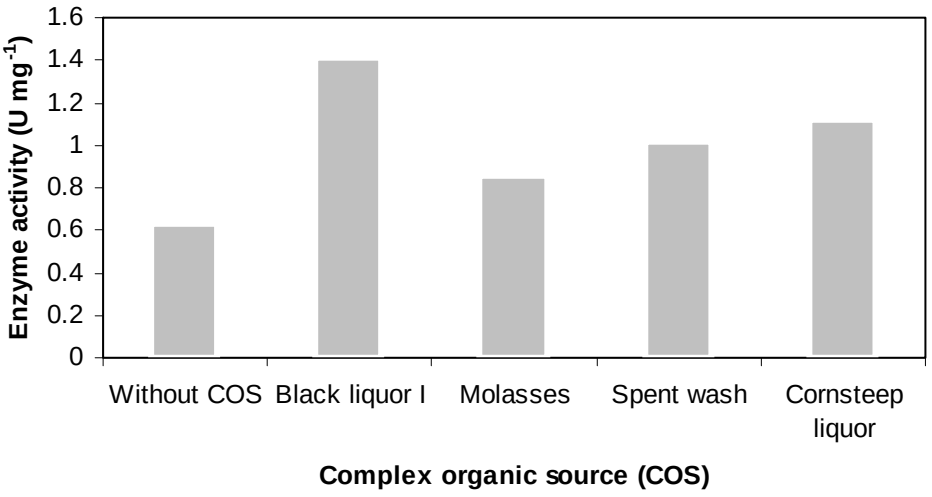
2.4. Results and discussion

The annual consumption of penicillin G acylase is around 10 - 30 million tons and to meet this huge demands there is a need for the development of cost-effective production process [14]. Earlier, the different aspects related to the reduction of production cost such as screening of modified microorganisms and modifications to the fermentation, immobilization techniques have been extensively studied [14]. However, the studies related to the search of low cost microbial growth substrate in PA production are negligible [5]. Therefore, in the present study we have used industrial west material as COS in penicillin G acylase production. We have also used industrial west material without any pretreatment because the pretreatment may contributes in the increase of overall production cost. One of the reason behind the search of new low cost COS is that the CSL a conventional COS need pretreatment such as the adjustment of pH, heating and clarification. This may consume lot of energy and time at industrial scale operation.

The 4% concentration of different industrial waste material as COS was used to study their effect on penicillin G acylase production (Fig. 1). Use of 4% concentration of CSL as a COS has been reported earlier [15, 16]. In comparison with the without COS, 2.25, 1.36, 1.62 and 1.78 fold higher penicillin G acylase activity was obtained with black liquor I, molasses, spent wash and CSL respectively (Fig. 1), this indicates the need of COS in penicillin G acylase production. However, almost 26% higher penicillin G acylase activity was obtained with black liquor I in comparison with CSL a conventional COS. The black liquor I also showed higher specific activity (1.42 ± 0.08 U mg^{-1}) in comparison with the entire COS. This specific activity is comparable with the published work. It was reported that the PGA released from *E. coli* cells through mechanical cell disruption which is used in the present study showed low (0.1

– 0.3 IU mg protein^{-1}) specific activity [17]. However, the specific activity obtained in the present study is higher (1.42 ± 0.08 U mg^{-1}). This may be due to the presence of black liquor because in the reported study yeast extract was used as COS for penicillin G acylase production [17]. These results clearly indicate that the black liquor is suitable for penicillin G acylase production. This may help to introduce black liquor in penicillin G acylase production without making any change in the existing process. To evaluate this, we have studied the effect black liquor on four basic parameters such as concentration, pH, temperature and growth period for penicillin G acylase production.

Figure 1: The effect of different complex organic source on the production of penicillin G acyalse.

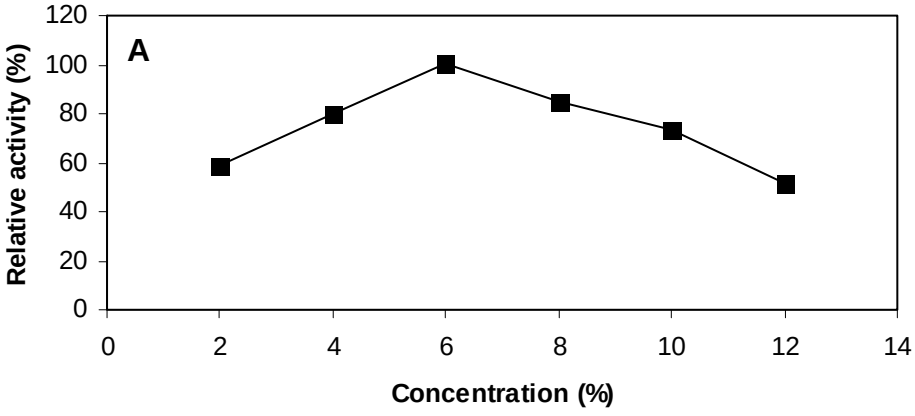


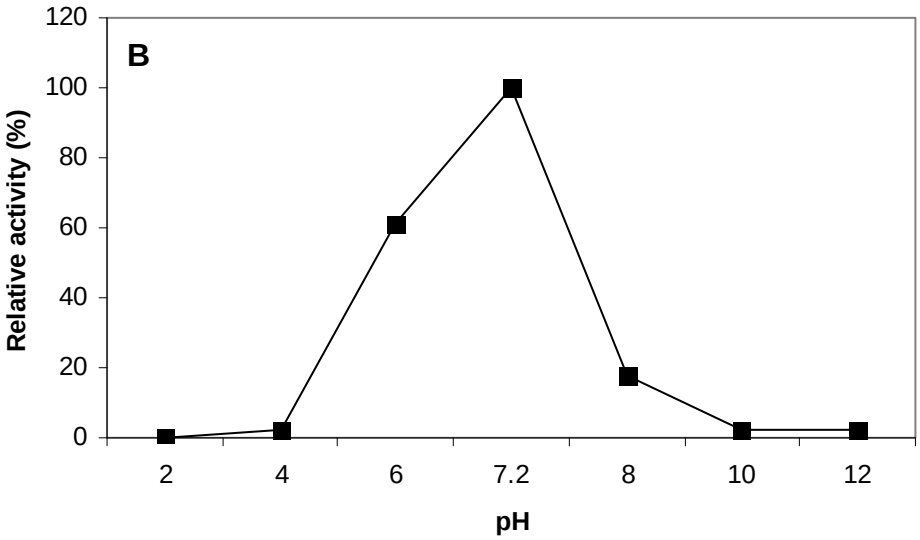
The different concentration of black liquor I as COS was used to study its effect on penicillin G acylase production (Fig. 2 A). The enzyme activity was observed at all the tested concentration. However, higher specific activity (1.76 ± 0.07 U mg^{-1}) was

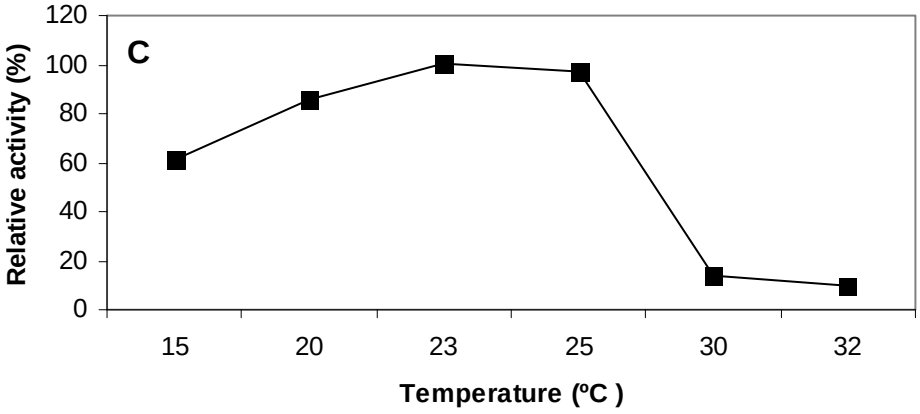
obtained at 6% concentration. This indicates that the 6% (w/v) black liquor concentration as COS may be required to obtained higher penicillin G acylase production. The effect of pH, temperature and growth period on PGA production was studied using 6% (w/v) black liquor I concentration.

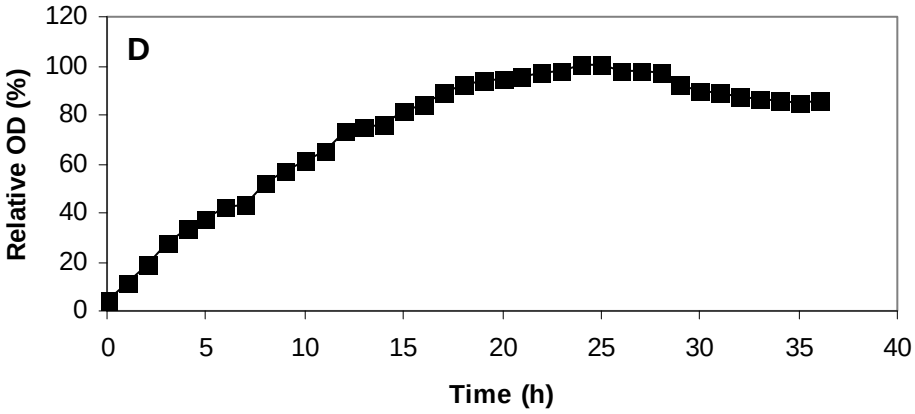
Figure 2: The effect of different parameters such as concentration (A), pH (B), temperature (C) and growth period (D) on the production of penicillin G acyalse using black liquor.

[The higher values obtained for the enzyme activity and microbial growth measured at 660 nm was considered as 100% for the calculation of relative %]




B
Relative activity (%)
120
100
80
60
40
20
0
2
4
6
7.2
8
10
12
pH


C
Relative activity (%)
120
100
80
60
40
20
0
15
20
23
25
30
32
Temperature (ºC)

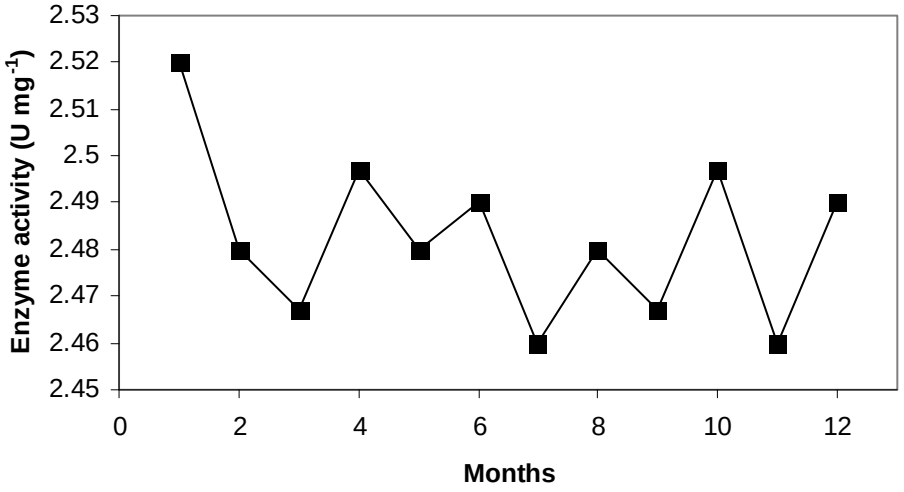


There was almost negligible enzyme activity at pH 4 and pH 8 to pH 12 (Fig. 2 B). The higher enzyme activity was obtained at pH 7.2. The pH 7.2 was used earlier for penicillin G acylase production using CSL as a COS [15]. In general, the conventional pH of growth medium based on CSL for the penicillin G acylase production was reported to be around pH 7.0 [6]. This indicates that the black liquor do not have any effect on the conventional pH pattern of penicillin G acylase production. Similarly, most of the penicillin G acylase production studies using CSL as a COS have been carried out at 23 ºC and 24 ºC for the period of 24 h [15, 18]. In the present study, the higher specific activity (1.76 ± 0.08 U mg^{-1}) was also obtained at 23 ºC and optimum growth period as 24 h (Fig. 2 C). These results indicate that there is no need to change conventional production parameters such as pH, temperature and growth period to introduce black liquor in the existing penicillin G acylase production process.

As the properties of black liquor may vary from industry to industry, it is essential to understand the effect of source of black liquor on penicillin G acylase production.

Because the concentration of the respective component present in the black liquor depends on the raw material and processing process the respective industry used. Therefore, the effect of source of black liquor on the PGA production was studied using black liquor II. The black liquor II was diluted 10 times prior to use because it has higher COD and pH (18359 ± 114 mg l^{-1}; pH 10) in comparison with the black liquor I (COD 3135 ± 47 mg l^{-1}; pH 9). The black liquor II showed higher specific activity (2.5 ± 0.02 U mg^{-1}), which was almost 30% higher than the black liquor I. However, the reason behind the higher activity is obscure, it needs further investigations. The possible reason may be the presence of growth ingredients probably in higher quantity in black liquor II than the black liquor I. However, the identification of the ingredients which boost the penicillin G acylase production is out of the scope of present study. The black liquor II was stored at around 8 ºC and used for the production of PGA for almost 12 months. However, no any significant reduction in the specific activity (2.48 ± 0.017 U mg^{-1}) was observed (Fig. 3). This indicates that the black liquor of any source can be used as a COS for penicillin G acylase production.

Figure 3. The effect of black liquor storage period on the production of penicillin G acyalse



2.5. Conclusion

It is evident from the above results that the black liquor a waste of pulp and paper industries can be used as a COS in the production of penicillin G acylase. As the industrial production of 6-APA (precursor of the semisynthetic penicillins) using penicillin acylase is around 9000 tons per year [19] and it may keeps on increasing in coming years. At such a large scale, the use of industrial waste as a nutrient may help in the reduction of overall production cost as well as in the management of environmental pollution. However, further study is needed to verify this using microbial strain being used at industrial level.

2.6. References

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Chapter: 3

Purification of penicillin G acylase using ultrafiltration polymeric membrane

Chapter: 3

3.1. Summary

The aim of the present study was to search the utility of membranes in penicillin G acylase purification, as in general membranes exhibit an intrinsically high efficiency of energy utilization. In the present study, different ultrafiltration membranes were used to understand their role in the purification of penicillin G acylase. Almost 4.2 fold purification with 31% recovery of penicillin G acylase was obtained using 300,000; 100,000 and 30,000 molecular weight cutoff membranes. Permeate of 300,000; 100,000 and retentate of 30,000 molecular weight cutoff membrane showed similar pattern on the SDS-PAGE. The results indicate the possibility of obtaining high purification and recovery of penicillin G acylase using 300,000 and 30,000 molecular weight cutoff membrane. It can be concluded that the adaptation of present purification method at industrial level will give significant economical advantage.

3.2. Introduction

Penicillin G acylase (E.C. 3.5.1.11) is an enzyme used commercially for the production of β-lactam antibiotics. The β-lactam antibiotics have around $15 billion annual sales, which make up 65% of the total current antibiotics market and the consumption of an enzyme is estimated to be in the range of 10–30 million tons [1]. The enzyme is also involved in synthesis of the anti-platelet agent and in enzymatic activation of pro-drugs in cancer therapy [2]. Because of industrial importance, development of efficient and economic production process to purify large quantities of penicillin G acylase remains a major thrust area of research. The conventional purification process such as ion exchange [3], aqueous two-phase [4], and immobilized metal affinity chromatography [5] has been widely studied. The use of

polymeric membrane in protein purification is also reported where penicillin G acylase purification was studied using immobilized metal affinity membranes [5]. Recently, affinity membrane filtration has emerged as an alternative to affinity column chromatography. The major advantage of using membrane filtration is that the high flow rates at low pressure drops can be achieved. This significantly improves the washing, elution and regeneration processes in comparison with the columns chromatography. Easy scale up is another advantage of increasing use of membrane in biotechnological operations. In the present study, we have tried to evaluate the suitability of ultrafiltration in the purification of PGA which is not reported earlier. Ultrafiltration is a pressure-driven, size-exclusion membrane process and its principle application is for removal of particulate and microbial matter greater in size than the molecular weight cutoff. Today, ultrafiltration has become a common unit operation in biotechnological industries and its application even in partial purification of penicillin G acylase may results in significant economical advantage at industrial level operations.

3.3. Materials and methods

3.3.1. Materials

Ultrafiltration polyethersulfone membrane of 300,000 MWCO (molecular weight cutoff), regenerated cellulose membrane of 100,000 MWCO and regenerated cellulose membrane of 30,000 MWCO were obtained from Millipore Corporation (USA). All other chemicals used were of ACS grade.

3.3.2. Enzyme production

Escherichia coli (NCIM 2400) was obtained from the National Collection of Industrial Microorganisms, Pune, and maintained routinely on nutrient agar slants. Cells were grown in the defined medium g L^{-1} (3 g peptone, 3 g yeast extract, 3.5 g NaCl, 3 g K_2HPO_4, 0.3 g $KH_2 PO_4$, 60.0 g black liquor, 1 g phenylacetic acid and 0.1 g polypropylene glycol). The pH of the medium was adjusted to 7.2. The inoculum was developed in 250 mL conical flask containing 50 mL medium incubated at 23^0C for 24 h on a rotary shaker. The inoculum was transferred aseptically into a 1L Gallenkamp fermenter with magnetic stirring containing 500 mL medium. The fermentation was carried out at 23 ºC under agitation at 300 rpm and 0.5 vvm aeration for 24h. After fermentation the cells were collected by centrifugation (10,000 g) using Remi C24. The harvested cells were suspended in 50 mM sodium phosphate buffer pH 7 and then disrupted in a Branson sonifier. The disrupted material was centrifuged at 10,000 g for 15 min and the supernatant was treated as crude extract of an enzyme penicillin G acylase.

