L-Glutaminase production by an immobilized marine *Pseudomonas* sp. BTMS -51

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Certificate

This is to certify that the work presented in the thesis entitled "L-Glutaminase production by an immobilized marine *Pseudomonas* sp-BTMS-51" is based on the original research done by Mr. Rajeev Kumar S, under my guidance and supervision, at the Department of Biotechnology, and no part of the work has been included in any other thesis for the award of any degree.

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1. INTRODUCTION

Biotechnology is professed to catalyse positively the socio-economic development of mankind in the new millennium and among the various biotechnologies, enzyme biotechnology has a major role to play. Enzymes are the central dogma of life and catalyse thousands of biochemical reactions both inside the cell and outside the cell in the environment and hence there is no life without enzyme. From the role of an inevitable biocatalyst in any living cell mediating life, they have assumed greater significance in the day to day activities of mankind. Consequently enzymes have become one of the major commodity today and their manufacture and applications, in the recent past, has emerged as one of the major industry with a global market exceeding US$ 500 million and is expected to record further growth in the coming years.

Enzymes are frequently used, as biocatalyst for process improvement, to enable utilisation of new types of raw materials, production of metabolites through biotransformations, texture improvement in textiles, leather processing, detergents, flavour improvement of food, food processing, waste treatment and several other applications. Enzymes also have tremendous potential as therapeutic agents. Among the industries, food industry is by far the largest consumer of commercial enzymes (Lonsane & Ramakrishna, 1989, Bhotmange and Shastri, 1994) followed by textile, detergent and leather industries.

Enzyme based analytical protocols like ELISA have revolutionised the biomedical field and highly sensitive biosensors based on immobilized enzymes (Karube, 1988., Brueckel *et al*, 1990) have replaced conventional analytical procedures used in the health care industry. Enzyme based biosensors have also been employed in food technology (Wagner & Schmid, 1990), fermentation control (Bradley *et al*, 1989), and environmental monitoring (Rawson *et al*, 1989). With the overwhelming increase in biotechnology-based industries and research world wide, the demand for speciality enzymes like nucleases and polymerases has increased, and enzyme based analyses and diagnostic protocols are rapidly developing. This is expected to cause a boost in the enzyme industry especially in the production of high-cost analytical grade enzymes. Klein & Langer (1986) had reviewed the

application of immobilized enzymes in clinical medicine and proposed a bright future for enzyme based drug therapies.

Developments in enzyme-based processes and their applications in several fields have resulted in an increase in the quest for novel enzymes with improved activities in the recent years and a wide variety of sources are being explored for enzymes with commercial applications. Although animal, plant and microbial enzymes have been used in industries in the past, current trend is replacement of animal and plant enzymes with microbial enzymes. This is mainly due to the ease with which microbial enzymes can be obtained, and as they are generally more stable. Furthermore, the economic production, consistency, ease of process optimisation, and modification, along with the possibility of enzyme production using genetically engineered microorganisms with enhanced yield have qualified microorganisms as the apt sources of industrial enzymes. Often extracellular enzymes are preferred over intracellular or cell-bound enzymes, as they are easier to isolate (Harrison, 1987). About 80 % of the industrial enzymes are produced from microorganisms, owing to the fact that a wide spectrum of enzymes are elaborated by them, which offer an opportunity to select specific enzymes for specific purposes (Bhotmange & Shastri, 1994). In fact, intensive screening is perused all over the world for enzymes with novel properties and functions from various microorganisms inhabiting different environments (Fogarty & Kelly, 1990).

Interest in amidohydrolases such as L-asparaginase and L-glutaminase started with the discovery of their antitumour properties (Broome, 1961., Roberts *et al*, 1970., Bauer *et al*, 1971). L-glutaminase apart from its use as a therapeutic enzyme, also earns its importance in food industry as a flavour enhancing agent (Yokotsuka, 1985., Koibuchi *et al*, PT- WO 9960104, 1999). Recently, several other applications has been proposed for L-glutaminase and the enzyme is used in biosensors for determination of glutamine and glutamate in pharmaceutical formulations (Botre *et al*, 1993), mammalian cell cultures (White *et al*, 1995., Mulchandani & Bassi, 1996), liquid samples (Luong *et al*, PT-CA 2109896, 1994), and hybridoma cultures (Meyerhoff *et al*, 1993). Glutaminase is also used in the industry for manufacture of speciality chemicals like threonine (PT- JP5068578, 1993., JP11225789, 1999) and other γ-glutamyl alkylamides (PT-JP05284983, 1993., Tachiki *et al*, 1998). Immobilized glutaminase is also used in conversion of glutamine to glutamate

specifically employed in flavour enhancement of liquid seasonings (Koseko *et al*, 1994). Since the industrial sources of L-glutaminase are limited and mainly confined to few species of *Bacilli*, *Yeast*, and *Apsergillus oryzae*, the search for potential microorganisms that hyper produce the enzyme with novel properties, and economically viable bioprocesses for their industrial production is perused all over the world (Nagendraprabhu & Chandrasekaran, 1995).

Marine biosphere is one of the richest of earth's innumerable habitats, and yet is one of the least characterised. Because of the diversity and scale, it offers enormous opportunities for non-destructive exploitation within many facets of modern biotechnology (Sabu, 1999). Although the oceans cover more than two thirds of the world's surface, the knowledge on marine microbes is still very limited, and they remain as untapped sources of many metabolites with novel properties (Faulkner, 1986, Chandrasekaran, 1996). Marine bacteria and their enzyme systems are useful in several industrial applications due to their increased tolerance to salinity, and consequently are being intensively screened for products of economic importance (Moriguchi *et al*, 1994).

In view of the tremendous potential of marine microbes, as sources of useful metabolites, there exist an urgent need to develop viable bioprocess technologies for efficient utilisation of them, besides appropriate selective isolation methods and screening programs for novel metabolites. Efforts have recently been made in this direction and there are reports on marine microorganisms, which are potent producers of adhesives (Abu *et al*, 1992) roslipins (Tomoda *et al*, 1999), pyrostatins (Aoyama *et al*, 1995), siderophores (Martinez *et al*, 2000), Hydrogen (Matsunaga *et al*, 2000) anti-HIV compounds (Schaeffer & Krylov, 2000) and several enzymes (Farrell & Crosa, 1991., Moriguchi *et al*, 1994., Kim *et al*, 1999, Araki *et al*, 1999., Sabu *et al*, 2000). With the emerging interest in marine bioresources, researchers world-wide have started to look upon marine microorganisms as potent sources of enzymes with improved and novel properties important for the industry, pharmaceuticals and research. Reports are available on the production of industrially important enzymes like protease (Michels & Clark, 1997), amylase (Brown & Kelly, 1993), lipase (Ando *et al*, 1991, 1992), cellulase (Bronnenmeier *et al*, 1995), agarase (Vera *et al*, 1998), DNA polymerase (Lundberg *et al*, 1991), chitinase (Suresh & Chandrasekaran, 1999), glutaminase (Nagendraprabhu & Chandrasekaran, 1995.,

Sabu *et al*, 2000), and several other enzymes. Niehaus & Antranikian (1997) had recently reviewed the production and application of heat stable enzymes from marine microorganisms. Nevertheless, much of these efforts were focussed on basic studies and as such very little information is available on the large-scale production of marine microbial enzymes.

Marine bacteria are recognised for their ability to colonise immersed surfaces (Austin, 1988) and most of them are able to obtain sufficient nutrients, only when they grow as *aufwuchs*, a condition during which the bacteria remain adsorbed onto solid particles (Chandrasekaran, 1996). The unique property of marine bacteria to adsorb on solid supports make them ideal candidates for immobilized cell processes and indeed a *Marinobacter* sp. adsorbed on porous glass beads was tested for degradation of a hydrophobic C-18 isoprenoid ketone (Bonin *et al* 2001). In spite of the fact that marine bacteria possess the ability to colonise immersed surfaces and consequently become ideal candidates for immobilized whole cell processes, such processes has rarely been attempted in the production of extracellular enzymes by marine microorganisms.

Conventionally, commercial scale production of microbial metabolites are carried out by submerged fermentation (SmF) which allows a better process control and automation together with reduced risk of contamination. There is resurgence in interest, in the use of solid state fermentation (SSF) for large-scale manufacture of microbial metabolites (Lonsane, 1994). The application of immobilized living microbial cells represents a new, fascinating and rapidly growing trend in microbial technology. Immobilization of microbial cells represents the transfer of the cells from a free state to a state of confinement or localisation in certain defined region of space with retention of catalytic activity and often with retention of viability so that the cells can be used repeatedly or continuously (Klein & Wagner, 1983). Natural biofilms also qualify as immobilized cultures, and adsorbed cells are increasingly being used in bioprocesses for metabolite production (Truck *et al*, 1990a,b., Linko *et al*, 1996) and waste treatment (Wong *et al*, 1993., Fuji *et al*, 2000). Compartmentalisation cells implies that the cell density in solid phase is very high, with cell loading greater than 10^{10} cells per cm^3 of support matrix not an uncommon occurrence (Chibata, 1979., Klein & Wagner, 1983); a very attractive feature in increasing the productivity per unit reactor volume.

In contrast with the batch or continuous submerged fermentations, where free cells are utilised for metabolite production, immobilized cells offer several advantages (Kolot, 1981), which include the acceleration in reaction rate due to increased cell density per unit reactor volume and feasibility for using high dilution rates, as wash out of cells is not a problem with immobilised systems. Cell metabolism (Galazzo & Bailey, 1989) and cell wall permeability (Fletcher & Marshall, 1982) is increased upon immobilization, and as the cells are able to multiply on or inside the support matrix, they can be activated on site if needed. Also, use of immobilized cells eliminate the need of costly steel fermenters, and the production plants can be designed to be smaller in size, comprising of columns packed with immobilized cells. As a result, better process control could be achieved (Kolot, 1981).

Cell immobilization technology is particularly suited for production of extracellular enzymes and there is a growing interest in applying cell immobilization techniques for continuous production of enzymes (Ramakrishna & Prakasham, 1999). This offers several advantages over the conventional processes, which include the easy separation and reuse of cells, high cell concentrations, flexibility in reactor design, and operation as well as stabilisation of several cell functions (Dervakos and Webb, 1991).

Immobilized whole cell systems have been successfully employed in the production of several extracellular enzymes by terrestrial microbes e.g. α-amylase (Duran-Paramo *et al*, 2000), glucoamylase (Fiedurek & Szczodark, 1995), Lipase (Ferrer & Sola, 1992), Protease (el-Aassar *et al*, 1990), Cellulase (Xin and Kumakura, 1993), Xylanase (Mamo & Gessesse, 2000), and Ribonuclease (Manolov, 1992).

Several methods of immobilization have been attempted and by far the commonest methods used in immobilization of whole cells is gel entrapment in alginate or carrageenan and physical adsorption on solid supports, probably because these methods are less harsh on the living cells. The supports used for cell adsorption are wide and varied, which includes ceramics (Shiraishi *et al*, 1989), porous glass (Jager & Wandrey, 1990), polyurethane foam (Haapala *et al*, 1994), Nylon web (Linko *et al*, 1996) stainless steel biomass support particles (Webb *et al* 1986), and *Luffa* sponge (Ogbonna *et al*, 1994). However, the industrial use of immobilized

viable microbial cells is limited to few processes, the most important of which is ethanol production.

Though the immobilized cell processes offer several advantages over the conventional fermentation technologies, a complete exploitation of these potential advantages in industrial applications will strongly depend on the wise selection of a set of processing parameters allowing for high productivity combined with extended operational stability. This set includes; immobilization method, mode of operation (repeated batch vs continuous), aeration and mixing, bioreactor configuration, medium composition (including feeding of substrates, precursors or additional nutrients), temperature, pH, and whenever required, in situ product and/or excess biomass removal (Freeman and Lilly, 1998). A proper understanding of the influence and interactions of these parameters, cell physiology, and behaviour under immobilized conditions, productivity, and operational stability is required for a rational and systematic design and evaluation of new processes.

With the pressing need and demand for information on immobilization of marine bacteria and processes for metabolite production by them, in the present study an effort was made to study the production of extracellular L-glutaminase by marine *Pseudomonas* sp. BTMS-51 under immobilized conditions.

OBJECTIVES OF THE PRESENT STUDY

From the ongoing review of literature it is understood that there exists a dearth of knowledge on use of immobilized cell processes for metabolite production and production of exoenzymes employing marine bacteria despite the fact that they are very much amenable to immobilization due to their adhesive properties. Hence, it was proposed to study in detail the process for the immobilization of marine *Pseudomonas* sp, BTMS 51, and extracellular L- glutaminase production by the immobilized cells.

The primary objective of the study was to evaluate the commonest whole cell immobilization protocols towards extracellular L-glutaminase production by the marine *Pseudomonas* sp. under immobilized condition and to develop a viable bioprocess technology employing the immobilized cells.

The specific objectives of the present study include

1. Process optimisation for immobilization of whole cells of *Pseudomonas* sp. in Ca-alginate beads and L-glutaminase production by immobilized bacteria under batch mode operation
2. Evaluation of the reusability of immobilized cell beads under repeated batch operation
3. Process development for continuous production of the enzyme by Ca-alginate immobilized bacteria in a Packed bed reactor(PBR) and a Circulating bed reactor(CBR)
4. Process optimisation for immobilization of *Pseudomonas* sp. BTMS -51 on preformed carriers and evaluation of enzyme production by cells immobilized on three different carrier systems
5. Optimisation of the enzyme production by adsorbed cells using response surface methodology and generating information on the interaction between process variables
6. Evaluation of the reusability of the cell adsorbed carrier particles in L-glutaminase production under repeated batch operation
7. Continuous production of the enzyme in packed bed reactor with cells adsorbed onto a bed of polystyrene, PUF or Nylon web.

2. REVIEW OF LITERATURE

2.1 Immobilization of microbial cells

Immobilization of cells can be defined as the transfer of cells from a free state to a state of confinement or localisation in a certain defined region of space with the retention of viability and catalytic activity (Flint, 1987). This implies the attachment of cells or their inclusion in a distinct solid phase that permits exchange of substrates, products, inhibitors etc., at the same time separating the catalytic cell biomass from the bulk phase containing substrates and products (Ramakrishna & Prakasam, 1999).

Immobilization of the microbial cells are reported to change the physiological state of microorganism (Hamer, 1990., Hahn-Hagerdal, 1990), and often there are changes in the cell permeability (Vijayalakshmi *et al*, 1979., Fletcher & Marshal, 1982), changes in the respiration rate of cells (Novarro & Durand, 1977) and even changed gene expression (Marshall, 1994). These changes can be brought about by cell surface interactions and interactions with the micro environment of the cells (Hahn-Hagerdal, 1982., Anderson, 1986., Rouxhet and Mozes, 1989). The development and application of immobilized cell technology is posing many new, interesting but difficult problems concerning the understanding and control of cell physiology. However, many of the changes in cell physiology are also advantageous and improvements in processes have been a consequence of such changes (Anderson, 1986).

Immobilization of microbial cells can occur as a natural phenomenon of adhesion and biofilm formation and by artificial methods (Webb, 1987., Ramakrishna & Prakasam, 1999). Eversince the application of immobilized bacteria for production of a commodity chemical (Acryamide) by an industry was first reported (Nitto Chemical Industries Co., Nishikawa *et al*, 1987), the interest in application of immobilized cells for production of commercial products have overwhelmingly increased, and studies on production of industrially important compounds have been undertaken both by industries and academics (eg. Linko and Hujanen, 1990., Keshavarz *et al*, 1990., Rymowicz *et al*, 1993).

Whole cell immobilization has been attempted world-wide, as a viable alternative to conventional microbial fermentations, and various methods and numerous carrier materials were tried over the years for immobilization of viable

microbial cells. Several comprehensive reviews have been published on the general and specialised topics in this field demonstrating the extent of interest in this rapidly developing area. (Durand and Navarro, 1978., Kolot, 1981., Chibata & Tosa , 1981., Scott, 1987., Furusaki & Seki, 1992., Gerbsch & Buchholz, 1995., Freeman & Lilly, 1998, Ramakrishna & Prakasham, 1999).

Processes based on immobilized living microbial cells have several advantages over those employing free cells (Kolot, 1980), and these aspects has been reviewed by Dervakos & Webb (1991). In principle, cell immobilization allows biological particles of any size, shape and density to be produced for a wide variety of prokaryotes. One of the major features of most immobilization processes is the very high concentration of cells that can be achieved, and this combined with the ability to handle immobilized cells distinguishably from the liquid phase, offers a number of possibilities for process engineering improvement (Webb, 1987). Many advanced bioreactor systems require that the biocatalyst (microorganism) be immobilized into or onto a solid support material in order to reduce cell wash out, and increasing significantly the biocatalyst concentration while providing for optimum contact with the support (Scott, 1987). The remarkable advantage of the system is the ability to determine cell density prior to fermentation and it also facilitates operation of microbial fermentation in the continuous mode without cell wash out (Ramakrishna & Prakasham, 1999).

Despite the several potential advantages of immobilized cell systems, an extensive exploitation of these methods demands the proper understanding of the process parameters that govern such operations. This includes immobilized cell physiology, mode of operation, aeration and mixing, bioreactor configuration, medium composition, temperature, pH and their interactions (Freeman & Lilly, 1998). Several studies have been made towards improvement of immobilized cell processes which include methods to improve oxygenation (Ogbonna *et al*, 1991), increase of surface area per unit volume of matrix (Jager & Wandrey, 1990., Truck *et al*, 1990., Muscat *et al*, 1996., Senturban *et al*, 1996), modes of mixing (Jager & Wandrey, 1990., Pakula & Freeman, 1996) and medium optimisation (Kloosterman & Lilly, 1985., Tsay and To, 1987., Michel *et al*, 1990., Feijoo *et al*, 1995) . Among the important parameters affecting performance of an immobilized cell system,

perhaps the most important are the method of immobilization and the type of carrier employed for immobilizing microbes.

2.2 Methods of immobilization

Microbial cell mobility can be restricted by aggregating the cells or by confining them into, or attaching them to, a solid support (Scott, 1987). The choice of the method for immobilization is governed primarily by the desired physiological state of the cells and the purpose to which they are to be employed (Webb, 1987). The numerous techniques available for immobilizing microbial cells may be classified based on the nature of the physical process involved namely attachment, entrapment, containment, and aggregation. Nevertheless, these techniques may be categorised broadly into (1) attachment, where the microorganisms adhere to a surface or other organisms by self adhesion or chemical bonding, and (2) entrapment, where the organisms are caught in the interstices of fibrous or porous materials, or are physically restrained within, or by a solid porous matrix such as a stabilised gel, or a membrane (Scott, 1987). The techniques available for immobilization of microbial cells have been a subject of extensive review and several reports are available on the supports used and methods of immobilization (Durand & Navarro, 1978., Kolot, 1980, Klein & Vorlop, 1985., Scott, 1987., Gerbesch & Buchholz, 1995, Ramakrishna & Prakasham, 1999).

2.2.1 Attachment of cells

Most microorganisms in natural and man made habitats possess the ability to adhere to surfaces (Marshall, 1976) or to other microorganisms forming cell aggregates (Scott, 1987). This attachment, either natural or induced, can form the basis for an inexpensive but effective immobilization technique.

2.2.1.1 Aggregation/ Cell to cell crosslinking

The simplest way of achieving cell aggregation is by flocculation. Some bacterial flocs are stable against high shear fields used in column reactors such as fluidized beds (Scott, 1983). Fungal mycelia also form aggregates in the shape of spherical pellets (Metz & Kossen, 1977). Various yeast strains are known to

flocculate and the resultant aggregates have been successfully employed in tower fermenters (Bu'lock *et al*, 1984., Kuriyama *et al*, 1985., Netto *et al*, 1985).

Flocculation is a key feature of activated sludge process for wastewater treatment (Foster *et al*, 1985). However, few microorganisms are available with sufficiently high flocculating activity, and a more applicable way to enhance flocculation and stabilise cell aggregates is by addition of crosslinking agents (Klein & Vorlop, 1985). Addition of polyelectrolytes is an efficient method to achieve cell aggregation and had been used to enhance flocculation (Tsumara & Kasumi, 1976., Cizinska *et al*, 1985). Application of oppositely charged polymers (van Keulen, 1981) and even aeration (Deverell & Clark, 1985) have been tried for inducing microbial aggregation. However, this method has not been widely used for immobilization in spite of its simplicity, possibly due to the denaturing effect of the procedure (Klein & Vorlop, 1985).

2.2.1.2 Adsorption/Attachment to surfaces

Adsorption of cells on a preformed carrier, a classical method for cell immobilization, is probably the earliest method of immobilized cell application and has been employed for vinegar production since 1823 (Klein & Vorlop, 1985). The method has been widely used in environmental control technology, where naturally occurring microbial biofilms are used for waste treatment and pollution control (Holm *et al*, 1992., Pons & Fuste, 1993., Wong *et al*, 1993., Jayachandran *et al* 1994., van Schie and Fletcher, 1999). A preformed carrier of variable structure is mixed with cell suspension and the cells adhere to the surface in a more or less complete way. Since the carrier is usually inert and as no additional chemicals are involved in the process, the immobilization is carried out under the same physiological conditions in which the cells are kept in suspension. This approach offers minimum hindrance to the cell physiology, and allows cell detachment and relocation with potential establishment of equilibrium between adsorbed and freely suspended cell populations often advantageous for extended operational stability (Freeman & Lilly, 1998). The mechanisms involved in cell adsorption on supports are complex and varied, and have been the subject of several extensive studies (Fletcher, 1987., Busscher *et al*, 1992., Marshall, 1992, Fletcher, 1994., van Schie & Fletcher, 1999). The implications of microbial adhesion in biotechnological processes are reviewed by Marshall (1994).

The choice of carrier is an important consideration in cell immobilization by adhesion. Since the carrier is preformed and its preparation does not interfere with the immobilization step, there exist considerable freedom in the selection of a suitable carrier. Some of the carrier systems tried and their applications are listed in Table 2.1.

The limiting factors of the technique of adsorption have been identified as adhesion capacity and adhesive strength. To obtain a high cell loading capacity the inner, as well as the outer, surface of the porous carrier material has to be accessible for adsorption (Klein & Vorlop, 1985). Therefore, the pore diameter has to be small enough to give a large surface area, and on the other hand large enough to allow cell penetration and exchange of materials without loosing their mechanical stability (Freeman & Lilly, 1998). Hence, it often becomes necessary that the carrier has an optimum pore size, and indeed this can be determined (Navarro & Durand, 1977., Messing *et al*, 1979). The second point of concern is the adhesive strength. Interaction forces are generally not very strong, with the exception of those species, which have developed special mechanism for surface anchored growth. Information are available on the thermodynamics of adhesion (Gerson & Sajic, 1979), influence of pH (Navarro & Durand, 1977., Marcipar *et al*, 1979), ionic strength (Vijayalakshmi *et al*, 1979), and support composition (Marcipar *et al*, 1979). Electrostatic forces are also believed to be involved in microbial adhesion, apart from van der Waals forces and more and more data is becoming available on the subject (Marshall, 1994). However, the experimental data available so far has not been conclusive in a mechanistic sense and further data on the surface properties of cells and carriers are required to have a better understanding.

Notwithstanding the fact that an in depth information is warranted on the mechanisms of cell adhesion and surface interactions, several processes have found success in metabolite production employing the process. More and more carriers are being tried as supports, and the processes based on adhered microbial cells are rapidly being developed together with a better understanding of the process of cell adhesion. Table 2.1 summarises few applications of microbial cells immobilized by adhesion

Table 2.1
Carrier systems employed in immobilization by adsorption and potential applications of adsorbed microbial cells

Support	Organism	Application / Product	Reference
Anion exchanger	*Bacillus licheniformis*	Protease	Okita & Kirvan, 1987
Celite	*Penicillium chysogenum*	Penicillin G	Gbewonyo *et al*, 1987
Celite	*Penicillium chysogenum*	Penicillin G	Keshavarz *et al*, 1990
Cellulosic fabric	*Trichoderma reesei*	Cellulase	Kumakura, *et al*,1989
Ceramics	*Gluconobacter suboxydans*	Gluconic acid	Shiraishi *et al*, 1989
Coal fly ash blocks	*Synechococcus sp.*	Biomass	Matsumoto *et al*, 2000
Cotton fibers	*Zymomonas mobilis*	Ethanol	Anon, 1981
Duralite Noire®	*Mixed culture*	Waste water Treatment	Messing, 1982
Fuyolite®	*Vibrio alginolyticus*	Biofilter for removal of NH_4	Kim *et al*, 2000
Glass	*Steptomyces tendae*	Nikkomycin	Truck *et al*, 1990a, b
Loofa sponge	*Saccaromyces cerevisiae*	Ethanol	Ogbonna *et al*, 1994
Nylon web	*Steptomyces cellulophillum*	Cellulase	Linko *et al*, 1996
Nylon web	*Phanerochaete chrysosporium*	Lignin peroxidase	Linko 1988
Polypropylene	*Clitocybula dusenii*	Manganese peroxidase	Ziegenhagen & Hofrichter, 2000
Polyurethane foam	*Trichoderma reesei*	Glucanase Xylanase	Haapala *et al*, 1995
Stainless Steel Biomass support	*Trichoderma viridae*	Cellulase	Webb *et al*, 1986

2.2.1.3 Covalent Bonding

Covalent bonding, another way of attaching cells to the surface of a preformed carrier, is based on the formation of covalent bonds between the cell surface and the inorganic support, and requires the use of a binding (cross-linking) agent (Kolot, 1980., Valuev *et al*, 1993., Ramakrishna & Prakasham, 1999). Carriers which have the reacting groups as epoxy and halocarbonyl (Nelson, PT-US 3957580, 1976),

Zr(IV) oxide (Kennedy *et al*, 1976), Ti(IV) oxide (Kennedy, 1978), Cynuric chloride (Gainer *et al*, 1980), Glutaraldehyde (Nelson, PT-US 3957580, 1976) aldehyde, oxirane and amine groups (Zurkoba *et al*, 1978) carbodimide (Jack & Zajic, 1977), and polyethylene oxide (van der Sluis *et al*, 2000) have been tried. Silanization, coupling by glutaraldehyde (Navarro & Durand, 1977), isocyanate (Navarro, 1975) metal hydoxide(Kennedy *et al*, 1976), were attempted for immobilization of yeast cells. However, the use of this technique has not yet been successfully exploited in any immobilized cell applications due to lack of detailed literature on the method and its feasibility. The potential advantages of higher bonding stability achieved by covalent binding is overshadowed by the disadvantages of toxicity while using chemical coupling agents and a reduced number of possible carriers due to specific requirements for functionalized surfaces (Klein & Vorlop, 1985).

2.2.2 Entrapment

Physical entrapment in porous polymeric carriers is by far the most widely used technique for whole cell immobilization. This represents a more definite means of immobilization that does not have a significant dependence on cellular properties (Scott, 1987). It is clear, however, that the support network has to be formed in presence of the cells to be entrapped, and that the network forming reactions therefore have to be adapted to the physiological requirements of the stability of the cells (Klein & Vorlop, 1985). The reaction conditions are characterised by the solvent, temperature, possible addition of catalysts, and to a great extent by the chemical composition, functionality and size of the network precursor molecules. These determine not only the toxicity and rate of crosslinking, but also the stability of the polymeric carrier under the operational conditions. Entrapment methods usually adopted include micro-encapsulation, gelation of polymers, precipitation of polymers, ionotropic gelation of polymers, polycondensation, and polymerization. A detailed review with extensive coverage of all these methods is out of scope of the present study and the review is therefore restricted to the commonest of all these techniques - Ionotropic gelation. Reviews covering the other methods may be found elsewhere (Klein & Vorlop, 1985).