3.3.3. Enzyme assay

Enzyme assay was carried out using 3.72% of penicillin G K^+ salt (Hindustan Antibiotics Ltd. Pimpri, India) as a substrate in 0.1 M phosphate buffer pH 7.0. To the mixture of 0.2 mL sample containing enzyme and 0.8 mL 0.1 M phosphate buffer pH 7.0, 1.0 mL substrate was added and then incubated at 40 ºC for 1 h. The reaction was terminated by pipetting 0.2 mL of the reaction mixture into 3.8 mL of citrate-phosphate buffer, pH 2.5. The enzymatic activity was estimated by determining the amount of 6-aminopenicillanic acid (6-APA) formed using p-dimethylaminobenzaldehyde reagent [6]. The specific activity of the free enzyme was

expressed as μmoles of 6-APA formed per minute per mg of protein at pH 7.0 and 40 ºC (U mg^{-1}). Protein was determined using folin-ciocalteau's reagent [7].

3.3.4. Enzyme Purification

3.3.4.1. 1st step of purification

The crude enzymatic extract was purified by using Amicon stirred cell having a capacity of 50 mL with 13.4 cm^2 membrane area (Data Sheet - PF1050EN00; Millipore Corporation, USA). The 1st step of purification was carried out by using 300,000 MWCO membrane. Prior to the loading of enzyme sample, membrane was washed with buffer (0.1 M phosphate buffer pH 7.0) and flux was measured. The 50 mL feed (crude enzymatic extract) was loaded in a stirred cell and filtration was carried out at 0.5 bar pressure. After the collection of 50 mL filtrate as permeate, another 50 mL feed was loaded and total 100 mL permeate was collected. Then 50 mL buffer was loaded and collected as permeate. The total volume of collected permeate in the first step of purification was 150 mL. After the collection of total permeates, 5 mL buffer was loaded and allowed to stir for 10 min without applying any pressure and collected as retentate from the top of the stirred cell. The enzyme activity and protein was estimated from the samples collected as feed, permeate and retentate.

3.3.4.2. 2nd step of purification

The 2nd step of purification was carried out by using 100,000 MWCO membrane. Prior to loading of enzyme sample, membrane was washed with buffer and flux was measured. The 150 mL feed (permeate of 1st step of purification) was loaded in a stirred cell and filtration was carried out at 0.5 bar pressure. After the collection of

150 mL filtrate as permeate, 20 mL 50 mL buffer was loaded and collected as permeate. The total volume of collected permeate in the 2nd step of purification was 170 mL. After the collection of total permeates, 5 mL buffer was loaded and allowed to stir for 10 min without applying any pressure and collected as retentate from the top of the stirred cell The enzyme activity and protein was estimated from the .samples collected as feed, permeate and retentate.

3.3.4.3. 3rd step of purification

The 3rd step of purification was carried out by using 30,000 MWCO membrane. Prior to loading of enzyme sample, membrane was washed with buffer and flux was measured. The 170 mL feed (permeate of 2nd step of purification) was loaded in a stirred cell and filtration was carried out at 0.5 bar pressure. After the collection of 155 mL filtrate as permeate, 15 mL of remaining feed was collected as retentate from the top of the stirred cell. The enzyme activity and protein was estimated from the .samples collected as feed, permeate and retentate.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples collected at the each step of purification were analyzed by SDS-PAGE [8] using a 3.9% stacking gel and 8% resolving gel and gels were stained by Coomassie brilliant blue G-250.

3.3.5. Treatment of experimental data

Every experimental point reported in the table represents the average value of three experiments performed under the same conditions. The experimental errors did not exceed 5%.

3.4. Results and discussion

In comparison to column process, membrane process is easy to be scaled up industrially and has the advantage of large surface area, short diffusion path and low pressure drop [5]. However, the use of membrane process to obtained highly purified biomolecule appears to be difficult. Because of concentration polarization, a dynamic or secondary layer (secondary membrane) of rejected particles is formed on the membrane surface during filtration. This secondary membrane controls its separation performance [9]. However, membrane process can be effectively used for the purification of biomolecules after proper evaluation. The membrane process is much more appropriate in the case of penicillin G acylase. As the semi-purified preparation of penicillin G acylase can be used for the preparation of immobilized enzyme suitable for industrial application. It was reported that the immobilization of highly purified penicillin G acylase resulted in a less stable preparation [10]. Therefore it was thought to evaluate the suitability of ultrafiltration membrane in penicillin G acylase purification. The ultrafiltration appears to be more economical at industrial level operation in comparison with the other purification methods. The major advantage of ultrafiltration is that it can be performed without changing any physical condition of biomolecule such as pH, temperature and ionic strength which helps in enhancing the stability of biomolecule. The purification method widely studied so far such as ionexchage and metal affinity chromatography require changing of original pH, temperature and ionic strength of biomolecule to be purified [3, 5]. The change in original physical condition of biomolecule also occur in aqueous two-phase purification method and it has a limitation on the loading concentration of biomolecule to be purified which may be uneconomical at industrial level [4]. The requirement of dialysis to restore the original physical condition of purified

biomolecule to achieve high stability is very common in conventional purification methods.

In the present study, different ultrafiltration membranes were used to understand their role in the purification of PGA. We have used 300,000 MWCO membrane as a 1st step of purification because it was reported that the molecular weight of *Escherichia coli* penicillin G acylase is around 89.5 kDa, it is a heterodimer with a 20.5 kDa α-subunit and a 69 kDa β-subunit [11]. It was thought that the significant rejection of high molecular weight proteins will be achieved using 300,000 MWCO. The second 2nd step of purification was carried out using 100,000 MWCO membrane to reject further the high molecular weight protein as to enhance the purification. And the 3rd step of purification was carried out using 30,000 MWCO membrane to concentrate the penicillin G acylase. This was done as the additional buffer was added at the end of 1st and 2nd step of purification to achieve higher permeation of penicillin G acylase.

Table 1 shows the penicillin G acylase purification pattern obtained with different MWCO membrane. Around 63% recovery with 1.42 fold purification of an enzyme was obtained with 300.000 MWCO membrane. Whereas around 54% recovery and 1.48 fold purification of an enzyme was obtained with 100,000 MWCO membrane. However, 30,000 MWCO membrane showed 4.2 fold purification with 31% recovery of an enzyme. The purification of penicillin G acylase obtained with the present method is comparable with the other established methods. In the literature, different recovery and purification fold of penicillin G acylase has been reported for the different methods. Ion-exchange method gives 48% recovery and 5.7 fold purification whereas aqueous two-phase processes shows 97% recovery and 3.5 fold purification

[4]. On the contrary, penicillin G acylase purified using immobilized metal affinity membranes shows 97.9% recovery and 4.63 fold purification while using NH_4Cl as eluent [5].

Table 1: Penicillin G acylase purification pattern obtained with different MWCO membrane.

Sample	Volume (mL)	Total enzyme activity (U)	Total protein (mg)	Specific activity ($U\ mg^{-1}$)	Recovery (%)	Purification (Fold)
Feed	100	129	370	0.348	100	1.0
Permeate of 300,000 MWCO	150	82.0	165.5	0.495	63.56	1.42
Permeate of 100,000 MWCO	170	69.5	134.3	0.517	53.87	1.48
Retentate of 30,000 MWCO	15	40.0	27.0	1.48	31.00	4.25

Table 2: Rejection pattern obtained with different MWCO membrane.

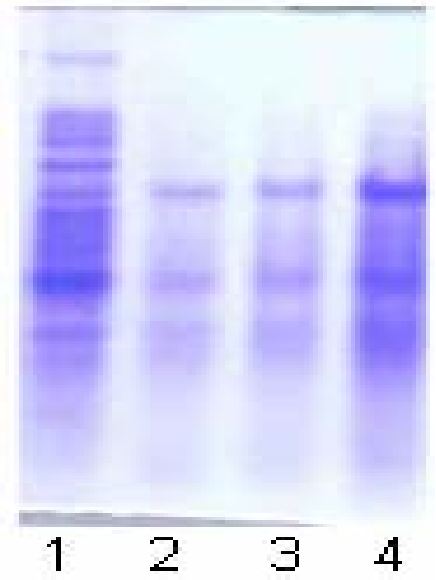
Membrane	Enzyme rejection (%)	Protein rejection (%)
300,000 MWCO	84.3	95.6
100,000 MWCO	51.0	54.4
30,000 MWCO	100.0	61.5

[Rejection (%) = (Concentration in feed – Concentration in permeate / Concentration in feed) x 100]

Table 2 shows the rejection pattern obtained with different MWCO membrane. Almost 84% enzyme rejection and 95% protein rejection was observed for the 300,000 MWCO membrane. However the enzyme rejection was not 100%, although the molecular weight of the penicillin G acylase was significantly lower (89.5 kDa) than the MWCO of membrane (300.000). This may be attributed to the effect of secondary membrane which controls the separation performance of the membrane [9]. This effect was more pronounced with the 100,000 MWCO membrane. It shows significantly lower rejection of an enzyme (51%) and protein (54%). This may be attributed to the less difference in MWCO of membrane (100,000) and molecular weight of an enzyme (89.5 kDa). However, 100% rejection of an enzyme and 61% rejection of protein were obtained with the 30,000 MWCO membrane. This may be attributed to the much lower MWCO of membrane (30,000) than the molecular weight of an enzyme (89.5 kDa). On the contrary, SDS-PAGE showed almost similar pattern of purification with all the MWCO membrane (Fig. 1). The intensity of band is different because of loading concentration of the protein. Most of the higher molecular weight protein was rejected by the 300,000 MWCO membrane. The permeate of 300,000 and 100,000 MWCO membrane as well as retentate of 30,000 MWCO membrane showed three band. However, *Escherichia coli* penicillin G acylase purified using mixed ion exchange supports also showed two major and one minor band on SDS-PAGE [3]. This indicates that the present method of purification is comparable with the established methods of penicillin G acylase purification.

Figure 1. SDS-PAGE pattern obtained for different steps of the purification

[Lane 1, feed; lane 2, permeate of 300,000 MWCO; lane 3, permeate of 100,000 MWCO; lane 4, retentate of 30,000 MWCO. The protein quantities of samples loaded were as follows: lane 1 (30 µg); 2 (8.0 µg); 3 (11.0 µg); 4 (25 µg)].



3.5. Conclusion

As there is no much difference in the purification fold obtained at the 1st (1.42) and 2nd step (1.48) of the purification and similar SDS-PAGE pattern was obtained with the 100,000 MWCO and 300,000 MWCO membrane. This strongly suggests the possibility of obtaining high purification and recovery of penicillin G acylase using 300,000 MWCO and 30,000 MWCO membrane. It can be concluded that the adaptation of present purification method at industrial level will give significant economical advantage.

3.5. References

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Chapter: 4

Studies for the preparation of cellulose based porous membrane suitable for biotech applications

Chapter: 4

4.1. Summary

The aim of the present study was to develop a process for the preparation of cellulose based porous membrane suitable for biotech applications. The different experiments were carried out to develop a novel membrane casting solution. The developed membrane casting solution comprises a mixture of cellulose based water insoluble polymer, water soluble polymer, amine and two water miscible solvent. The membrane casting solution was spread on the glass plate using glass rod and then membrane was recovered by immersing the plate in water. The prepared membrane showed high flux, good hydrophilicity, excellent film-forming ability, good mechanical properties, and high chemical reactivity. The involvement of heating, cooling and highly hazardous chemicals is almost negligible in the developed process of membrane preparation.

4.2. Introduction

An advantage of using membranes in bioseparation, bioconversion or related operations of biotech industries is that the high flow rates at low pressure drops can be achieved. This improves the washing, elution, and regeneration processes and decreases the probability of deactivation of the biomolecules by shortening their exposure to an unfavorable medium. In general, two approaches have been employed to prepare membranes suitable for the biotech applications. In the most common method, porous membranes are prepared from polyethylene, polypropylene, nylon, polysulfone, and glass. However, these membranes are usually hydrophobic and relatively inert, and hence require modifications. Some of the membranes may require amplification of the number of active groups. To overcome these drawbacks, a second

approach has been employed wherein membranes are prepared that have preincorporated functional groups. However, the problems with this type of membranes include hydrophobicity (poly glycidyl methacrylate-co-ethylene dimethacrylate membrane), brittleness, and solubility in acids (cellulose acetate membrane). Another drawback with both of the above methods is that the pore size of the membrane cannot be easily controlled. Materials commonly used for making porous membranes are polycarbonates, polyamides (nylon 6, nylon 6,6, nylon 610, nylon 13), polysulfones, cellulose derivatives (for example, cellulose, cellulose dictate, cellulose triacetate, cellulose nitrate), polyacrylonitrile and copolymers, polypropylene, polytetrafluoroethylene, alumina, silica, carbon, polyvinylidene fluoride, high and low density polyethylene, polypropylene, polystyrene, polyvinyl chloride, acrylonitrile-butadiene-styrene terpolymers, styrene-acrylonitrile and styrene-butadiene copolymers, polyvinylacetate, polyvinylidene chloride, ethylenevinylacetate copolymers, ethylene-acrylic copolymers, polymethylacrylates, and oxidation polymers such as polyphonylencoxide.

However, in comparison to synthetic polymers, use of cellulose appears to be more environments friendly. As with the increased loss of crude oil, renewed interest has been expressed in cellulose globally. Cellulose membranes could be superior to many of the synthetic membranes if they could be produced in a manner, which is economically and environmentally superior to the techniques now available. This would reduce the consumption of non-renewable petrochemical resources. Cellulose is one of the most ideal membrane materials since it is a kind of most abundant organic resource, naturally degradable, biology compatible, hydrophilic, foul resistant and it has good resistance to acid, alkali and organic solvents. Cellulose and its

derivatives, in particular, esters are widely used in the manufacture of membranes. The most common commercial cellulose esters are cellulose acetate (CA), cellulose acetate propionate (CAP) and cellulose acetate butyrate (CAB) [1 – 4]. CAB is considered as one of the good membrane materials because of its good resistance to fouling, chlorine tolerance, chemical stability and the ability to control the molecular weight cut-off (MWCO) [5, 6].

The different methods have been invented for the preparation of cellulose membranes using either cellulose or its derivatives such as cellulose diacetate, cellulose triacetate, and cellulose nitrate. The U.S. Patent No. 6,929,884 described a method for manufacture of cellulose-based films which slowly releases solids. This membrane was prepared by dissolving microcrystalline cellulose in a solvent such as an alkali metal salt in a polar solvent such as dimethylacetamide (DMAC), dispersing the solid in the solution and gelling the solution with water to form a film. The another patent filed by Sartorius Ag, Germany (DE 202004008615 U1; 2004) described a method for the preparation of cellulose microporous membranes by dispersing cellulose triacetate in methyl formate 3 h at 20°C, keeping 90 min at –40C°, heating up to room temp, adding a dispersion of cellulose diacetate in methyl formate (ratio cellulose diacetate/triacetate 1:1), coating onto glass substrate, drying 90 min under N_2 to obtained a membrane. However, the involvement of cooling and heating operations may pose extra energy requirement as well as solvent exposure to the surrounding. Secondly, the chemical composition of the membrane may impose restriction on the development of chemically active membrane. A typical process is described in U.S. Patent No. 3,412,184 which, involves casting a solution of a cellulose ester as a thin film, evaporating a portion of the casting solvent, leaching with an organic solvent, optionally immersing in a hot water bath to subject the film to a heating step, and then

recovering the product membrane. A more advanced, continuous process for the production of cellulose ester membranes is described in U.S. Patent No. 3,792,135. This process involves the following steps, (1) coating a film of a "dope" of a cellulose ester on a web; (2) permitting solvent to evaporate into the atmosphere, to cause incipient formation of a skin or "active" layer; and (3) immersing the film in a hot aqueous bath, to gel the film in the form of an asymmetric membrane. The operation such as immersion of the film in hot water bath and solvent evaporation in to the atmosphere may cause serious concern for environmental point of view as well as overall product cost.

In biotech applications, the membrane should have the properties like hydrophilicity, excellent film-forming ability, good mechanical properties, and high chemical reactivity. In comparison to other cellulose derivatives, ethyl cellulose appears to be more suitable for this purpose because of its better chemical stability. However, there is very negligible literary evidence where ethyl cellulose based membrane have been used in biotech applications. The present study has given major thrust in the modification of membrane casting solution, which may help in the development of a chemically more reactive novel membrane. Prior art do not show any attempt where a major thrust has been given on the modification of casting solution to obtained more chemically reactive cellulose based porous membrane. The U.S. Patent No. 4,840,819 described a method for preparing composite membrane for enhanced gas separation. Where, the ethyl cellulose solution was prepared by dissolving 1.0% ethyl cellulose in isopropanol. The use of ethyl cellulose membrane for the separation of hydrazine has been also reported where approximately 15 wt % clear solution of ethyl cellulose in toluene was prepared and cast on a clean glass plate as a dense membrane. The cast

film was evaporated to dryness in open air at room temperature and the film was vacuum dried for a period of 5 h at ambient temperature to remove any traces of solvent [7]. In the present study we have tried to develop cellulose based porous membrane which has the advantages such as higher flux, good hydrophilicity, excellent film-forming ability, good mechanical properties, and high chemical reactivity as to make it suitable for biotech applications. We have also tried to develop a process where the use of heating, cooling and highly hazardous chemicals is almost negligible.

4.3. Materials and methods

4.3.1. Materials

Ethyl cellulose, polyethylene glycol, polyethylemine, diethylaminoethyl chloride (Aldrich); Epichlorohydrin, glutaraldehyde, chloroacetic acid, sodium borohydride, hydrazine hydrate (Loba); Ethanol (Omnis); 1-4 Dioxane (Merck) were used as received. All other chemicals used were of ACS grade.

4.3.2. Membrane

The membrane was prepared as per the procedure described in an Indian patent filed by us [8].

4.3.3. Membrane characterization

To check the chemical and physical structure of the membrane, membrane was cast using membrane cast solution on the glass plate by spreading cast solution using glass rod and recovering the membrane by immersing the plate in water. Sequential drying

of the membrane was carried out using 10% to 50% ethyl alcohol followed by air drying for approximate 40 min and hot oven drying at 70ºc for approximate 3 hr. Scanning electron microscopy (SEM) was employed to investigate the morphology of the membranes and the attenuated total reflectance fourier transform infrared (ATR/FT-IR) spectra to understand the chemical structure of the membrane.

4.3.4. Membrane adsorption

The study of adsorption was carried out using following procedure -

1) Membrane was fixed in a Amicon stirred cell having 13.4 cm^2 surface area and washed with distilled water followed by equilibration with 10 ml of adsorption buffer (0.1M Phosphate buffer pH 7.4) for 15 min.

2) Then diluted normal horse serum (NHS) was loaded on the membrane in a stirred cell and stirred for 15 min. Dilution of the NHS was done using adsorption buffer.

3) Three washes were given for three times sequentially with 5 ml adsorption buffer by stirring 5 min each.

4) This was followed by elution with 5 ml elution buffer (1M NaCl + 0.1M Phosphate buffer pH 7.4) after stirring for10 min.

5) Lastly the membrane was washed with 5 ml 0.1N NaOH after stirring for10 min. Estimation of the protein concentration from the samples collected at each step was carried out using Lowry method [9].

4.3.5. Treatment of experimental data

Every experimental point reported in the table represents the average value of three experiments performed under the same conditions. The experimental errors did not exceed 5%.

4.4. Results and discussion

In general, the different chemical modifications are required to make a membrane suitable for biotech applications. For instance, the membrane suitable for filtration of biomolecules may not be suitable for enzyme immobilization or selective bioseparation. Thus, the membrane suitable for biotech applications requires the properties such as higher flux, good hydrophilicity, good biocompatibility, good mechanical properties, and high chemical reactivity. In search of this, cellulose was selected as a base polymer and different experiments were carried out to develop a novel membrane suitable for biotech applications.

The selection of suitable polymer for membrane preparation depends on its insolubility in aqueous media and film formation property. Table 1 shows the effect of different cellulose based polymer on its film formation quality. The polymer was dissolved in different solvent and thin smear was prepared on the glass plate. Then this glass plate was immersed separately in water, 1N NaOH, 1N HCl and the quality of film was checked. In comparison, ethyl cellulose showed good film formation properties, thus it was selected for the further study.

Table 1: Effect of different polymer on the film formation

Polymer	Concentration (%)	Solvent	Film quality
Ethyl cellulose	10	Dioxane	Good
Ethyl cellulose	5	Cuprammonium solution	Fair
Methyl cellulose	5	Dimethylsulphoxide	Fair
Cellulose microcynstalline	5	Cuprammonium solution	Fair

Table 2 shows the effect of different concentration of ethyl cellulose on film quality. This was done to select the most suitable concentration of ethyl cellulose for the preparation of membrane. Different concentration of ethyl cellulose was dissolved in 1, 4-dioxan and thin smear was prepared on the glass plate and film quality was checked. The very good film quality was obtained with the 10% ethyl cellulose. This concentration was taken as a base for the further study. However, the water flux of the membrane prepared using 10% ethyl cellulose was very poor. The water flux of the membrane was measured at 0.2 bar by using stirred cell.

Table 2: Effect of ethyl cellulose concentration on film formation

Ethyl cellulose Concentration (%)	Film Quality
5	Fair
6	Fair
7	Good
8	Good
9	Better
10	Very good

To improve the flux of the membrane, it was thought to add some water soluble polymer in membrane cast solution. Table 3 shows the effect of water soluble polymer incorporated in the membrane cast solution on flux. Polyethylene glycol (PEG) was used as a water soluble polymer. The different concentration of PEG and 9% ethyl cellulose was dissolved in 1, 4-dioxan and membrane was prepared on the glass plate by spreading cast solution using glass rod and recovering the membrane by immersing the plate in water. The water flux of the membrane was measured at 0.2 bar by using stirred cell after treating the membrane with 0.1N NaOH. The higher flux was obtained with the 2% PEG, this concentration was selected for the further study.

Table 3: Effect of different concentration of PEG on the flux of the membrane

PEG Concentration (%)	Relative Flux (%)*
1	65
2	100
3	60
4	50
5	30

* - Higher flux obtained was considered as 100% for the calculation of relative flux (%).

Table 3 clearly shows the significant effect of the incorporation of water soluble polymer in the membrane cast solution on membrane flux. Therefore, it was thought to verify the role of ethyl cellulose concentration below 10% on the membrane flux. Table 4 shows the effect of ethyl cellulose concentration below 10% on flux. The different concentration of ethyl cellulose and 2% PEG was dissolved in 1,4-dioxan and membrane was prepared on the glass plate by spreading cast solution using glass

rod and recovering the membrane by immersing the plate in water. The water flux of the membrane was measured at 0.2 bar by using stirred cell. The higher flux was observed at 5% ethyl cellulose concentration, this suggests the significant role of PEG in membrane preparation.

Table 4: Effect of lower concentration of ethyl cellulose on flux

Ethyl cellulose concentration (%) + 2% PEG	Relative Flux (%)*
4	**67**
5	**100**
6	**52**
7	**40**

* - Higher flux obtained was considered as 100% for the calculation of relative flux (%).

Table 4 indicates the significant effect of PEG in lowering the ethyl cellulose concentration in membrane cast solution. Therefore, it was thought to optimize all the ingredients of the membrane cast solution. However, the purpose of the present study was to develop a membrane suitable for biotech applications. Thus, flux can not be the only criteria for the development of membrane. The presences of reactive groups on the membrane surface are essential to carry out different chemical modifications as to make it suitable for biotech applications. Therefore, the criterion of blood protein adsorption on the membrane was selected to optimize the ingredients of membrane cast solution. Table 5 shows the effect of different ingredients of membrane cast solution on protein adsorption.

Table 5: Effect of different membrane cast solution on membrane quality and protein adsorption

Membrane cast solution (type)	Membrane quality	Relative protein adsorption (%)*
I (Ethyl cellulose + 1,4-dioxan)	Bad [breaks easily]	32
II (Ethyl cellulose + PEG + 1,4-dioxan)	Good [not smooth]	17
III (Ethyl cellulose + PEG + Amine + Ethyl alcohol + 1,4-dioxan)	Best [White, sooth and tough; Flux – 2500 LMH (L m^{-2} h^{-1}) at 0.5 bar; Dry weight - 49.25 g / m^{-2}]	100

* - Higher adsorption was considered as 100% for the calculation of relative flux (%).

To check the chemical and physical structure of the membrane, membrane was cast using membrane cast solution type III (Table 5) on the glass plate by spreading cast solution using glass rod and recovering the membrane by immersing the plate in water. Sequential drying of the membrane was carried out using 10% to 50% ethyl alcohol followed by air drying for approximate 40 min and hot oven drying at 70ºc for approximate 3 hr. Scanning electron microscopy (SEM) was employed to investigate

the morphology of the membranes. Figure 1 show that most of the pores are distributed uniformly. Figure 2 shows the attenuated total reflectance fourier transform infrared (ATR/FT-IR) spectra. The spectra reveal the chemical structure of the membrane, which is different form the membrane so far developed. It shows several characteristic peaks, the peak at 3475 cm^{-1} corresponds to the -OH groups of closed ring structure of the polymer unit. The peak at 2869 cm^{-1} corresponds to C-H bond whereas the peak at 1374 cm^{-1} corresponds to C-N bond. The sharp peak at 1074 cm^{-1} corresponds to –C-O (stretch). This indicates the presence of reactive groups available for chemical modification of membrane as to make it suitable for biotech applications.

Figure 1: SEM of the membrane

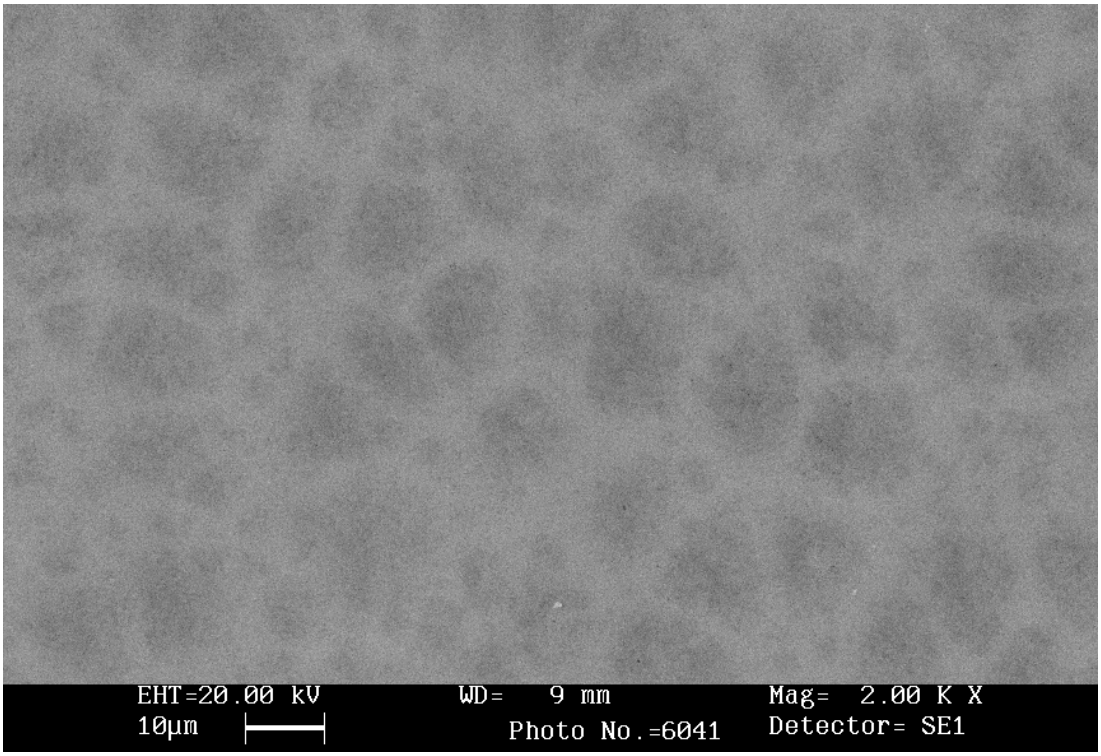
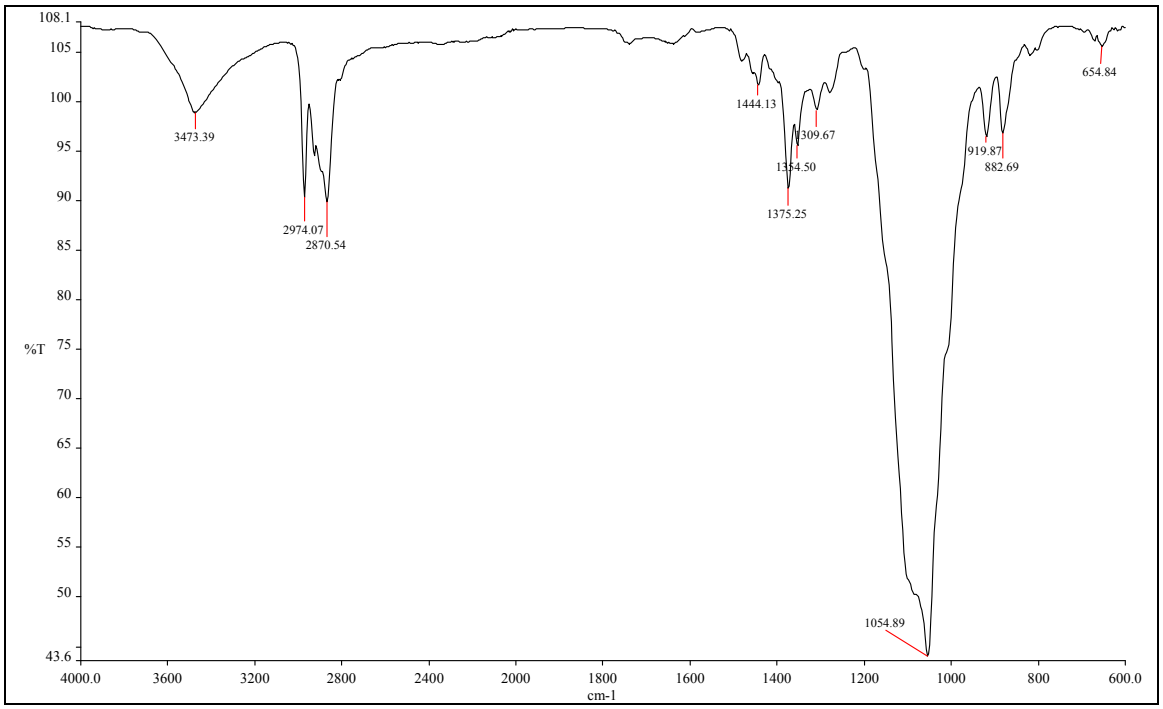


Figure 2: Attenuated total reflectance Fourier Transform Infrared (ATR/FT-IR) spectrometry of the membrane.



To check the suitability of the membrane for chemical modification, the membrane was treated with NaOH at 60ºC in the presence of bisoxirane and epichlorohydrin, which primarily used for the activation of membrane in ligands binding. The membrane was cast using membrane cast solution type III (Table 5) on the glass plate by spreading cast solution using glass rod and recovering the membrane by immersing the plate in water. The membrane was treated with different chemical treatment (I&II, Table 6) and the blood protein adsorption was checked using stirred cell. The flux of the membrane was measured using water prior to adsorption of protein (Initial flux) and after the completion of the experiment (End flux). Table 6 shows the results, which indicates the membrane stability at different chemical treatment and the reduction in flux suggest the binding of the proteins. These qualities are very much required for the ligand binding, which commonly used in bioseparation and enzyme immobilization. Figure 3 shows the photograph of the membrane treated with different chemicals. This indicates the present membrane has excellent chemical reactivity and stability.

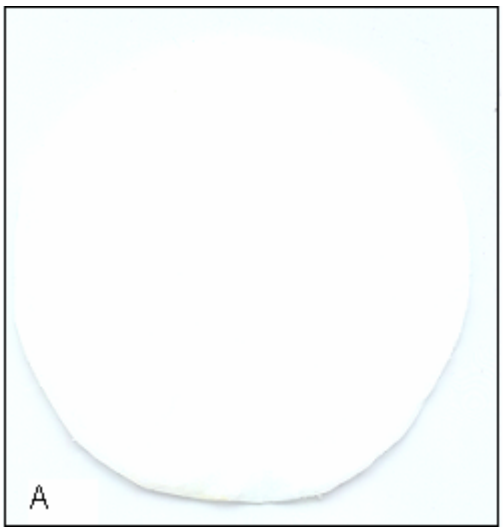
Table 6: Effect of different chemical treatment on the membrane

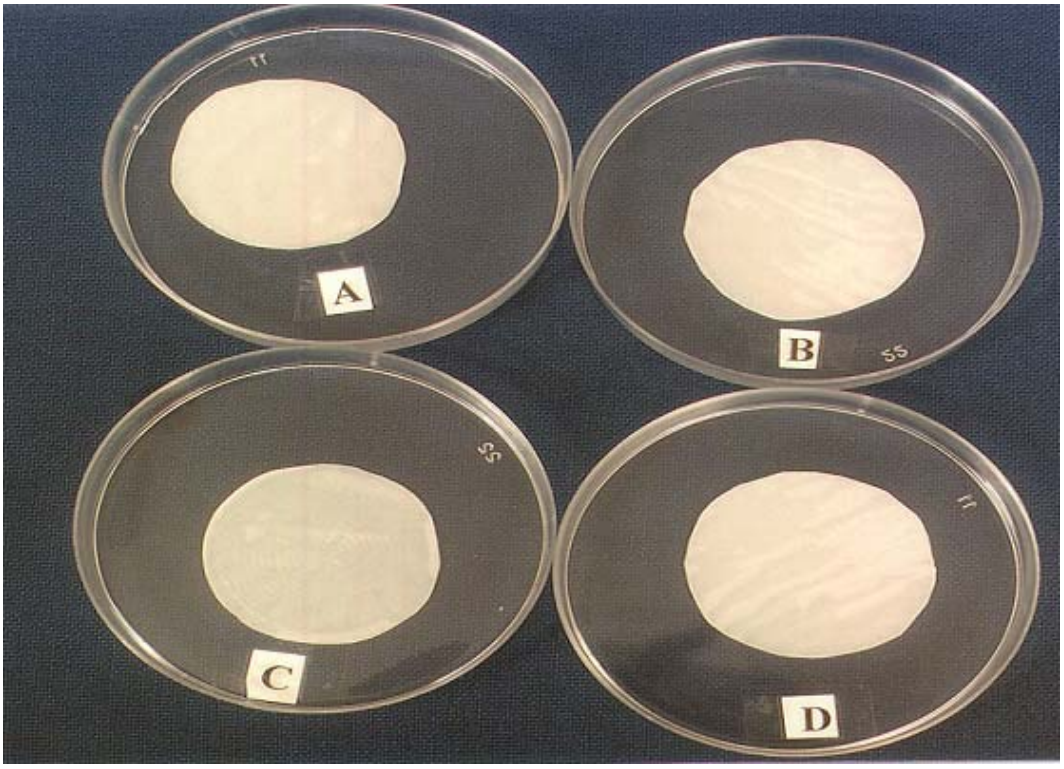
Chemicals Treatment	Initial flux (LMH)	End flux (LMH)	Decrease in flux (%)	Protein Adsorption (%)
I. 5% Bis+0.2% BH in 1N NaOH at 60ºC for 1 hour under mild stirring	2194	1343	38.78	40.33
II 0.5% EPI in 1N NaOH at 60ºC for 1 hour under mild stirring	2015	1657	17.76	34.57

LMH – L m^{-2} h^{-1}; Bis- Bisoxirane; BH - Sodium borohydride; EPI – Epichlorohydrin; % Adsorption = 100 - [Protein concentration in wash x 100 / Protein concentration in feed].