2.2.2.1 Ionotropic gelation

If a water-soluble polyelectrolyte is mixed with the appropriate, usually multivalent, counter-ions, solidification is achieved through formation of polysalts. The term "ionotropic gelation" was introduced in connection with the formation of highly water-swollen structures of controlled morphology (Klein & Vorlop, 1985). The most well known example is the Ca-alginate gel, obtained by gelation of a Na-alginate solution in a $CaCl_2$ bath. A large variety of polymers with different structure and functional groups exist with regard to polyanions and the most important and most widely used products are Alginate and κ- carrageenan. Chitosan as a polycation is also promising in the immobilization of cells by ionotropic gelation. Smidsrod & Skjak-Braek (1990), Martinson *et al*, (1989., 1992) and Kierstan & Bucke (2000) have reviewed cell immobilization in Ca-alginate gels.

Though entrapment by polymerisation in polyacrylamide network is an extensively studied method for whole cell immobilization, the application is practically limited to laboratory studies, with few exceptions (Chibata *et al*, 1974., Lee & Chang, 1990). With regard to technical applications in food and health-related areas, a definite trend is observed towards the use of natural polymers (Klein & Vorlop, 1985).

The κ- carrageenan method

The κ- carrageenan method was introduced by Chibata and his group in 1977 (Yamamoto *et al*, 1977., Tosa *et al*., 1979). κ- Carrageenan is a naturally occurring polysaccharide isolated from seaweed and widely used as food additive. The polymer is easily available and is non toxic. Gelation of κ- carrageenan can be brought about by cooling the solution from 40 ^{0}C to 10 ^{0}C, or by the use of gel inducing agents such as various metal ions (K^+, Rb^+, Cs^+, Ca^{2+}, Al^{3+}), aliphatic amines, aromatic diamines, aminoacids and their derivatives or water miscible organic solvents. However, gelation with K^+ is found to be the best of the methods (Klein & Vorlop, 1985).

In a typical procedure, κ- carrageenan is dissolved in physiological saline previously warmed to 70-80 ^{0}C, at a concentration between 2% and 5 %, and this solution is kept at 40 ^{0}C. After mixing the cell suspension with this, the slurry is dropped from a sampler pipette/syringe into a cold 0.1M KCl solution for gel formation (Tosa *et al*, 1979). The method is mild on the living cells, except for the

heating step, but most of the microbes are able to tolerate the operation temperature (40-45 ^{0}C) and are immobilized live, maintaining their ability to grow as demonstrated by Chibata (1979). Post gelation treatments are also reported for increasing the stability of the beads (Tosa *et al*, 1979, 1982). The method had gained wide popularity even in its infancy and has found applications in industry (Chibata, 1979., Chibata & Tosa, 1981)

The alginate method

The application of alginate for whole cell immobilization was first recognised in 1975, when aluminium was used as the counter ion (Hackel *et al*, 1975). Kierstan & Bucke (1977) later introduced the most successful Ca-alginate combination. Alginates are natural polymers and similar to κ-carrageenan are derived from seaweed and find several applications in food industry. Alkali and magnesium alginates are soluble in water, whereas, alginic acids and the salts of polyvalent cations are insoluble. Simple dropping of sodium alginate solution into a solution of $CaCl_2$ results in the formation of rigid near-spherical gels by ionotropic gelation. For immobilization of microbes, alginate solution and the cell suspension are mixed under sterile conditions, followed by gelation through dropping into $CaCl_2$ solution, which results in the formation of beads of a predetermined diameter range (Groboillot *et al*, 1994).

The procedure has found extensive applications ever since its conception, and not surprisingly so, because of the great amount of flexibility offered by the technique. Ca-alginate beads can be prepared in a broad range of particle sizes and shapes, cell loading can be varied up to 30% wet weight (Klein & Vorlop, 1985), bead stability and property may be altered by judicious choice of the concentration of alginate, counter ions and cell loading (Fukushima & Hanai, 1982., Kumar *et al*, 2001), post gelation treatments (Ruggeri *et al*, 1991) etc., and also equipment are available for large scale bead preparation. Several attempts were made in improving mass transfer in the Ca- alginate beads (Sada *et al*, 1981., Ogbonna *et al*, 1991., Pilkington *et al*, 1998) and the large-scale production of microbeads (Hunik & Tramper, 1993., Serp *et al*, 2000., Champagne *et al*, 2000) indicating the wide popularity of the technique. In fact it is one of the very few whole cell immobilization

techniques, which had found industrial applications (eg. Nagashima *et al*, 1984., Eikmeier & Rehm, 1984., Fukushima & Motai, 1990)

Since the entrapment in either Ca-alginate or κ- carrageenan of a controlled amount of cells is very simple and generally non-toxic, various cells can be immobilized with a complete preservation of viability. This is the key for the success of these techniques over the other methods and an increased use in the industry. Some of the immobilized cell applications where ionotropic gelation technique was employed are given in Table 2.2

Table 2.2
Application of ionotropic gelation in the entrapment of microbial cells

Matrix	*Microorganism*	*Product/Application*	*Reference*
κ- carrageenan	*Escherichia coli*	β-lactamase	Ryan & Parulekar, 1991
κ- carrageenan	*Corynebacterium sp*	L(+)Tartaric acid	Zhang & Quian, 2000
κ- carrageenan	*Zymomonas mobilis*	Ethanol	Krishnan *et al*, 2000
Al- copolystyrene maleic acid	*Candida tropicalis*	Phenol degradation	Klein *et al*, 1979
Ca-alginate	*Aureobasidium pullulans*	Glucoamylase	Federici *et al*, 1990
Ca-alginate	*Penicillium chrysogenum*	Protease	el-Asssar *et al*, 1990
Ca-alginate	*Bacillus cereus*	Cyclodextrin glucosyl transferase	Jamuna *et al*, 1993
	Bacillus polymyxa	α-amylase	Mohandass & Chandrasekaran, 1994
Ca-alginate	*Pseudomonas putida*	Phenol degradation	Bandhopadhyay *et al*, 1999
Chitosan - $K_4[Fe(CN)_6]$	*Pseudomonas sp.*	Methanol+glycine/ L-serine	Behrendt, 1981
Chitosan-polyphosphate	*Escherichia coli*	L-tryptophan	Vorlop & Klein, 1981
Mg-Pectinate	*Saccaromyces cerevisiae*	Ethanol	Giordano *et al*, 2000
Polyethyleneimine Ba-alginate	*Steptomyces clavuligerus*	Penicillin G conversion to Cephalosporin G	Demain & Baez-Vasquez, 2000

2.3 Applications of immobilized whole cells

A wide variety of bulk and speciality chemicals can be produced by immobilized whole cell biocatalysts. As yet few are in commercial use, the main reason of which being the cost of change over from existing petrochemical base. (Flint, 1987).

At the present time most of the studies on immobilized microorganisms are purely of academic interest, but for the few processes within the chemical industry producing fine or speciality chemicals on a large scale. Flint (1987) and Ramakrishna & Prakasham (1999) has reviewed several of these applications either adapted by the industry or have been investigated by researchers with the view of future applications. Table 2.3 summarises some of the important studies made in this respect.

Table 2.3

Applications and potent industrial uses recognised for immobilized microbial cells

Product/Application	*Microorganism*	*Immobilization method*	*Reference*
Speciality chemicals			
Acetyl Co-A	*Brevibacterium ammoniagenes*	Entrapped in Polyacryamide	Samejima *et al*, 1978
Acrylamide	*Brevibacterium sp.*	Entrapped in Polyacryamide	Lee & Chang, 1990
ATP	*Yeast*	Photocrosslinking of resin prepolymer	Asada *et al*, 1979
Glutathione	*E coli and Saccaromyces cerevisiae*	Entrapped in k- carrageenan	Murata *et al*, 1980
Menthol	*Rhodotorula minuta*	Photocrosslinking of resin prepolymer	Omata *et al*, 1981
NADP	*Achromobacter*	Entrapped in Polyacryamide	Uchida *et al*, 1978
Aminoacids			
Arginine	*Serratia marcescens*	Entrapped in k-carragenan	Fujimura *et al*, 1984
Aspartate	*E. coli*	Entrapped in Polyacryamide	Chibata *et al*, 1974 Sato *et al*, 1979

Glutamine	*Corynebacterium glutamicum*	Entrapped in Polyacryamide	Slowinsky & Charm, 1973
Tryptophan	*E.coli*	Entrapped in Polyacryamide	MarechaL *et al*, 1979
Tyrosine	*Erwinia hericole*	Entrapped in Collagen	Yamada *et al*, 1978
Antibiotics			
Cephalosporin	*Cephalosporium acremonium*	Entrapment in modified Ba-alginate	Park & Khang, 1995
Cyclosporin	*Tolyplocadium inflatum*	Adsorbed on celite	Lee *et al*, 1996
Nikkomycin	*Streptomyces tendae.*	Adsorbed on CP glass	Truck *et al*, 1990a,b
Penicillin G	*Penicillium chrysogenum*	Adsorbed on celite	Keshavarz *et al*, 1990
Commodity chemicals			
Acetic acid	*Acetobacter acetii*	Entrapped in k-carrageenan	Osuga *et al*, 1984
		Entrapped in Ca-alginate & Adsorbed on Cellulose	Krisch & Szajani, 1996
Citric acid	*Yarrowia lipolytica*	Entrapped in Ca-alginate	Rymowicz *et al*, 1993
Ethanol	*Saccaromyces cerevisiae*	Adsorbed on ceramic	Zhang *et al*, 1996
		Entrapped in Ca-alginate	Giordano *et al*, 2000
	Zymomonas mobilis	Entrapped in k-carrageenan	Krishnan *et al*, 2000
Fructose	*Actinoplanes missouriensis*	Entrapped in Cellulose fibers	Linko *et al*, 1977
Lactic acid	*Lactobacillus delbruckii*	Entrapped in Ca-alginate	Linko, 1980
Tartaric acid	*Corynebacterium sp.*	Entrapped in k- carrageenan	Zhang & Qian, 2000

Steroid transformations			
Hydrocortisone - Prednisolone	*Arthrobacter simplex*	Entrapped in Polyacryamide	Kloosterman and Lilly, 1986.
Hydrocortisone- Prednisolon	*Arthrobacter simplex*	Entrapped in Polyacryamide	Silbiger & Freeman, 1991
Progesterone-11 hydroxyprogesterone	*Rhizopus nigricans*	Agar entrapped	Maddox *et al*, 1981
	Aspergillus ochraceus		Dutta & Samanta, 1999
Testosterone - dehydrotestosterone	*Pseudomonas testosteroni*	Entrapped in urethane prepolymer	Yang & Studebaker, 1978
Waste management			
Ammonia removal	*Vibrio alginolyticus*	Adsorbed on synthetic packing matrial	Kim *et al*, 2000
Benzene degradation	*Pseudomonas putida*	Entrapped in Polyacryamide	Somerville & Ruffell, 1977
Phenol degradation	*Pseudomonas putida*	Entrapped in Ca-alginate	Bandhopadhyay *et al*, 1999
Rubber effluent treatment	*Mixed culture Acinetobacter sp.*	Entrapment in Entrapped in Ca-alginate	Jayachandran, 1998

Biosensors

Chemicals assayed and microbes used in microbial biosensors

Chemical	*Organism*	*Detection method*	*Reference*
Acetic acid	*Trichosporon brassicae*	Oxygen electrode	Hikuma *et al*, 1979
Cholesterol	*Nocardia erythropolis*	Oxygen electrode	Wollenberger *et al*, 1980
Creatinine	*Nitrosomonas sp. & Nitrobacter sp.*	Oxygen electrode	Karube, 1988
Glutamine	*Sarcina flava*	Ammonia electrode	Rechnitz *et al*, 1978
Phosphate	*Chromatium sp.*	Hydrogen current	Matsunaga *et al*, 1984
Alcohol	*Candida vimi*	Oxygen electrode	Mascini *et al.* 1989

2.4 Extracellular enzyme production by immobilized microorganisms

Cell immobilization technology is a much-suited technique for production of extracellular enzymes (Ramakrishna & Prakasham, 1999) and several attempts has been made on whole cell immobilization for enzyme production. Among the

microbial enzymes, starch degrading enzymes, α-amylase and glucoamylase have been studied extensively. α- Amylase production was attempted even before 30 years (Kokubu *et al*, 1978) and since then, several workers have succeeded in production of the enzyme by a variety of immobilized microorganisms and employing various methods of immobilization. Ramakrishna *et al* (1993) immobilized *Bacillus cereus* in Ca-alginate and used both packed bed and fluidized bed reactors for continuous production of the enzyme. Mohandass & Chandrasekaran (1994) immobilized *Bacillus polymyxa* in Ca-alginate and used packed bed reactor for continuous production of the α- amylase. Blieva (1982) immobilized *Aspergillus* sp. on floating filter plates for production of α-amylase and the process recorded an operational stability near two months. Tonkova *et al*, (1994), used membrane bound and agar entrapped *Bacillus licheniformis* cells were employed for thermostable amylase production. Recently an immobilized cell process was reported to give 385 % higher production of the enzyme compared to the free cell process (Duran-Paramo *et al*, 2000).

Aspergillus strains have been widely attempted as sources of extracellular glucoamylases and several immobilized cells processes are reported for its production (Bon & Webb, 1989., Kuek *et al*, 1991). Li *et al* (1984) obtained a stable production of the enzyme by *A.niger* for over 1000h under repeated batch cultivation in shake flasks. Emilia- Abraham *et al* (1991), reported a 350 operation period in an aerated packed bed reactor. Fiedurek and Szczodark (1995) attempted passive immobilization by adsorption on natural carriers. Process optimisation for glucoamylase production by *Aureobasidium pullulans* in a fluidised bed reactor was reported by Fedirici *et al* (1990).

Immobilized whole cells of microorganisms were also used in the production of important industrial enzymes like proteases, lipases, and cellulases. Okita and Kirwan (1987) reported the use of a continuous stirred tank reactor for protease production by *Bacillus licheniformis* immobilised by binding with an anion exchanger. However, a stable operation could be achieved only for 150h. A higher rate of production of fibrinolytic enzymes and other proteases compared to free cell process, was observed in immobilized cultures of *Penicillium chrysogenum* (el-Aassar *et al*, 1990). Immobilized cell processes for lipase production by various

microbial cells eg- *Rhizopus chinensis* (Nakashima *et al*, 1990), *Aspergillus niger* (Jamil & Omar, 1992), *Candida rugosa* (Ferrer & Sola, 1992), *Sporotrichum thermophile apinis* (Johri *et al*, 1990) are reported in the literature. Both entrapment and adsorption methods were tried with a great amount of success.

Production of cellulolytic and xylanolytic enzymes by immobilized *Trichoderma reesei*, using a wide range of carriers including κ- carrageenan (Frein *et al*, 1982), stainless steel biomass support particles (Webb *et al*, 1986), polyurethane foam (Turker & Mavintuna, 1987), cellulosic fabric (Kumakura *et al*, 1989) and copolymer covered paper (Xin & Kumakura, 1993) are reported. Several other microbes have also been tried for cellulase production using different reactor configurations. These include bubble column reactor (Kang *et al*, 1995), sprouted bed reactor (Webb *et al*, 1986) and a rotating biological contractor (Linko *et al*, 1996).

Other important enzymes produced using immobilized microorganisms are Xylanases (Haapala *et al*, 1994 &1995., Mamo & Gessesse, 2000), Lignin peroxidase (Linko, 1988., Bonarme & Jeffries, 1990., Jager & Wandrey, 1990), manganese peroxidase (Ziegenhagen & Hofritchter, 2000), cyclodextrin glucosyl transfrease (Jamuna *et al*, 1993., Saswathi *et al*, 1995), pectinase (Blieva, 1982) and ribonuclease (Manolov, 1992).

2.5 Bioreactors for immobilized cell processes

Probably the first rational development of an immobilized cell reactor was that of Pasteur who proposed an acetifier where *Acetobacter* cells were immobilized on the surface of wood chips (Webb, 1987). These types of reactors, -the tricking bed reactors are still in use for vinegar production and for wastewater treatment. The majority of all current bioreactors are stirred tanks operated in batch mode. However, the very concept of cell immobilization is intrinsically linked to continuous operation (Webb, 1987). Further, the use of immobilized cells in a stirred tank often leads to particle break up. In this context, there is a need to develop novel reactor configurations suited for use with immobilized cells. In contrast with the large amount of literature published on techniques for whole cell immobilization, there are only few detailed and systematic reports available on immobilized cell reactors.

A complex array of factors influences the choice of reactor type for a particular process. These include the immobilization method, characteristics of the support matrix, nature of substrate, inhibitory effects and hydrodynamic and economic considerations. Each type of reactor has its own advantages and disadvantages and the choice of the ideal reactor type for a process is subject to a proper understanding of the process and the microbe involved. Several recent attempts have been directed towards these goals and Freeman & Lilly (1998) present an overview of the developments.

2.5.1 Stirred tank reactors (STR)

Even though STRs are not that common in immobilized cell processes there are reports on its use in such processes (Amin *et al*, 1993., Ogbonna *et al*, 1994., de Gooijer *et al*, 1996). The major problem encountered with the use of immobilized cell particles in STR is related to the harsh treatment to which the particles are exposed. A high shear rate may have severe damaging effects, especially in the case of gel particles. Modifications in the classical design (eg., Ogbonna *et al*, 1994) and use of a very low agitation rate (eg. Amin *et al*, 1993) may allow mixing without destruction of immobilized cell particles. However, these adaptations are not as satisfactory as the rational design of a purpose built reactor.

2.5.2 Fixed bed reactors

Packed bed reactors are the commonest of these reactor types although other fixed bed rector types like Rotating biological contactors (RBC), Trickling bed filters (TBF) and Parallel flow sheet-reactors are also used in immobilized cell processes. Plug flow packed bed reactors have been extensively used in such immobilized cell operations (Branyik *et al*, 2000., Mamo & Gessesse, 2000., van Iersel *et al*, 2000). PBRs operated on a once-through basis may offer high rates of reaction, but at the same time have relatively poor mass and heat transfer coefficients due to low liquid velocities. Reactors with recycling shows improved mass and heat transfer characteristics, and improved controllability (Webb, 1987). The size of particulate carrier is an important consideration in PBR as it affects both pressure drop and internal diffusion resistance. The size distribution should be as uniform as possible since the pressure depends on bed voidage (Webb, 1987).

Particle compression caused by static weight of the bed and the pressure due to flow is usually a severe drawback. Compression results in a considerable decrease in bed voidage and consequent increase in pressure drop across the bed. There can also be a drastic reduction in activity due to a decrease in specific bed area caused by bed compression (Webb, 1987).

Plugging and channelling is another major problem when using particulate substrates, and PBR also present considerable drawbacks in three-phase operation. Gas liquid contact is restricted, as is the release of gas from the voids. This can cause gas flooding which in turn produces poor liquid distribution and hence poor performance (Ghose and Bandhopadhayay, 1980).

The rotating disc fermenter or rotating biological contactor is an attached film fermentor type developed originally for cultivation of mycelial fungi (Blain *et al*, 1979). The mycelia grow on the surfaces of a series of vertical discs (usually of polypropylene) attached to a shaft, and which rotate half submerged in the culture medium. RBC has also been used with bacteria (Jayachandran, 1998) but most of the applications are in wastewater treatment. However, the system can also be operated aseptically and a good performance in the production of citric acid by *Aspergillus niger* has been reported (Anderson *et al*, 1980).

Trickling bed fermenters are one of the earliest to be used in immobilized cell applications such as vinegar production. The TBFs have been successfully employed in large-scale treatment of wastewater with sand or gravel as the packing material. Though the present applications of TBF are largely related to wastewater treatment, it is impressive to see that such reactor types are even employed in antibody production by immobilized mammalian cells (Phillips *et al*, 1992).

One of the impressive design concepts in fixed bed reactor types was produced by the Research association for petroleum alternative development (RAPAD) Japan, for continuous production of power alcohol from molasses (Oda *et al*, 1983). One of the two pilot scale processes developed employs yeast cells immobilized on photo cross-linkable resin formed in the shape of sheets. These are arranged vertically in a parallel flow type reactor. The design allows efficient release of gas and prevents blockage, and therefore gives better productivities in spite of a lesser surface area compared to particulate beads

2.5.3 Fluidised bed reactors

Fluidised bed reactors are one of the most widely studied reactor types used in immobilized cell processes. Here the particles become suspended as a result of the upflow of the fluid phase (usually a mixture of gas and liquid). Biological fluidised bed treatment of wastewater is a particular application of interest, where solid particles can be used (Cooper & Atkinson, 1981). FBRs were recently reported in the degradation of phenol (Bandhopadhyay *et al*, 1999., Branyik, 2000), benzoic acid (Hecht *et al*, 2000), sulphate reduction (Nagpal *et al*, 2000), desulpherisation (Chu *et al*, 2000) and waste water treatment (Segar *et al*, 1997., Hirata *et al*, 2000). Use of FBRs have also been explored for aseptic systems in the production of antibiotics (Truck *et al*, 1990b., Park & Khang, 1995., Sarra *et al*, 1999), glucoamylase (Federici *et al*, 1990), cyclodextrin glucosyl transferase (Jamuna *et al*, 1993), ethanol (Sun *et al*, 1997., Krishnan *et al*, 2000), and in beer fermentation (Tata *et al*, 1999) besides several other metabolites.

FBRs combine some of the advantages of STR and packed bed, and have few of their disadvantages. The attractive features include good mixing and mass transfer properties. In three-phase operation, gas-liquid contact and gas removal are facilitated as compared to packed beds. Thus higher volumetric oxygen transfer coefficients can be attained and gas flooding can be eliminated and the overall performance of FBRs may be higher despite having a low cell density per unit reactor volume. A specialised form of fluidised bed particularly suitable for use with mycelial fungi is the sprouted bed fermenter. The increased shear occurring at base of the bed of dense particles provide for attrition of the tough mycelial tissue, enabling steady control of biomass to be achieved (Webb *et al*, 1986)

2.5.4 Pneumatically mixed reactors

The use of gas to circulate the contents of a fermenter through an external tube or internally using a draft tube is a convenient mean of achieving good mixing and aeration. Such "airlift" fermenters are of simple construction and are low consumers of power. Consequently, they are very attractive for large-scale operation. The hydrodynamic behaviour of mass transfer characteristics of gas mixed reactors have been studied by Blenke (1979) and Schugerl(1982). Air lift reactors were employed for production of cyclosporin A (Foster *et al*, 1983), prednisolone (Kloosterman &

Lilly, 1986), citric acid (Rymowicz *et al*, 1993), actinomycin D (Dalili & Chau,1988), penicillinG (Keshavarz *et al*, 1990)and a range of other products.

Another important type of gas mixed reactor is the bubble column fermenter. Manolov (1992) used the bubble column reactor for continuous production of ribonuclease by immobilized *Aspergillus clavatus* and the process reported an operational stability of 960h. Other important applications described for bubble column include the production of antibiotics (Archuri *et al*, 1986., Gbewonyo *et al*, 1987., Mahmaud & Rehm, 1987), enzymes (Suresh, 1998., Manolov, 1992., Linko *et al*, 1996), and steroid transformations (Silbiger & Freeman, 1991., Dror & Freeman, 1995).

Black *et al* (1984) described a novel type of gas mixed fermenter -the "circulating bed" fermenter (CBF), which facilitates the mixing of biomass carriers of essentially neutral buoyancy. Particle motion is induced here, by introducing air and/or recycled gas below the distributor. The CBF was successfully operated continuously for extended periods for the production of alcohol by yeast cells.

Radical design concepts in reactor continue to be investigated for use with immobilized cells, and most of these include modifications in the existing designs and original concepts are also being perused.

2.6 Operation conditions and media in immobilized cell applications

An effective utilisation of the potential advantages of immobilized whole cell processes depends strongly on the wise selection of a set of processing parameters allowing for high productivity combined with extended operational stability. This set includes the immobilization method, mode of operation, aeration and mixing, bioreactor configuration, medium composition and operational conditions. Freeman and Lilly (1998) have reviewed the effect of processing parameters on the feasibility and operational stability of immobilized viable microbial cells. As the concept of the use of immobilized cells is very closely linked to continuous production/applications (Webb, 1987), the important concepts in continuous operation has to be carefully examined for a successful, process design. There had been studies on the effect of H/D ratio of reactors (Rymowicz *et al*, 1993), dilution rates (Dalili & Chau, 1988., Para & Baratti, 1988., Manalov, 1992), and aeration rates (Mahmoud & Rehm , 1987,

Shiraishi *et al*, 1989., Manalov, 1992) on reactor performance almost in all cases identifying an empirical optimum. Data on the impact of operational aspects such as non-homogenous mixing resulting from air bubble coalescence in reactors (Chen, 1990), effect of sparger type and location (Wu & Hsiun, 1996), biofilm detachment (Moreira *et al*, 1996., Tijhuis *et al*, 1996) thermal conductivity, and efficient pH control on operational stability though available are still very limited.

Medium composition affects operational stability through its effect on the cell growth and *de novo* protein synthesis, as well as creating conditions for prolonged product synthesis (Freeman & Lilly, 1998). Most of the media used in immobilized cell processes for metabolite production are nutritionally limited, compared to the growth medium as in several of these processes growth of the cells is not the primary concern. This is due to the fact that there is often sufficient biomass in/on the carrier and the production medium serves only to supply the maintenance requirements and provide the necessary substrates, inducers and micronutrients for formation of the desired product. However, the use of whole medium is routinely employed for regeneration of the primary population of immobilized cells, so as to maintain extended operational stability. This is accomplished usually by switching alternatively between richer and poorer medium as applied in several instances of immobilized cell operations (Morikawa *et al*, 1980., Deo & Gaucher, 1985., Ogaki *et al*, 1986., Truck *et al*, 1990a). It is also proposed that the composition of the reactivation medium and its mode of operation should also be optimised, eg., whole medium composition (Deo & Gaucher, 1985), and the introduction of the starvation-adaptation period (Deo & Gaucher, 1984). In continuous mode of operation, maintenance of stable level of nutrients required a change in the dilution rate due to the observed gradual change in cell load (Arcuri *et al*, 1986., Keshavarz *et al*, 1990).

Optimisation of the production medium has often resulted in improved product yields (Jager *et al*, 1985., Kirk *et al*, 1986., Tsay & To, 1987., Feijoo *et al*, 1995). Modern concepts in medium optimisation like the factorial designs and response surfaces have been tried recently in processes employing immobilized cells (Suvarnalatha *et al*, 1993., Azanza-Teruel *et al*, 1997, Escamilla *et al* 2000). This is particularly advantageous in understanding the interactions of various process parameters. Greasham & Inamine (1986) and Bull *et al* (1990) have discussed the applications of such designs in microbial processes. More generalised uses in process

improvement are described by Hicks (1993) and Myers & Montgomery (1995). Nevertheless, these radical concepts in process optimisation have not frequently been used with immobilized microbial systems. Use of these modern methods for process improvement may surely be expected to increase in the coming years, and along with a better understanding of the process and the interaction of operational variables.