Figure 3: Effect of different chemical treatment on membrane

[A – Non-treated; B – Treated with 1 N NaOH + 1,4-Butandioldiglycidyl ether for 48 h at room temperature; C – Treated with 1 N NaOH for 48 h at room temperature; D – Treated with 1 N HCl for 48 h at room temperature]





4.5. Conclusion

In the present study, the major thrust was given in the modification of membrane casting solution, which helps in the development of a chemically more reactive novel membrane. Prior art do not show any attempt where a major thrust has been given on the modification of casting solution to obtained more chemically reactive cellulose based porous membrane. The present study relates to a process for the preparation of cellulose based porous membrane suitable for biotech applications. The membranes in accordance with the present study possess the advantages such as high flux, good hydrophilicity, excellent film-forming ability, good mechanical properties, and high chemical reactivity. This suggests the presence of chemically reactive molecules in membrane casting solution, which help in the enhancement of these properties. The membrane preparation process does not involve heating, cooling and highly hazardous chemicals. Another advantage is of high flux after binding of chemically reactive molecules, which require in biotech applications. This property of the developed membrane makes it suitable for commercial exploitation. As it is a charged membrane, it may be used without any further amplification of the number of active groups, in various applications such as bioseparation, enzyme immobilization etc. It can also be modified easily to alter the charged either positively or negatively without major reduction in flux. All these properties of the developed membrane make it suitable for biotech applications such as membrane bioreactors, chromatographic separation, affinity chromatography or related unit operations generally carried out in biotech industries.

4.6. References

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Chapter: 5

Studies of penicillin G acylase immobilization using highly porous cellulose based polymeric membrane

Chapter: 5

5.1. Summary

The aim of the present study was to investigate the suitability of the developed novel cellulose based porous membrane (Chapter 4) in 6-APA production. The different ionic molecules/compounds were used as a ligand for the immobilization of penicillin G acylase on the highly porous cellulose based polymeric membrane prepared as described in chapter 4. The immobilized enzyme activity around 250 U_{App} was obtained with the ligand such as proline, tryptophan, casein acid hydrolysate and brilliant green. Comparatively, proline showed less IMY% (percentage immobilization yield - 58) but higher RTA% (percentage of activity retention - 71) and specific activity (145 $U_{App}\ g^{-1}$). However, the crosslinked preparation of brilliant green obtained using glutaraldehyde showed 82 ± 2.7% immobilized enzyme activity after the completion of successive five cycles. In comparison with the free enzyme, the enzyme immobilized on the brilliant green coupled membrane showed around 2.4 fold increase in Km value (47.4 mM) as well as similar optimum pH (7.2) and temperature (40 ºC). The immobilized enzyme retained almost 50% activity after 107 days and 50 cycles of operation. Almost 50% decrease in buffer flux after enzyme immobilization was observed. At the end of the 30 cycles, flux pattern shows around 38% decrease in buffer flux however, after 16 cycles of operation flux moves closer towards the steady state. The scale up studies carried out using 250 mL operating volume indicates the suitability of developed enzyme immobilized membrane for industrial applications.

5.2. Introduction

Penicillin G acylase (E.C. 3.5.1.11) is an enzyme used commercially for the production of β-lactam antibiotics. The β-lactam antibiotics have around $15 billion annual sales, which make up 65% of the total current antibiotics market and the consumption of an enzyme is estimated to be in the range of 10–30 million tons [1]. The enzyme is also involved in synthesis of the anti-platelet agent and in enzymatic activation of pro-drugs in cancer therapy [2]. Because of industrial importance, the search of suitable carrier for the enzyme immobilization always remains a major thrust area of research. However, the mass transfer limitations in the carrier remain a major barrier to further improvement of the enzyme performance at industrial level [3]. The accumulation of product and generation of pH gradients are the major drawback in the conventional form of the carrier such as bead [3]. However, most of the recent studies indicate the immobilization trend of using carrier with bead configuration [4 – 8]. Comparatively, the use of unconventional source of carrier for enzyme immobilization is not widely reported. There are some reports where polymeric membranes such as nylon [9] and porous polymer with oxirane groups [10] were used for enzyme immobilization. In comparison with the conventional sources, polymeric membranes appear to be suitable carrier for the enzyme immobilization. Because substrate molecules can be convected through the membrane support, rather than having to diffuse to a bead or particle to reach the active site of an enzyme, this may enhance the mass transfer efficiency significantly. However, irrespective of advantages such as easy scale up, high throughput etc., the fouling of polymeric membrane remains a major problem which reduces productivity and increases maintenance and operating costs [11]. Particularly when the carries has to undergo different chemical reaction to immobilize the enzyme, this may results in the

reduction of pore size and enhance the fouling. Therefore, it is desirable that the carrier should have the high pore size, chemical and mechanical stability and biodegrability to overcome the after use disposal problem. In this work, we have used a highly porous cellulose based polymeric membrane for enzyme immobilization to overcome the problem of mass transfer, fouling and biodegrability. The different ionic molecules/compounds generally used for the protein adsorption were screened to select most appropriate molecules/compounds as a ligand for enzyme immobilization and its effect on the stability and functional properties of the enzyme were studied. The crude enzyme preparation and adsorption-crosslinking method [12] was used for the enzyme immobilization. This may enhance the selective binding of enzyme to the carrier and also help in the reduction of overall process cost.

5.3. Materials and methods

5.3.1. Materials

Epichlorohydrin, glutaraldehyde , chloroacetic acid, sodium borohydride, hydrazine hydrate (Loba, India); proline , tyrosine, tryptophan, gelatin, casein, peptone, casein acid hydrolysate, beef extract**,** meat extract, soya peptone**,** tryptone **(**Himedia, India); polyethylemine, diethylaminoethyl chloride, procion red (Aldrich Chemicals); brilliant green (Fluka Chemicals.) were used as received. All other chemicals used were of ACS grade.

5.3.2. Enzyme production

Escherichia coli (NCIM 2400) was obtained from the National Collection of Industrial Microorganisms, Pune, and maintained routinely on nutrient agar slants. Cells were grown in the defined medium g L^{-1} (3 g peptone, 3 g yeast extract, 3.5 g

NaCl, 3 g K_2HPO_4, 0.3 g KH_2PO_4, 60.0 g black liquor, 1 g phenylacetic acid and 0.1 g polypropylene glycol). The pH of the medium was adjusted to 7.2. The inoculum was developed in 250 mL conical flask containing 50 mL medium incubated at 23^0C for 24 h on a rotary shaker. The inoculum was transferred aseptically into a 1L Gallenkamp fermenter with magnetic stirring containing 500 mL medium. The fermentation was carried out at 23 ºC under agitation at 300 rpm and 0.5 vvm aeration for 24h. After fermentation the cells were collected by centrifugation (10,000 g) using Remi C24. The harvested cells were suspended in 50 mM sodium phosphate buffer pH 7 and then disrupted in a Branson sonifier. The disrupted material was centrifuged at 10,000 g for 15 min and the supernatant was used as an enzyme penicillin G acyalse without any further treatment.

5.3.3. Enzyme assay

Enzyme assay was carried out using 3.72% of penicillin G K^+ salt (Hindustan Antibiotics Ltd. Pimpri, India) as a substrate in 0.1 M phosphate buffer pH 7.0. To the mixture of 0.2 mL sample containing enzyme and 0.8 mL 0.1 M phosphate buffer pH 7.0, 1.0 mL substrate was added and then incubated at 40 ºC for 1 h. The reaction was terminated by pipetting 0.2 mL of the reaction mixture into 3.8 mL of citrate-phosphate buffer, pH 2.5. The enzymatic activity was estimated by determining the amount of 6-aminopenicillanic acid (6-APA) formed using p-dimethylaminobenzaldehyde reagent [13]. The specific activity of the free enzyme was expressed as μmoles of 6-APA formed per minute per g of protein at pH 7.0 and 40 ºC (U g^{-1}). Protein was determined using folin-ciocalteau's reagent [14].

5.3.4. Enzyme Immobilization

5.3.4.1. Membrane

The membrane was prepared as per the procedure described in chapter 4 and in a Indian patent filed [15].

5.3.4.2. Membrane modification

The chemical modification of membrane was carried out to coupled ligand comprising different ionic molecules/compounds such as DEAE, chloroaceticacid, dyes, amino acids and proteins as ligand to check their effect on enzyme immobilization (Table 1). After complete washing of the modified membrane to remove unbound chemicals, it was equilibrated with 50 mM sodium phosphate buffer pH 7 prior to enzyme immobilization. Enzyme immobilization was carried out by loading 5 mL of enzyme on a modified membrane (13.4 cm^2) fixed in an Amicon stirred cell under mild stirring for 2 hours. To remove unbound enzyme, sequentially four washing with 5 ml of 50 mM sodium phosphate buffer pH 7 and finally with 5 ml 1M NaCl was carried out. Sample of each washing was collected to estimate the enzyme activity and protein (Enzyme assay).

5.3.4.3. Immobilized enzyme activity

As mentioned above (Membrane modification), the enzyme immobilized membrane was equilibrated with buffer and then 10 mL substrate (Enzyme assay) was loaded and allowed to react at 40 ºC for 1 h. After 1 h, sample was removed to measure the 6-APA (Enzyme assay) and termed as test. The blank was treated similarly and the difference between test and blank was taken for the calculation of immobilized enzyme activity (Test - Membrane + Ligand + Enzyme; Blank - Membrane + Ligand). The apparent catalytic activity (U_{App}) was expressed as the μmoles of 6-APA

produced per minute per square meter of membrane at pH 7.0 and 40 ºC (μmoles min^{-1} m^{-2}). The specific activity (U_{App} g^{-1}) was expressed as the μmoles of 6-APA produced per minute per g of protein adsorbed on the membrane at pH 7.0 and 40 ºC (μmoles min^{-1} g^{-1}). These experimental conditions were always applied, except when indicated otherwise.

5.3.4.4. Treatment of experimental data

Every experimental point reported in the table and figures represents the average value of three experiments performed under the same conditions. The experimental errors did not exceed 5%.

5.4. Results and discussion

In the present study we have used a crude enzyme preparation for immobilization because this may enhance the stability as well as it will help in the reduction of overall process cost. It was also reported that the immobilization of highly purified penicillin G acylase resulted in a less stable preparation [16]. The enzyme immobilization to the membrane was carried out using adsorption-crosslinking method [12]. Because, this may facilitate the selective binding of an enzyme to the carrier. As well as this may compensate the use of crude enzyme for immobilization to some extent. An enzyme immobilization using the combination of adsorption and crosslinking is a well known method. The different carriers such as ion exchangers, hydrophobic adsorbents and biospecific adsorbents can be used for this purpose [12]. Earlier, this concept have been used for the immobilization of penicillin G acylase on different carriers such as gelatin [17], chitosan-based support [18], derivitised cross-linked agarose, pheoxyacetylcellulose, oxirane-polyacrlamide resin, polyacrylamide

foam, cross-linked polymethyl methacrylate/glycol dimethacrylate copolymer, cross-linked polystyrene, and porous glass, DEAE-cellulose, cellulose, CM-cellulose, ion exchangers, acrylamide gel, polyamides, silica gel, and Al_2O_3 [19]. However, the studies involving polymeric membrane for penicillin G acylase immobilization using the adsorption-crosslinking method are negligible. In the present paper, we have used different ionic molecules/compounds as ligand to modify the cellulose based highly porous membrane to study penicillin G acylase immobilization. Most of these ionic molecules/compounds have been used earlier for an enzyme immobilization such as DEAE, chloroaceticacid (CM: Carboxymethyl) [19], hydrazine hydrate [20], different dyes [12], polyethylimine [21], amion acid as ligand [22], proteins such as albumin [23] and gelatin [17]. The purpose of using these ionic molecules/compounds as a ligand was also to avoid harsh chemical reactions for membrane modification and enzyme immobilization. This may help in developing a relatively inexpensive and nontoxic chemistry for enzyme immobilization.

5.4.1. Effect of different ionic molecules/compounds on immobilization

The binding of different ionic molecules/compounds as ligand to the membrane was carried out to generate functional groups on the membrane for enzyme adsorption. Each step of the treatment was optimized using enzyme activity as the screening criteria. Although the different treatment was given to the membrane for the binding of different ligands but the enzyme immobilization procedure was uniform. The pH and ionic strength of the buffer was also not changed for the enzyme immobilization. Therefore any change in enzyme activity after immobilization may be solely due to the interactions between carrier and enzyme. In Table 1 the enzyme activity and protein adsorption obtained for different ligands are presented. Table 1 show that

there is no relation between the amount of protein adsorbed on the membrane and enzyme activity. The membrane without any modification showed significant protein adsorption but negligible enzyme activity. This suggests the need of modification of membrane to make it suitable for enzyme immobilization. On the other hand, DEAE showed comparatively higher protein adsorption but significantly less enzyme activity. The reason particularly in the case of DEAE may be the high amino-group concentration. It was reported that the carriers having high amino-group concentration results in multi-point bonding with penicillin G acylase which deactivate the retained activity [24]. Another reason may be the presence of non-favorable interactions between the enzyme and the supporting matrix and microenvironment in which the immobilized enzyme is operating. Interestingly, the enzyme activity around 250 U_{App} was obtained with the ligand such as proline, tryptophan, casein acid hydrolysate and brilliant green, this is not reported earlier. Table 2 shows the effect of these four ligand on IMY%, RTA% [24] and specific activity of immobilized enzyme. Comparatively, proline showed less IMY% but higher RTA% and specific activity. This indicates that the proline is most suitable for selective binding of penicillin G acylase to the carrier. The selective binding of penicillin G acylase to the proline may be due to the presence of secondary α-amino group which is unique among the 20 protein-forming amino acids.

Table 1: Effect of different ionic molecules/compunds (ligands) coupled to membrane on enzyme immobilization

Ligands	Treatment	Treatment conditions	Preparation prior Enzyme loading	Protein Adsorbed (g m^{-2})	Immobilized Enzyme Activity (U_{App})
Nil	Membrane without any Modification	Nil	Washing with DW & Buffer	1.9	24.87
DEAE	A. Membrane +Epichlorohydrin (0.5% v/v) prepared in 0.1 N NaOH + 0.2% sodium borohydride	40ºC for 1 h at mild stirring	Washing with DW	5.62	44.77
	B. Membrane after treatment A + 2- diethylaminoethyl chloride hydrochloride (5.0% wt/v) prepared in 0.1N NaOH + 0.2% sodium borohydride	40ºC for 1 h at mild stirring	Washing with 0.1N NaOH followed by 0.1N HCl, DW & Buffer		
Chloro - aceticacid	Membrane + Chloroacetic acid 10% (wt/v) prepared in 0.1 N NaOH	60ºC for 1 h at mild stirring	Washing with DW & Buffer	2.68	176.61

[U_{App} - µmoles min^{-1} m^{-2} of membrane; DW - distilled water; RT – room temperature (28±2°C); Mild stirring - ~100 rpm; Buffer - 50 mM sodium phosphate buffer pH 7]

Continue Table 1:

Ligands	Treatment	Treatment conditions	Preparation prior Enzyme loading	Protein Adsorbed (g m^{-2})	Immobilized Enzyme Activity (U_{App})
Polyethyl -imine	A. Membrane +2 N HCl prepared in DW	60ºC for 1 h at mild stirring	Washing with DW	2.85	194.27
	B. Membrane after treatment A + polyethylimine (1.0% wt/v) prepared in DW	RT for 1 h at mild stirring	Washing with DW & Buffer		
Hydrazine hydrate	A. Membrane +10 mL Hydrazine prepared in DW	RT for 24 h at mild stirring	Washing with DW	4.32	42.28
	B. Membrane after treatment A + glutaraldehyde (5% vol/vol)	37ºC for 24 h	Washing with DW & Buffer		
Procion Red	Membrane + Procion Red MX-5B (2.5% wt/vol) prepared in 0.1 N NaOH	60ºC for 1 h at mild stirring	Washing with DW followed by 2M NaCl, 10% methanol, DW & Buffer	2.46	144.40
Brilliant Green	Same as Procion Red	Same as Procion Red	Same as Procion Red	3.2	316.29

Continue Table 1:

Ligands	Treatment	Treatment conditions	Preparation prior Enzyme loading	Protein Adsorbed ($g\ m^{-2}$)	Immobilized Enzyme Activity (U_{App})
Tyrosine	A. Membrane + 2 N HCl prepared in DW	60ºC for 1 h at mild stirring	Washing with DW	3.16	131.09
	B. Membrane after treatment A + glutaraldehyde (5% vol/vol) prepared in DW	RT for 24 h at mild stirring	Washing with DW		
	C. Membrane after treatment B + Tyrosine (5 mg / mL) prepared in 0.1 N NaoH	RT for 24 h at mild stirring	Washing with DW& Buffer		
Proline	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	2.34	341.29
Tryptophan	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	4.15	254.10
Gelatin	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	1.93	57.21
Casein	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	3.46	149.37

Continue Table 1:

Ligands	Treatment	Treatment conditions	Preparation prior Enzyme loading	Protein Adsorbed (g m^{-2})	Immobilized Enzyme Activity (U_{App})
Peptone	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	2.6	141.91
Casein acid hydrolysate	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	3.5	249.11
Beef extract	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	3.0	199.29
Meat extract	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	1.7	99.50
Soya Peptone	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	4.0	167.53
Tryptone	Same as Tyrosine	Same as Tyrosine	Same as Tyrosine	2.4	161.69

[U_{App} - µmoles min^{-1} m^{-2} of membrane; DW - distilled water; RT – room temperature (28±2°C); Mild stirring - ~100 rpm; Buffer - 50 mM sodium phosphate buffer pH 7]