2.7 Marine bacteria as sources of enzymes

Marine microorganisms take active part in the mineralisation of complex organic matter through degradative pathways of their metabolism in the marine environment. Much of the organic and inorganic wastes expelled by the human population and industries find their way into oceans which become their final resting place. Biodegradation of these complex materials are accomplished eventually by the microorganisms in the marine habitat through the action of their enzymes. Also it is widely accepted that the oceans harbour several compounds and microbes as yet uncharacterised. All these factors pose the marine microorganisms as potent sources of several enzymes with novel and improved properties, which have current importance in the industry. An extensive review of the sources, applications and production of marine microbial enzymes is presented by Chandrasekaran & Kumar (2001) in a recent review. Some of these important enzymes and their microbial sources are given in Table 2.4

Table 2.4
Marine microorganisms as enzyme sources

Enzyme	Source	*Reference*
Agarase	*Bacillus cereus*	Kim et al, 1999
	Vibrio sp	Araki et al, 1998
	Alteromonas sp	Leon et al, 1992
Amylase	*Pyrococcus furiosus*	Koch et al, 1991
	Vibrio alginolyticus	Kim et al, 1999 b
β-glucosidase	*Pyrococcus furiosus*	Kengen et al, 1993
Aryl sulphatase	*Alteromonas carrageenovora*	Barbeyron et al, 1995
Carrageenase	*Cytophaga sp*	Sarwar et al, 1985
Cellulase	*Thermotoga maritima*	Bronnenmeier et al, 1995
Chitinase	*Therococcus chitinophagus*	Huber et al, 1995
	Beauveria bassiana	Suresh & Chandrasekaran, 1999
DNA polymerase	*Thermococcus sp*	Southworth et al, 1996
Endoglucanase	*Pyrococcus furiosus*	Bauer et al, 1999
Glutamate dehydrogenase	*Pyrococcus furiosus*	Consalvi et al, 1991
L-asparginase	*Mucor sp*	Mohapatra et al, 1997
L-glutaminase	*Vibrio sp. &*	Renu &
	Pseudomonas sp.	Chandrasekaran,1992
	Micrococcus luteus	Moriguchi et al, 1994
	Vibrio sp	Nagendraprabhu & Chandrasekaran,1996, 1997
	Beauveria sp.	Sabu et al, 2000
	Pseudomonas sp.	Kumar et al, 2001
Lipase	*Vibrio sp.*	Ando et al, 1991, 1992
Mannanase	*Vibrio sp*	Tamaru et al, 1995
Nuclease	*Vibrio sp*	Maeda & Taga, 1976 b
Protease	*Pseudomonas sp*	Makino et al, 1981
	Vibrio anguillarum	Farrel & Crossa, 1991
	Nocardiopis sp.	Dixit & Pant, 2000
Pullulanase	*Pyrococcus furiosus*	Dong et al, 1997
	Thermotoga maritima	Bibel et al, 1998
Terminal oxidase	*Pseudomonas nautica*	Simpson et al, 1997
Xylanase	*Thermotoga maritima*	Wintehalter & Liebl, 1995
	Vibrio sp	Araki et al, 1999

2.8 Immobilization of marine microbes

Immobilization of marine microbes for biotechnological applications has been rarely attempted despite the fact that they are extremely suited for immobilization due to the adhesive properties exhibited by them. There has been several studies on the adhesion of marine bacteria to immersed surfaces (Fletcher, 1987., Yu *et al*, 1991., Marshall, 1992.) but most of these studies deal with the basic phenomena involved in adhesion and studies on the practical applications of immobilised marine microorganisms are limited. However, there have been some recent attempts on the use of immobilized marine microbes for removal of ammonia (Sakairi *et al*, 1996., Kim *et al*, 2000), production of mareninn (Lebeau *et al*, 2000),and β-carotene (Bhosle & Gagdre, 2001). Production of L-glutaminase using marine *Vibrio costicola* adsorbed on polystyrene (Nagendraprabhu & Chandrasekaran, 1997), Ca-alginate immobilized marine *Pseudomonas* (Kumar *et al*, 2001), and fungus *Beauveria sp.* (Sabu *et al*, 2001) were reported. Valuev (1993), described the immobilization of photoluminescent marine microbes in synthetic hydrogels, and proposed the future applications of immobilized marine bacteria and yeasts. The prospects of using immobilized marine microbes for production of useful metabolites are bright, and radical improvements in this field may be expected in the near future.

2.9. Sea water as base for fermentation media

Nutritional requirements of marine bacteria are quite different from their terrestrial counterparts and information on this aspect is very limited. Seawater composition can be markedly different depending upon the sources and the location. It contains several microelements, and a large amount of organic matter due to the suspended debris of live and dead microbes, and higher organisms, making it rich soup of nutrients. The presence of high salt concentration limits the use of seawater in the cultivation of terrestrial microbes, which are least adapted to such conditions. However, the same conditions may be advantageous in cultivation of marine microorganisms, and several of them show a strict dependence on certain elements present in the seawater.

Maeda & Taga (1976) compared the production of nucleases by several bacteria in seawater and distilled water based media, and found that a marine *Vibrio* sp. was able to synthesise DNAase only when seawater was used as base. The

bacteria showed a strict dependence on seawater even for growth, and magnesium ions were required by the organism for DNAase production. The effect of seawater salts on production of carrageenase by a marine *Cytophaga* sp. was studied by Sarwar *et al* (1985) and it was observed that NaCl and $MgCl_2$ could induce *de novo* synthesis of the enzyme. Seawater based media were successfully employed in the production of L-glutaminase by marine *Vibrio* (Nagendraprabhu & Chandrasekaran, 1997) and marine fungus (Sabu *et al*, 2000) under solid state fermentation, and also employing Ca-alginate immobilized marine *Pseudomonas* (Kumar *et al*, 2001) and *Beauveria* (Sabu *et al*, 2001). Blamey *et al* (1999) used a marine water based medium for cultivation of the deep-sea microbes -*Thermococcus* sp. and *Pyrococcus* sp and observed that Co, Ni, Zn, Mo, Mn and Mg ions were essential for both the organisms. Maeda *et al* (2000) described the use of a seawater medium in the production of hydrogen by marine photosynthetic bacteria. The process however recorded a stable operation for only 10 days with gradual decline in production. Bhosale & Gadre (2001) used seawater-based medium for production of β-carotene by a marine *Rhodotorula glutinis*. A two-fold increase in yield of the pigment was recorded in the seawater medium compared to a distilled water based medium of the same composition, besides easy extraction of the pigment when seawater was used as medium.

However, these studies do not give an insight into the physiological conditions of the marine microbes that enable them to perform better in the seawater based media, or the molecular nature of their dependence on seawater salts. Though seawater may continue to be used as a basal fermentation medium for marine microbes, detailed studies on the molecular nature of these adaptations alone will allow an efficient use of it, in view of future process modelling and design of media for marine microbial fermentations.

2.10 L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2)

Glutaminases are ubiquitous in the biological world (Ohshima *et al*, 1976) and organisms ranging from bacteria to human beings have the enzyme. L-glutaminase (EC.3.5.1.2) is an amidohydrolase which catalyses the hydrolytic deamidation of L-glutamine resulting in the production of L-glutamic acid and ammonia. Glutaminase has a central role in cellular nitrogen metabolism. Two types of glutaminases have been recognised in almost all mammalian tissues (Errera & Greenstien, 1949). These are generally categorised as the kidney type and the liver type glutaminases and both the types have been purified and characterised (Svenneby *et al*, 1973., Cuthroys *et al*, 1976., Heini *et al*, 1987). Interest in amidohydrolases started with the discovery of their anti-tumour properties (Broome, 1961., el-Asmar & Greenberg, 1966., Santana *et al*, 1968., Roberts *et al*, 1970) and since then, lot of efforts has gone into extensive studies on microbial glutaminases with the intention of developing them as anti-tumour agents. A parallel interest on microbial glutaminases stemmed from its application in food flavouring, especially in the soy sauce and related industries of the orient, which initiated the quest for industrial sources of the enzyme. With the development of biotechnology, microbial glutaminases found newer applications in clinical analyses and even in manufacture of metabolites. This led to extensive studies on glutaminase despite the fact that modern biotechnological techniques suggest alternative and specific methods for the treatment of cancers, where amidohydrolases used to be employed.

2.11 Microbial sources and production of L-glutaminase

Since the discovery of its anti-tumour properties, glutaminases have been in prime focus and microbial sources of the enzyme were sought. Wade *et al*, (1971) and Imada *et al* (1973) had tested the amidase activities of thousands of microbes. This was followed by the isolation and purification of glutaminases (Katsumata etal, 1972., Bascomb *et al*, 1975., Eremenkov *et al*, 1975., Lebedeva *et al*, 1981) and their structure determinations (Wlodawer *et al*, 1977., Tanaka *et al*, 1988., Ammon *et al*, 1988) with the ultimate aim of developing these enzymes as a therapeutic agent. Interest in the food related applications also led to a wide screening for microbial sources of the enzymes which subsequently resulted in the discovery of glutaminases with novel properties like pH (PT- JP09149787, 1997.) and temperature tolerance

(PT- JP 113332553, 1999, Sato *et al*, 1999), and active at high salt concentrations (Moriguchi *et al*, 1994, Sato *et al*, 1999), the features particularly desired in the industry. Several species of bacteria, yeasts and fungi are reported to produce glutaminase and an overview of the attempted sources is given in Table 2.5

Table 2.5

Microbial sources of L-glutaminase

T -Terrestrial
M-Marine

Microorganism	*Habitat*	*Reference*
Bacteria		
Acinetobacter glutaminisificans	T	Holchenberg, 1985
Bacillus licheniformis	T	Cook *et al*, 1981
Bacillus subtilis	T	Harayama & Yuasuhara, 1991
	T	Shimazu *et al*, 1991
Erwinia cartowora	T	Wade *et al*, 1971
Micrococcus luteus	M	Moriguchi *et al*, 1994
Pseudomonas 7A	T	PT-WO 9413817, 1994
Pseudomonas aurantica	M	Lebedeva *et al*, 1981, 1997
Pseudomonas fluorescens	T	Eremenkov *et al*, 1975
Pseudomonas nitroreducens	T	Tachiki *et al*, 1996
Pseudomonas sp	M	Kumar *et al*, 2001
Vibrio costicola	M	Nagendraprabhu & Chandrasekaran, 1996
Fungi		
Actinomucor elegans	T	Chou *et al*, 1993
Actinomucor taiwanensis	T	Chou *et al*, 1993
	T	Lu *et al*, 1996
Aspergillus awamori	T	Jones & Lovitt, 1995
Aspergillus oryzae	T	Choi *et al*, 1991
	T	Yano *et al*, 1991
	T	Koibuchi *et al*, PT-W09960104, 1999
Aspergillus sojae	T	Ushijima *et al*, 1987
Aspergillus sp	T	van den Broek & Affolter, PT-W09902691, 1999
Beauveria sp.	M	Sabu *et al*, 2000

Yeasts		
Candida sp	T	PT-J63173586, 1988
Candida utilis	T	Kakinuma *et al*, 1987
Cryptococcus albidus	T	Fukushima & Motai, 1990
Cryptococcus laurentii	T	Kakinuma *et al*, 1987
Cryptococus nodaensis	T	Sato *et al*, 1999
Rhodosporidium toruloides	T	Ramakrishnan & Joseph, 1996
Saccaromyces cerevisiae	T	Abdumalikov *et al*, 1967
Sporomyces sp	T	PT-J63173586, 1988
Torulopsis candida	T	Kakinuma *et al*, 1987

Eventhough several microorganisms, as given above, have been tested as sources of L-glutaminase, feasibility of production technologies were tried only with a few of them. One of the earlier attempts in this direction was that of Eremenkov *et al*, (1975) who observed asparaginase-glutaminase activities under continuous cultivation of *Pseudomonas*. The researchers at Kikkoman Corporation, Japan performed some of the remarkable works on production of the enzyme. The research group at the company used mutation and protoplast fusion techniques for improving glutaminase production by the Koji mould *Aspergillus sojae* (Ushijima *et al*, 1987). A submerged fermentation process for production of thermostable glutaminase by *Cryptococcus nodaensis*, and its purification was developed by the company (Sato *et al*, 1999) and the process was patented (PT-JP11332553, 1999). Another important process describes the production of enzyme from the yeasts *Cryptococcus* sp., *Candida* sp., *Saccaromyces* sp. and *Sporomyces* sp (PT-J63173586, 1988). The process claims to yield high titres of glutaminase, which may be exploited commercially. There had been several promising laboratory scale studies on production of L-glutaminase most of which describing the empirical optimisation of production conditions under submerged and solid state fermentation. (eg., Nagendraprabhu & Chandrasekaran, 1996., 1997., Keerthi *et al*, 1999., Sabu *et al*, 2000, Sabu *et al*, 2001) These studies dealt basically with marine microorganisms and the glutaminases produced are salt-tolerant. One of the enzymes from *Pseudomonas*

sp is even found to be thermo-tolerant besides being able to tolerate salinity (unpublished results). Several new processes like solid state fermentation on inert supports (Nagendraprabhu & Chandrasekaran, 1997) and continuous production employing immobilized fungal spores (Sabu *et al*, 2001) were attempted for the first time. Nevertheless currently the major industrial sources of glutaminase remains to be species of *Bacilli* (Table 2.6).

Table 2.6
Commercial production of microbial L-glutaminase

Manufacturer	*Brand name*	*Source*
Amano enzymes Inc. Japan	Glutaminase F "Amano 100"	*Bacillus subtilis*
Biocatalysts, UK	Flavorpro B73P	*Bacillus sp*
Kikkoman Corporation, Japan	GLN	*Bacillus sp.*
Ajinomoto Co Inc Japan	Glutaminase	*
Enzyme Development Corporation	Enzeco	*Bacillus subtilis*

*Source not disclosed

2.12 Applications of L-glutaminase

Microbial glutaminases have found several potent applications in the recent years. The enzyme, though originally identified as a potent anti cancer drug with possible applications in enzyme therapy, has been used in food industry for flavour enhancement. Recent applications of the enzyme include its use in biosensors and in the manufacture of speciality chemicals by enzymatic transformations. The important applications are discussed below under the categories- therapeutic applications, applications in food industry, analytical applications, and manufacture of fine chemicals.

2.12.1 Therapeutic applications of glutaminase

High rates of glutamine consumption is a characteristic of cancerous cells (Lazarus & Panasci, 1986) and experimental therapies have been developed based on depriving tumour cells of glutamine (Roberts *et al*, 1970., Rosenfeld & Roberts, 1981;Keerthi,1999). Tumour inhibition is mediated by inhibition of both protein and

nucleic acid biosynthesis in the cancerous cells. Inhibition of the tumour cell uptake of glutamine is one of the possible ways to check growth and this is best accomplished by the use of glutaminase, which breaks down glutamine. This in fact results in a selective starvation of the tumour cells because unlike normal cells they lack properly functioning glutamine biosynthetic machinery (Tanaka *et al*, 1988). Several studies were made towards using glutaminase in cancer therapy (Santana *et al*, 1968., Roberts *et al*.1970., Bauer *et al*, 1971., Hersh 1971., Schmid *et al*., 1974., Spiers *et al*, 1979., Warrell, *et al*., 1982). Clinical trials were performed by Warrel *et al* (1982) against adult leukaemia. One of the major problems encountered in the treatments with microbial glutaminases is the development of immune responses against the enzyme. Also the enzyme introduced intravenously has to act at the tumour site within the short span of time it remains in circulation before being cleared at the kidneys. The problems faced in clinical applications of *Achromobacter* glutaminase as a drug in the treatment of leukaemia was described by Spiers *et al* (1979), while, Holchenberg(1979) described the human pharmacology and toxicology of *Acinetobacter* glutaminase. Davis *et al* (1980) tried immobilization of the enzyme in polyethylene glycol and found that the enzyme was active, had a longer half-life in circulation, and is non -immunogenic compared to their free counterparts. More over, the enzyme could be absorbed from the peritoneal cavity. Giordano *et al* (1981) proposed an extracorporeal administration of the enzyme in acute lymphoblastic leukaemia. However, it may be noted that alternative methods for checking the glutamine metabolism of tumour cells are coming up which includes the use of phenyl acetate to deplete glutamine (PT-US 5 877 213, 1999., US 6 037 376, 2000) and the use of gene therapy. Phenyl acetate was proposed as a drug in the treatment of human cancer even before 30years (Neish, 1971). The latter approach was recently tried by use of an antisense mRNA for phosphate activated glutaminase in Ehrlich ascites tumour (Lobo *et al*, 2000). Such improvements in cancer therapy may soon obviate the role of microbial glutaminases as an anti-tumour drug.

One of the most promising therapeutic applications ever proposed for L-glutaminase is in the treatment of HIV. Roberts *et al*, (PT-WO 9413817, 1994) has patented a therapy for HIV, where glutaminase from *Pseudomonas* sp 7A is administered so as to inhibit HIV replication in infected cells. The enzyme can also bring about inhibition of tumour (melanoma) and DNA biosynthesis in affected cells.

2.12.2 Applications of glutaminase in food industry

Glutamate is the most important amino acid in food manufacture for a delicious taste (O'Mahony & Ishi, 1987., Fukushima & Motai, 1990). The pleasant and palatable tastes of oriental fermented foods like soy sauce, *miso* and *sufu* is considered to be related to the content of glutamic acid in them (Kirzimura *et al*, 1969., Takeuchi *et al*, 1972., Chou & Hwan 1994) accumulated due to the hydrolysis of a protein component catalysed by proteolytic enzymes, including proteases and peptidases (Lu *et al*, 1996). Hydrolysis of glutamine by glutaminase may also contribute significantly to the high content of glutamate in these products (Takeuchi & Yokoo 1974., Yamamoto & Hirooka, 1974., Yano *et al*, 1988., Nakadai & Nasuno, 1989., Chou *et al*, 1993).

Several attempts were made to improve the quality of soy sauce and *miso* utilising the action of microbial glutaminases (Takeuchi *et al*, 1972., Takeuchi & Yokoo, 1974., Yokotsuka *et al*,1987., Nakadai & Nasuno, 1989., Harayama & Yahuhira, 1991). Koji mould with highly active glutaminase was used for increasing the glutamate content of soy sauce by Yamamoto & Hirooka (1974), whereas glutaminase of *Cryptococccus albidus* was used for the purpose by Yokotsuka *et al* (1987) and Iwasa *et al* (1987). In another method, the Japan Tokko Koho company used peptidoglutaminase of *Bacillus circulans* to improve the flavour of soy sauce (Kikuchi *et al* PT-S-48-35459, 1973). Glutaminase from *Actinomucor taiwanensis* was used to increase the glutamate content of Sufu by Chou & Hwan (1994).

Impressive studies were undertaken at the Kikkoman Corporation, Japan for production of glutaminase and its applications. Ushijima & Nakadai (1987) used mutation and protoplast fusion techniques for improving glutaminase production by *Aspergillus sojae* employed in *shoyu* fermentation. Fukushima & Motai (1990) achieved continuous conversion of glutamine in liquid seasonings using an immobilized *Cryptococcus albidus*. Sato *et al*, (1999) described a fermentation procedure for glutaminase production by *Cryptococcus nodaensis* in 30L fermenter and an effective method for purification of the enzyme. Recently these processes for glutaminase production and purification have been patented by the company (Sato *et al*, PT- JP11332553, 1999, PT- US 6063 409, 2000).

Several other reports are available on the use of microbial glutaminases in food flavouring (Hamada, 1991., PT- JP09149787, 1997., PT-WO9902691,1999, PT-

US 6 007 851, 1999). Processes for continuous conversion of glutamine to glutamate in food preparations, employing either immobilized glutaminase or whole cells of glutaminase producing microbes are also reported by industries and research institutions (eg., Fukushma & Motai, 1990., PT-US7544562, 1991., Hamada , 1991., Koseko *et al*, 1994).

Salt tolerant glutaminases are very valuable in the industrial processes that require high salt environments like the soy sauce fermentation. Glutaminases from conventional sources (eg., *Aspergillus oryzae*) are markedly inhibited by high salt concentrations as demonstrated by Yano *et al*(1988). Salt tolerant glutaminases were patented for use in industrial processes (PT-JP J63094975, 1987., JP02261379., JP 1133253, 1999). Moriguchi *et al* (1994) has proposed the use of salt tolerant glutaminase from marine bacteria as a possible alternative, since their enzymes could be halophilic rather than halotolerant allowing for the use of high salt concentrations.

2.12.3 Analytical applications

Analysis of glutamine and glutamate levels of body fluids is important in clinical diagnostics and health monitoring. Enzymatic determination of glutamine and glutamate is more accurate and reliable compared to the older techniques like Nesslerization followed by determination of ammonia. Lund (1986) described an efficient spectrophotometric method for determination of L-glutamine and L-glutamate using L-glutaminase and L-glutamate dehydrogenase. Glutaminases are used currently both as free enzyme, or immobilized on membranes of biosensors for monitoring glutamine and glutamate levels of fluids. The application of glutaminase in clinical analyses have led to a tremendous boost in the search for glutaminases which are stable over longer periods for use in biosensors, and companies have started to manufacture highly purified glutaminase enzyme specifically for the purpose. However, the clinically used glutaminases largely come from mammalian sources with only few exceptions. One of the important producers of analytical grade microbial glutaminase is the Kikkoman Corporation, Japan. The *Bacillus sp.* glutaminase produced by the company is used in clinical analyses for determination of glutamine in conjunction with L-glutamate oxidase and peroxidase (Dr Nobuyoshi Sato, Kikkoman Co., personal communication).

An amperometric enzyme electrode probe with membrane immobilized glutaminase was described by Villarta *et al*, (1992) for determination of serum glutamine levels of humans. Botre *et al* (1993) used a glutaminase-based biosensor for determination of glutamine and glutamate in pharmaceutical formulations. The enzyme has also been widely employed in the monitoring of glutamine and glutamate levels in mammalian cell culture media (Huang *et al*, 1995., Mulchandani & Bassi, 1996) and hybridoma culture media (Meyerhoff *et al*, 1993., Campmajo *et al*, 1994) by flow injection analysis using biosensors.

Free enzyme was used in the determination of glutamine in insect cell culture media by Wang *et al* (1993). Important applications are also proposed for glutaminase based biosensors in the online monitoring of fermentation (Schuegerl *et al*, 1991., Matuszewski *et al*, 1991., Rosario *et al*, 1992).

2.12.4 Manufacture of Fine-chemicals

One of the most important emerging applications of glutaminase in industry comes from its use in the manufacture of γ-glutamyl alkamides the most important of which is threonine. L-γ-glutamyl alkamides are prepared by γ-glutamyl transfer from a donor such as glutamine or glutathione to a glutamyl acceptor like ethylamine, methyl amine or glycyl glycine. Glutaminases are found to catalyse these reactions and several processes based on the microbial glutaminase catalysed synthesis of γ-glutamyl alkamides have come up in the recent years. Researchers at the Taiyo Kagaku Co., Ltd., Japan, devised a method for continuous production of threonine employing immobilized *Pseudomonas nitoreducens* as source of glutaminase (Abelian *et al*, 1993). The process reported a threonine yield of 95% on the basis of glutamine consumed.

Patents have been filed for the threonine production process employing glutaminase from *Pseudomonas nitroreducens*, *P.adapta* and *P.denitrificans* (PT-JP 5068578, 1993) and *Bacillus* sp (PT-JP05284983). Tachiki *et al* (1998) used the glutaminase from *P. nitroreducens* for the production of γ-glutamyl- methylamide in addition to threonine by using methylamine as the γ-glutamyl acceptor. A method for production of threonine using the *P. nitroreducens* glutaminase and for producing the enzyme was described (PT-JP 11225789, 1999).

3.MATERIALS AND METHODS

3A. MICROORGANISM

Pseudomonas sp. BTMS 51 was used throughout the study

3A.1 SOURCE

Pseudomonas sp. BTMS-51 was isolated from marine sediments of the Cochin coast (Renu, 1992), and is available as stock culture in the culture collection of the Department of Biotechnology, Cochin University of Science and Technology, India

3A.2 MAINTENANCE OF CULTURE

The culture is maintained on Zobell's marine agar (HImedia®) slants and subcultured at regular intervals. One set of the culture stock is maintained as lyophilised and another set layered with mineral oil. A culture set preserved in refrigerator and routinely subcultured was used as the working culture for various studies.

3B SUBSTRATE

L-Glutamine (SRL, India) was used as the substrate for glutaminase production by the immobilized cells of *Pseudomonas* sp. Also known as L-2-Amino glutaramic acid or α-amino γ-carbamido butyric acid, L-glutamine is a non-essential amino acid in mammals, which plays a significant role in body metabolism. It has molecular weight of 146.15 and the molecular formula is $C_5H_{10}N_2O_5$. Glutamine occurs as optimal isomers with D and L (Greenstein & Winitz, 1961). Common natural sources of the amino acid are wheat gluten hydrolysate and beet sugar molasses.

3C L-GLUTAMINASE PRODUCTION BY Ca-ALGINATE IMMOBILIZED WHOLE CELLS OF *PSEUDOMONAS* SP.

Whole cells of *Pseudomonas* sp. were immobilized by gel entrapment in Ca-alginate beads under conditions, which were selected arbitrarily, and subsequently optimised. The beads with immobilized whole cells of *Pseudomonas* sp. were used for enzyme production studies. Five different media were tested, initially for

selection of a suitable enzyme production medium, and the medium that supported the maximum enzyme yield was selected for further studies. Optimisation of process parameters that influence preparation of stable immobilized cell beads with maximal enzyme yield was carried out. Criteria for selection of optimal conditions were based on the stability of beads and the ability to yield maximal enzyme activity under batch mode in shake flask cultures. The results were incorporated and a repeated batch production study was performed to assess the reusability of the immobilized cell beads. Continuous production of the enzyme by the immobilized cells was studied both in packed bed and in circulating bed reactors, and the efficiency and operational stability of the reactors were evaluated.

3C.1 L-GLUTAMINASE PRODUCTION BY Ca-ALGINATE IMMOBILIZED WHOLE CELLS OF *PSEUDOMONAS* SP. UNDER BATCH MODE

3C. 1.1 Immobilization

3C.1.1.1 Preparation of cells for immobilization

3C.1.1.1.1 Growth Medium

Nutrient Broth was used for cell growth and biomass production. Since the organism is from a marine source, the medium was supplemented with an additional 1% of NaCl. Further, the growth medium contained 0.1% L-glutamine.

The final composition of the medium was

Nutrient Broth (Himedia, India)	-13g
NaCl	-10g*
L-glutamine	-1g
Distilled water	- 1000ml
pH	- 6

* Final NaCl concentration in the medium was 1.5% (w/v)

3C.1.1.1.2 Biomass production

1. A loopful of 18-24h old Zobell's marine agar slant culture was transferred to 10ml of the growth medium taken in a test tube and was incubated in a shaker incubator, at 35 ^{0}C and 200 rpm, for 24h.

2. The test tube grown culture was used for inoculating 100ml of sterile growth medium in 250ml Erlenmeyer flasks. The flasks were incubated as above for 18h.

3. After incubation, 150ml of the culture broth was used to inoculate 1.5L growth medium (10%v/v inoculum) in a 2.5L laboratory fermentor (Eyela, Japan). The fermentor was aerated at 1vvm level with filtered air (0.22μ Millipore® air filter) and agitation was provided by a turbine impeller at 300rpm.Temperature was maintained at 35 ^{0}C and foam control was achieved using a mechanical foam breaker attached to the impeller shaft. Alternatively, 100 ml growth medium in 250ml Erlenmeyer flasks were inoculated at 10% v/v level and were incubated in a shaker incubator, at 35 ^{0}C and 200 rpm. In either of the cases, the incubation was continued for 18h to recover the mid log phase culture, unless otherwise stated.

4. Cells were harvested by centrifugation, under sterile conditions, at 10000 rpm and 4^{0}C for 10 minutes in a refrigerated centrifuge (Kubota, Japan). The cell pellets were washed twice in sterile physiological saline and the wet weight of the pellets was determined. Known weight of the cells was suspended in sterile saline and was used for immobilization.

3C.1.1.2 Gel Entrapment of Cells

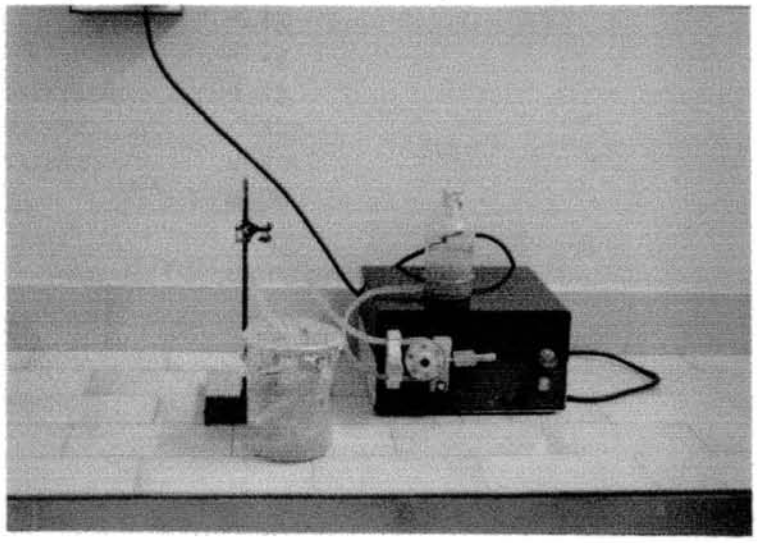
Immobilized viable cell (IVC) beads were prepared by mixing a known quantity of the cell suspension prepared as above, with a sterile Sodium alginate solution of defined concentration, so that the final slurry contained the desired concentration of cells and Sodium alginate. The mixture was pumped through a sterile silicon tube (3mm internal diameter) with a micropipette tip attached at the tip, using a peristaltic pump, into a sterile $CaCl_2$ solution of known concentration, such that spherical beads of an average diameter 4mm were formed (Fig 3.1). The beads were cured in the same solution for the desired period to increase their physical stability, washed, and stored at 4 ^{0}C in sterile physiological saline containing 0.1% $CaCl_2$ until required.

3C.1.2 Activation of beads

The storage of immobilized viable cell (IVC) beads at low temperature slows down the metabolic activities of the cells. Hence, before use in enzyme production the cells have to be acclimatised to the production medium. So the cells in the beads

were activated by incubating the beads in a solution of 1% glutamine in physiological saline for 12h, at room temperature, unless otherwise stated.

Fig 3.1 Experimental set up for gel entrapment of cells



3C.1. 3 Medium selection

Five different media were evaluated to select the most ideal enzyme production medium (EPM) that could support maximal enzyme production by immobilized cells. These include,

1. Medium 1: Mineral Salts Glutamine medium (MSG) (Renu & Chandrasekaran, 1992)

Glutamine	- 10g
Glucose	- 5g
NaCl	- 30g
KH_2PO_4	- 1g
$MgSO_4.7H_2O$	-0.5g
$CaCl_2$	-0.1g
$NaNO_3$	-0.1g
tri Sodium citrate	-0.1g
Distilled water	- 1L
pH	-6.00

2. Medium 2: MSG in 50% Seawater

Glutamine	- 10g
Glucose	- 5g
KH_2PO_4	- 1g
$MgSO_4.7H_2O$	-0.5g
$CaCl_2$	-0.1g
$NaNO_3$	-0.1g
tri Sodium citrate	-0.1g
Aged seawater	-500ml
Distilled water	-500ml
pH	-6.00

3. Medium 3: Seawater Glutamine medium (SWG)

Glutamine	- 10g
Glucose	- 5g
Aged seawater	- 1L
pH	- 6.00

4. Medium 4: SWG in 50% seawater

Glutamine	- 10g
Glucose	- 5g
Aged seawater	-500ml
Distilled water	-500ml
pH	- 6.00

5. Medium 5: Distilled water glutamine medium (DWG)

Glutamine	- 10g
Glucose	- 5g
Distilled water	- 1L
pH	- 6.00

3C.1.4 Enzyme production and recovery

Ca-alginate beads with entrapped *Pseudomonas* sp. cells were prepared and activated as mentioned under sections 3C.1.1.2 and 3C.1.2 respectively. 20g of beads were incubated in 50ml of enzyme production medium (EPM) selected based on the studies mentioned under section 3C.1.3, in 250ml Erlenmeyer flasks at 35 ^{0}C with mild agitation (100rpm) in a shaker incubator for 24h. After incubation, the medium was decanted, centrifuged at 6000rpm at 4 ^{0}C for 10 minutes, and the supernatant was used as the crude enzyme preparation.

3C.1.5 Optimisation of cell immobilization and enzyme production by the immobilized viable cells

The various parameters affecting immobilization of the whole cells of *Pseudomonas* sp. BTMS 51 by Ca-alginate gel entrapment were optimised initially followed by optimisation of parameters for activation and enzyme production by the immobilized cells. The stability of beads as well as the enzyme production efficiency of the immobilized cells were evaluated in order to determine the ideal level of the parameter under test, towards optimisation of the immobilization conditions. However, enzyme yield alone was evaluated while optimising activation and production conditions. Consecutive evaluation of parameters (Sandhya & Lonsane, 1994) was performed. The parameters were varied one at a time and the optimised result was incorporated in the subsequent studies.

3C.1.5.1 Optimisation of immobilization conditions

Factors such as support concentration, initial cell loading, age of cells and concentration of $CaCl_2$ used for bead preparation etc., directly affect the physical properties of beads in terms of its stability, strength, solubility, pore size, mass transfer efficiency etc., besides enzyme yield. Hence, these parameters were optimised towards production of immobilized cell beads with maximal enzyme yield and stability. The parameters studied were in the order - Support (Na-alginate) concentration, inoculum concentration, age (growth stage) of the cells used for immobilization, concentration of $CaCl_2$ in the solution used for immobilization, and curing time. Beads were prepared by varying the levels of these parameters as mentioned under section 3C.1.1.2, and activated as mentioned under section 3C.1.2. The activated beads were subjected to enzyme production studies as outlined under section 3C.1.4 in the EPM, selected from studies conducted under section 3C.1.3. The optimal level of each parameter was determined based on the enzyme yield, and the stability of the beads against solubilization in citrate buffer.

3C.1.5.1.1 Support concentration

Sodium alginate was used as the support for preparation of beads. Optimal support (Na-alginate) concentration was determined by using solutions with varying concentrations of Na-alginate (1%, 2%, 3%, 4% and 5% w/v), and cell concentration

of 1% (w/v) for preparation of beads. Cell suspension in saline was mixed with Na-alginate solution of the desired concentration so that the final mixture contained 1% w/v cells and the desired alginate concentration. The ideal concentration of alginate in solution was determined based on stability and enzyme yield of the immobilized cell beads.

3C.1.5.1.2 Cell loading

Optimal cell loading for the preparation of stable beads with maximal enzyme yield was determined by using different concentrations of cells (0.25%, 0.5%, 0.75%, 1%, and 1.5% wet weight/v) for preparation of beads

3C.1.5.1.3 Age of the inoculum

Ideal age of the cells used for immobilization, which supports maximum enzyme production, was determined using cells at different phases of growth for immobilization. Suspensions prepared from cells incubated for various time intervals (12h, 18h, 24h, 30h, 36h, 42h and 48h) were used for immobilization.

3C.1.5.1.4 Concentration of $CaCl_2$

Optimal concentration of $CaCl_2$ in the solution used for bead preparation and curing was determined by using solutions having different concentrations (0.025M, 0.05M, 0.1M, 0.15M, 0.2M, 0.25M, and 0.3M).

3C.1.5.1.5 Curing time

The influence of curing (hardening) time on production of immobilized-cell beads with maximal stability and enzyme yield was determined by allowing the beads to harden in $CaCl_2$ solution for the varying time intervals (0h, 1h, 2h, 3h, 4h and 5h) after preparation.

3C.1.5.2 Optimisation of Activation Parameters

Optimal conditions that promote activation of the immobilized cells in the stored beads were determined by evaluating them at different levels. Beads prepared under the optimised conditions (section 3C.1.5.1) were subjected to varying process conditions of activation, and the effect was evaluated by estimating the enzyme yield (3C.1.4) in the medium selected (section 3C.1.3). Initially five media were tried as

the activation medium and the ideal medium was selected, based on the enzyme yield. This was followed by further optimisation of the selected medium and activation conditions. The parameters studied were - medium for activation, substrate concentration in the medium, pH of the medium, temperature of activation, and duration of activation in the order of mention.

3C.1.5.2.1 Selection of Activation medium

Five different media selected arbitrarily were evaluated as the activation medium include.

1. Medium 1: 1% w/v glutamine in Physiological saline
2. Medium 2: 1% w/v glutamine in Distilled water
3. Medium 3: 1% w/v glutamine in Sea water
4. Medium 4: Enzyme production medium
5. Medium 5: 1% Glucose in seawater

3C.1.5.2.2 Substrate concentration

Optimal L- glutamine concentration in the activation medium for maximal enzyme yield by the immobilized cells was determined by varying the concentration of glutamine (0.125%, 0.25%, 0.5%, 0.75%, 1%, 1.5% and 2% w/v) in the activation medium.

3C.1.5.2.3 pH of activation medium

Optimal pH of the activation medium that could support activation of immobilized cells for maximal enzyme yield was evaluated by using activation media with pH adjusted to the desired levels (4, 5, 6, 7, 8, 9, and 10)

3C.1.5.2.4 Activation temperature

The ideal incubation temperature that favours activation of immobilized cells for maximal enzyme yield was determined by performing the bead activation at different temperatures (25 ^{0}C, 27 ^{0}C, 30 ^{0}C, 35 ^{0}C, 37 ^{0}C, 40 ^{0}C, and 45 ^{0}C).

3C.1.5.2.5 Activation time

Optimal duration of activation for maximal enzyme production by the immobilized cells was evaluated by incubating the beads for varying time intervals (0h, 6h, 12h, 18h, 24h, 30h, and 36h) in the activation medium followed by estimating enzyme yield in the EPM.

3C.1.5.3 Optimisation of parameters affecting enzyme production

Beads with immobilized viable cell beads were prepared and activated under the optimised conditions (section 3C.1.5.& 3C.1.5.2 respectively) Enzyme production and recovery were performed as mentioned under section 3C.1.4, with SWG as the basal enzyme production medium (selected based on studies performed under section 3C.1.3). Various process parameters influencing enzyme synthesis by the Ca-alginate immobilized cells were optimised towards maximal enzyme production. The parameters tested included pH of the enzyme production medium, temperature of incubation, substrate concentration in the medium, additional carbon sources, additional nitrogen sources, and the time of retention. These were varied one at a time to the desired levels and the effects on enzyme production by the immobilized cells were studied. The conditions, which gave maximal enzyme yield, were considered to be optimal and incorporated in the subsequent studies.

3C.1.5.3.1 pH of enzyme production medium

Initial pH of the enzyme production medium was adjusted to the desired levels (4, 5, 6, 7, 8, 9, and 10) using 1N HCl or 1N NaOH and enzyme production by immobilized cells in these media was evaluated.

3C.1.5.3.2 Incubation Temperature

The influence of incubation temperature on enzyme production was determined by incubating the immobilized cells with enzyme production medium at different temperatures (25 ^{0}C, 27 ^{0}C, 30 ^{0}C, 35 ^{0}C, 37 ^{0}C, 40 ^{0}C and 45 ^{0}C), and analysing the enzyme yield.

3C.1.5.3.3 Substrate concentration

Substrate concentration in the medium that promoted maximal enzyme synthesis by the immobilized cells was determined by varying the amount of L-glutamine added to the enzyme production medium. The concentrations tested include 0.25 %, 0.5% 1%, 1.5% 2%, 2.5% and 3% w/v.

3C.1.5.3.4 Additional carbon sources

Though the substrate L-glutamine can act both as a carbon and nitrogen source, the need for additional carbon source in the enzyme production medium for

enhanced synthesis of the enzyme was evaluated by incorporating the different carbon sources (glucose, galactose, lactose, maltose, sucrose, mannitol and sorbitol) at 0.03M level in the medium. A control was also run with only glutamine added to the medium.

After determination of the ideal additional carbon source, the optimal concentration of the same, for maximal enzyme production by the immobilized cells, was evaluated by incorporating it in the medium at different levels (0.125%, 0.25%, 0.5%, 1%, 1.5% and 2% w/v).

3C.1.5.3.5 Additional nitrogen sources

L-glutamine can serve as a nitrogen source in the medium, and the aged seawater, which was used as the base, contains several undefined organic compounds. However, the requirement of additional nitrogen sources other than L-glutamine in the medium for maximal enzyme synthesis by the immobilized cells, was tested by incorporating different organic nitrogen sources (peptone, tryptone, yeast extract, beef extract, malt extract and soy bean meal) at 0.3% (w/v) level in the EPM. The medium after incubation with immobilized cells was assayed for enzyme activity and protein content. Cell leaching from the beads was measured as change in Optical Density (OD_{600}) of the medium.

3C.1.5.3.6 Retention time

The optimal retention time (time of incubation) for the immobilized cells, which supported maximal enzyme synthesis was determined by incubating the beads in the EPM for a total period of 42h with periodic sampling. Samples were withdrawn as whole flasks at 6h intervals starting from the 6^{th} hour, and the recovered medium was assayed for enzyme activity.

3C.2 PRODUCTION OF L-GLUTAMINASE BY IMMOBILIZED CELLS OF *PSEUDOMONAS* SP. UNDER REPEATED BATCH OPERATION

The prospects for repeated use of the immobilized cells of *Pseudomonas* sp. for enzyme production was explored using the cells repeatedly, for several batches of operation. Beads were prepared and activated as mentioned under sections 3C.1.1.2 and 3C.1.2 respectively. 20g of the activated beads were introduced into 50ml of the optimised EPM (section 3C.1.5.3) in 250ml Erlenmeyer flasks and incubated at 35

^{0}C for 12h (the optimised retention time), in a shaker incubator with a mild agitation of 100 rpm. Medium after incubation was collected, and one half of the medium was used for determination of cell leaching and growth as free cells in the medium (measured as total cell protein). Other half was centrifuged at 6000 rpm, 4 ^{0}C for 10minutes to recover the supernatant which was used for the enzyme assay. The beads were recovered aseptically, washed in sterile saline and were transferred to 50ml of fresh EPM to continue the next cycle of operation. At the end of each cycle, 5 beads were withdrawn and biomass in the beads was estimated as total protein. The repeated batch operation was continued for 20 cycles and the performance of immobilized cells on repeated use was evaluated.

3C.3 CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY Ca-ALGINATE IMMOBILIZED *PSEUDOMONAS* SP. IN A PACKED BED REACTOR

3C.3.1 Medium

The Seawater glutamine medium (SWG) optimised for synthesis of L-glutaminase by the immobilized cells under batch mode, with the following composition, was used as the enzyme production medium for continuous production of L-glutaminase. The substrate (L-glutamine) concentration was varied wherever indicated.

SWG -Composition

L-Glutamine	- 20g
D-Glucose	- 10g
Aged seawater	- 1L
pH	- 6.00

3C.3.2 Immobilization and activation of cells

Immobilization and activation of cells were performed as mentioned under section 3C.1.1.2 and 3C.1.2 respectively, incorporating the conditions optimised for both.

3C.3.3 Reactor set-up

3C.3.3.1 Reactor specifications

A glass column reactor of height 45 cm and internal diameter 3.6 cm was used for the experiment. The reactor column had a bottom support of perforated glass disc at a height of about 2.5cm from the bottom. The bottom also contained an outlet (bottom port), which opened vertically down. Immediately beneath the glass disc and above the bottom port was a nozzle sparger, which entered the column from the side. The reactor also had sampling ports at 5cm intervals from the level of the bottom disc. (Fig 3.2)

3C.3.3.2 Sterilisation and packing of the reactor

All ports of the reactor were closed and the column was sterilised by dry heat in a hot air oven. Tubing and adapters were sterilised by autoclaving and were connected to the appropriate ports under aseptic conditions. All ports and the sparger nozzle were closed except the bottom port and the sampling port at 30cm height. The sampling port was used as the influent port and the bottom port as the effluent port. Activated beads were introduced into the reactor under aseptic conditions and packed to a height of 30cm from the bottom disc. A perforated Teflon disc was positioned over the bed to prevent bed expansion during operation.

3C.3.4. Determination of void volume

Sterile physiological saline was introduced through the influent port using a peristaltic pump and was allowed to fill the reactor till it just reached the top of the Teflon disc. The bottom port was opened and saline was allowed to drain till the liquid level in the column was just below the bottom plate. The volume of the drained saline was measured. The process was repeated five times and the average volume was taken as the void volume of the packed bed reactor. The total working volume of the reactor was 250cc.

3C.3.5 Reactor operation.

The reactor was operated as a top fed column. Medium was introduced through the influent port at 30cm height using a peristaltic pump and allowed to fill the column till the liquid level was just above the bed. The effluent port was opened and the rate of flow of effluent was adjusted to be equal to that of the influent, so that

the liquid level in reactor remained constant at 30cm height. The reactor was run for a minimum of 6-8 h at the required flow rate to attain equilibrium before studying enzyme production at the desired feed rate and substrate concentration. The experimental set up is shown in Figure 3.2. Samples were collected from the effluent port at fixed intervals and subjected to various analyses.

3C.3.6 Effect of Operational parameters on reactor performance

The performance was rated based on the efficiency of the reactor for continuous L-glutaminase production by the immobilized cells. Packing and operation was performed as mentioned above (sections 3C.3.3.2 and 3C.3.6 respectively) with SWG medium containing different concentrations of the substrate (0.5%, 1%, 1.5% and 2% w/v). For each medium the reactor was run at five different flow rates - 60, 80, 100, 120 and 140 ml/h, and at each flow rate the reactor was operated continuously for a minimum of 8-12h after equilibration. Samples were drawn every 2h, and the enzyme production as well as substrate concentration in the influent and effluent was analysed. From these data the volumetric productivity and substrate conversion efficiency of the reactor was calculated

3C.3.6.1 Calculation of volumetric productivity and % substrate conversion

Volumetric productivity = Enzyme Activity (U/ml) x Dilution rate (U/ml/h)

Where, dilution rate $= \frac{1}{\text{Residence time}}$

Residence Time = V_0 / Flow rate , where V_0 is the void volume

Substrate conversion efficiency was measured as percentage substrate conversion

% Substrate conversion =

$$\frac{[\text{substrate conc. in feed} - \text{substrate conc. in effluent}]}{[\text{substrate conc. in feed}]} \times 100$$

3C.3.7 Operational stability of the reactor

The suitability of the reactor over prolonged operation in the continuous mode was evaluated at the substrate concentration and feed rate, which gave maximum substrate conversion efficiency. The reactor was operated continuously for a period of 120h (5days) after equilibration and the performance was evaluated.

3C.4 CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY Ca-ALGINATE IMMOBILIZED *PSEUDOMONAS* SP. IN A CIRCULATING BED REACTOR

3C.4.1 Medium

The Seawater glutamine medium (SWG) with the composition given under section 3C.3.1 was used as the enzyme production medium for continuous production of the enzyme. The substrate (L-glutamine) concentration was varied wherever indicated.

3C.4.2 Immobilization and activation of cells

Immobilization and activation of cells were performed as mentioned under section 3C.1.1.2 and 3C.1.2 respectively, incorporating the conditions, optimised for both.

3C.4.3 Reactor set-up

A glass column reactor with the specifications as given under section 3C.3.3.1 was used for the study. All ports of the reactor were closed and the column was sterilised by dry heat in a hot air oven. Tubing and adapters were autoclaved and connected to the appropriate ports under aseptic conditions. All ports were closed except the bottom port and the 30cm height sampling port. The sparger nozzle was connected to an air compressor (Elico, India) through a 22μ Millipore® air filter and a flow meter (Eyela®, Japan). The sampling port was used as the influent port and the bottom port as the effluent port.

3C.4.4 Reactor operation.

50cc of activated beads were introduced into the reactor under aseptic conditions. The reactor was operated as a top fed column. Medium was introduced through the influent port at 30cm height using a peristaltic pump till 250cc of medium entered the column (liquid level ≅ 25cm from bottom). The effluent port was opened and the rate of flow of effluent was adjusted to be equal to that of the influent, so that the liquid level in reactor remained constant at 250cc. The bed of immobilized cell beads was fluidized pneumatically with filtered air from the compressor at 150cc/minute, monitored by a flowmeter (Fig 3.3).

Fig 3.2 Experimental setup for continuous production of L-glutaminase by Ca-alginate immobilized *Pseudomonas* sp. in a packed bed reactor

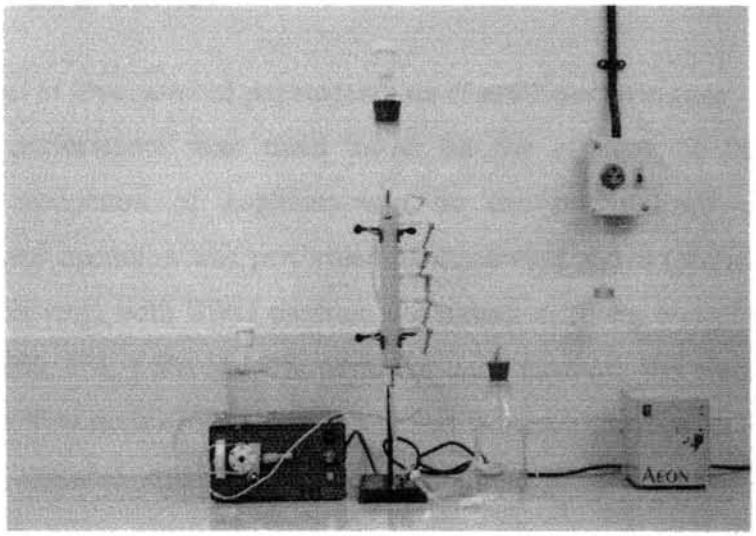
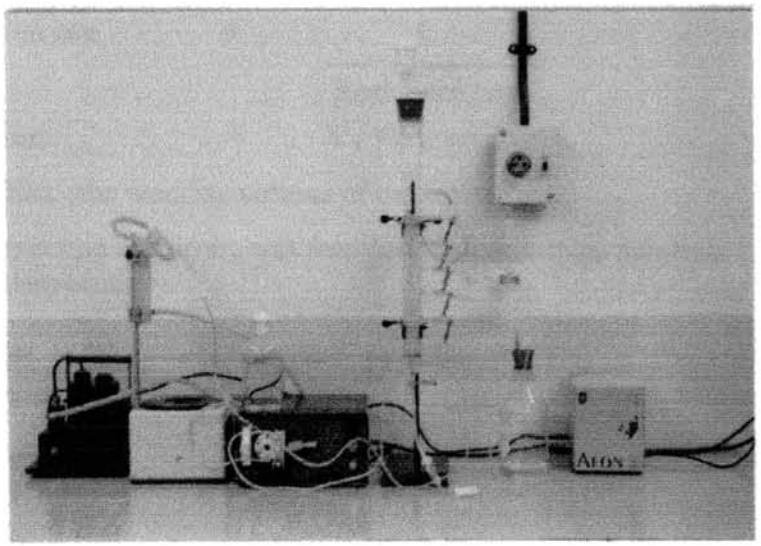


Fig 3.3 Experimental setup for continuous production of L-glutaminase by Ca-alginate immobilized *Pseudomonas* sp. in a Circulating bed reactor



The reactor was run for a minimum of 6-8 h at the required flow rate to attain equilibrium before studying enzyme production at the desired feed rate and substrate concentration. Samples were collected from the effluent port at fixed intervals and subjected to various analyses.

3C.4.5. Effect of Operational parameters on reactor performance

The performance was rated based on the efficacy of the reactor for continuous production of L-glutaminase by the immobilized cells. Reactor preparation and operation was performed as mentioned above (sections 3C.4.3 and 3C.4.4 respectively) with SWG medium containing different concentrations of the substrate (0.5%, 1%, 1.5% and 2% w/v). For each medium, the reactor was run at five different flow rates - (60, 80, 100, 120 and 140 ml/h), and at each flow rate the reactor was operated continuously for a minimum of 8-12h after equilibration. Samples were taken every 2h, and the enzyme production as well as substrate concentration in the influent and effluent was analysed. From these data the volumetric productivity and substrate conversion efficiency of the reactor was calculated as given below

Volumetric productivity = Enzyme Activity (U/ml) x Dilution rate (U/ml/h)

Where, dilution rate $= \frac{1}{\text{Residence time}}$

Residence Time = V / Flow rate,

Where, V=250cc (the working volume of the reactor)

Substrate conversion efficiency was measured as percentage substrate conversion

% Substrate conversion =

$$\frac{[\text{substrate conc. in feed} - \text{substrate conc. in effluent}]}{[\text{substrate conc. in feed}]} \times 100$$

3C.4.6 Operational stability of the reactor

The suitability and stability of the circulating bed reactor for prolonged operation in the continuous mode was evaluated at the substrate concentration and feed rate, which gave maximum substrate conversion efficiency. The reactor was operated

continuously for a period of 120h (5days) after equilibration and the performance was evaluated.

3C.5 ANALYTICAL METHODS

3C.5.1 Enzyme Activity

L-glutaminase activity was determined as the amount of ammonia liberated upon incubation of the sample with buffered substrate (L-glutamine) solution, essentially following the method of Imada *et al* (1973), with minor modifications.

1. A 0.2ml aliquot of the sample was mixed with 0.5ml of a 0.04M L-glutamine solution, in presence of 0.5ml Na-phosphate buffer (0.1M, pH 8.00) and 0.5ml distilled water.

2. The mixture was incubated in a water bath at 37 ^{0}C for 20 minutes

3. The reaction was arrested by the addition of 0.5ml of a 1.5M solution of Trichloroacetic acid (TCA)

4. 0.1ml of the reaction mixture was transferred to 3.7ml of distilled water in another test tube and mixed using a cyclomixer.

5. 0.2ml of Nessler's reagent was added to the test tube, when the ammonia in the reaction mixture gave a pale yellow colour. The amount of ammonia was quantified by measuring the absorbance of the solution at 450nm in a UV-Visible spectrophotometer (Spectronic Genesis 5, USA) exactly 10minutes after addition of Nessler's reagent against suitable blanks and comparing with a standard graph plotted with ammonium chloride as standard.

6. A unit of L-glutaminase activity was defined as the amount of enzyme that liberates 1μM of ammonia under standard assay conditions. Enzyme activity was expressed in units per millilitre (U/ml) of the sample.

3C.5.2 Protein

Protein content in the sample was estimated by the Lowry's method (Lowry *et al* 1951) using Bovine serum albumin (BSA) as the standard and the values were expressed in mg/ml.

Reagents

A) 2% solution of Sodium carbonate (Na_2CO_3) in 0.1N NaOH
B) 0.5% solution of Cupric sulphate ($CuSO_4.5H_2O$) in distilled water.
C) 1% solution of Sodium Potassium tartrate in distilled water.
D) Mix equal volumes of reagents B and C. Prepared fresh before use
E) To one ml of reagent D, add 50ml of reagent A. Prepared fresh before use.

Folin and Ciocalteau's phenol reagent: Dilute 1:1 with distilled water. Prepared fresh

Assay

5ml of freshly prepared reagent E was added to 1ml of the sample, mixed well, and kept for 10 minutes. 0.5ml of Folin's phenol reagent was added and incubated for 30 minutes followed by measuring the absorbance at 750nm in a UV - Visible spectrophotometer (Spectronic Genesis 5, USA).

3C.5.3 Total Cell Protein

Total cell protein was measured using the method of Herbert *et al* (1971), which essentially is a modified Lowry's method.

Reagents

A) 0.5% Sodium carbonate (Na_2CO_3) solution in distilled water.
B) 0.5% Cupric sulphate solution ($CuSO_4.5H_2O$) in 1% Sodium Potassium tartrate (prepared fresh)
C) 50ml of reagent A is mixed with 2ml of regent B. The reagent was prepared immediately before use.
D) Folin and Ciocalteau's Phenol reagent: Diluted 1:1 in distilled water.

Assay

0.5ml of sample was added to a test tube containing 0.5ml of 1N NaOH. The tubes were closed with aluminium foil and the contents were boiled for 5 minutes by keeping the tubes in a water bath at 100 ^{0}C. The mixture was cooled rapidly under tap water and 2.5ml of reagent C is added. The tubes were kept for 10minutes which

was followed by addition of 0.5ml of reagent D. A blank containing 0.5ml of DW instead of sample, and a set of standard protein (Bovine serum albumin) solutions were treated in the same way including the heating stage. After keeping for 30 minutes allowing full colour development, the absorbance was measured at 750nm in a UV-Visible spectrophotometer (Spectronic Genesis 5, USA) against the blank.

3C.5.4 L-Glutamic acid (L-glutamate)

L-glutamate was measured by the modified glutamate dehydrogenase method of Lund (1986). Reduction of NAD to NADH upon enzymatic conversion of glutamate to α- keto glutarate, (which is proportional to the amount of glutamate that is oxidised), was measured for quantification of L-glutamate concentration.