5.4.2. Effect of glutraldehyde on the enzyme stability

The stability of immobilized enzyme is a critical factor from its practical application point of view. To verify this, further study was carried out using the ligands which showed relatively higher activity of the immobilized enzyme such as proline, tryptophan, casein acid hydrolysate and brilliant green (Table 2). During repeated use, the possibility of leakage of adsorbed enzyme from the carries is evident. To prevent the crosslinking of adsorbed enzyme on proline, tryptophan, casein acid hydrolysate and brilliant green with glutaraldehyde was carried out. Glutaraldehyde is a bifunctional crosslinking reagent and it can be used for post-immobilization cross-linking to avoid enzyme leakage [25]. It was reported that the higher retention of an enzyme activity and operational stability could be achieved using affinity adsorption of the enzyme on the carrier surface and then crosslinking with glutaraldehyde [26, 27]. Figure 1 show a comparative study carried out using crosslinked and non crosslinked enzyme preparation. The enzyme activity pattern of crosslinked and non crosslinked preparations were studied up to five cycles carried out successively using batch mode of operation. This was done to select most appropriate ligand for further study. Comparatively, the non crosslinked preparations of proline and brilliant green showed almost 75% immobilized enzyme activity up to two cycles and then it slide down below 10% around five cycles (Fig.1A). On the contrary, the crosslinked preparations of

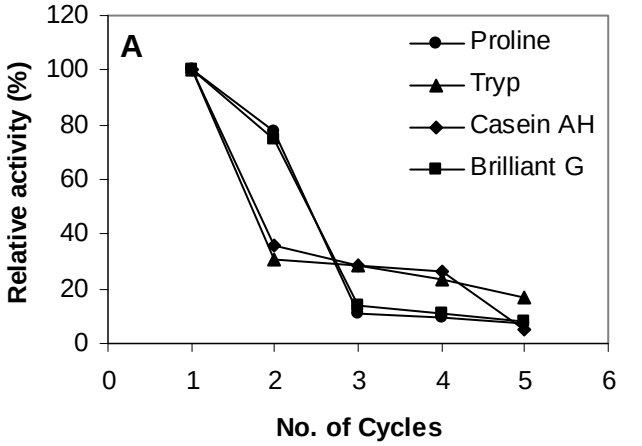
Table 2: Effect of ionic molecules/compunds (ligands) coupled to membrane on IMY%, RTA% and specific activity of immobilized enzyme

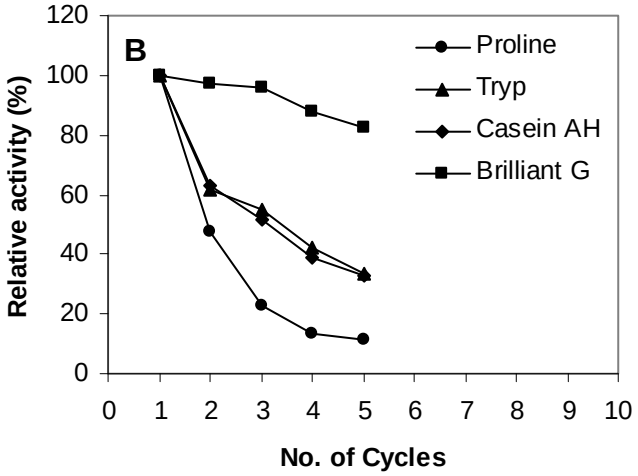
Ligands	IMY%	RTA%	Specific activity (U_{App} g^{-1})
Proline	58.22	71.74	145.85
Tryptophan	82.43	21.30	61.22
Casein AH	79.62	25.58	71.17
Brilliant green	71.72	39.41	98.84

[AH - acid hydrolysate; U_{App} g^{-1} - μmoles min^{-1} $g^{-1}$of protein adsorbed on the membrane;
Activity of free enzyme – 348.64 U g^{-1};
IMY% (percentage immobilization yield) =
(Activity of free enzyme - Activity of immobilized enzyme) / Activity of free enzyme x 100];
RTA% (percentage of activity retention) = (Activity of free enzyme - Activity of immobilized enzyme) x 100]

Figure 1: Effect of glutraldehyde on the enzyme stability

[A - non crosslinked; B - crosslinked; Glutaraldehyde treatment – membrane + ligand + 5 mL 2% (v/v) glutaraldehyde prepared in buffer (50 mM sodium phosphate buffer pH 7) was allowed to react for 1 h under mild stirring; Enzyme acticity – ezyme activity was estimated as mentioned in materials and methods (Immobilized enzyme activity) after washing with buffer.]





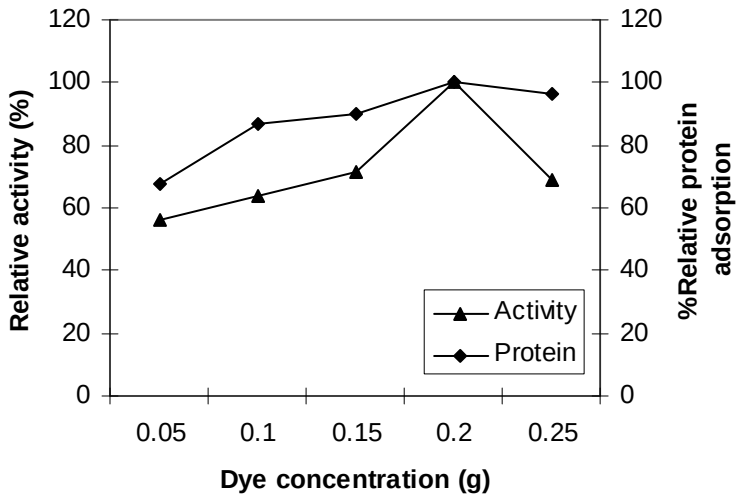
brilliant green showed 82 ± 2.7% immobilized enzyme activity after the completion of successive five cycles (Fig.1B). This indicates that the brilliant green coupled membrane has the potential to be a suitable carrier for penicillin G acylase immobilization. Although the RTA% (Table 2) was significantly less (~39) for brilliant green than the proline (~71) however, it was comparable with the others results. The penicillin G acylase immobilized on chemically grafted nylon particles showed 30% retention of activity [24]. In conclusion, crosslinking with glutaraldehyde fail to protect the immobilized enzyme activity in case of proline, tryptophan and casein acid hydrolysate. This may be due to the lack of reactive groups which require to facilitate the crosslinking and forming the network to prevent enzyme leakage during repeated use.

5.4.3. Optimization of immobilization and characterization

As brilliant green appears to be most suitable for penicillin G acylase immobilization which is also not reported earlier, it is essential to evaluate its suitability for practical application. Therefore, the optimization of immobilization and characterization have been studied to evaluate the most relevant factors such as brilliant green concentration, immobilization time, enzyme loading, effect of pH and temperature, effect on kinetic parameters, effect on storage and operational stability and flux pattern of the enzyme immobilized membrane.

Figure 2: Effect of brilliant green concentration

[Dye treatment – membrane was treated with different concentration of brilliant green as mentioned in Table 1; Enzyme activity – enzyme activity was estimated as mentioned in materials and methods (Immobilized enzyme activity) after glutaraldehyde treatment]



5.4.4. Effect of brilliant green concentration

This study was carried out to select the optimum brilliant green concentration for the immobilization of an enzyme. The different concentarion of brilliant green (0.05 to 0.25 g) was loaded on the membrane and the immobiliztion of an enzyme was checked. The higher enzyme activity and protein adsorption was obtained with 0.2 g brilliant green concentration (Fig. 2). The further study was carriedout using 0.2 g brilliant green concentration.

5.4.5. Optimization of enzyme loading

To evaluate the amount of enzyme required to obtain higher activity, different volume of enzyme which corresponds to different amount of protein (2 – 33 g m^{-2}) was loaded on the membrane coupled with brilliant green. The enzyme activity increased with increase in protein loading from 2 to 22 g m^{-2} and there after it decreased (Fig. 3), the higher activity was obtained at 22 g m^{-2}. It appears that the higher loading of protein lead to embedding of active sites of enzyme during the immobilization process which results in the decrease of enzyme activity at higher loading. The further study was carried out using 22 g m^{-2} amount of protein for loading on the brilliant green coupled membrane.

5.4.6. Optimization of enzyme loading time

The time required for loading of enzyme was optimized by measuring protein from the feed at various time intervals (Fig. 4). The 20% adsorption of protein was obtained within 10 min however, it reaches to equilibrium in 170 min which

Figure 3: Optimization of enzyme loading

[This experiment was carried out using membrane prepared under identical conditions. Different volume of enzyme comprising 7.4 to 44.4 mg protein which corresponds to 5.5 to 33 g m^{-2} was loaded on the membrane coupled with brilliant green (0.2 g) and allowed to react for 1 h under mild stirring. After 1 h enzyme activity was estimated as mentioned in materials and methods (Immobilized enzyme activity) after glutaraldehyde treatment]

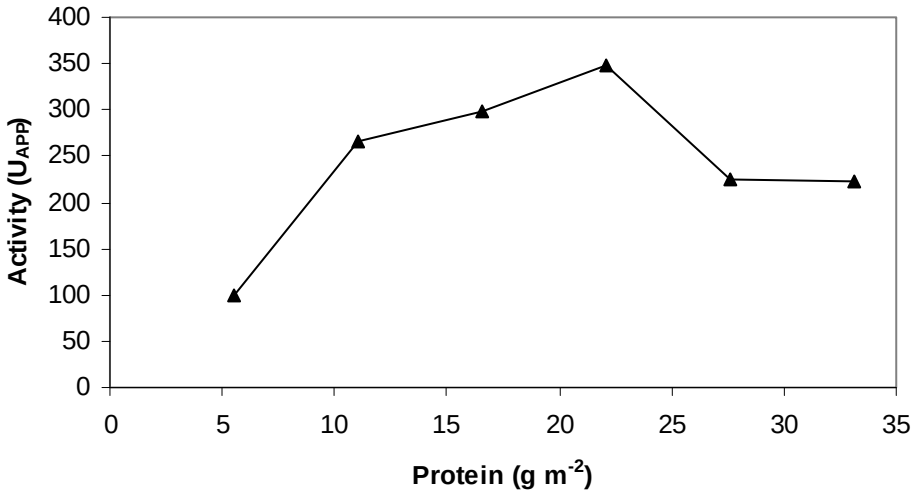
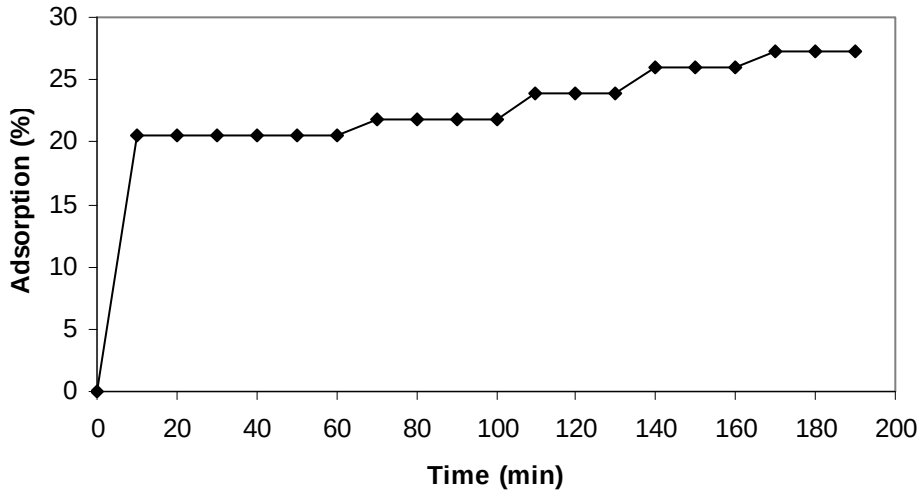


Figure 4: Optimization of enzyme loading time

[The enzyme comprising 29.6 mg protein which corresponds to 22 g m^{-2} was loaded on the membrane coupled with brilliant green (0.2 g) under mild stirring and 0.2 mL sample was removed from the top of the amicon flow cell after every 10 min to estimate the protein. Protein was estimated as mentioned in materials and methods (Enzyme assay); Adsorption (%) = 100 – (Protein concentration in sample x 100 / Protein concentration in feed)]



showed 27% adsorption. The short time (10 min) required for the significant enzyme adsorption (20%) may be due to the presence of ionic interactions between brilliant green and enzyme. This might have results in the enhancement of specific adsorption of the enzyme. As well as in the rejection of non specific protein as the crude enzyme preparation was used for the immobilization. The short time required for adsorption will be more beneficial at industrial applications where increase in operation time results in the increase of product cost. As there was no increase in adsorption up to 60 min, it was decided to carry out further study using 1h as enzyme loading time on the membrane.

5.4.6. Effect of pH

Generally, the environmental conditions such as pH have significant effect on enzyme activity. In comparison to the free enzyme, the changes in optimum pH and pH activity curve of immobilized enzyme depends on the enzyme and or the support charges. These changes are responsible for the generation of partition effects in the microenvironment of the immobilized enzyme and in the domain of the bulk solution [9]. The activities of free and immobilized enzyme were examined at different pH values and the results are presented in Figure 5. The optimum pH for free and immobilized occurred at the same pH 7.2. The pH 7.2 was also used for the production and assay of the enzyme. These results suggested that the microenvironments around the catalytic site of the immobilized and in the bulk remain unaltered. No change in pH pattern of free and immobilized PGA was also reported earlier [28, 29]. The curve pattern of free and immobilized enzyme was almost similar however, immobilized enzyme curve was slightly broader than the free

enzyme. This indicates that the immobilized enzyme is less sensitive to pH than the free enzyme.

Figure 5: Effect of pH

[This experiment was carried out using membrane prepared under identical conditions. The immobilized enzyme activity was estimated using substrate (Enzyme assay) prepared in different pH, following similar procedure as mentioned in materials and methods (Immobilized enzyme activity). The free enzyme activity was carried out as mentioned in materials and methods (Enzyme assay). Relative activity (%) was calculated using higher enzyme activity obtained as 100%.The following buffers were used in the indicated pH ranges; at pH 3.0 – 6.0, 0.1 M citrate; pH 7.2, 0.05 M phosphate; and at pH 8.0 – 10.0, 0.1M borate.]

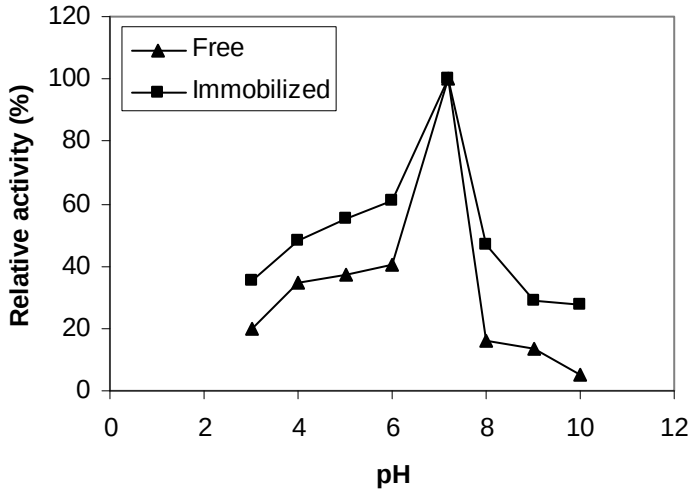
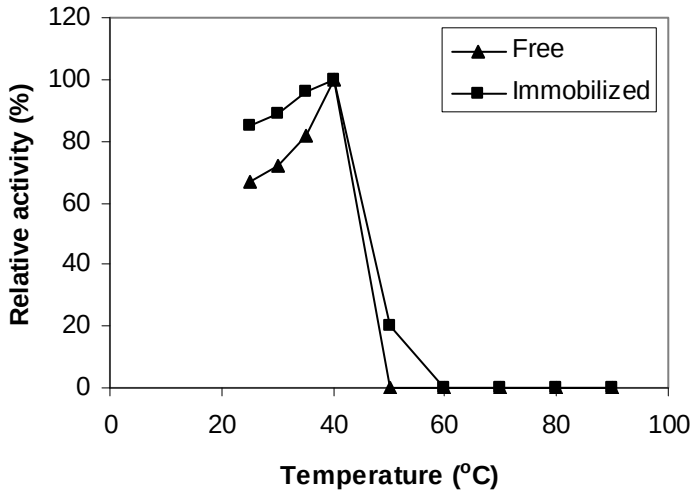


Figure 6: Effect of the temperature

[This experiment was carried out using membrane prepared under identical conditions. The immobilized enzyme activity was estimated at different temperature following similar procedure as mentioned in materials and methods (Immobilized enzyme activity). The free enzyme activity was carried out as motioned in materials and methods (Enzyme assay). Relative activity (%) was calculated using higher enzyme activity obtained as 100%.]



5.4.7. Effect of the temperature

The activities of free and immobilized enzyme were examined at different temperature and the results are presented in Figure 6. The free and immobilized enzyme shows the same optimal temperature at about 40 ºC. The same temperature was also used for enzyme assay. The similar results were reported earlier for free and immobilized enzyme using different support [30]. The curve pattern of free and immobilized enzyme was almost similar however, immobilized enzyme curve was

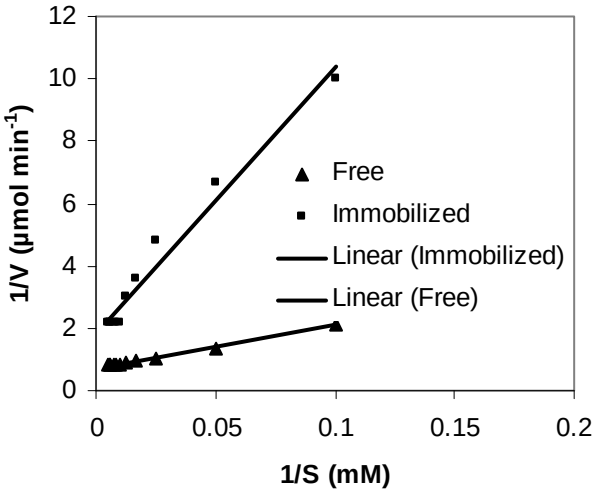
slightly broader than the free enzyme curve below the optimum temperature. The immobilized enzyme also showed around 20% activity at 50 ºC. This indicates that the immobilized enzyme is less sensitive up to50 ºC than the free enzyme.

5.4.8. Effect on kinetic parameters

Generally, the kinetic parameters (K_m & V_{max}) of an enzyme undergo variations after immobilization which indicates change in affinity for the substrate. These variations may occur due to several factors such as protein conformational changes induced by the attachment to the support, steric hindrances and diffusional effects. Effect of these factors results in decrease or increase of the value of apparent K_m. The decrease in the K_m indicates faster reaction rate, whereas an increase of the K_m suggests the requirement of higher substrate concentration to achieve the same reaction rate observed for the free enzyme [31]. To evaluate the effect of immobilization on the kinetic parameters, the activity of enzyme was studied as a function of substrate concentration and the K_m & V_{max} values were determined from the Lineweaver–Burk plots (Fig. 7). After immobilization, an increase in Km value of 19.52 mM to 47.4 mM was observed which corresponds to around 2.4 fold higher than the free enzyme. However, the V_{max} value showed the decrease from 1.4 (μmol min^{-1}) to 0.55 (μmol min^{-1}) which corresponds to around 2.5 fold lower than the free enzyme. This indicates that the immobilization results in lowering the affinity for the substrate with respect to free enzyme. Around 2.5 fold increase in K_m value after immobilization was also reported earlier [32].

Figure 7: Lineweaver–Burk plot

[Kinetic parameters were determined using different concentration of substrate (Enzyme assay). The immobilized enzyme activity was estimated as mentioned in materials and methods (Immobilized enzyme activity). The free enzyme activity was carried out as mentioned in materials and methods (Enzyme assay)].

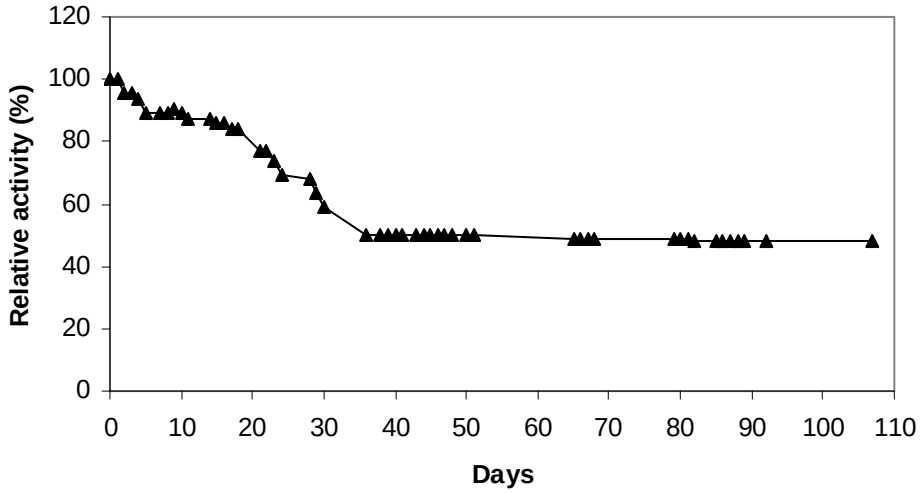


5.4.9. Effect of storage on operational stability

The stability of enzyme activity over longer period of time is essential from its practical application point of view. To evaluate this, the enzyme activity was measured in a batch mode of operation and the gap between two batches was minimum 24h. The effect of storage on operational stability of immobilized enzyme is shown in Figure 8. Total 50 cycles of operations were carried out over 107 days and 84.05%±11.25% average enzyme activity for first 30 days and 22 cycles was obtained, which corresponds to around 16% loss of enzyme activity. Whereas, 48.88%±1.33% average enzyme activity for next 77 days and 28 cycles was observed, this corresponds to around 50% loss of enzyme activity. This indicates that the immobilized enzyme retained almost 50% activity after 107 days and 50 cycles of operation which appears to be significant. In general, the retention of operational stability varies from carrier to carrier. It was reported that the penicillin G acylase immobilized onto Amberlite XAD-7 showed a gradual activity loss upon repeated reuse and after modification its operational life time extended to >400 h at 37 ^{o}C. Whereas, penicillin G acylase immobilized on Eupergit C showed operational stability around 60% over >800 cycles [33]. On the other hand, penicillin V acylase immobilized on the Amberlite CG-50 showed 52% loss of activity over 68 cycles [34].

Figure 8: Effect of storage on operational stability

[The immobilized enzyme activity was estimated every day under identical experimental conditions as mentioned in materials and methods (Immobilized enzyme activity). After completion of the experiment, membrane was washed with buffer (50 mM sodium phosphate buffer pH 7) and stored at 4 ºC. Relative activity (%) was calculated using higher enzyme activity obtained as 100%.]

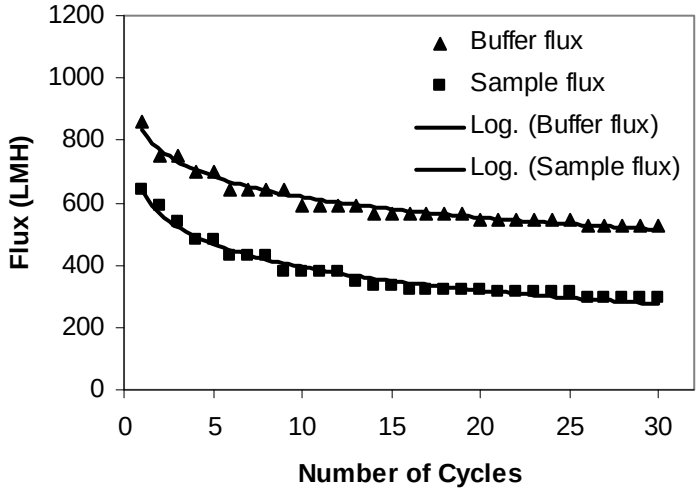


5.4.10. Effect on flux pattern

As an enzyme was immobilized on the membrane therefore it was essential to understand the flux pattern, this will help to asses its operating life. Figure 9 shows the results obtained for 30 cycles of operations. Almost 50% decrease in buffer flux after enzyme immobilization was observed. This indicates that the immobilization procedure has significant effect on the pore size of the membrane. The average buffer flux obtained for the first 1 to 15 cycle was 655±83 LMH whereas for 16 to 30 cycles it was 545±15 LMH. The average sample flux observed for the first 1 to 15 cycle was 436±94 LMH whereas for 16 to 30 cycles it was 310±11 LMH. These results suggest that as the number of cycle increases the deviation in flux value decreases and it moves closer towards the steady state. At the end of the 30 cycles, flux pattern shows almost 38% decrease in buffer flux which means around 60% active flux of the membrane remains intact. This suggests the good operating life of the membrane.

Figure 9: Effect on flux pattern

[LMH - ; L m^{-2} h^{-1}; Buffer flux – flux measured prior to start of the experiment using buffer (50 mM sodium phosphate buffer pH 7); Sample flux – flux measured after completion of the experiment and during the removal of reacted substrate.]



5.4.11. Scale up studies

This study was carried out to understand the behavior of the immobilized enzyme at higher operating volume. This will also helps in understanding the suitability of enzyme immobilized membrane for industrial applications. The enzyme was immobilized on the brilliant green coupled membrane having surface area 72 cm^2. The flat sheet membrane module was used for the construction of membrane reactor (Fig.10). The enzyme activity was estimated using 250 mL different concentration of the substrate (10 to 200 mM) to determine the K_m & V_{max} values of the reactor. The substrate was recicultaed at the cross flow rate of 64 mL min^{-1} for the period of 1 h at 40 ºC which was maintained using water bath. The reactor showed K_m 65.12 mM and V_{max} 4.22 (µmol min^{-1}) towards the substrate penicillin G K^+ salt (Fig.11). In comparison to the kinetic parameters obtained for the 10 mL operating volume (5.4.8. Effect on kinetic parameters), reactor showed 1.37 fold higher K_m and 7.67 fold higher V_{max}. The higher V_{max} obtained at 25 fold higher operating volume clearly indicates that the effect of mass transfer has minimized by changing the operating conditions. This indicates the suitability of developed enzyme immobilized membrane for industrial applications.

Figure 10: Schematic diagram of membrane reactor

[I – Feed (Substrate); II – Peristaltic Pump; III – Membrane module; IV – Permeate; V – Retentate]

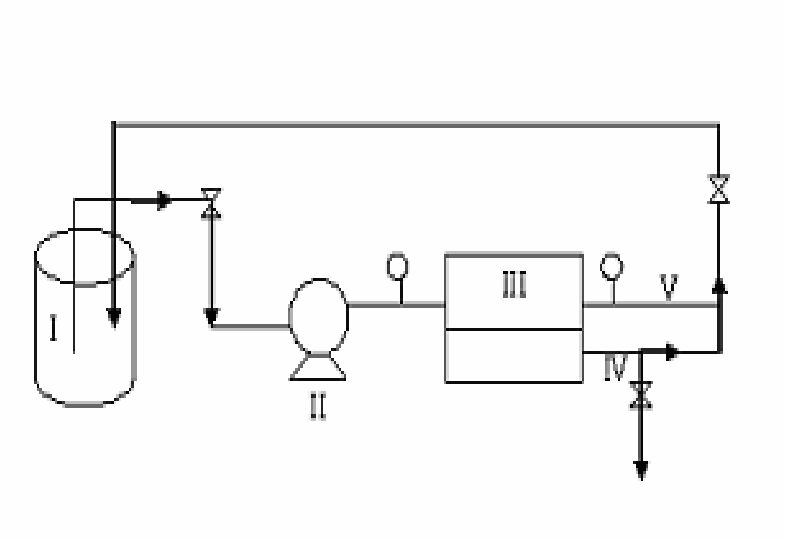
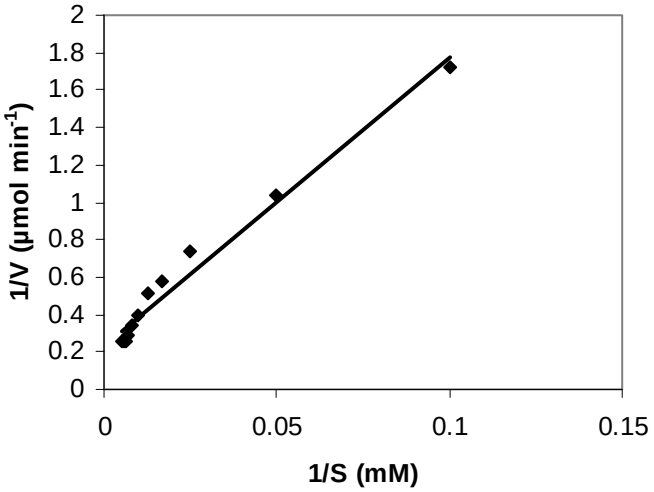


Figure 11: Lineweaver–Burk plot

[Kinetic parameters were determined using flat sheet membrane module and 250 mL operating volume.]



5.5. Conclusion

Penicillin G acylase immobilization was studied using highly porous cellulose based membrane modified with different ionic molecules/compounds. The novelty of the results was the findings of proline and brilliant green for penicillin G acylase immobilization. Proline showed selective adsorption of penicillin G acylase but with less stability, improvement in binding chemistry may enhance the stability. Comparatively, penicillin G acylase immobilized on the brilliant green coupled membrane showed significant activity retention as well as storage and operational stability and flux pattern. The use of enzyme obtained from the modified microbial stain for immobilization will make this technology suitable for industrial applications.

5.6. References

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Chapter: 6

Studies of *Escherichia coli* whole cell immobilization for the production of 6-aminopenicillanic acid: A comparative study

Chapter: 6

6.1. Summary

The evaluation of different parameters was carried out using different form of *Escherichia coli* whole cell. The whole cell obtained using black liquor (6%) in a growth medium showed optimum pH, temperature and K_m comparable with the soluble penicillin G acylase but the operational stability was very poor. The whole cell permeabilized using chloroform and treated with glutaraldehyde showed 93.48%±6.0% average enzyme activity over 5 cycles. Whereas, 86.47%±6.07% average enzyme activity was obtained for 25 days. The permeabilized whole cells entrapped using gelatin and Na-alginate matrix showed 93.23%±4.56% and 90.24%±6.88% average enzyme activity over 5 cycles and for 25 days respectively. The similar optimum pH and temperature was observed for whole cell without any treatment, glutaraldehyde treated whole cell and entrapped whole cells. However, 2.0 fold higher K_m was obtained for entrapped whole cells in comparison with the K_m of glutaraldehyde treated whole cell. The whole cells bind to the cellulose based membrane modified with DEAE showed 4.0 fold higher enzyme activity than unmodified membrane but very poor operational stability. The results indicate that the glutaraldehyde treated whole cell appears to be most suitable for industrial applications.

6.2. Introduction

The whole cell immobilization has a wide range of applications in biological processes such as ethanol production to phenol degradation [1]. The whole cell immobilization has many advantages over immobilized free enzyme however, there

are some disadvantages such as low activity, diffusional restriction and the leaching of cellular components into the reaction mixture [2]. These disadvantages restrict the reuse of biocatalyst which is a basic purpose of immobilization. Therefore, continuous efforts are going on to overcome these problems as to make it suitable for industrial applications [3]. The methodologies so far developed for the whole cell immobilization mainly comprises confining them behind membranes, binding them to a support or entrapping them within a matrix [1]. However, there is a need to develop a cost effective method for the immobilization of whole cell with higher operational and storage stability as to make it suitable for industrial applications.

Penicillin G acylase (E.C. 3.5.1.11) is an enzyme being used commercially for the production 6-aminopenicillanic acid (6-APA) an intermediate for the production of β-lactam antibiotics. The β-lactam antibiotics have around $15 billion annual sales, which make up 65% of the total current antibiotics market and the annual consumption of an enzyme is estimated to be in the range of 10–30 million tons [4]. Thus the use of whole cell may reduce the overall production cost of 6-APA as well as minimizes the problem related to the disposal of biocatalyst upon exhaustion. So far, different techniques have been studied for the immobilization of whole cells with penicillin G acylase activity. [2, 5, 6]. However, not much emphasis was given in the evaluation of operational and storage stability [2]. In the present study we have evaluated different techniques for the immobilization of *Escherichia coli* (*E. coli*) cells with penicillin G acylase activity to obtain higher operational and storage stability.

6.3. Materials and methods

6.3.1. Materials

Benzene, Toluene, Xylene, Ethyl acetate, Diethyl ether, Chloroform, Dichloromethane, Dimethylsulfoxide, Isopropyl alcohol, Acetone, Methanol (Merck, India); Gelatin (Himedia, India); Sodium alginate, Glutaraldehyde (Loba, India); Calcium chloride (SRL, India). All other chemicals used were of ACS grade.

6.3.2. Whole cell production

Escherichia coli (NCIM 2400) was obtained from the National Collection of Industrial Microorganisms, Pune, and maintained routinely on nutrient agar slants. Cells were grown in the defined medium g L^{-1} (3 g peptone, 3 g yeast extract, 3.5 g NaCl, 3 g K_2HPO_4, 0.3 g KH_2PO_4, 60.0 g black liquor, 1 g phenylacetic acid and 0.1 g polypropylene glycol). The pH of the medium was adjusted to 7.2. The inoculum was developed in 250 mL conical flask containing 50 mL medium incubated at 23^0C for 24 h on a rotary shaker. The inoculum was transferred aseptically into a 1L Gallenkamp fermenter with magnetic stirring containing 500 mL medium. The fermentation was carried out at 23 ºC under agitation at 300 rpm and 0.5 vvm aeration for 24h. After fermentation the cells were collected by centrifugation (10,000 g) using Remi C24. The harvested cells were suspended in 50 mM sodium phosphate buffer pH 7.2 (buffer) and the slurry treated as whole cell.

6.3.3. Whole cell penicillin G acylase activity assay

Whole cell penicillin G acylase activity was carried out using 3.72% of penicillin G K^+ salt (Hindustan Antibiotics Ltd. Pimpri, India) as a substrate prepared in 0.1 M

phosphate buffer pH 7.2. The mixture of 2 g slurry containing whole cells and 5.0 mL buffer was prepared using cyclomixer and 0.2 mL mixture was withdrawn to measure OD at 660 nm. Then this mixture was centrifuged (10,000 g) to collect the pellets. The cell concentration was determined from optical density measurements at 660 nm and converted to wet weight with a standard curve. The pellets were mixed with 1.0 mL buffer and then 1.0 mL substrate was added and incubated at 40 ºC for 1 h using shaking water bath (100 rpm). After 1 h, the reaction mixture was centrifuged and 0.2 mL supernatant was removed for the estimation of 6-APA using p-dimethylaminobenzaldehyde reagent [7]. The control was prepared by incubating the mixture of slurry and buffer in a boiling water bath for 5 minutes prior to centrifugation and then treated equally. The whole cell activity was expressed as μmoles of 6-APA formed per minute per g wet weight of cell at pH 7.0 and 40 ºC (U g^{-1}).

6.3.4. Determination of whole cell K_m and stability

The whole cell K_m and stability experiments were carried out in an Amicon stirred cell using micro filtration membrane of 0.45 μm pore size and 13.4 cm^2 surface area. The cell pellets (3.0 g wet weight) were added in to the stirred cell with the help of buffer. The cells were washed three times sequentially with 10 mL buffer. Buffer was removed from stirred cell by applying pressure (0.5 bar). Then 20 mL substrate was added and allowed to react at 40°C for 1 h under mild stirring. The reacted substrate was removed by applying pressure (0.5 bar) for the estimation of 6-APA (6.3.3.). The next run was started for the determination of operational stability after washing the cells with 10 mL buffer three times sequentially. Whereas, for the determination of

storage stability, stirred cell was stored at 4°C containing cells and 10 mL buffer till the next run.

6.3.5. Effect of solvent

Effect of solvent on whole cell penicillin G acylase activity was studied using same methodology as mentioned above (6.3.3.) except the initial step of mixture preparation. The test was prepared using 2 g slurry containing whole cells, 4.5 mL buffer and 0.5 mL respective solvent. The control was prepared using 2 g slurry containing whole cell and 5.0 mL buffer. Both the samples were allowed to mix on cyclomixer for 5 min and then centrifuge. To check the effect of solvent on the leakage of an enzyme penicillin G acylase, the 1.0 mL sample was collected from the supernatant after centrifugation and the estimation of 6-APA was carried out as mentioned above (6.3.3.). The activity in the supernatant was expressed as μmoles of 6-APA formed per minute per mL of supernatant at pH 7.0 and 40 ºC (U mL^{-1}).