Reagents

1. *Tris EDTA Hydrazine buffer (pH 8.00)*

   Stock solutions of 1M Tris (hydroxymethyl aminomethane) pH 8.00 and 0.5M EDTA (pH 8.00) were prepared and mixed in appropriate concentration so as to obtain the Tris EDTA buffer which is 0.1M with respect to Tris and 0.002M with respect to EDTA and having a pH of 8.00. Hydrazine hydrate was added to the buffer in the ratio 1ml hydrazine per 19ml of buffer.

2. *0.1M ADP*

   A 0.5M stock solution was prepared by dissolving 1.068g of Adenosine 5' Diphosphate (Disodium salt) in de-ionised distilled water and making up to 5ml. 1ml of the stock solution was diluted 5 times before use.

3. *30mM NAD*

   A 0.25M stock solution was prepared by dissolving 1.659 g of β-Nicotinamide Adenine Dinucleotide (free acid) in de-ionised distilled water and making up to 10ml. 1.2ml of the stock solution was made up to 10ml to obtain the 30mM working solution.

4. *1mM L-Glutamic acid (L-glutamate)*

   14.713 mg of L-glutamic acid was dissolved and made up to 100ml in de-ionised distilled water

5. *L-Glutamate Dehydrogenase (GLDH)*

100mg (4500 IU) of lyophilised bovine liver Glutamate dehydrogenase (glutamic dehydrogenase EC.1.4.1.3., Merck, Germany) was dissolved in 4ml of 0.01M Na-phosphate buffer (pH 7.9), so that 1ml of the enzyme solution contained 1125 units of activity.

Assay

1. 250 µl of appropriately diluted sample was added to a micro centrifuge tube that contained 0.5ml of Tris EDTA hydrazine buffer, 50 µl of NAD solution, and 5 µl of ADP. To this mixture was added 185 µl of de- ionised distilled water.

2. The micro centrifuge tubes were closed and the contents mixed gently by inverting.

3. Absorbance was measured at 340nm in a UV-Visible spectrophotometer (Spectronic Genesis 5, USA) to obtain the background reading.

4. 10 µl of GLDH was added to the tubes and mixed gently by inversion.

5. The reaction mixture was incubated at room temperature for 40minutes and the absorbance was measured at 340nm until the absorbance remained constant. The background absorbance was subtracted from this to obtain net absorbance.

6. Concentration of L-glutamic acid (L-glutamate) in the sample was determined from a standard graph constructed using known concentrations of glutamate and performing the assay as above.

3C.5.5 Substrate (L-glutamine) concentration

L-glutamine concentration in the medium was measured in a two step reaction following the method of Lund (1986). L-Glutamine was enzymatically deaminated to L-glutamate and the amount of L-glutamate was measured.

Reagents

1. *0.5M Acetate buffer, pH 5.00*
2. *2mM L-glutamine*
3. *L-Glutaminase*
   Lyophilised Bovine liver L-glutaminase EC.3.5.1.2 (Sigma -Aldrich, USA) was dissolved in reagent 1 diluted 1:10 in deionized distilled water, so as to obtain a 10U/ml solution.

Assay

1. 250µl of the appropriately diluted test sample is added to a microcentrifuge tube containing 450µl of deionized distilled water and 200µl of 0.5M acetate buffer (pH 5.00).

2. 100µl of L-glutaminase is added and mixed gently by inversion. The tubes are incubated for 1h at 37 ^{0}C when the L-glutamine is converted to L-glutamate.

3. 250µl of the reaction mixture was used for determination of L-glutamate content as mentioned under section 3C.5.4

4. A standard curve was prepared by performing the reaction as above with known concentrations of glutamine.

5. For samples containing both glutamine and glutamate (as is the case with the EPM) endogenous glutamate concentration was determined initially as in section 3C.5.4 and the values were subtracted from glutamate concentration derived from deamination of glutamine.

3C.5.6. Bead Stability

Stability of beads with immobilized cells of *Pseudomonas* sp. was evaluated in terms of their ability to withstand dissolution in citrate buffer. 200mg of beads (5beads) were suspended in 10ml of 0.1M citrate buffer (pH 5.00) and subjected to mild agitation on a rotary shaker at 100rpm. Time required for complete dissolution of beads was noted and the beads, which lasted longer, were considered as more stable. Bead stability was expressed in terms of dissolution time in minutes.

3C.5.7 Biomass in the beads

Biomass content in the beads was measured in terms of the total cell protein content. 5 beads (200mg) were washed in saline and dissolved in 10ml of 0.1M citrate buffer (pH 5.00). Total cell protein in the solution was estimated as outlined under section 3C.5.3 and expressed in terms of mg protein /g bead (mg/g).

3C.5.8. Cell leaching and Cell growth in medium

Cell leaching from the beads and growth as free cells in the medium was measured as the biomass in the medium. The medium was assayed for total cell protein following the method given under section 3C.5.3 and expressed in terms of mg protein/ml medium (mg/ml).

3D. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON POLYSTYRENE BEADS

The natural phenomenon of bacterial adherence and biofilm formation on surfaces immersed in aquatic environments was exploited for immobilization of *Pseudomonas* sp. by physical adsorption onto polystyrene beads. This was achieved by allowing the cells to grow in a medium containing pre-treated polystyrene beads. Initially the conditions required for growth and colonisation of the cells on polystyrene beads were optimised under batch mode and the enzyme yield from the immobilized cells was evaluated. The medium and conditions ideal for maximal enzyme synthesis by the immobilized cells was then optimised under batch mode using a response surface methodology. The results were incorporated and repeated batch studies conducted to explore the potential for repeated use of the cell-immobilized beads. Continuous synthesis of L-glutaminase by the immobilized cells was studied in a packed bed reactor and the operational stability of the reactor was evaluated.

3D.1 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON POLYSTYRENE BEADS UNDER BATCH OPERATION

3D.1.1 Support matrix and pre-treatment

Polystyrene (poly(1-phenylethylene), which is a commercially available insulating and packaging material was used as the inert support for immobilization of

bacterial cells. Polystyrene beads purchased as a single lot from local market were subjected to pre-treatment, before use as the carrier matrix (Nagendraprabhu & Chandrasekaran, 1995). Beads of 4-5mm diameter was autoclaved at 121 ^{0}C, 15 lbs for 20minutes during which they collapsed and reduced in size (Brydson, 1982). Reduced beads of uniform size (≅ 2.5mm, 0.0084cc) were selected for the immobilization studies (Fig 3.4a). The beads were washed in distilled water and dried at 70 ^{0}C in a hot air oven. The dried beads were sterilised before use by autoclaving at 121 ^{0}C, 15 lbs for 20 minutes.

3D.1.2 Immobilization

3D.1.2.1 Inoculum preparation

1. A loopful of 18h old agar slope culture of *Pseudomonas* sp. was inoculated to 10ml of growth medium (3C.1.1.1.1) in a test tube and incubated at 35 ^{0}C and 200rpm agitation, for 24h in shaker incubator.

2. The test tube grown culture was inoculated to 100ml of the same medium in 250ml Erlenmeyer flasks and was incubated as above for 18h.

3. The cells were harvested by centrifugation at 10000 rpm and 4 ^{0}C for 10minutes under aseptic conditions in a refrigerated centrifuge (Kubota, Japan)

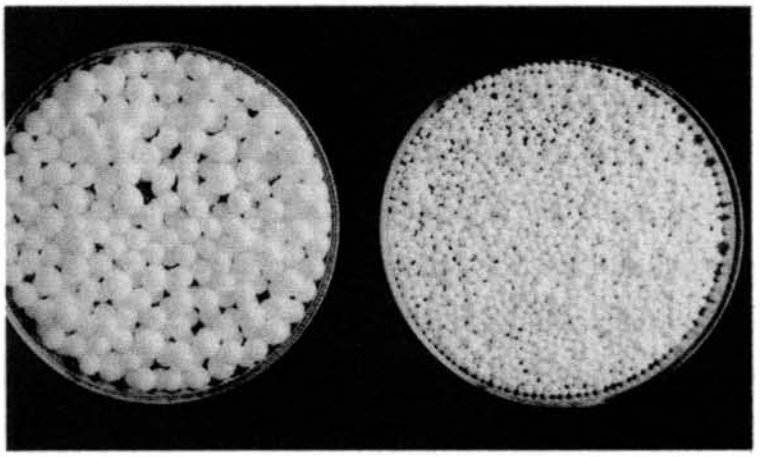
4. The cell pellets were washed in sterile physiological saline and a known weight of the cells were suspended in saline to make a 1% w/v (10mg/ml) cell suspension which was used as the inoculum.

3D.1.2.2 Immobilization by cell attachment and growth on polystyrene beads

Immobilization of the whole cells of *Pseudomonas* sp. on polystyrene beads was performed by growing the cells along with pre-treated polystyrene beads in an immobilization medium. The cells during their growth were attached to the support matrix (polystyrene beads) and colonised the surface as a biofilm. 2ml of the inoculum (20mg cells) prepared as above (section 3D.1.2.1) was used to inoculate 100ml of immobilization medium containing 50cc (21.786g, ~5952 Nos.) of polystyrene beads in 250ml Erlenmeyer flasks. The flasks were incubated in a shaker incubator at 35 ^{0}C with a mild agitation of 80 rpm for the desired time to facilitate cell growth and adsorption on the support.

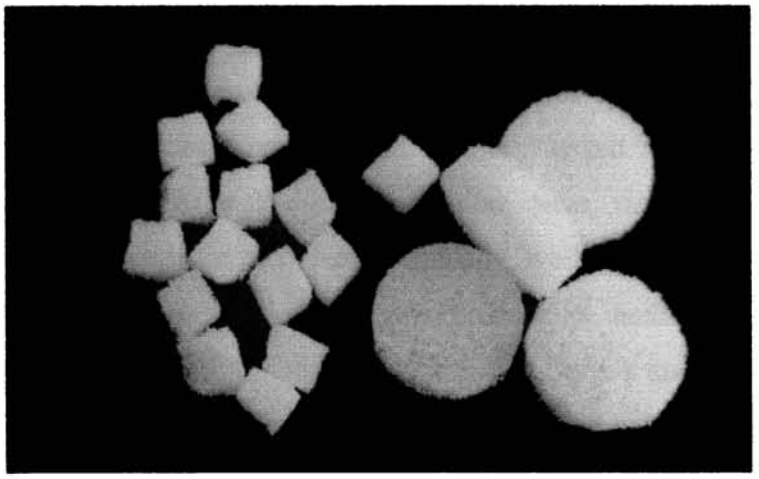
Fig 3.4 Inert synthetic supports used in immobilization of *Pseudomonas* sp.

a) Polystyrene beads

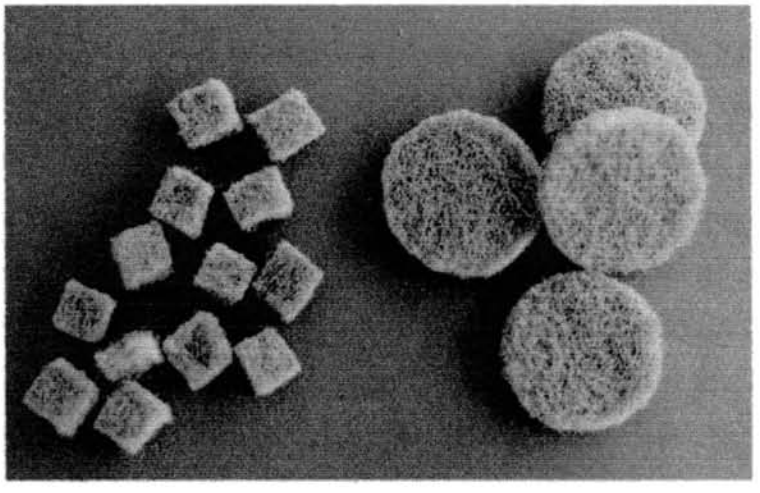


A-Before treatment, B-After treatment

b)PUF cubes and discs used for immobilization



c)Nylon web cubes and discs



3D.1.3 Selection of immobilization medium and incubation time.

Growth medium and the incubation period were considered important for cell growth and adsorption on polystyrene beads. Two media (MSG medium [section 3C.1.3] and Zobell's marine broth [Himedia®, India] were tested as the growth (immobilization) medium. Immobilization experiments were performed, as mentioned under section 3D.1.2.2, using each of the media as the immobilization medium. Efficiency of the media and ideal time for incubation to effect maximal cell adsorption along with growth was evaluated simultaneously by monitoring cell growth in the medium and cell adsorption on polystyrene beads, at 6h intervals for a total period of 48h. 1ml of the growth medium and 250 (2.1cc) beads of polystyrene were recovered aseptically at these intervals and biomass estimations (measured as total cell protein) were performed to assess growth as free cells and cell adsorption.

3D.1.4 Enzyme production and recovery

Cells were immobilized on polystyrene beads, as outlined under section 3D.1.2.2, using the medium and incubation time selected from studies performed under section 3D.1.3. After the immobilization cycle, the growth medium was decanted and the beads were recovered aseptically, washed in sterile saline and 50cc of it were transferred to 50ml of enzyme production medium (EPM) in 250ml Erlenmeyer flasks. The flasks were incubated at 35 ^{0}C with a mild agitation of 80rpm in a shaker incubator unless otherwise indicated. After incubation the medium was decanted, centrifuged at 6000rpm, and 4 ^{0}C, for 10minues in a refrigerated centrifuge (Kubota, Japan), and the supernatant was used as the crude enzyme preparation.

3D.1.5 Selection of enzyme production medium and incubation time

Mineral salt glutamine medium (MSG) and seawater glutamine medium (SWG) with the compositions given under section 3C.1.3 were tested for their suitability as the enzyme production medium (EPM). Enzyme production studies were performed as mentioned above (section 3D.1.4) with either of the media as the EPM. Samples were withdrawn at 6h intervals for a total period of 24h and assayed for enzyme activity, cell biomass on support, and cell detachment from support (free cell growth in medium measured as total protein). The medium that supported maximal enzyme synthesis by the immobilized cells, and the ideal incubation time

were determined and further studies were performed in the selected EPM and incubation time.

3D.1.6 Optimisation of parameters for enzyme production by *Pseudomonas* sp immobilized on polystyrene beads

The EPM and the important environmental parameters affecting enzyme synthesis by the immobilized cells were optimised using a response surface type δ Box-Behnken (Box & Behnken, 1960) model experimental design. The treatments considered on the design were concentration of substrate (L-glutamine) carbon source (D-glucose) and nitrogen source (Yeast extract) in the medium, pH of the medium, and incubation temperature.

3D.1. 6.1 Design of experiment

Box-Behnken design model, is a second-order design that allows estimation of quadratic effects, and is based on combining a two-level factorial design with an incomplete block design. This design was used for creating the quadratic response model. For such designs, factors need to be measured at three levels.

The design model assumed that L-glutaminase production by the immobilized cells was a function of the five selected factors (concentration of L-glutamine, glucose, and yeast extract, pH of medium and incubation temperature) through multinomials of appropriate degree. A design model with 46 runs in 2 blocks of 23 cases was used as exhibited in Table 3.1, and each independent variable was tested at three levels. The levels were coded in standardised units with the values -1, 0 and 1 representing the lower middle and higher levels respectively. The following quadratic model was chosen to represent the relationship fitted between the above five variables.

$$Y = b_0 + \sum_{i=1}^{5} b_i x_i + \sum_{i=1}^{5} \sum_{j=1}^{5} b_{ij} x_i x_j$$

Or in the expanded form,

$$\begin{aligned} Y = {} & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + \\ & b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + \\ & b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 \end{aligned}$$

Table 3.1

Box -Behnken design for 5 variables at 3 levels -2 blocks and 46 runs

BLOCK	RUN	X1	X2	X3	X4	X5
1	1	-1	-1	0	0	0
1	2	1	-1	0	0	0
1	3	-1	1	0	0	0
1	4	1	1	0	0	0
1	5	0	0	-1	-1	0
1	6	0	0	1	-1	0
1	7	0	0	-1	1	0
1	8	0	0	1	1	0
1	9	0	-1	0	0	-1
1	10	0	1	0	0	-1
1	11	0	-1	0	0	1
1	12	0	1	0	0	1
1	13	-1	0	-1	0	0
1	14	1	0	-1	0	0
1	15	-1	0	1	0	0
1	16	1	0	1	0	0
1	17	0	0	0	-1	-1
1	18	0	0	0	1	-1
1	19	0	0	0	-1	1
1	20	0	0	0	1	1
1	21	0	0	0	0	0
1	22	0	0	0	0	0
1	23	0	0	0	0	0
2	1	0	-1	-1	0	0
2	2	0	1	-1	0	0
2	3	0	-1	1	0	0
2	4	0	1	1	0	0
2	5	-1	0	0	-1	0
2	6	1	0	0	-1	0
2	7	-1	0	0	1	0
2	8	1	0	0	1	0
2	9	0	0	-1	0	-1
2	10	0	0	1	0	-1
2	11	0	0	-1	0	1
2	12	0	0	1	0	1
2	13	-1	0	0	0	-1
2	14	1	0	0	0	-1
2	15	-1	0	0	0	1
2	16	1	0	0	0	1
2	17	0	-1	0	-1	0
2	18	0	1	0	-1	0
2	19	0	-1	0	1	0
2	20	0	1	0	1	0
2	21	0	0	0	0	0
2	22	0	0	0	0	0
2	23	0	0	0	0	0

In this model, Y represents the dependent variable =Enzyme yield, X_1, X_2, X_3, X_4 and X_5 are the independent variables denoting glutamine conc., glucose conc., yeast extract conc., pH of medium and incubation temperature respectively, b_1, b_2, b_3, b_4 and b_5 are linear coefficients, b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35} and b_{45} are second order interaction coefficients, and b_{11}, b_{22}, b_{33}, b_{44} and b_{55} are the quadratic coefficients. The design of experiments in terms of actual factors is given in Table 3.2.

The levels tested were L-glutamine- 0.5, 1 and 1.5% w/v; glucose - 0.5, 1 and 1.5% w/v; Yeast extract 0, 0.25 and 0.5% w/v; pH 4, 6 and 8; and incubation temperature 30^0C, 35^0Cand 40^0C. Media were prepared with the desired concentration of the components and adjusted to the desired initial pH. Enzyme production studies were conducted as outlined under section 3D.1.4 using each of the above media as the EPM, at the desired incubation temperature and incubating for 12h (selected based on earlier studies mentioned under 3D.1.5). The experimental runs were performed in a randomised sequence with three replications of each combination of factors. The average of enzyme yield, obtained for the three trials was considered as the response for each experimental run.

3D.1.6.2 Data analysis and Optimisation

The responses obtained from the trials conducted as above following the Box Behnken design model for five variables, was used to estimate the coefficients of the polynomial model using standard regression techniques. The estimate of "Y" was used to generate an optimal combination of factors that can support maximal enzyme synthesis by the immobilized cells using predictive models from response surface methodology. The software "Design-Expert ®" (Stat-Ease Inc., USA) was used to fit the response surface -Box Behnken model to the experimental data.

Table 3.2

Box Behnken design model for optimisation of L-glutaminase production by *Pseudomonas* sp. immobilized on polystyrene

Block	Run #	pH	Temp. (^{0}C)	Glutamine. Conc. (%w/v)	Glucose. Conc. (%w/v)	Yeast Extract Conc. (%w/v)
		X_1	X_2	X_3	X_4	X_5
1	1	8	40	1	1	0.25
1	2	8	30	1	1	0.25
1	3	4	40	1	1	0.25
1	4	4	30	1	1	0.25
1	5	6	35	1.5	1.5	0.25
1	6	6	35	1.5	0.5	0.25
1	7	6	35	0.5	1.5	0.25
1	8	6	35	0.5	0.5	0.25
1	9	6	40	1	1	0.5
1	10	6	40	1	1	0
1	11	6	30	1	1	0.5
1	12	6	30	1	1	0
1	13	8	35	1.5	1	0.25
1	14	8	35	0.5	1	0.25
1	15	4	35	1.5	1	0.25
1	16	4	35	0.5	1	0.25
1	17	6	35	1	1.5	0.5
1	18	6	35	1	1.5	0
1	19	6	35	1	0.5	0.5
1	20	6	35	1	0.5	0
1	21	6	35	1	1	0.25
1	22	6	35	1	1	0.25
1	23	6	35	1	1	0.25
2	24	6	40	1.5	1	0.25
2	25	6	40	0.5	1	0.25
2	26	6	30	1.5	1	0.25
2	27	6	30	0.5	1	0.25
2	28	8	35	1	1.5	0.25
2	29	8	35	1	0.5	0.25
2	30	4	35	1	1.5	0.25
2	30	4	35	1	0.5	0.25
2	32	6	35	1.5	1	0.5
2	33	6	35	1.5	1	0
2	34	6	35	0.5	1	0.5
2	35	6	35	0.5	1	0
2	36	8	35	1	1	0.5
2	37	8	35	1	1	0
2	38	4	35	1	1	0.5
2	39	4	35	1	1	0
2	40	6	40	1	1.5	0.25
2	40	6	40	1	0.5	0.25
2	42	6	30	1	1.5	0.25
2	43	6	30	1	0.5	0.25
2	44	6	35	1	1	0.25
2	45	6	35	1	1	0.25
2	46	6	35	1	1	0.25

3D.2 PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON POLYSTYRENE BEADS UNDER REPEATED BATCH OPERATION

The suitability of the cells adsorbed on polystyrene beads for repeated use in enzyme production was evaluated by reusing the polystyrene beads for several cycles of operation. Cells were immobilized as mentioned in section 3D.1.2.2 using the immobilization medium and incubation time selected from studies performed under section 3D.1.3. 50 cc of the beads with immobilized cells washed in sterile saline, was introduced into 50ml of the optimised EPM (section 3D.1.6) in 250ml Erlenmeyer flasks. The flasks were incubated with mild agitation (80rpm) at 35 ^{0}C in a shaker incubator for 12h (selected based on studies described under section 3D.1.5). After incubation, the polystyrene beads were recovered aseptically, washed in sterile saline and introduced into 50ml of fresh EPM for the next cycle of operation as described above. The medium was recovered after each cycle, and one portion was used for estimation of cell detachment and growth as free cells (cell biomass measured as total protein). The other portion was centrifuged at 6000 rpm and 4 ^{0}C for 10minutes in a refrigerated centrifuge (Kubota Japan) and the supernatant was used for enzyme assays. At the end of each cycle, 250 beads (2.1cc) of polystyrene beads were recovered and cell biomass attached to them was measured as total cell protein. The beads recovered were replaced with an equal amount of beads from a parallel run to maintain the total volume constant. The repeated batch experiment was continued for 20 cycles and the reusability of beads was assessed in terms of their ability to maintain the attached biomass in the enzyme production phase.

3D.3. CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP IMMOBILIZED ON POLYSTYRENE IN A PACKED BED REACTOR

3D.3.1 Medium

The Seawater glutamine medium (SWG) optimised for L-glutaminase production under batch mode (3D.1.6.2) was used as the EPM with minor modifications (substrate concentration) for continuous production of the enzyme. The substrate (L-glutamine) concentration was varied wherever indicated.

SWG -Composition

L-Glutamine	- 15.0g
D-Glucose	- 12.4g
Yeast Extract	- 3.0g
Aged seawater	- 1.0L
pH	- 7.8

3D.3.2 Reactor set-up

3D.3.2.1 Reactor

A glass column reactor with the specification given under section 3C.3.3.2 was used.

3D.3.2.2 Sterilisation and packing of the reactor

All ports of the reactor were closed and the column was sterilised by dry heat in a hot air oven. Tubing and adapters were autoclaved and connected to the appropriate ports under aseptic conditions. All ports and the sparger nozzle were closed except the bottom port and the 20cm height sampling port. The sampling port was used as the influent port and the bottom port as the effluent port. Polystyrene beads pre-treated and sterilised as in section 3D.1.1 were introduced into the reactor under aseptic conditions and packed to a height of 20cm from the bottom disc. A perforated Teflon disc was positioned over the bed to prevent bed expansion during operation.

3D.3.3. Determination of void volume

Reactor void volume was determined as outlined under section 3C.3.4.

3D.3.4. Cell immobilization on the polystyrene bed

Whole cells of *Pseudomonas* sp. were immobilized on the polystyrene bed packed within the column by circulating an actively growing culture of the bacterium through the bed. 15ml of a cell suspension inoculum, prepared as in section 3D.1.2.1, was used to inoculate 1.5L of the immobilization medium (selected based on studies mentioned under section 3D.1.5) in a 2.5L laboratory fermentor (Eyela, Japan) and cells were cultured at 35 ^{0}C. The fermentor was aerated at 1vvm level with filtered (0.22μ Millipore air filter) air and agitation was provided by a turbine impeller at 300rpm. Foam control was achieved using a mechanical foam breaker attached to the

impeller shaft. Medium from the fermentor was pumped into the glass column reactor at a flow rate of 80ml/min using a peristaltic pump. The medium was introduced through the influent port and allowed to fill column till it reached the top of the bed. The bottom port was opened and the medium was allowed to flow out at the same flow rate (using a valve) as the influent stream so that the liquid level remained constant just above the bed. The effluent was reintroduced into the fermentor so as to maintain a re-circulating stream of actively growing cells. The experimental set up is shown in Figure 3.5. The circulation of growing cells through the bed was continued for a total period of 36h when the cells in the well-mixed immobilization medium from the fermentor were attached to the bed by adsorption eventually colonising the bed surface. The progress of cell adsorption on bed surface was evaluated by sampling the bed at 6h intervals. 100 beads were recovered aseptically and the biomass attached to them was estimated as total cell protein. The bed volume was maintained constant by replacing an equal amount of immobilized cell beads from a parallel run maintained under batch mode as shake flask culture. Cell growth in the medium was also estimated as total cell protein by taking medium samples aseptically at 6h intervals from the influent port.

3D.3.5 Reactor operation.

Immobilization of cells on the polystyrene bed was performed as detailed above (3D.3.4). The bed was washed by circulation of sterile physiological saline at a flow rate of 80ml/h for 1h, which approximately was the residence time at that flow rate for the column. For studies on continuous production of L-glutaminase, the reactor was operated as a top fed column (Fig 3.6). EPM was introduced through the influent port using a peristaltic pump and allowed to fill the column till the liquid level reached the top of the bed. The effluent port was opened and the rate of flow of effluent was adjusted to be equal to that of the influent, so that the liquid level in reactor remained constant just above the bed. The reactor was run for a minimum of 6-8 h, at the required flow rate, to attain equilibrium before studying enzyme production at the desired feed rate and substrate concentration. Samples were collected from the effluent port at regular intervals and assayed for enzyme activity.

Fig 3.5 Experimental setup for immobilization of *Pseudomonas* sp on a packed bed of Polystyrene in a column reactor

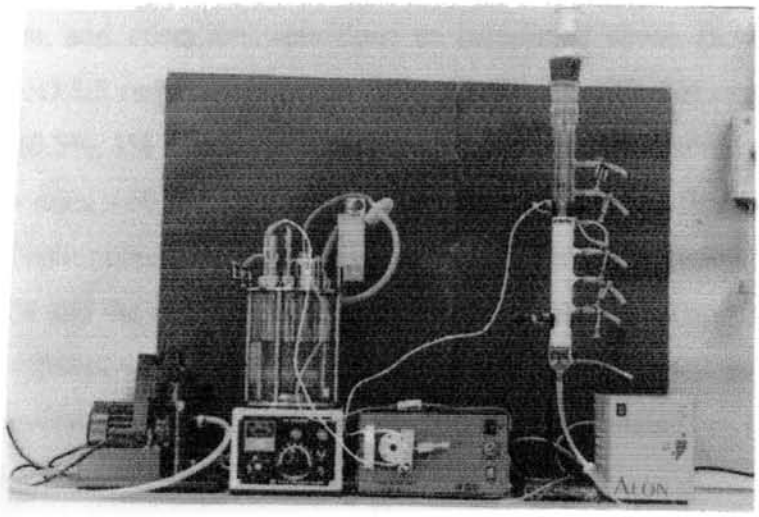
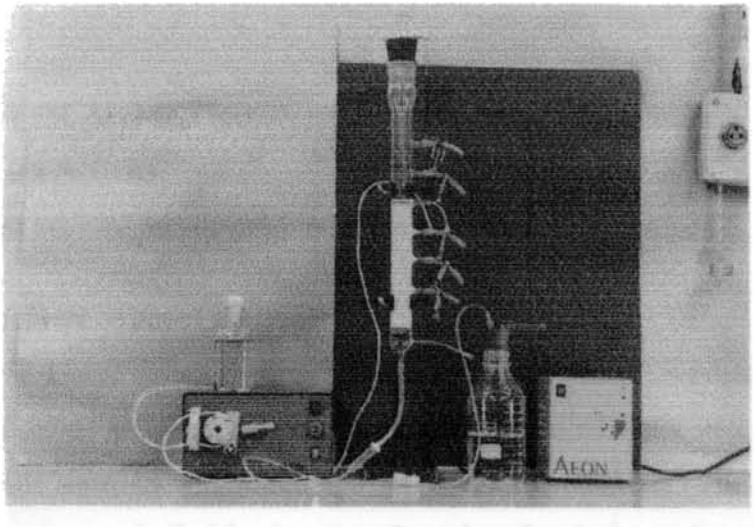


Fig 3.6 Experimental setup for continuous production of L-glutaminase by *Pseudomonas* sp. adsorbed on polystyrene in a PBR



3D.3.6 Effect of Operational parameters on reactor performance

The reactor performance was evaluated based on the efficiency of the reactor for continuous L-glutaminase production by the immobilized cells. Packing, immobilization, and operation were done as mentioned above (sections 3D.3.3.2 ,3D.3.4, and 3D.3.5 respectively) with EPM containing different concentrations of the substrate (0.5%, 1% and 1.5% w/v). For each medium the reactor was run at five different flow rates :- 60, 80, 100, 120 and 140 ml/h, and at each flow rate the reactor was operated continuously for a minimum of 8-12h after equilibration. Samples were taken every 2h and the enzyme production, as well as substrate concentration, in the influent and effluent were analysed. From these data the volumetric productivity and substrate conversion efficiency of the reactor were calculated as detailed under section 3C.3.6.1

3D.3.7 Operational stability of the reactor

The suitability of the packed bed reactor for prolonged operation in the continuous mode was evaluated at the substrate concentration and feed rate, which gave maximum substrate conversion efficiency. The reactor was operated continuously for a period of 72h (3days) after equilibration and the performance was evaluated.

3D.4 ANALYTICAL METHODS

3D.4.1 Enzyme Activity

L-glutaminase activity was determined as described under section 3C5.1

3D.4.2 Biomass on support matrix

250 (2.1 cc) beads with immobilized cells were washed in 5ml of physiological saline and were suspended in 5ml of distilled water in a test tube. 5ml of a 1M NaOH solution was added and mixed in a cyclomixer. The solution with suspended beads was boiled by keeping the tubes for 5minues in a boiling water bath, after which they were cooled rapidly in water. The protein released by alkali lysis of the adsorbed cells was measured as detailed under section 3C.5.3. Concentrations were expressed as mg/cc of polystyrene.

3D.4.3 Growth as free cells/biomass in medium.

Cell biomass in the medium was estimated as total cell protein to assess the growth of bacteria as free cells in the medium. Medium was diluted to the required level and 1ml of the diluted medium was used for analysis of total cell protein as mentioned under section 3C.5.3, and was expressed as mg/ml of medium.

3D.4.4 Substrate concentration

Glutamine concentration in sample was measured as mentioned under section 3C.5.5

3E. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON POLURETHANE FOAM

Whole cells of *Pseudomonas* sp. were immobilized onto polyurethane foam by physical adsorption exploiting the natural phenomena of cell attachment and biofilm formation on immersed surfaces. Immobilization was performed by growing the cells in a medium containing pre treated polyurethane foam (PUF), when the cells adhered onto the support matrix and colonised it as a biofilm. Initially, the conditions required for growth and colonisation of the cells on PUF were optimised under batch mode and the enzyme production efficiency of the immobilized cells were evaluated. The medium and conditions ideal for maximal enzyme synthesis by the immobilized cells was then optimised under batch mode using a response surface methodology. The results were incorporated and repeated batch studies were conducted in order to explore the potential for repeated use of the cell-immobilized beads. Continuous synthesis of L-glutaminase by the immobilized cells was studied in a packed bed reactor and the operational stability of the reactor was evaluated.

3E.1 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON PUF UNDER BATCH MODE

3E.1.1 Support matrix and pre-treatment

Polyurethane foam, which is a commercially available insulating and packaging material, was used as the inert support for immobilization of bacterial cells. PUF sheets purchased from local market, were cut (dimension of 1 X 1 X 0.7 cm) so as to form cubes with a volume of 0.7cc (Fig 3.4b). The cubes were washed in distilled water and autoclaved at (121 ^{0}C and 15lbs for 20minutes) before use.

3E.1.2 Immobilization

3E.1.2.1 Inoculum preparation

Inoculum was prepared using *Pseudomonas* sp. at a concentration of 10mg/ml as mentioned under section 3D.1.2.1

3E.1.2.2 Immobilization by cell attachment and growth on PUF cubes

Immobilization of *Pseudomonas* sp on PUF was carried out by growing the cells along with pre-treated cubes of PUF in an immobilization medium. The cells, during their growth, were attached to the support matrix and colonised the surface as a biofilm. 2ml of the inoculum (20mg cells) prepared as detailed above (section 3E.1.2.1) was used to inoculate 100ml of immobilization medium containing 50cc (71 Nos.) of PUF cubes in 250ml Erlenmeyer flasks. The flasks were incubated in a shaker incubator at 35 ^{0}C with a mild agitation of 80 rpm, for the desired time to facilitate cell growth and adsorption on the support.

3E.1.3 Selection of immobilization medium and incubation time.

Two media were tested as the growth (immobilization) medium, which were MSG medium (section 3C.1.3) and Zobell's marine broth (Himedia, India). Immobilization experiments were performed, as said in section 3E.1.2.2, using each of the media as the immobilization medium. Efficiency of the media and ideal time for incubation to effect maximal cell adsorption along with growth was evaluated simultaneously by monitoring cell growth in the medium and cell adsorption on PUF cubes at 6h intervals for a total period of 48h. 1ml of the growth medium and 3 cubes (2.1 cc) of PUF were recovered aseptically at the said intervals and biomass estimations (measured as total cell protein) were performed to assess growth as free cells and cell adsorption.

3E.1.4 Enzyme production and recovery

Cells were immobilized on PUF cubes as outlined under section 3E.1.2.2 using the medium and incubation time selected from studies performed under section 3E.1.3. After the immobilization cycle, the growth medium was decanted, and the cubes were recovered aseptically, washed in sterile saline, and 50cc of it was transferred to 50ml of enzyme production medium (EPM) in 250ml Erlenmeyer flasks. The flasks were incubated at 35 ^{0}C, with a mild agitation of 80rpm, in a

shaker incubator unless otherwise indicated. After incubation the medium was decanted, centrifuged at 6000 rpm at 4 ^{0}C, for 10minues in refrigerated centrifuge (Kubota, Japan), and the supernatant was used as the crude enzyme preparation.

3E.1.5 Selection of enzyme production medium and incubation time

Mineral salt glutamine medium (MSG) and seawater glutamine medium (SWG) (compositions given under section 3C.1.3) were tested for their suitability as the enzyme production medium (EPM). Enzyme production studies were conducted as mentioned above (section 3E.1.4), with either of the media as the EPM. Samples were withdrawn at 6h intervals, over a total period of 24h, and assayed for enzyme activity, biomass on support, and cell detachment from support (free cell growth in medium measured as total cell protein). The medium that supported maximal enzyme synthesis by the immobilized cells, and the ideal incubation time were determined and further studies were performed in the EPM and incubation time selected, based on their ability to support maximal enzyme production.

3E.1.6 Optimisation of parameters for enzyme production by *Pseudomonas* sp immobilized on PUF

The EPM and the important environmental parameters that influence enzyme synthesis by the immobilized cells were optimised using a response surface type δ Box-Behnken model experimental design. The variables considered significant were the concentration of substrate (L-glutamine), carbon source (D-glucose), and nitrogen source (Yeast extract) in the medium, pH of the medium, and incubation temperature.

Design of experiment was performed as mentioned under section 3D.1.6.1. A design model was constructed with 46 runs in 2 blocks of 23 cases and each independent variable was tested at three levels. The levels tested were L-glutamine- 0.5, 1 and 1.5% w/v; glucose - 0.5, 1 and 1.5% w/v; Yeast extract 0, 0.25 and 0.5% w/v; pH 4, 6 and 8; and incubation temperature 30 ^{0}C, 35 ^{0}Cand 40 ^{0}C (Table 3.2). Media were prepared with the desired concentration of the components and adjusted to the desired initial pH. Enzyme production studies were performed as outlined under section 3E.1.4 using each of the above media as the EPM, and using the desired incubation temperature and an incubation time of 12h (selected from studies

mentioned under section 3E.1.5). The experimental runs were performed in a randomised sequence with three replications of each combination of factors. The average of enzyme yield obtained from the three trials was used as the response for each run.

3E.1.6.1 Data analysis and Optimisation

The responses obtained from the trials conducted as detailed above following the Box Behnken design model for five variables, was used to estimate the coefficients of the polynomial model using standard regression techniques. The estimates of the coefficients were substituted in the model to generate an optimal combination of factors that can support maximal enzyme synthesis by the immobilized cells using predictive models from response surface methodology. The software-Design-Expert ® (Stat-Ease Inc., USA) was used to fit the response surface - Box Behnken model to the experimental data.

3E.2 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON PUF UNDER REPEATED BATCH OPERATION

The suitability of the cells adsorbed on PUF for repeated use in enzyme production was evaluated by reusing the PUF cubes for several cycles of operation. Cells were immobilized (3E.1.2.2) using the immobilization medium and incubation time selected from studies performed earlier (3E.1.3). 50cc of the cubes with immobilized cells were washed in sterile saline, and were introduced into 50ml of the optimised EPM (section 3E.1.6) in 250ml Erlenmeyer flasks. The flasks were incubated with mild agitation (80rpm) at 30 ^{0}C in a shaker incubator for 12h. After incubation, the PUF cubes were recovered aseptically, washed in sterile saline and introduced into 50ml of fresh EPM to perform the next cycle of operation as detailed above. The medium was recovered after each cycle and one portion was used for estimation of cell detachment and growth as free cells (cell biomass measured as total protein). The other portion was centrifuged at 6000 rpm and 4 ^{0}C for 10 minutes in a refrigerated centrifuge (Kubota Japan) and the supernatant was used for enzyme assays. At the end of each cycle, 3cubes (2.1cc) of polystyrene beads were recovered and used for determination of cell biomass attached to them . The cubes recovered were replaced with an equal amount of PUF cubes from a parallel run to maintain the total volume constant. The repeated batch experiment was continued for 20 cycles

and the reusability of PUF cubes was assessed in terms of their ability to maintain the attached biomass in the enzyme production phase.

3E.3. CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON PUF IN A PACKED BED REACTOR

3E.3.1 Medium

The Seawater glutamine medium (SWG) optimised for L-glutaminase production by the cells immobilized on PUF under batch operation was used as the EPM for continuous production of the enzyme. The substrate (L-glutamine) concentration was varied wherever indicated.

SWG -Composition

L-Glutamine	- 15.0g
D-Glucose	- 15.0g
Yeast Extract	- 0.1g
Aged seawater	- 1.0L
pH	- 6.0

3E.3.2 Support matrix.

Polyurethane foam was used in the form of discs for this experiment (Fig 3.4b). PUF sheets with a thickness of 7mm were cut as circular discs of diameter 3.4cm(volume 6.352cc),pre-treated (section 3E.1.1), and used.

3E.3.3 Reactor preparation and packing

3E.3.3.1 Reactor

A glass column reactor with the specification given under section 3C.3.3.2 was used for the experiment.

3E.3.3.2 Sterilisation and packing of the reactor

All ports of the reactor were closed and the column was sterilised by dry heat in a hot air oven. Tubing and adapters were autoclaved and connected to the appropriate ports under aseptic conditions. All ports and the sparger nozzle were closed except the bottom port and the 20cm height sampling port. The sampling port was used as the influent port and the bottom port as the effluent port. Pre-treated and sterilised circular discs of PUF with thickness 7mm were introduced aseptically into

the glass column, and stacked one above another up to a height of 20cm from the bottom plate. A perforated Teflon disc was positioned over the bed to prevent bed expansion during operation.

3E.3.3. Determination of void volume

Reactor void volume was determined as outlined under section 3C.3.4.

3E.3.4. Cell immobilization on the PUF bed.

Immobilization of *Pseudomonas* sp. in the PUF bed was achieved by circulation of an actively growing culture of the bacterium through the bed (3D.3.4) The experimental set up is shown in Figure 3.7. The circulation of growing cells through the PUF bed was continued for a total period of 36h when the cells in the well-mixed immobilization medium from the fermentor were attached to the bed by adsorption eventually colonising the bed surface. The progress of cell adsorption on bed surface was evaluated by sampling the bed at 6h intervals. One disc was recovered aseptically and the biomass attached to the disc was estimated as total cell protein. The bed volume was maintained constant by replacing the disc with another from a parallel run maintained under batch mode as shake flask culture. Cell growth in the medium was estimated as total cell protein by taking medium samples aseptically at 6h intervals from the influent port.

3E.3.5 Reactor operation.

Immobilization of cells in the PUF bed was performed as above (3E.3.4). The bed was washed by circulation of sterile physiological saline at a flow rate of 80ml/h for 1h, which approximately was the residence time at that flow rate for the column. For studies on continuous production of L-glutaminase, the reactor was operated as a top fed column (Fig 3.8). EPM was introduced through the influent port using a peristaltic pump and allowed to fill the column till the liquid level reached the top of the bed. The effluent port was opened and the rate of flow of effluent was adjusted to be equal to that of the influent, so that the liquid level in reactor remained constant just above the bed. The reactor was run for a minimum of 6-8 h, at the required flow rate to attain equilibrium before studying enzyme production at the desired feed rate and substrate concentration. Samples were collected from the effluent port at fixed intervals and assayed for enzyme activity.

Fig 3.7 Experimental setup for immobilization of *Pseudomonas* sp on a packed bed of PUF in a column reactor

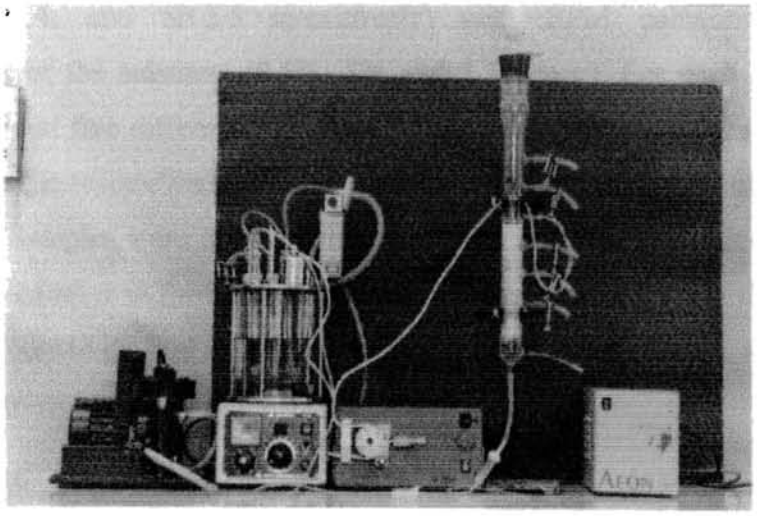
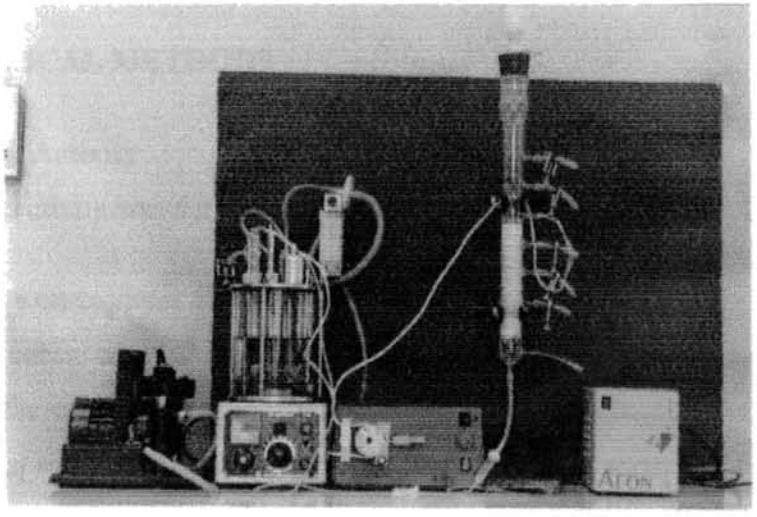


Fig 3.8 Experimental setup for continuous production of L-glutaminase by *Pseudomonas* sp. adsorbed on PUF in a PBR



3E.3.6 Effect of Operational parameters on reactor performance

The reactor performance was evaluated based on the efficiency of the reactor for continuous L-glutaminase production by the immobilized cells. Packing of discs, immobilization and operation were performed as mentioned above (sections 3E.3.3.2, 3E.3.4, and 3E.3.5 respectively) with EPM containing different concentrations of the substrate (0.5%, 1% and 1.5% w/v). For each medium the reactor was run at five different flow rates (60, 80, 100, 120 and 140 ml/h), and at each flow rate the reactor was operated continuously for a minimum of 8-12h after equilibration. Samples were taken every 2h and the enzyme production as well as substrate concentration in the influent and effluent was analysed. From these data the volumetric productivity and substrate conversion efficiency of the reactor were calculated as given under section 3C.3.6.1

3E.3.7 Operational stability of the reactor

The suitability of the packed bed reactor for prolonged operation under continuous mode was evaluated, at the substrate concentration and feed rate, which gave maximum substrate conversion efficiency. The reactor was operated continuously for a period of 72h (3days) after equilibration.

3E.4 ANALYTICAL METHODS

3E.4.1 Enzyme Activity

L-glutaminase activity was determined as mentioned under section 3C5.1

3E.4.2 Biomass on support matrix

Three cubes of PUF (2.1cc) for the batch experiment or one PUF disc (6.352cc) for the continuous production experiment were recovered, washed in 5ml of physiological saline and was suspended in 5ml of distilled water in a test tube. 5ml of a 1M NaOH solution was added and mixed in a cyclomixer. The contents were boiled by keeping the tubes for 5minues in a boiling water bath, after which they were cooled rapidly under tap water. The protein released by alkali lysis of the cells adsorbed on the disc was measured as in section 3C.5.3 and expressed as mg/cc of PUF.

3E.4.3 Biomass /growth as free cells in medium.

Biomass estimations in the medium was performed as detailed under section 3D.4.3

3E.4.4 Substrate concentration

L-glutamine concentration in sample was measured as mentioned under section 3C.5.5

3F. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON NYLON WEB

Whole cells of *Pseudomonas* sp. cells were immobilized on Nylon web matrix by physical adsorption. Immobilization was performed by growing the cells in a medium containing pre treated Nylon web, allowing the cells to adhere on the support matrix and colonise it as a biofilm. Initially the conditions required for growth and colonisation of the cells on Nylon web were optimised under batch mode, and the enzyme production efficiency of the immobilized cells were evaluated. The medium and conditions ideal for maximal enzyme synthesis by the immobilized cells were then optimised under batch mode employing a response surface methodology. The results were incorporated and repeated batch experiments were conducted to evaluate the potential for repeated use of the cell-immobilized beads. Continuous synthesis of L-glutaminase by the cells immobilized on Nylon web was studied in a packed bed reactor and the operational stability of the reactor was evaluated.

3F.1 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON NYLON WEB UNDER BATCH MODE

3F.1.1 Support matrix and pre-treatment

The widely used scrubber pads from Scrotch Brite India, were used as the inert support for immobilization of cells after vigorous pre-treatment. The "Scrotch-Brite®" scrubber pads (Dimension 7.5 X 7.5 X 0.7 cm) made of compressed Nylon web sheet, were purchased from the local market. The sheets were kept overnight in 0.1N NaOH after which they were recovered and washed in 0.1N HCl to neutralise the alkali. The sheets were then washed repeatedly in distilled water to remove the traces of acid. This was followed by autoclaving the sheets in distilled water at 121^0C and 15lbs for 20min (Ferrer and Sola, 1992). The water was poured off and fresh

water was added to repeat the process. The treated sheets were allowed to air dry at room temperature. For the immobilization experiments the sheets were cut into pieces (dimension 1 X 1 X 0.7 cm) with a volume 0.7cc (Fig 3.4c), which were sterilised before use by autoclaving at 121 ^{0}C and 15lbs for 20minutes

3F.1.2 Immobilization

3F.1.2.1 Inoculum preparation

A 10mg/ml suspension of *Pseudomonas* sp. cells prepared as detailed under section 3D.1.2.1 was used as the inoculum.

3F.1.2.2 Immobilization by cell attachment and growth on Nylon web cubes

Immobilization of *Pseudomonas* sp cells on Nylon web was performed by growing the cells along with pre-treated cubes of Nylon web in an immobilization medium. The cells were allowed to grow and attach to the support matrix colonising the surface as a biofilm. 2ml of the prepared inoculum (20mg cells) was used to inoculate 100ml of immobilization medium containing 50cc (71 Nos.) of Nylon web cubes in 250ml Erlenmeyer flasks. The flasks were incubated in a shaker incubator at 35 ^{0}C with a mild agitation of 80rpm for the desired time to facilitate cell growth and adsorption on the support.

3F.1.3 Selection of immobilization medium and incubation time.

Two media were tested as the growth (immobilization) medium, (MSG medium [section 3C.1.3] and Zobell's marine broth [Himedia, India]). Immobilization experiments were conducted as mentioned under section 3F.1.2.2 with either of the media as the immobilization medium. Efficiency of the media and incubation time for maximal cell adsorption on the support matrix along with cell growth was evaluated simultaneously by monitoring cell growth in the medium and cell adsorption onto Nylon web cubes at 6h intervals up to 48h. 1ml of the growth medium and 3 cubes (2.1 cc) of Nylon web were recovered aseptically at these intervals and biomass was estimated (measured as total cell protein) to assess growth as free cells and cell adsorption on support.

3F.1.4 Enzyme production and recovery

Cells immobilized on Nylon web cubes (section 3F.1.2.2) using the selected medium and incubation time (section 3F.1.3), were evaluated for enzyme production.. After the immobilization cycle, the growth medium was decanted and the cubes were recovered aseptically, washed in sterile saline and 50cc of the same were transferred to 50ml of enzyme production medium (EPM) in 250ml Erlenmeyer flasks. The flasks were incubated at 35 ^{0}C with a mild agitation of 80 rpm in a shaker incubator unless otherwise indicated. After incubation the medium was decanted, centrifuged at 6000rpm, at 4 ^{0}C for 10minues in refrigerated centrifuge (Kubota, Japan), and the supernatant was used as the crude enzyme preparation.

3F.1.5 Selection of enzyme production medium and incubation time

The Mineral salt glutamine medium (MSG) and seawater glutamine medium (SWG) (section 3C.1.3) were tested as the EPM. Studies on enzyme production by immobilized cells were conducted as mentioned above (section 3F.1.4) with either of the media as the EPM. Samples were withdrawn at 6h intervals, up to 24h, and assayed for enzyme activity, biomass on support and cell detachment from support (free cell growth in medium measured as total cell protein). The medium that supported maximal enzyme synthesis by the cells immobilized on Nylon web, and the ideal incubation time were determined from this study and subsequent experiments were conducted in the EPM and incubation time, selected based on their ability to support maximal enzyme production.

3F.1.6 Optimisation of parameters for enzyme production by *Pseudomonas* sp immobilized on Nylon web

The optimisation of EPM and production conditions for maximal enzyme production by cells immobilized on Nylon web was done as mentioned earlier (section 3E.1.6). A δ Box-Behnken model response surface methodology was used to design the experiment as in section 3D.1.6.1. The design model had 46 runs in 2 blocks of 23 cases and each independent variable was tested at three levels. The variables and levels tested were L-glutamine- 0.5, 1 and 1.5% w/v; glucose - 0.5, 1 and 1.5% w/v; Yeast extract 0, 0.25 and 0.5% w/v; pH 4, 6 and 8; and incubation temperature 30 ^{0}C, 35 ^{0}C and 40 ^{0}C (Table 3.2). Media were prepared with the

desired concentration of the components and adjusted to the desired initial pH. Enzyme production studies were performed as detailed under section 3F.1.4, using each of the above media as the EPM, and using the desired incubation temperature and an incubation time of 12h.The experimental runs were performed in a randomised sequence with three replications of each combination of factors. The average of enzyme yield obtained from the three trials was used as the response for each run.

3F.1.6.1 Data analysis and Optimisation

The responses obtained from the trials conducted as above following the Box Behnken design model for five variables, was used to estimate the coefficients of the polynomial model using standard regression techniques. The estimates of the coefficients were substituted in the quadratic response model and was used to generate an optimal combination of factors that can support maximal enzyme synthesis by the immobilized cells using predictive models from response surface methodology. The software "Design-Expert ®" (Stat-Ease Inc., USA) was used to fit the response surface -Box Behnken model to the experimental data.

3F.2 PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON NYLON WEB UNDER REPEATED BATCH OPERATION

The suitability of the cells adsorbed on Nylon web for repeated use in enzyme production was evaluated by reusing the Nylon cubes for several cycles of operation. Cells were immobilized (section 3F.1.2.2) using the immobilization medium and incubation time selected earlier (section 3F.1.3). 50cc of the cubes with immobilized cells were washed in sterile saline, and was introduced into 50ml of the optimised EPM (section 3F.1.6) in 250ml Erlenmeyer flasks. The flasks were incubated with mild agitation (80rpm) at 30 ^{0}C in a shaker incubator for 12h. After incubation, the PUF cubes were recovered aseptically, washed in sterile saline and introduced into 50ml of fresh EPM for the next cycle of operation. The medium was recovered after each cycle and one portion was used for estimation of cell detachment and growth as free cells (biomass measured as total protein). The other portion was centrifuged at 6000rpm and 4 ^{0}C for 10minutes in a refrigerated centrifuge (Kubota Japan) and the supernatant was used for enzyme assays. At the end of each cycle, 3cubes (2.1cc) of

Nylon web cubes were recovered and used for determination of biomass attached to them. The cubes recovered were replaced with an equal amount from a parallel run to maintain the total volume constant. The repeated batch experiment was continued for 20 cycles and the reusability of Nylon web cubes was assessed in terms of their ability to maintain the attached biomass in the enzyme production phase.

3F.3. CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON NYLON WEB IN A PACKED BED REACTOR

3F.3.1 Medium

The Seawater glutamine medium (SWG) optimised for batch mode production of L-glutaminase by the cells immobilized on Nylon web, with minor modifications (substrate concentration), was used as the enzyme production medium for continuous production of the enzyme. The substrate (L-glutamine) concentration was varied wherever indicated.

SWG -Composition

L-Glutamine	-	15.0g
D-Glucose	-	8.1g
Yeast Extract	-	3.7g
Aged seawater	-	1.0L
pH	-	6.0

3F.3.2 Support matrix.

Nylon web was used in the form of discs for this experiment (Fig 3.9). Sheets of Pre-treated Nylon web (section 3F.1.1), with a thickness of 6mm were cut as circular discs of diameter 3.4cm (volume 5.445cc). The discs were sterilised before use by autoclaving at 121 ^{0}C and 15lbs for 20minutes

3F.3.3 Reactor set-up

3F.3.3.1 Reactor

A glass column reactor with the specification given under section 3C.3.3.2 was used

3F.3.3.2 Sterilisation and packing of the reactor

Sterilisation of the reactor and packing were done as mentioned under section 3E.3.3.2

3F.3.3. Determination of void volume

Reactor void volume was determined as outlined under section 3C.3.4.