6.3.6. Treatment of cell with glutaraldehyde

Treatment of whole cell with glutaraldehyde was studied by mixing 2 g slurry containing whole cells, 4.5 mL buffer and 0.5 mL chloroform for 5 min on cyclomixer and then centrifuge to collect the pellets. The pellets were washed sequentially three time using 5 mL buffer. After washing, the pellets were suspended in a 5 mL glutaraldehyde (2% v/v) and allowed to react for 2 h and then centrifuge. The glutaraldehyde treated pellets were washed sequentially with three time using 5 mL buffer prior to the estimation of activity. The control was prepared by incubating the mixture of glutaraldehyde treated pellets and buffer in a boiling water bath for 5

min prior to the estimation of activity. The K_m and stability experiments were carried out using stirred cell and 3.0 g (wet weight) glutaraldehyde treated whole cells (6.3.4).

6.3.7. Whole cell entrapment in gelatin and Na-alginate matrix

The method described elsewhere was used with some variations for the entrapment of whole cell in gelatin and Na-alginate matrix [8]. The 5.0 g chloroform treated cells (6.3.5) were mixed with 100 mL aqueous solution of 6% w/v gelatin and 2% w/v Na-alginate using magnetic stirrer at 40°C. This mixture was added drop wise using peristaltic pump and silicon tube connected to the microtip into stirred cold 1.0 M calcium chloride solution. After complete addition, the mixture was allowed to stand as it is for 1.5 h and then beads were washed with the buffer till the washing becomes clear. Further, the beads were treated with glutaraldehyde (2% v/v) prepared in buffer for 3 h and then washed with distilled water for several times. The beads obtained were of uniform size and shape. The activity was estimated using 1.0 g beads (wet weight) as mentioned above (6.3.3.). The K_m and stability experiments were carried out using stirred cell and 3.0 g (wet weight) beads (6.3.4.).

6.3.8. Whole cell binding to cellulose based membrane

The cellulose based membrane was prepared as per the procedure described in chapter 4 and in a published paper [9]. The chemical modification of membrane was carried out to study the whole cells binding to the membrane (Table 1). The modified membrane (13.4 cm^2) was fixed in stirred cell and 3 g (wet weight) chloroform treated cells (6.3.5) were added. The cells were kept for 1 h under mild stirring and then removed from the top. Then the membrane was washed with 10 mL buffer three times sequentially. After washing, 20 mL substrate was added and allowed to react at 40°C

for 1 h under mild stirring. The reacted substrate was removed from the bottom side of the stirred cell by applying pressure (0.5 bar) and the estimation of 6-APA (6.3.3.) was carried out. The cell binding to the membrane was estimated by measuring OD at 660 nm before and after loading of the cells (6.3.3.).

6.3.9. Treatment of experimental data

Every experimental point reported in the table and figures represents the average value of three experiments performed under the same conditions. The experimental errors did not exceed 5%.

6.4. Results and discussion

6.4.1. Whole cell without any treatment

Penicillin G acylase is an intracellular enzyme therefore permeabilization of cell membrane is required to facilitate the permeation of substrate and product through the external membrane. So far, different detergents such as triton X-100, tweens, N-cetyl-N,N,N-trimethylammoniumbromide (CTAB) and organic solvents has been used for the permeabilization of cell membrane [6]. However in a present study, significant amount of black liquor (6%) was used in a growth medium (6.3.2.) as a complex organic source. Black liquor is a waste product of pulp and paper industry which contain different volatile organic compounds [10]. Therefore, it was thought that the presence of black liquor in a growth medium might have permeabilized cell membrane up to some extent. This observation was further confirmed as 3.0 fold higher whole cell penicillin G acylase activity was obtained with black liquor in comparison with the growth medium without black liquor. Thus, the evaluations of

different parameters were studied using whole cells without using any conventional permeabilizer to check its suitability for industrial applications.

The whole cells collected after the growth were used as it is to examined penicillin G acylase activity at different pH, temperature, substrate concentration, number of cycle and the results are presented in Fig. 1 - 4.

Figure 1: Effect of pH on penicillin G acylase activity

[The activity was estimated using substrate prepared in different pH, following similar procedure as mentioned in Materials and methods (6.3.3. Whole cell penicillin G acylase activity assay). Relative activity (%) was calculated using higher enzyme activity obtained as 100%.The following buffers were used in the indicated pH ranges; at pH 3.0 – 6.0, 0.1 M citrate; pH 7.2, 0.05 M phosphate; and at pH 8.0 – 10.0, 0.1M borate.]

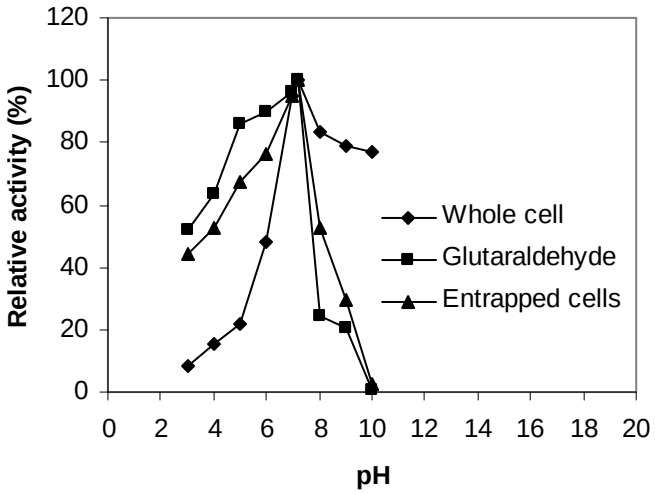


Figure 2: Effect of the temperature on penicillin G acylase activity

[The activity was estimated at different temperature, following similar procedure as mentioned in Materials and methods (6.3.3. Whole cell penicillin G acylase activity assay). Relative activity (%) was calculated using higher enzyme activity obtained as 100%.]

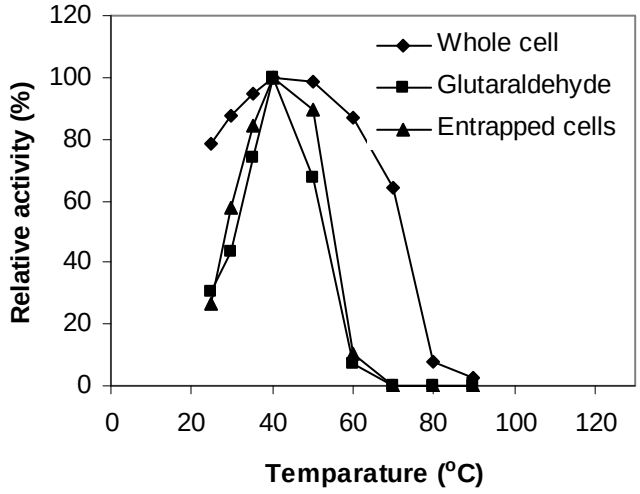


Figure 3: Lineweaver–Burk plot

[Kinetic parameters were determined using different concentration of substrate, following similar procedure as mentioned in Materials and methods (6.3.4. Determination of whole cell K_m and stability)].

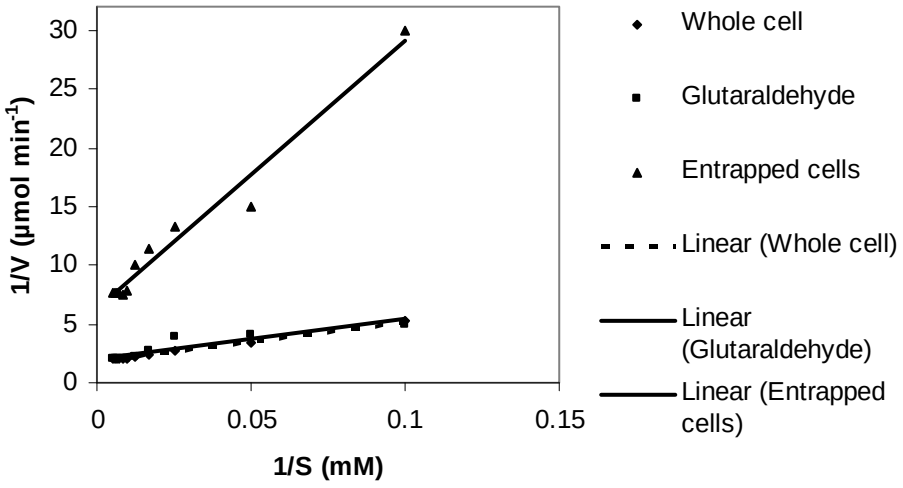
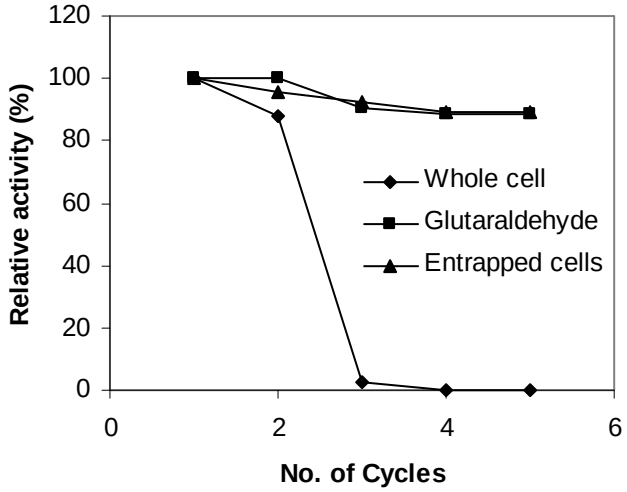


Figure 4: Effect of number of cycles on penicillin G acylase activity

[The activity was estimated sequentially one run after another, following similar procedure as mentioned in Materials and methods (6.3.3. Whole cell penicillin G acylase activity assay)].



The pH 7.2 was obtained as an optimum pH which was similar to the soluble penicillin G acylase as mentioned in chapter 5 and in a published paper [9]. This indicates that the microenvironment around the catalytic site of whole cell enzyme is similar to the soluble enzyme. However, there is marked difference in curve pattern of whole cell and soluble enzyme. The whole cell curve is significantly border in alkaline range (Fig. 1.) in comparison to soluble enzyme [9]. This indicates that the whole cell enzyme is less sensitive to alkaline pH than the soluble enzyme. Similarly, the same optimum temperature (40°C) was observed for whole cell and soluble enzyme. However, the whole cell curve is significantly border in temperature range above 40°C (Fig. 2.) in comparison with the soluble enzyme [9]. This indicates that the whole cell enzyme is less sensitive to the temperature above 40°C than the soluble enzyme. These results clearly indicates that the cell membrane plays a critical role in providing higher resistance to penicillin G acylase deactivation at pH 7.0 to 10.0 and temperature 40°C to 70°C. Interestingly, the whole cell K_m (18.06 mM) determined from the Lineweaver–Burk plots (Fig. 3) was very close to the K_m (19.52 mM) obtained for the crude soluble enzyme [9]. This may be due to the presence of similar microenvironments in and around the active site of whole cell and soluble enzyme. This also suggests that the cell membrane has marginal effect on the substrate affinity. However, the V_{max} value obtained for the whole cell (0.55 μmol min^{-1}) was around 2.6 fold lower than the soluble enzyme. This indicates that the cell membrane has significant effect on the reaction rate. Although the parameters such as optimum pH, temperature and K_m obtained for the whole cell were comparable with the soluble enzyme, the operational stability was very poor (Fig. 4). This indicates that the whole cell needs further modifications as to make it suitable for industrial applications.

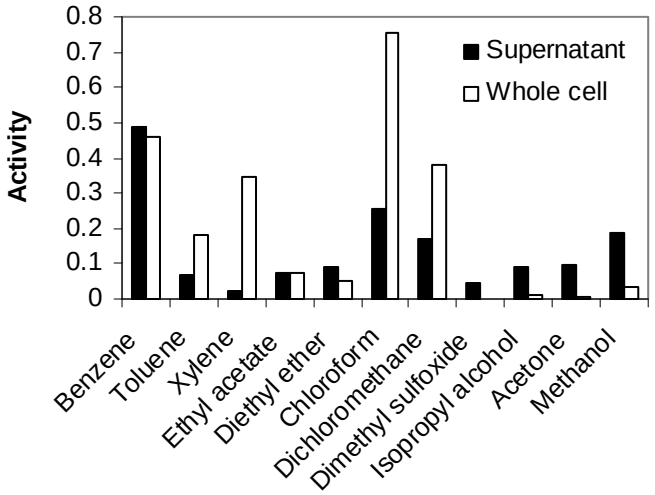
6.4.2. Effect of solvent

This is evident from the above results (6.4.1.) that the further treatment of whole cell is essential for the enhancement of operational stability. In general, immobilization of whole cell can enhance the operational stability [2]. However, it needs different chemical treatments which may hamper the permeation of substrate and product across the cell membrane. Therefore, permeabilization of whole cell is essential prior to immobilization [6]. In the above study (6.4.1.) it was observed that the presence of black liquor in a growth medium can permeabilize whole cell up to some extent which may not be sufficient for immobilization. Thus, this study was carried out to further enhance the permeabilization of whole cell. We have used only solvents for permeabilization, because they are cheaper than the other reagents and may be eliminated by simple evaporation [6]. This will help in the significant reduction of overall process cost at industrial level. Figure 5 shows the effect of different solvents on the whole cell penicillin G acylase activity. The higher activity was obtained with the chloroform, which was 1.85 fold higher than the activity obtained with whole cell without any treatment (6.4.1.). The results obtained with the different solvents are comparable with the results reported earlier [6]. It was reported that the permeabilization of *E. coli* whole cells using chloroform resulted in a 3.48 fold increase of penicillin G acylase activity with marginal leakage in supernatant [6]. On the contrary, a 1.4 fold increase in *E. coli* whole cells penicillin G acylase activity was reported with the most commonly used reagent for microbial whole cells permeabilization such as N-cetyl-N, N, N-trimethylammoniumbromide [8]. However in the present study, the combine increase in activity obtained with black liquor (3.1.) and chloroform treated whole cell was 4.85 fold higher than the untreated cells. But, the activity leakage in the supernatant was relatively higher than the reported earlier

[6]. This may be due to the presence of black liquor in a growth medium which results in higher permeabilization of *E. coli* whole cells. The results clearly suggest that the chloroform treatment is sufficient to make whole cells suitable for immobilization.

Figure 5: The effect of solvents on penicillin G acylase activity

[The activity was estimated using different solvents as per the procedure as mentioned in Materials and methods (6.3.5. Effect of solvent; 6.3.3. Whole cell PGA activity assay; Supernatant activity - U mL^{-1}; Whole cell activity - U g^{-1}].

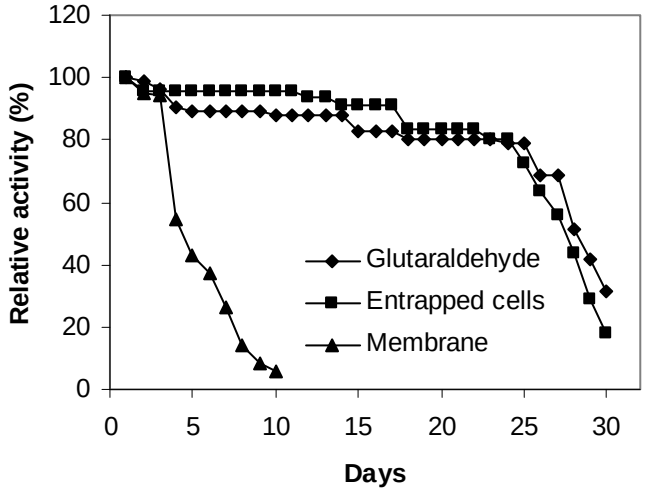


6.4.3. Treatment of cell with glutaraldehyde

Glutaraldehyde is a bifunctional crosslinking reagent, which covalently reacts with the amino groups and forms a good network, thus prevent the leakage of an enzyme [11]. It was reported that the glutaraldehyde treatment of permeabilized whole cells [5] or pore matrix in which permeabilized whole cells are entrapped [2, 8] can enhance the operational stability. In the present study the chances of enzyme leakage was higher due to the higher permeabilization (4.85 fold) of *E. coli* whole cells (6.4.2). Therefore, this study was carried out to check the effect of glutaraldehyde on the physico-chemical parameters and operational stability of permeabilized whole cell PGA. Figure 1 – 4 and 6 shows the effect of glutaraldehyde on permeabilized whole cell penicillin G acylase activity at different pH, temperature, substrate concentration, number of cycle and storage stability. The pH 7.2 was obtained as an optimum pH which was similar to the whole cell without any treatment (6.4.1.). This indicates that the microenvironments around the catalytic site in glutaraldehyde treated whole cell and whole cell without any treatment remain same. On the contrary, the glutaraldehyde treated whole cell curve is significantly broader in acidic range (Fig. 1.) whereas, it is in alkaline range for the whole cell without any treatment (Fig. 1.).

Figure 6: The effect of storage on penicillin G acylase activity

[The activity was estimated with the gap of 24 h between two runs, following similar procedure as mentioned in Materials and methods (6.3.4. Determination of whole cell K_m and stability)].