3F.3.4. Cell immobilization on the nylon bed.

Immobilization of *Pseudomonas* sp. in the nylon bed was done by circulation of an actively growing culture of the bacterium through the bed as outlined under section 3D.3.4. The experimental set up is shown in Figure 3.9. The circulation of growing cells through the bed was continued for a total period of 36h, when the cells in the well-mixed immobilization medium from the fermentor were attached to the Nylon discs by adsorption eventually colonising the bed surface. The progress of cell adsorption on bed surface was evaluated by sampling the bed at 6h intervals. One disc was recovered aseptically and the biomass content was estimated as total cell protein. The bed volume was maintained constant by replacing the disc with another from a parallel run maintained under batch mode as shake flask culture. Cell growth in the medium was also estimated as total cell protein by taking medium samples aseptically at 6h intervals from the influent port.

3F.3.5 Reactor operation.

Immobilization of cells in the Nylon bed was performed as above (3F.3.4). The bed was washed by circulation of sterile physiological saline at a flow rate of 80ml/h for 1.5h, which approximately was the residence time at that flow rate for the column. For studies on continuous production of L-glutaminase, the reactor operation was performed as in section 3E.3.5. The experimental setup for continuous glutaminase production by cells immobilized on nylon web is presented in Figure 3.10

3F.3.6 Effect of Operational parameters on reactor performance

The reactor performance was rated based on the efficiency of the reactor for continuous L-glutaminase production by the immobilized cells. Packing, immobilization, and operation were performed as mentioned above (sections 3F.3.3.2, 3F.3.4, and 3F.3.5 respectively) with EPM containing different concentrations of -

Fig 3.9 Experimental setup for immobilization of *Pseudomonas* sp. on a packed bed of nylon web in a PBR

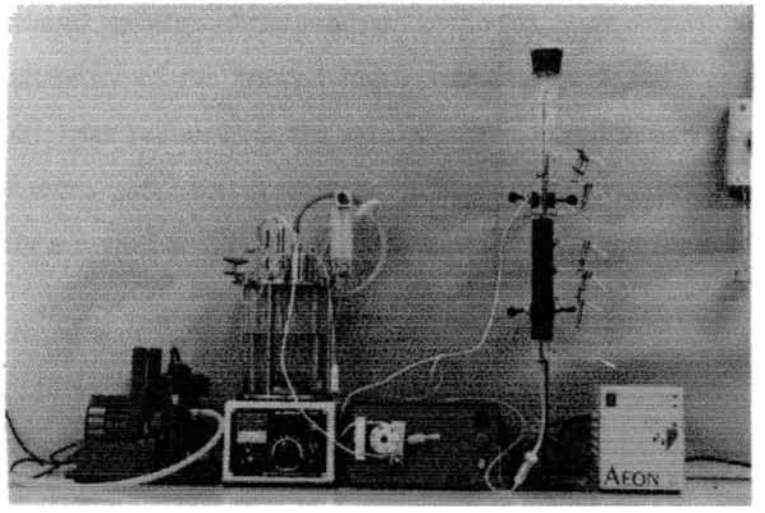
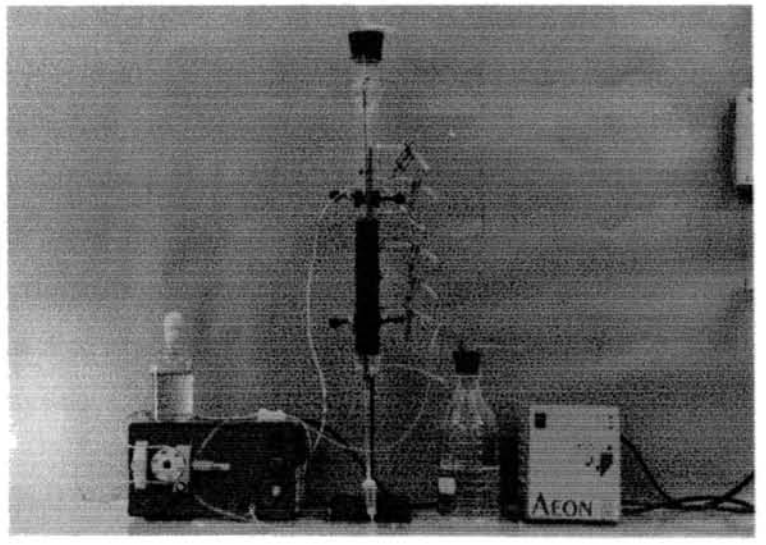


Fig 3.10 Experimental setup for continuous production of L-glutaminase by *Pseudomonas* sp. adsorbed on nylon web in a PBR



the substrate (0.5%, 1% and 1.5% w/v). For each medium the reactor was run at five different flow rates - 60, 80, 100, 120 and 140 ml/h, and at each flow rate the reactor was operated continuously for a minimum of 8-12h after equilibration. Samples were taken every 2h and the enzyme production as well as substrate concentration in the

influent and effluent was analysed. From these data the volumetric productivity and substrate conversion efficiency of the reactor was calculated as given under section 3C.3.6.1

3F.3.7 Operational stability of the reactor

The suitability of the packed bed reactor for prolonged operation in the continuous mode was evaluated at the substrate concentration and feed rate, which favoured maximum substrate conversion efficiency. The reactor was operated continuously for a period of 72h (3days) after equilibration and the performance was evaluated.

3F.4 ANALYTICAL METHODS

3F.4.1 Enzyme Activity

L-glutaminase activity was determined as outlined under section 3C5.1

3F.4.2 Biomass on support matrix

Three cubes of Nylon web (2.1cc) for the batch experiments or one disc of Nylon web (5.445cc) for the continuous production experiment was washed in 5ml of physiological saline and suspended in 5ml of distilled water in a test tube. 5ml of a 1M NaOH solution was added and mixed in a cyclomixer. The contents were boiled by keeping the tubes for 5minues in a boiling water bath, after which they were cooled rapidly under tap water. The protein released by alkali lysis of the cells adsorbed on the disc was measured as detailed under section 3C.5.3 and expressed as mg/cc of Nylon web.

3F.4.3 Biomass /growth as free cells in medium.

Biomass estimations in the medium was done as mentioned under section 3D.4.3

3F.4.4 Substrate concentration

L-glutamine concentration in sample was measured as detailed under section 3C.5.5

4. RESULTS

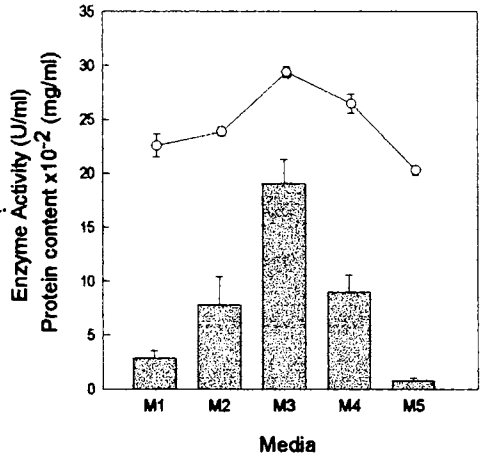
4A. L-GLUTAMINASE PRODUCTION BY Ca-ALGINATE IMMOBILIZED WHOLE CELLS OF *PSEUDOMONAS* SP.

4A.1 L-GLUTAMINASE PRODUCTION BY Ca-ALGINATE IMMOBILIZED WHOLE CELLS OF *PSEUDOMONAS* SP. UNDER BATCH MODE

4A.1.1 Medium selection

The basal enzyme production that could support maximal enzyme production by immobilized cells was first selected from among five media. Data presented in Fig 4.1 clearly indicated that sea water glutamine medium (SWG/Medium No.3) supported maximal enzyme synthesis (19.03 U/ml) by the immobilized cells while distilled water glutamine medium (DWG/Medium No. 5) supported the lowest level of glutaminase (0.749 U/ml). It was observed that the protein concentration in the medium was proportional to enzyme activity in general.

Fig 4.1 Selection of medium for L-glutaminase production by Ca-alginate immobilized *Pseudomonas* sp



M 1 - Mineral salts glutamine medium (MSG)
M 2 - MSG prepared in 50% seawater
M 3 - Sea Water Glutamine medium
M 4 - Seawater glutamine medium (half strength seawater)
M 5 - Distilled water + 1% Glutamine, 0.5 % glucose and 3% NaCl

4A.1.2 Optimisation of variables for cell immobilization and enzyme production by the immobilized viable cells.

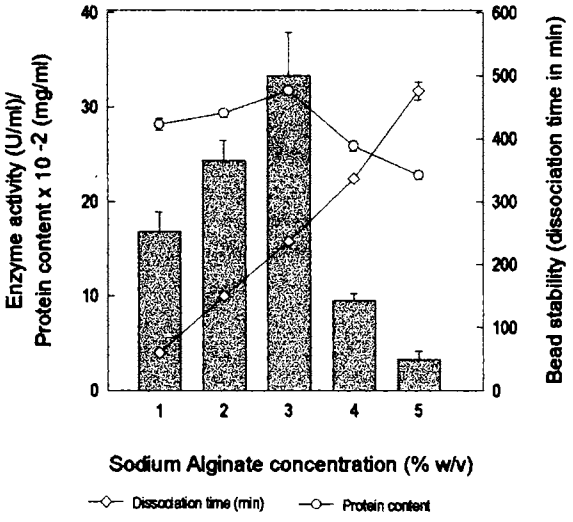
4A.1.2.1 Optimisation of immobilization

4A.1.2.1.1 Support concentration

Ideal concentration of sodium alginate as support for preparation of immobilized cell bead was determined. Results presented in Fig 4.2 suggest that 3% sodium alginate is ideal for preparation of beads since its supported the highest level of enzyme activity (33.33U/ml). Further ,it was noted that the enzyme production by

immobilized cells increased along with an increase in concentration of support up to 3% w/v level. However, with the higher levels tested, the enzyme production efficiency of the cells drastically declined (9.51 U/ml and 3.22U/ml for 4% and 5% alginate concentration respectively). Bead stability, measured as dissolution time showed a steady increase along with increase in support concentration with the beads being most stable (475 minutes for complete dissolution) at the highest alginate concentration tried. The beads were least stable at 1% alginate concentration (dissolution time 59 min) and were malformed. Nevertheless, at lower alginate concentrations the enzyme production ability of the cells did not suffer as evidenced by the enzyme yield obtained (16.85 U/ml for 1% and 24.34 U/ml for 2% alginate) when these concentrations were used for bead preparation.

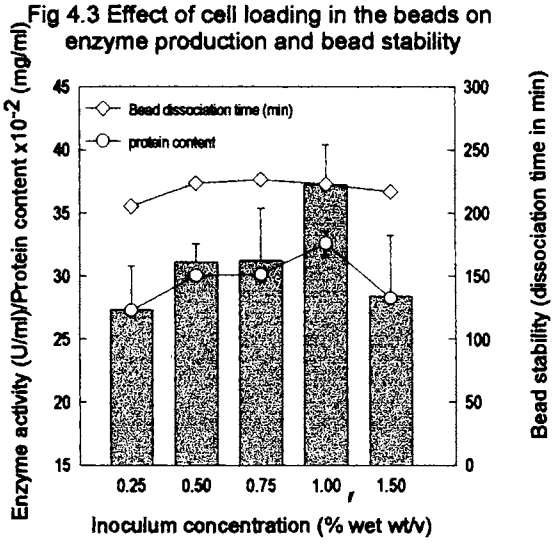
Fig 4.2 Effect of support concentration on enzyme production and stability of beads



4A.1.2.1.2 Cell loading

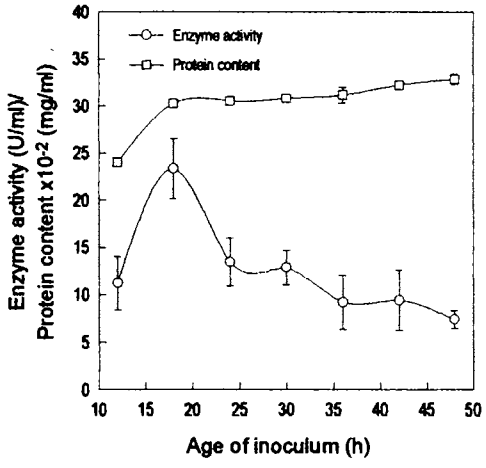
All levels of initial cell concentration of bacteria used supported considerable enzyme yield under immobilized conditions. The highest enzyme yield (37.23 U/ml) was obtained at a cell concentration of 1% w/v (Fig. 4.3), and the cells could synthesise glutaminase (25.84U/ml) even at a concentration of 0.25% w/v. However, the highest cell loading

Fig 4.3 Effect of cell loading in the beads on enzyme production and bead stability



tried (1.5% w/v) yielded only less enzyme (28.39 U/ml) compared to that obtained at 0.5%, 0.75% or 1% w/v cell concentration. The enzyme yield increased along with an increase in inoculum concentration from 0.25% up to 1%. The protein content also showed a concurrent increase indicating an enhanced synthesis of the enzyme. The density of cell loading did not affect the stability of the beads, as the bead dissolution times remained almost stable for the various levels tried.

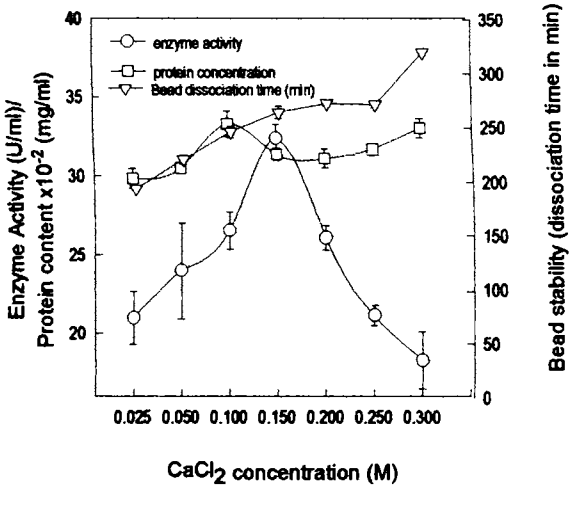
Fig 4.4 Effect of the inoculum age on enzyme production by immobilized *Pseudomonas* sp



4A.1.2.1.3 Age of the inoculum

The growth stage of the cells used for immobilization had a profound effect on enzyme production by the immobilized cells as evidenced by the data documented in Figure 4.4. Cells at their active growth phase recorded considerable enzyme yield compared to the other growth stages. Maximal enzyme yield (23.37 U/ml) was obtained with cells from an 18h old culture followed by cells from 24h and 12h old cultures (13.48 U/ml and 13.03 U/ml respectively). Protein content of the medium increased along with the use of older cells and did not show a direct relation to the enzyme activities obtained.

Fig 4.5 Effect of $CaCl_2$ concentration on enzyme production and bead stability



4A.1.2.1.4 Concentration of $CaCl_2$

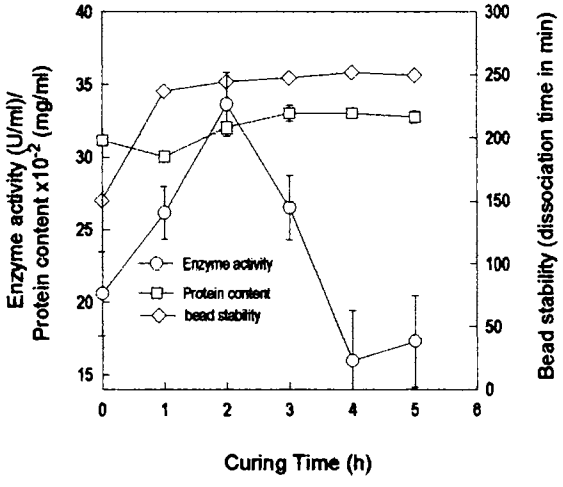
Concentration of $CaCl_2$ used for gel entrapment of cells had a direct impact on the stability of the beads, since the beads were more stable when a solution of higher concentration was used for their preparation. At the lower concentrations (0.025M and 0.05M) the beads were malformed and

susceptible to damage. The ability of immobilized cells for enzyme synthesis increased with the increase in concentration of $CaCl_2$ up to a concentration 0.15M when maximal enzyme yield (32.36 U/ml) was observed (Fig 4.5). However, the enzyme yield declined along with increase in $CaCl_2$ concentration above 1.5M. (26.06U/ml, 21.12 U/ml and 18.28 U/ml respectively for 0.2M, 0.25M and 0.3M $CaCl_2$ solutions respectively). Data indicated no direct relationship between enzyme activity and protein for $CaCl_2$ concentration above 0.1M

4A.1.2.1.5 Curing time

From the data presented in Figure 4.6, it was inferred that the immobilized cell beads require a minimal curing time of 1h for hardening. Curing for more than 2h did not had any effect on enhancing bead stability, since above this duration, the stability of beads remained almost constant irrespective of the curing time. However, enzyme production by immobilized cells was at a maximum (33.63U/ml) for the beads cured for 2h. Highest bead stability was observed with the beads cured for 4h (dissolution time 251min). Nevertheless, the dissolution times of beads cured for 2h and 3h (244min and 247min respectively) was comparable to this. Protein concentration in the medium did not show a direct relation with enzyme activity and increased along with increase in curing time.

Fig 4.6 Effect of bead curing time on enzyme production and bead stability



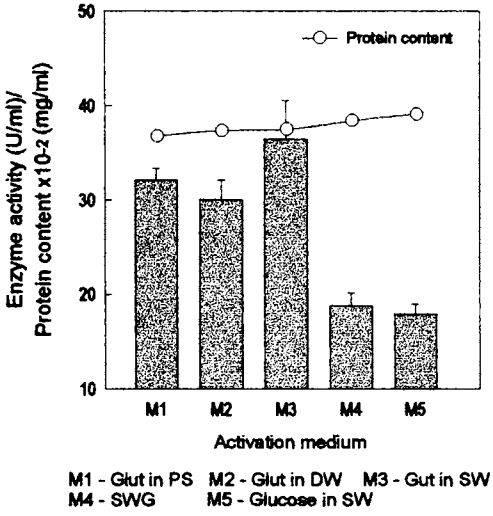
4A.1.2.2 Optimisation of Activation conditions

4A.1.2.2.1 Selection of Activation medium

Among the five media tested for their suitability as activation medium, Medium 3 was observed to be the ideal medium for activation of the immobilized cells (Fig 4.7). Immobilized cells activated in this medium gave a maximum yield of 36.48 U/ml. However, Medium 1 and Medium 2 supported enzyme yields of 32.13U/ml and 30.04 U/ml respectively, indicating that they are also effective as

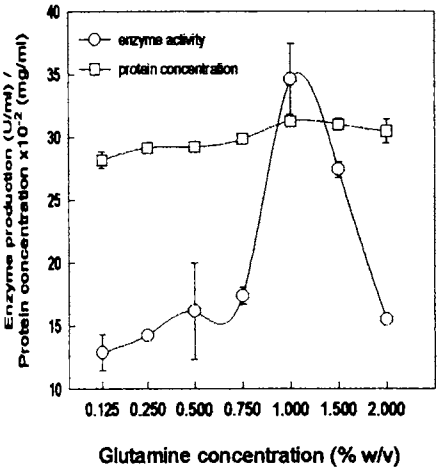
activation media. Interestingly, the enzyme production medium (Medium4), when used as activation medium, was not as effective as Medium 1, 2 or 3. Presence of L-glutamine in the activation media influenced the efficiency of activation medium as evidenced by the fact that Medium 5 which lacked glutamine did not support enhanced enzyme yield. Further, in this experiment also, the protein levels and enzyme yields did not show any direct relationship.

Fig 4.7 Selection of activation medium for enzyme production by immobilized *Pseudomonas* sp.



M1 - Glut in PS M2 - Glut in DW M3 - Gut in SW
M4 - SWG M5 - Glucose in SW

Fig 4.8 Effect of glutamine concentration in the activation medium on enzyme production by immobilized *Pseudomonas* sp.



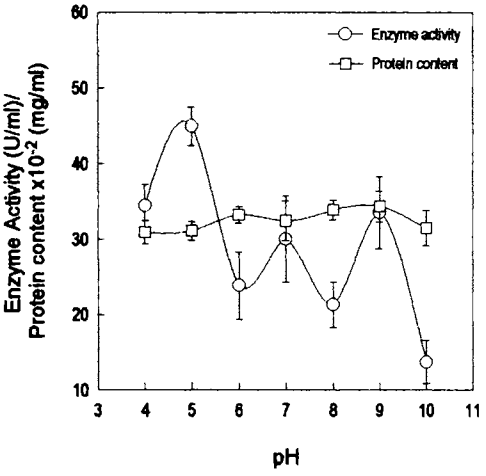
4A.1.2.2.2 Substrate concentration

Concentration of L-glutamine tested as substrate in the activation medium influenced greatly the enzyme yield by immobilized cells (Fig 4.8) Maximal enzyme yield (34.61 U/ml), was recorded, when the beads were activated in a solution containing 1% L-glutamine. There was a linear increase in enzyme yield along with increase in the concentration of the substrate used in the activation medium up to 1%, which however declined gradually with further increase in substrate concentration. Despite the variation in enzyme yield, protein levels remained almost stable for the various concentrations of L-glutamine tried.

4A.1.2.2.3 pH of the Activation medium

Of the various initial pH of activation medium tested, pH 5.0 supported a maximum of 44.94 U/ml of L-glutaminase (Fig 4.9). Although fluctuation in levels of enzyme yield was noted for the different initial medium pH tried, it was noted that the cells activated at pH 4 and 9 could also yield considerable amounts of enzyme (34.38U/ml and 33.48U/ml respectively) which interestingly were two extremes of pH, one acidic and the other alkaline. In general, enzyme yield was higher at acidic pH condition than alkaline. The immobilized cells activated at pH 9 also secreted more protein into the medium when used for enzyme production.

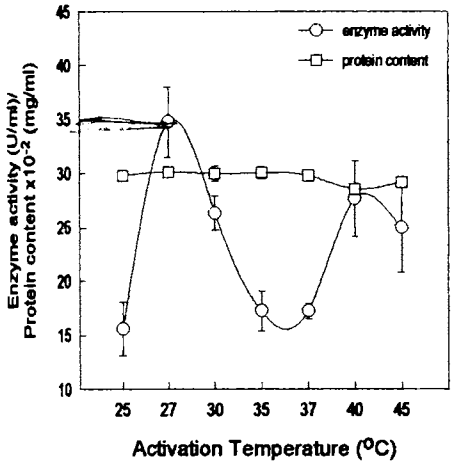
Fig 4.9 Effect of the pH of activation medium on enzyme production by immobilized *Pseudomonas* sp



4A.1.2.2.4 Activation temperature

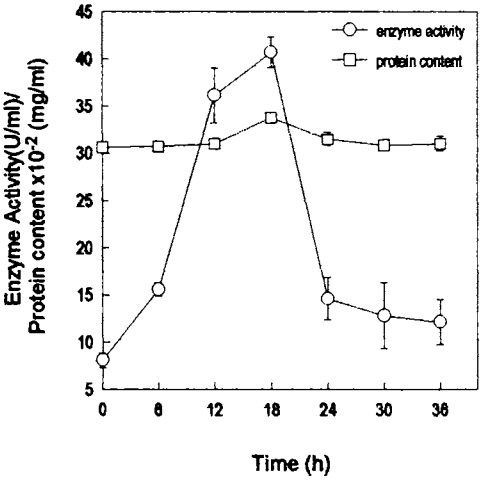
Data presented in Fig 4.10 indicate that activation temperature directly influence the immobilized cells in terms of their rate of enzyme synthesis. Thus, a maximum enzyme yield of 34.76 U/ml was recorded when the beads were activated at 27 ^{0}C, which declined drastically on further increase in activation temperature. However, the enzyme yield increased to 26.29 U/ml upon activation at 40 ^{0}C, and decreased again at 45 ^{0}C. Highest specific activity (115.392 U/mg protein), of L-glutaminase was also recorded at 27 ^{0}C. In general there was no direct relationship between enzyme yield and protein concentration for the activation temperatures tested.

Fig 4.10 Effect of Activation temperature on enzyme production by immobilized *Pseudomonas* sp



4A.1.2.2.5 Activation time

Fig 4.11Optimization of bead activation time

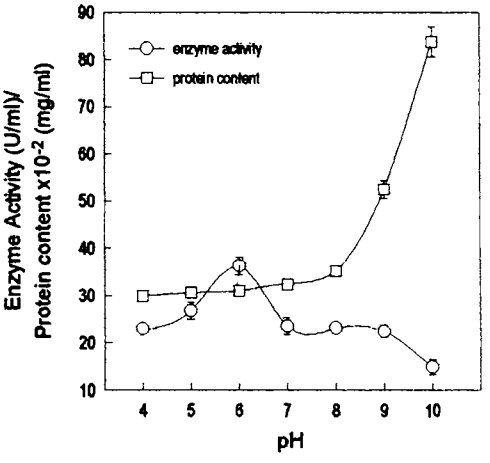


It was inferred from the data shown in Figure 4.11, that the activation of immobilized cells for a particular period is essential for an enhanced enzyme production. Although the immobilized cells were capable of L-glutaminase production even without any activation (8.09U/ml), an activation for 18h supported maximal enzyme yield (40.67 U/ml) followed by that for 12h (36.10 U/ml). With all the activation times tried, the enzyme yield was higher than that obtained for cells that were not activated. In this study there was no direct relationship between enzyme yield and protein concentration.

4A.1.2.3 Optimisation of parameters affecting enzyme production

4A.1.2.3.1 pH of enzyme production medium.

Fig 4.12 Influence of the pH of medium on enzyme production by immobilized *Pseudomonas sp*



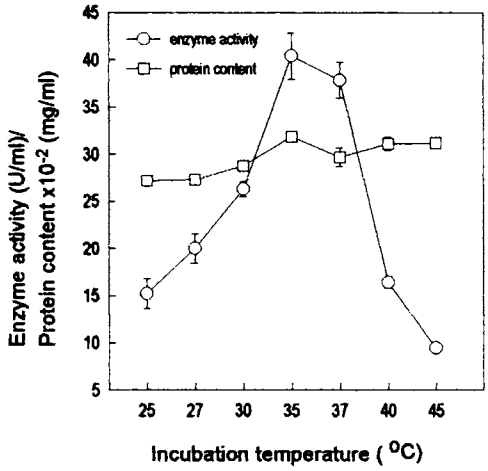
Immobilized cells showed a pH preference in the acidic range, for an enhanced enzyme production (Fig 4.12), since the highest enzyme production (36.25 U/ml) was recorded at a pH of 6.00 followed by that at pH 5.00 (26.82 U/ml). The enzyme yield showed a decline along with an increase in medium pH beyond 6.00. The enzyme yield was almost similar for the various pH tried, except for pH 10, when it was very low. Protein concentrations showed a steady increase along with an increase in pH of the medium, recording a maximum at pH 10. It was also

observed that an increase in pH below the neutral did not result in a linear increase in protein concentration, unlike that at alkaline conditions. However, there seemed to be no direct relationship between enzyme yield and protein concentration.

4A.1.2.3.2 Temperature of Incubation

Incubation temperature of 35 ^{0}C was observed to be ideal for maximal enzyme synthesis (40.37 U/ml) by the immobilized cells (Fig 4.13). Nevertheless, incubation at 37 ^{0}C, also resulted in considerable enzyme yield (37.83 U/ml). However, increase in incubation temperatures above 37 ^{0}C led to a sharp decline in enzyme production recording 9.44 U/ml at (45 ^{0}C). This decline in the enzyme yield was not correlated to the amount of protein secreted into the medium, which remained high at higher incubation temperatures.