This indicates that the glutaraldehyde treated whole cell enzyme is less sensitive to acidic pH. Similarly to the optimum pH, the same optimum temperature (40°C) was observed for glutaraldehyde treated whole cell and whole cell without any treatment (Fig. 2.). However, the glutaraldehyde treated whole cell curve is significantly sharp in comparison to the whole cell without any treatment. This indicates that the glutaraldehyde treated whole cell enzyme is more sensitive to the temperature above 40°C than the enzyme of whole cell without any treatment. These results clearly indicate that the glutaraldehyde treatment of whole cell after permeabilization may protect the enzyme at acidic pH but not at higher temperature. Surprisingly, the glutaraldehyde treated whole cell K_m (17.44 mM) determined from the Lineweaver–Burk plots (Fig. 3) was very close to the K_m (18.06 mM) obtained for the whole cell without any treatment. Similarly, the V_{max} value obtained for the glutaraldehyde treated whole cell (0.50 μmol min^{-1}) was very close to the V_{max} value (0.55 μmol min^{-1}) of whole cell without any treatment. This indicates that the higher permeabilization of whole cells has minimized the adverse effect of glutaraldehyde on substrate affinity and reaction rate. Secondly, the higher permeabilization of whole cells may have facilitated the maintenance of similar microenvironments which results in almost similar K_m and V_{max} values. On the contrary, about 30% decreased in whole cells penicillin G acylase activity has been reported due to the action of glutaraldehyde [2]. Although the parameters such as optimum pH, temperature and K_m obtained for the glutaraldehyde treated whole cell were comparable with whole cell without any treatment (3.1.) but there was significant difference in operation stability. Figure 4 shows the significant improvement in operation stability of glutaraldehyde treated whole cell. The average activity obtained for the 5 cycles was 93.48%±6.0%. It was reported that the glutaraldehyde treated permeabilized cells retain 100% penicillin G

acylase activity even after 8 cycles however, there was no information about storage stability [5].

The stability of whole cell penicillin G acylase activity over longer period of time is essential from its practical application point of view. To evaluate this, the whole cell penicillin G acylase activity was measured in a batch mode of operation and the gap between two batches was minimum 24 h. The effect of storage on operational stability of glutaraldehyde treated whole cell is shown in Figure 6. The significant stability of glutaraldehyde treated whole cell penicillin G acylase activity was observed up to 25 days. The average activity obtained for the 25 days was 86.47%±6.07%. The results clearly indicate that the glutaraldehyde treatment of permeabilized whole cell is essential to achieve the significant improvement in operational and storage stability of whole cell penicillin G acylase.

6.4.4. Whole cell entrapment in gelatin and Na-alginate matrix

The entrapment of microbial whole cells using polymeric matrices is the most common methodology studied so far [3]. In general, different kinds of matrices are used for the entrapment of whole cells [3] such as ionic hydrogels (calcium alginate, k-carrageenan etc.), thermogels (agar, agarose, cellulose), and synthetic polymers (polyacrylamide, polyvinyl alcohol, and polyurethane). The combination of naturally occurring polymeric matrix such as gelatin and Na-alginate has been reported for the entrapment *E. coli* whole cells containing penicillin G acylase, which showed low K_m toward penicillin G as a substrate [8]. However in this study, permeabilization of whole cells was carried out using N-cetyl-N, N, N-trimethylammoniumbromide reagent prior to the entrapment, which results in 1.4 fold increase in penicillin G

acylase activity [8]. Therefore, we have used the same matrix to check its effect on the performance of entrapped highly permeabilized (4.85 fold) whole cells (6.4.2.).

Figure 1 – 4 and 6 shows the effect of entrapment on the highly permeabilized whole cell penicillin G acylase activity at different pH, temperature, substrate concentration, number of cycle and storage stability. The pH 7.2 was obtained as an optimum pH which was similar to the whole cell without any treatment (6.4.1.). This indicates that the microenvironments around the catalytic site of entrapped whole cell and whole cell without any treatment remain same. On the contrary, the entrapped whole cell curve is broader in acidic range but less than the curve of glutaraldehyde treated whole cell (Fig. 1.). This indicates that the entrapped whole cell enzyme is less sensitive to acidic pH but not similar to the glutaraldehyde treated whole cell. Similarly to the optimum pH, the same optimum temperature (40°C) was observed for entrapped whole cell and whole cell without any treatment (Fig. 2.). However, the entrapped whole cell curve is significantly sharp in comparison to the whole cell without any treatment. It is also almost similar to the curve of glutaraldehyde treated whole cell (Fig. 2.). This indicates that the entrapped whole cell enzyme is more sensitive to the temperature above 40°C than the enzyme of whole cell without any treatment. These results clearly indicate that the entrapped whole cell after highly permeabilization may protect the enzyme at acidic pH but not at higher temperature. Interestingly, the entrapped whole cell K_m (35.99 mM) determined from the Lineweaver–Burk plots (Fig. 3) was almost 2.0 fold higher than the K_m (18.06 mM) obtained for the whole cell without any treatment. Similarly, the V_{max} value obtained for the entrapped whole cell (0.158 μmol min^{-1}) was around 3.48 fold lower than V_{max} value (0.55 μmol min^{-1}) of whole cell without any treatment. This indicates that the

entrapment has significant effect on the substrate affinity and reaction rate. This means that the entrapped whole cell failed to minimize the conventional problem of mass transfer associated with the entrapped whole cells [3]. Although the optimum pH and temperature obtained for the entrapped whole cells were similar to the whole cell without any treatment (6.4.1.) but there was significant difference in kinetic parameters and operation stability. Figure 4 shows the significant improvement in operation stability of entrapped whole cells, which was almost similar to the glutaraldehyde treated whole cell. The average activity obtained for the 5 cycles was 93.23%±4.56%. It was reported that the entrapped *E. coli* whole cells in a similar matrices showed decreasing trends of operational stability and it retain nearly 50% activity at the 6 cycles however, there was no information about storage stability [8]. The storage stability of entrapped whole cells was studied by measuring its penicillin G acylase activity in a batch mode of operation and the gap between two batches was minimum 24 h. The effect of storage on operational stability of entrapped whole cells is shown in Figure 6. The significant stability of entrapped whole cells penicillin G acylase activity was observed up to 25 days. The average activity obtained for the 25 days was 90.24%±6.88% which was slightly higher than the activity observed for glutaraldehyde treated whole cell (3.3). It was reported that the whole cells of *Alcaligenes faecalis* containing penicillin G acylase entrapped in a polyvinyl alcohol crosslinked with boric acid and glutaraldehyde showed retention of 100% activity after 15 cycles and about 65% enzyme activity at the end of the 31 cycle [2]. However, the results clearly indicate that the entrapment of whole cells in a combination of naturally occurring polymeric matrix such as gelatin and Na-alginate helps in the enhancement of operational and storage stability of whole cell penicillin G acylase.

6.4.5. Whole cell binding to cellulose based membrane

The higher K_m obtained for the entrapped whole cell in the above study (6.4.4.) indicates the effect of mass transfer limitations. It was reported that the accumulation of product and generation of pH gradients are the major drawback in the bead form of carrier, which has been mostly used for the penicillin G acylase immobilization [12]. This suggests the need for development of different type of carrier configuration for the whole cell/soluble penicillin G acylase immobilization. In comparison with the conventional sources, polymeric membranes appear to be suitable carrier for the whole cell/soluble enzyme immobilization. Because, substrate molecules can be convected through the membrane support rather than having to diffuse to a bead or particle to reach the active site of an enzyme. This helps in the enhancement of mass transfer efficiency significantly. Comparatively, the use of unconventional source of a carrier for whole cell/soluble penicillin G acylase immobilization is not widely reported. There are some reports where nylon [13] and cellulose based [9] polymeric membranes were used for soluble penicillin G acylase immobilization. Therefore, the whole cell binding to cellulose based membrane was studied.

Table 1 shows the different methodologies used for the binding of permeabilized whole cells containing penicillin G acylase to the membrane. In comparison with the unmodified membrane, the membrane (3B., Table 1) modified with HCl and then treated the adsorbed/bind whole cells with glutaraldehyde showed 1.73 fold increase in penicillin G acylase activity. Whereas, 4.0 fold increase in penicillin G acylase activity was observed where membrane (4C., Table 1) was modified with DEAE and then adsorbed/bind whole cells treated with glutaraldehyde. This significant increase

in penicillin G acylase activity indicates the suitability of cellulose based membrane for whole cell immobilization. However, its suitability for industrial applications depends on its operational stability. The effect of storage on operational stability of whole cell bind to the membrane is shown in Figure 6. Around 94% operational stability of whole cell penicillin G acylase activity was observed up to 3 days and then it decreased sharply.

Table 1: Effect of glutaraldehyde and DEAE on immobilization of whole cell to the membrane

Treatment	Treatment conditions	Preparation prior cell loading	Cell binding (g m^{-2})	Immobilized cell Activity (U_{App})
1. Membrane without any Modification + cell	Nil	Washing with Buffer	216±3	55.97±1.1
2. Membrane + cell + glutaraldehyde (5% vol/vol) prepared in Buffer	RT for 1 h at mild stirring	Washing with Buffer	287±7	74.62±1.5
3A. Membrane + 2 N HCl prepared in DW	60ºC for 1 h at mild stirring	Washing with DW & Buffer	-	-
3B. Membrane after treatment A + cell + glutaraldehyde (5% vol/vol) prepared in Buffer	RT for 1 h at mild stirring	Washing with Buffer	363±10	97.01±2.4
4A. Membrane + Epichlorohydrin (0.5% v/v) prepared in 0.1 N NaOH + 0.2% sodium borohydride	40ºC for 1 h at mild stirring	Washing with DW	-	-
4B. Membrane after treatment A + DEAE (5.0% wt/v) prepared in 0.1N NaOH + 0.2% sodium borohydride	40ºC for 1 h at mild stirring	Washing with 0.1N NaOH followed by 0.1N HCl DW& buffer	-	-
4C. Membrane after treatment A &B + cell +glutaraldehyde (5% vol/vol) prepared in buffer	RT for 1 h at mild stirring	Washing with Buffer	772±33	226.36±7.3

[U_{App} - µmoles min^{-1} m^{-2} of membrane; Cell binding (g m^{-2}) - wet weight of cell bind to membrane; DW - distilled water; RT – room temperature (28±2°C); Mild stirring - ~100 rpm; Buffer - 50 mM sodium phosphate buffer pH 7.2; DEAE - 2-diethylaminoethyl chloride hydrochloride]

The average activity obtained for the 1 -5 days was 77.25%±26.59% and for the 5 – 10 days was 22.47%±15.4%. The results clearly indicate that the whole cells bind to the membrane in the present form is not suitable for industrial applications. There is need for the development of suitable binding chemistry as to enhance the penicillin G acylase storage and operational stability of whole cells bind to the membrane.

6.5. Conclusion

In the present study we have evaluated the different form of whole cell such as whole cell without any treatment, glutaraldehyde treated whole cell, entrapped whole cells and whole cells bind to the cellulose based membrane. However, the glutaraldehyde treated whole cell and entrapped whole cells showed significant storage and operational stability. Comparatively, glutaraldehyde treated whole cell appears to be most suitable for industrial applications because of low preparation cost and K_m (17.44 mM) similar to the K_m (19.52 mM) of crude soluble enzyme [9]. However, the pilot scale trials using the permeabilized glutaraldehyde treated whole cell of microbial strain being used at industrial level is essential prior to any conclusion. But considering the commercial importance of penicillin G acylase, the use of permeabilized glutaraldehyde treated whole cell in 6-APA production at industrial level will significantly reduce the overall production cost. And also, this will minimize the after use disposal problem of annually consumed enzyme which is in the range of 10–30 million tons [4].

6.6. References

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Chapter: 7

General discussion and Conclusion

Chapter: 7

7.1. Discussion

In general, reduction in overall biotech product cost depends on the four basic factors such as 1. the microbes selected, adapted or engineered for higher yields of products; 2. the process selected, adapted or engineered for low capital and operating costs; 3. the microbial growth substrate or process feed stock adapted for low cost; and 4. the process byproducts minimal or managed as saleable products rather than as wastes [1]. In the present thesis, we have tried to cover 2^{nd} and 3^{rd} factor to achieve the overall reduction in 6-APA production cost. As well as, indirectly we have also covered 4^{th} factor for pulp and paper industry by using their waste product for the production of high value product such as penicillin G acylase.

In chapter 2, we have carried out different studies in order to invent low cost microbial growth substrate for the production of penicillin G acylase. Our findings clearly indicate the suitability of black liquor a waste product of pulp and paper industry [2] as a complex organic source for the production of penicillin G acylase, which was not reported earlier. The industrial production of 6-APA (precursor of the semisynthetic penicillins) using penicillin acylase is reported to be around 9000 tons per year [3], and it may keeps on increasing in coming years. At such a large scale, the use of black liquor as a microbial growth substrate may help in the reduction of overall production cost as well as in the management of environmental pollution.

In chapter 3, we have tried to achieve the advantages of the use of membrane such as large surface area, short diffusion path and low pressure drop [4] in the purification of

penicillin G acylase. Our findings clearly indicate the suitability of membranes in the purification of penicillin G acylase, which was not reported earlier. The adaptation of present purification method at industrial level will give significant economical advantage.

In chapter 4, we have described a method for the production of chemically more reactive novel cellulose based porous membrane suitable for biotech applications. Our membrane has the advantages such as high flux, good hydrophilicity, excellent film-forming ability, good mechanical properties, and high chemical reactivity. The membrane preparation process does not involve heating, cooling and highly hazardous chemicals. Another advantage is of high flux after binding of chemically reactive molecules, which require in biotech applications. This property of the developed membrane makes it suitable for commercial exploitation. As it is a charged membrane, it may be used without any further amplification of the number of active groups, in various applications such as bioseparation, enzyme immobilization etc. It can also be modified easily to alter the charged either positively or negatively without major reduction in flux. All these properties of the developed membrane make it suitable for biotech applications such as membrane bioreactors, chromatographic separation, affinity chromatography or related unit operations generally carried out in biotech industries.

In chapter 5, we have described the suitability of our invented membrane for the immobilization of penicillin G acylase. The novelty of our findings was the selective adsorption of penicillin G acylase obtained by the proline and suitability of brilliant green for penicillin G acylase immobilization, which was not reported earlier.

Penicillin G acylase immobilized on the brilliant green coupled membrane showed significant activity retention as well as storage and operational stability and flux pattern. This strongly indicates its suitability for industrial applications. The use of brilliant green coupled membrane for the immobilization of penicillin G acylase will certainly help in the reduction of 6-APA production cost at industrial level.

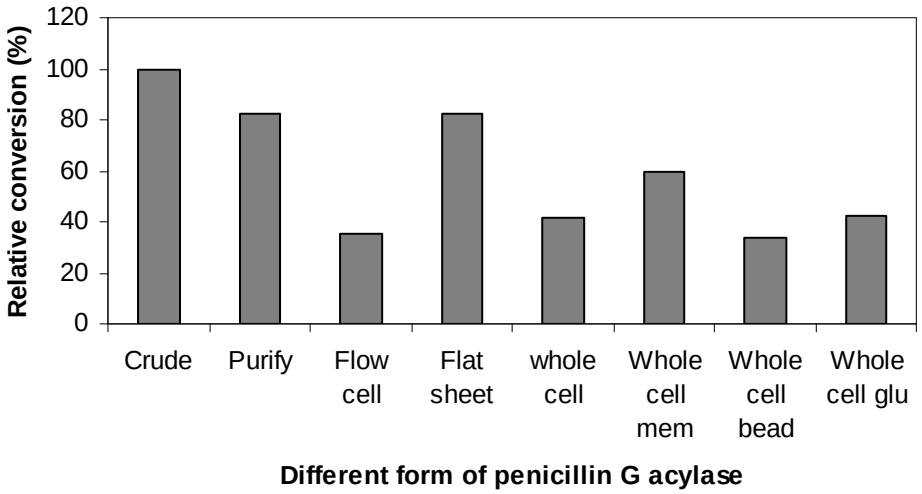
In chapter 6, we have evaluated the different form of whole cell such as whole cell without any treatment, glutaraldehyde treated whole cell, entrapped whole cells and whole cells bind to the cellulose based membrane. This was done to find out most suitable form of whole cell for industrial applications. As the use of whole cell as a biocatalyst in combination with the membrane at industrial level will be the potentially most economical unit operation. Our findings strongly suggest the use of permeabilized glutaraldehyde treated whole cell in 6-APA production at industrial level will significantly reduce the overall production cost. And also, this will minimize the after use disposal problem of annually consumed enzyme which is in the range of 10–30 million tons [5].

7.2. Conclusion

It can be seen from the above discussion that the over all approach of the present thesis was to develop an economical process for the 6-APA production suitable at industrial level. In the thesis, we have used different form of penicillin G acylase for the production of 6-APA. Table 1 shows the substrate conversion obtained by the different form of penicillin G acylase. In comparison to crude preparation, the higher and almost similar substrate conversion was obtained with the purified enzyme (82.32 ± 0.59) and flat sheet module study (82.35 ± 1.14) carried out in chapter 5 (5.4.11.

Scale up studies). It was also observed that the significant activity retention as well as storage and operational stability and flux pattern can be obtained using penicillin G acylase immobilized on the brilliant green coupled membrane (Chapter 5). This can be concluded that the method invented in the present thesis for the production of 6-APA using membrane has a great potential to be used at industrial level.

Table 1: Substrate conversion by different form of penicillin G acylase



7.3. References

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7.4. List of patent and publications from thesis

7.3.1. Patent filed

1. Adikane HV, Thakar DM, Kharul UK (2006) A process for the preparation of cellulose based porous membrane useful for the separation of biomolecules, Indian Patent (0849/DELI/2006).

7.31. Paper published

1. Adikane HV, Thakar DM (2009) Studies of Penicillin G Acylase Immobilization Using Highly Porous Cellulose-Based Polymeric Membrane. Applied Biochemistry and Biotechnology. Pages 1-16 (Available online).

7.3.2. Paper under communication.

1. "Studies of *Escherichia coli* whole cell immobilization for the production of 6-aminopenicillanic acid: A comparative study" H. V. Adikane and D. M. Thakar.

2. "Use of black liquor a waste product of pulp and paper industry for the production of *Escherichia coli* penicillin G acylase" H. V. Adikane and D. M. Thakar.

3. "Purification of penicillin G acylase using ultrafiltration polymeric membranes" H. V. Adikane and D. M. Thakar.