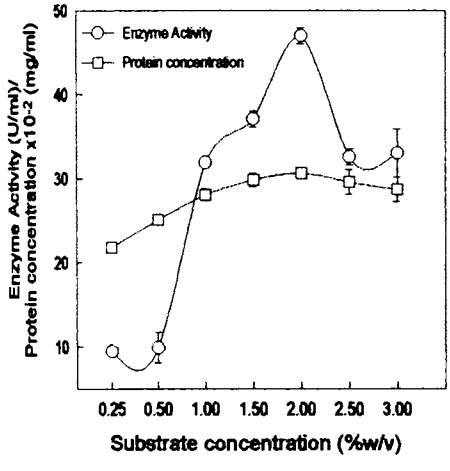
Fig 4.13 Optimization of incubation temperature



4A.1.2.3.3 Substrate concentration

Substrate concentration in the enzyme production medium directly influenced enzyme production by immobilized cells since enzyme yield increased considerably along with increase in the substrate concentrations up to 2% (Fig 4.14) when the a yield of 46.96 U/ml was recorded. Nevertheless, high levels of enzyme yield could also be obtained at substrate concentrations of 1.5% (37.08U/ml) and 1.0% (31.91 U/ml). The protein concentration in the medium recorded higher values where enzyme activity was higher, suggesting a positive relationship, indicating an enhanced synthesis of the enzyme at higher substrate concentration

Fig 4.14 Optimization of substrate concentration



4A.1.2.3.4 Additional carbon sources

Incorporation of additional carbon sources into the enzyme production medium at 0.03M level, resulted in a significant increase in the enzyme yield compared to the control (7.12U/ml), which contained only glutamine (Fig 4.15). Among the various carbon sources tested, D-glucose supported maximal enzyme yield (35.06 U/ml) followed by sucrose (30.49 U/ml). Mannitol and sorbitol also could support considerable enzyme production (26.74 U/ml and 28.83 U/ml respectively), even though the protein concentration in the medium was lowest with these as the carbon sources. Lactose was the least effective as a carbon source (9.44 U/ml). The optimum concentration of glucose for maximal enzyme synthesis (40.3 U/ml) was determined to be 1% w/v in a subsequent study (Fig 4.16).

Fig 4.15 Effect of additional carbon sources in the medium onenzyme production by immobilized *Pseudomonas* sp

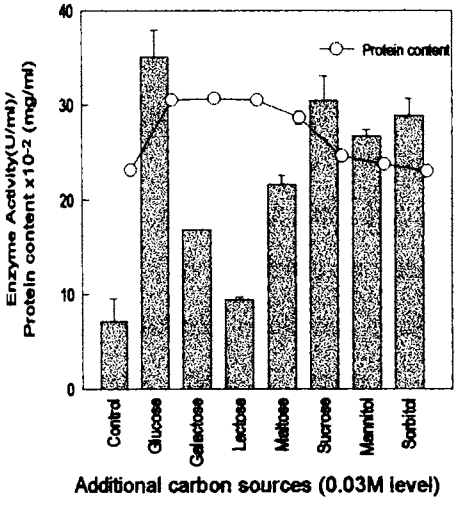
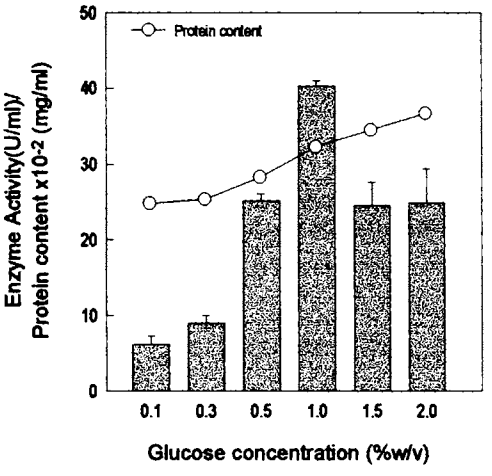


Fig 4.16 Optimization of Glucose concentration

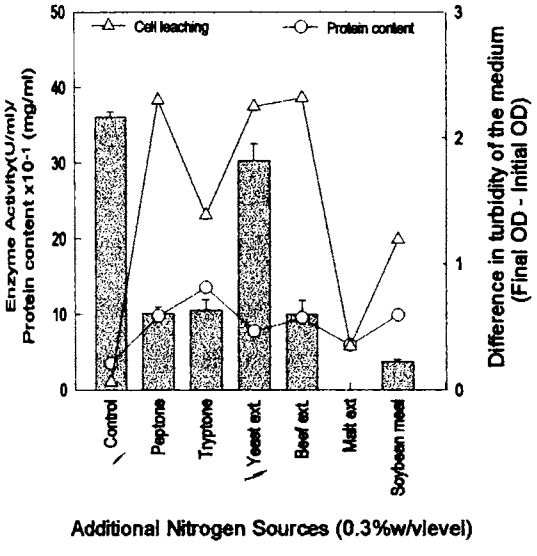


4A.1.2.3.5 Additional nitrogen sources

Presence of additional nitrogen sources other than L-glutamine at 0.3%w/v level, in the medium had a negative impact on enzyme synthesis by immobilized cells (Fig 4.17), since there was a drastic reduction in enzyme production compared to the control (36.1 U/ml). However, yeast extract when added to the medium could support an enzyme production of 30.34U/ml, which was marginally lesser than the control. Nevertheless, addition of any nitrogen source along with glutamine resulted

in cell growth within the beads, which led to cell leaching into the medium, and was observed as an increase in turbidity of the medium. Malt extract was the least effective of all nitrogen sources tested and addition of it resulted in a total seizure of enzyme production. Protein secretion into the medium did not show any correlation with enzyme production. Although presence of additional nitrogen sources resulted in a decreased enzyme yield, it supported increase in protein secreted out into the medium, compared to the control. High concentrations of protein were associated with Tryptone.

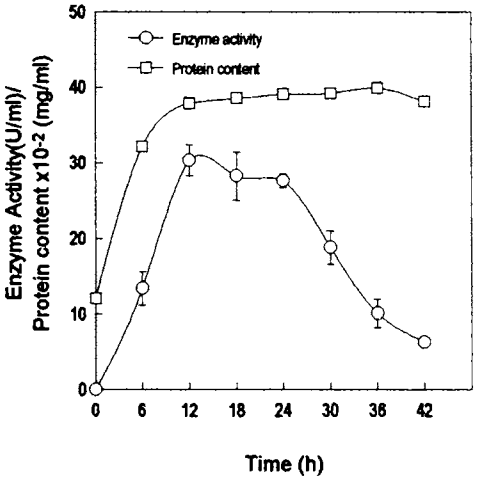
Fig 4.17 Influence of additional Nitrogen Sources in the medium on enzyme production by immobilized *Pseudomonas* sp



4A.1.2.3.6 Retention time

Data presented in Fig 4.18 suggest that 12h retention is ideal for obtaining maximal enzyme yield by immobilized cells, under batch operation, although similar enzyme yields could be obtained for retention periods of 18h and 24h. Protein concentrations also recorded higher values for retention periods ranging from 12h onwards up to 42h. Retention periods above 24h led to reduced enzyme yield in spite of a high protein concentration.

Fig 4.18 Optimization of retention time for enzyme production by immobilized *Pseudomonas* sp.



4A.2 L-GLUTAMINASE PRODUCTION BY Ca-ALGINATE IMMOBILIZED *PSEUDOMONAS* SP. UNDER REPEATED BATCH OPERATION

The data obtained for 20cycles of repeated batch operation shown in Figure 4.19 indicated that the immobilized cells were active and could be reused, since enzyme yield remained between 21 to 33 units over the entire period of operation (Table 4.1), and on an average 25 U/ml of L-glutaminase was synthesised per cycle. The enzyme yield varied from a minimum of 21.2 U/ml to a maximum of 32.81 U/ml during the course of study. The biomass in the beads increased with the batches of operation for the first few cycles and remained almost steady for the subsequent batches. (Average 3.3-mg protein/g bead). Growth as free cells did occur in the medium and the biomass varied between 2.94 and 4.52 mg protein/ml of medium. Enzyme production showed a positive relationship with the biomass content of the beads, with an enhanced enzyme yield, when biomass concentration in the beads was high. However no such relation was noted between biomass content in the medium and amount of enzyme secreted. The results suggest that the immobilized cells in the beads were able to synthesise L-glutaminase consistently, and could efficiently be reused without significant reduction in activity.

Fig 4.19 Repeated batch fermentation for L-glutaminase production by Ca-alginate immobilized *Pseudomonas* sp

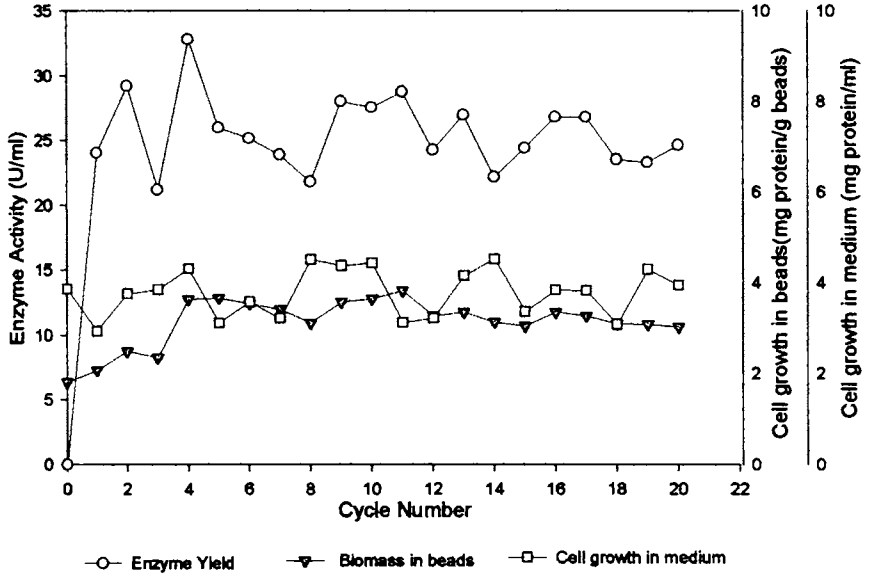


Table 4.1

Repeated batch production of L-glutaminase by Ca-alginate immobilized cells of *Pseudomonas* sp. Enzyme yield and biomass variations with batches of operation

Cycle #	Enzyme activity (U/ml)	Biomass in beads (mg protein/g bead)	Biomass in medium (mg protein/ml medium)
0	0	1.82	3.87
1	24.04	2.08	2.94
2	29.22	2.50	3.76
3	21.20	2.36	3.85
4	32.81	3.64	4.31
5	25.99	3.67	3.12
6	25.17	3.54	3.58
7	23.89	3.42	3.22
8	21.80	3.12	4.52
9	28.01	3.58	4.38
10	27.56	3.65	4.44
11	28.76	3.83	3.13
12	24.27	3.28	3.22
13	26.97	3.36	4.16
14	22.17	3.14	4.53
15	24.42	3.05	3.37
16	26.82	3.36	3.84
17	26.82	3.27	3.83
18	23.52	3.10	3.09
19	23.30	3.09	4.30
20	24.64	3.03	3.95

4A.3 CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY Ca-ALGINATE IMMOBILIZED *PSEUDOMONAS* SP. IN A PACKED BED REACTOR

The packed bed reactor had a void volume of 94.4cc. The dilution rates calculated for the flow rates 60, 80, 100, 120 and 140 ml/h were 0.64, 0.85, 1.06, 1.27 and 1.48 h^{-1} respectively. Table 4.2 shows the enzyme yield, volumetric productivities and substrate conversion rate recorded at different substrate concentrations and dilution rates for successive continuous runs. It was observed that, in general, the enzyme yield increased along with an increase in substrate concentration. The volumetric productivity increased along with increase in dilution rate as well as substrate concentration in most instances. Whereas, at all the substrate concentrations tried, increase in dilution rates resulted in a decrease in enzyme production as expected in continuous fermentations. Further, substrate conversion was most efficient at lower substrate concentration and dilution rates.

Table 4.2

L- Glutaminase production by Ca-alginate immobilized cells of *Pseudomonas* sp. in a packed bed reactor
Evaluation of performance under different operation conditions

Substrate Conc: (w/v)	F Flow rate (ml/h)	D=F/V Dilution rate (h^{-1})	R=1/D (V/F) Residence time (h)	Y= Enzyme yield (U/ml)	Std Dev	% substrate conversion	Std Dev	P=YD Productivity (U/ml/h)
	60	0.64	1.57	19.68	0.81	28.1	1.12	12.51
	80	0.85	1.18	15.43	1.89	23.5	1.2	13.08
0.50%	100	1.06	0.94	13.52	2.2	22.77	2.56	14.32
	120	1.27	0.79	10.97	0.84	22.53	1.2	13.94
	140	1.48	0.67	8.29	0.78	17.93	2.3	12.29
	60	0.64	1.57	27.56	2.64	18.05	1.21	17.52
	80	0.85	1.18	24.42	2.34	15.63	1.21	20.69
1.00%	100	1.06	0.94	20.49	1.73	14.39	1.45	21.71
	120	1.27	0.79	18.24	1.29	13.69	0.83	23.19
	140	1.48	0.67	15.32	3.4	13.2	0.46	22.72
	60	0.64	1.57	35.52	1.61	11.14	0.56	22.58
	80	0.85	1.18	30.22	1.89	10.09	0.72	25.61
1.50%	100	1.06	0.94	25.21	0.74	9.37	0.59	26.71
	120	1.27	0.79	22.86	1.35	9.13	0.61	29.06
	140	1.48	0.67	18.44	1.17	8.8	2.27	27.35

Table continued on next page

	60	0.64	1.57	36.05	1.61	8.84	0.58	22.91
	80	0.85	1.18	32.25	1.89	8.18	0.98	27.33
2.00%	100	1.06	0.94	27.27	0.74	7.69	1.76	28.89
	120	1.27	0.79	24.04	1.35	7.09	1.39	30.56
	140	1.48	0.67	20.52	1.17	6.12	0.7	30.43

4A.3.1 Effect of Operational parameters on Reactor performance.

The reactor performance was assessed in terms of enzyme yield, volumetric productivity, and substrate conversion efficiency upon continuous operation. The effect of varying the operational parameters, flow rate and substrate concentration on the above was evaluated and the results are given below.

4A.3.1.1 Effect of dilution rate and substrate concentration on enzyme yield

At all the substrate concentrations tried, the enzyme production by immobilized cells decreased with an increase in dilution rates (Fig 4.20a). While maximal enzyme yield (36.05 U/ml) was obtained at a dilution rate of 0.64 h^{-1} in the medium containing 2% substrate (L-glutamine), the lowest enzyme yield (8.29U/ml) was obtained at a dilution rate of 1.48 h^{-1} and in the medium containing the lowest substrate concentration (0.5% w/v). The enzyme yield showed a linear increase with increase in substrate concentration at all the dilution rates tried (Fig 4.20b)

4A.3.1.2 Effect of dilution rate and substrate concentration on volumetric productivity

Volumetric productivity of the enzyme increased along with an increase in dilution rates up to 1.27 h^{-1} for the substrate concentration 1%, 1.5% and 2%. Whereas, at a substrate concentration of 0.5%, this increase in productivity occurred only up to a dilution rate of 1.06 h^{-1} and decreased thereafter (Fig 4.21a). While the maximal productivity (30.56 U/ml/h) was obtained at a dilution rate of 1.27 h^{-1} in the medium containing 2% substrate, the lowest was recorded at a dilution rate of 0.64h^{-1} in the medium containing 0.5% substrate. However, at each of the dilution rates tried, the increase in productivity was directly proportional to the increase in substrate concentration, and the maximal volumetric productivity was obtained in media containing the highest substrate concentration, at all the individual dilution rates tested (Fig 4.21b).

4A.3.1.3 Effect of dilution rate and substrate concentration on substrate conversion

Maximum substrate conversion was obtained at lower dilution rates for all the concentrations of substrate tried. The decrease in efficiency for substrate conversion was linear with the dilution rates, irrespective of the concentration of substrate in the medium (Fig 4.22a). The highest percentage of substrate conversion that could be achieved was only 28.1, at a dilution rate of 0.64 h^{-1}, in the medium containing 0.5% substrate. With an increase in dilution rate, the substrate conversion rate dropped to the lowest (6.12 %), in the medium containing 2% substrate. In general, the effect of substrate concentration on substrate conversion efficiency showed an inverse relation, with the maximum % substrate conversion, being in the media with the lower substrate concentrations (Fig 4.22b)

Fig 4.20 Effect of operational parameters on continuous production of L-glutaminase by Ca-alginate immobilized *Pseudomonas* sp.in a PBR-Dependence of enzyme yield on operational variables

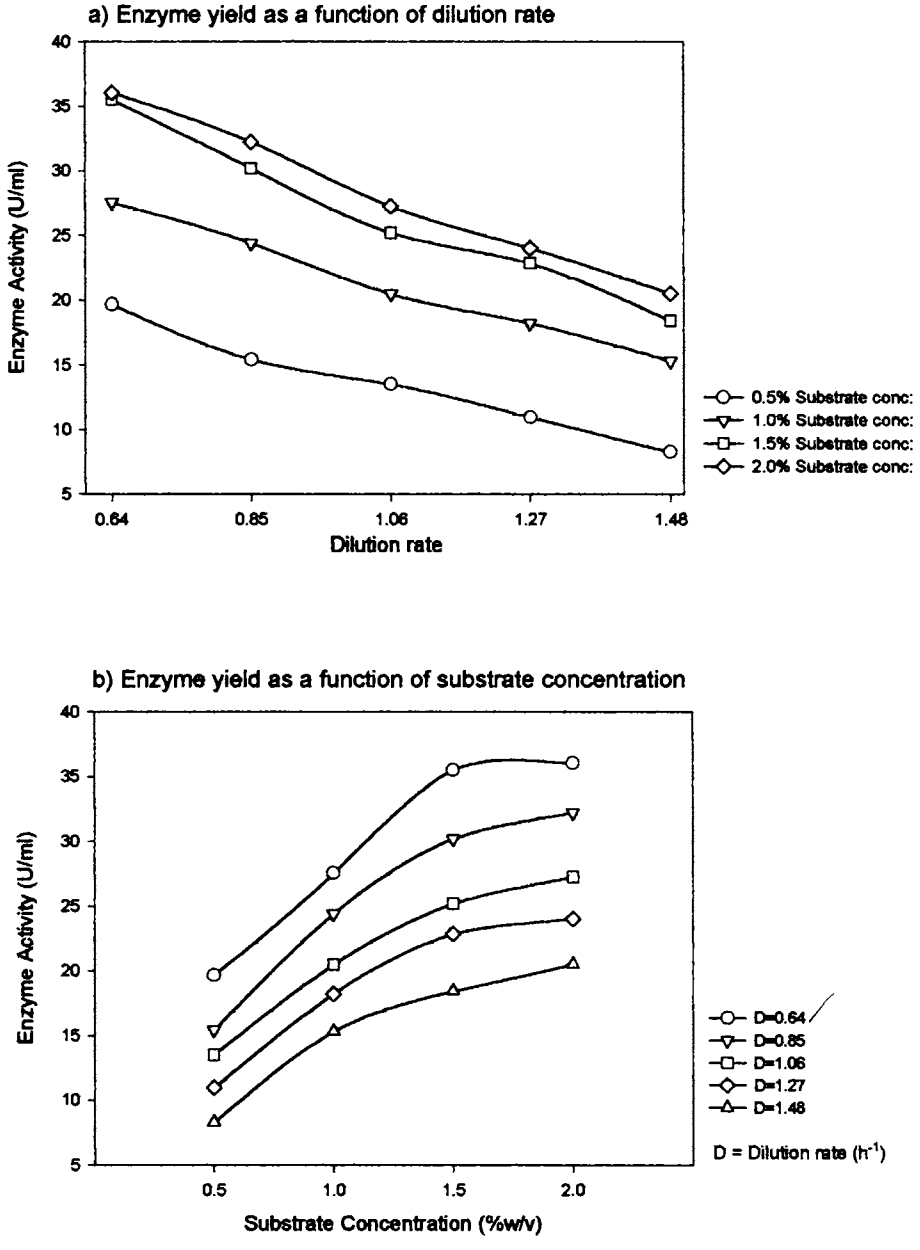


Fig 4.21 Effect of operational parameters on continuous production of L-glutaminase by Ca-alginate immobilized *Pseudomonas* sp. in a PBR Dependence of productivity on operational variables

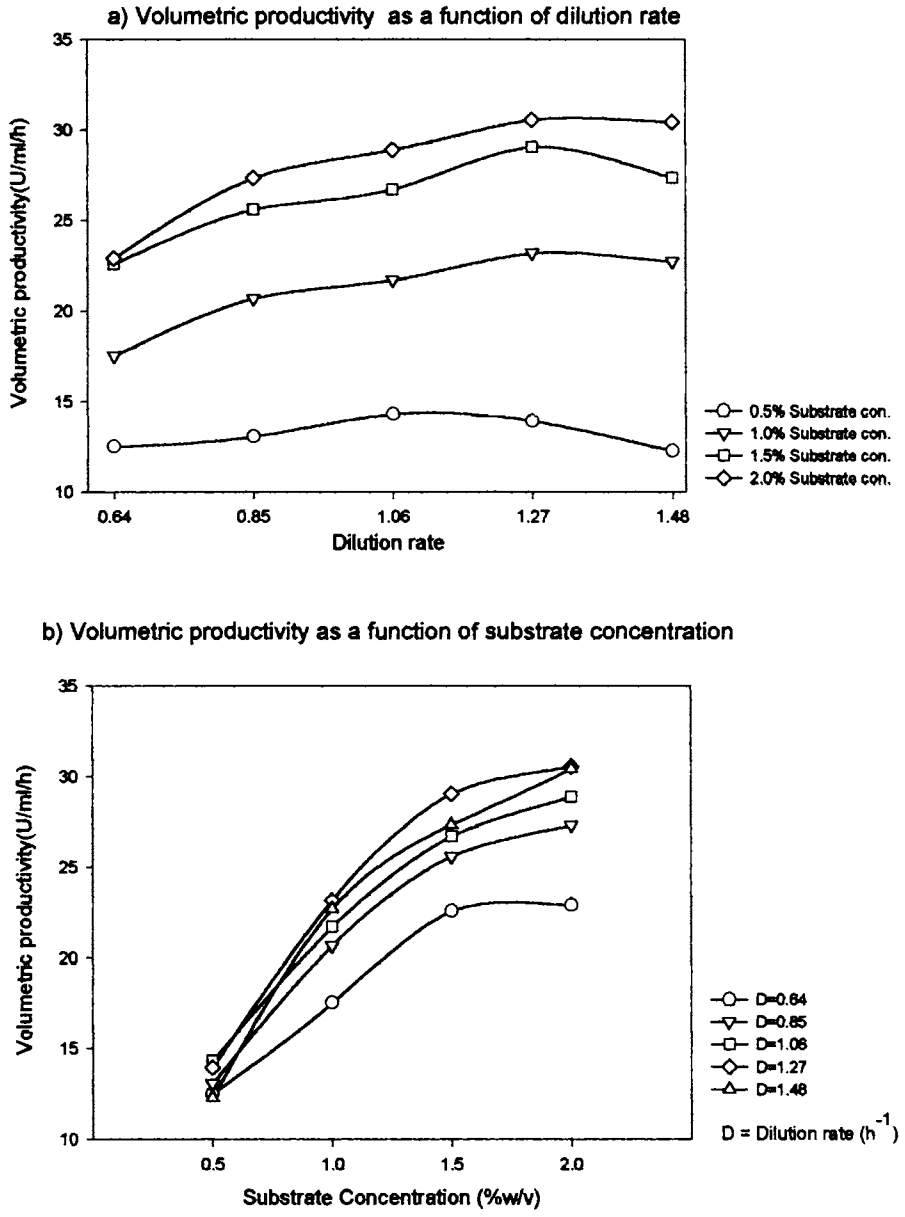
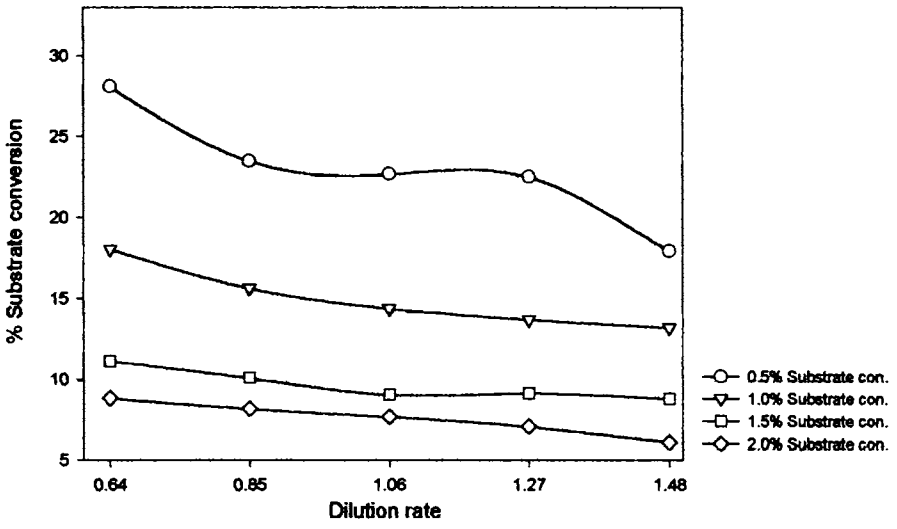
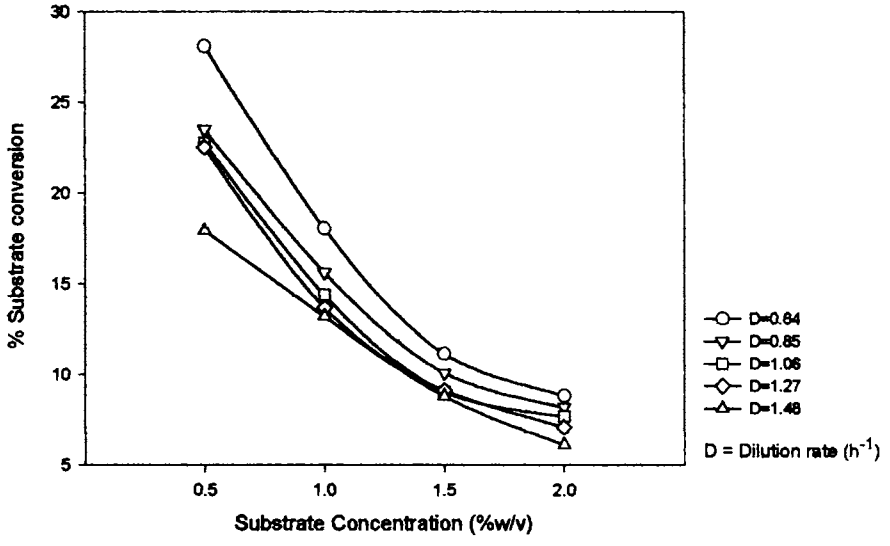


Fig 4.22 Effect of operational parameters on continuous production of L-glutaminase by Ca-alginate immobilized Pseudomonas sp. in a PBR
Dependence of %substrate conversion on operational variables

a) Substrate conversion as a function of dilution rate



b) Substrate conversion as a function of substrate concentration



4A.3.2 Operational stability of the reactor

The packed bed reactor was operated at the maximum substrate conversion efficiency (0.5% substrate and dilution rate 0.64 h^{-1}) for evaluation of the operational stability over a period of 120h. The immobilized cells in the PBR synthesised L-glutaminase consistently during the operation, as evidenced by the data shown in Figure 4.23 and Table 4.3. There was no apparent reduction in enzyme yield and an average enzyme yield of 21.07U/ml was recorded. The biomass in the beads remained fairly consistent, at an average value of 3.39mg protein/g of beads although there was cell growth and leaching into the medium indicated by growth of free cells in the medium. The biomass content in the effluent stream remained between 2.91 and 4.67 mg protein/ml, and the average volumetric productivity was 13.49U/ml/h

Fig 4.23 Packed bed reactor performance over prolonged operation up to 120h at 0.6 h^{-1} dilution rate and 0.5% substrate concentration

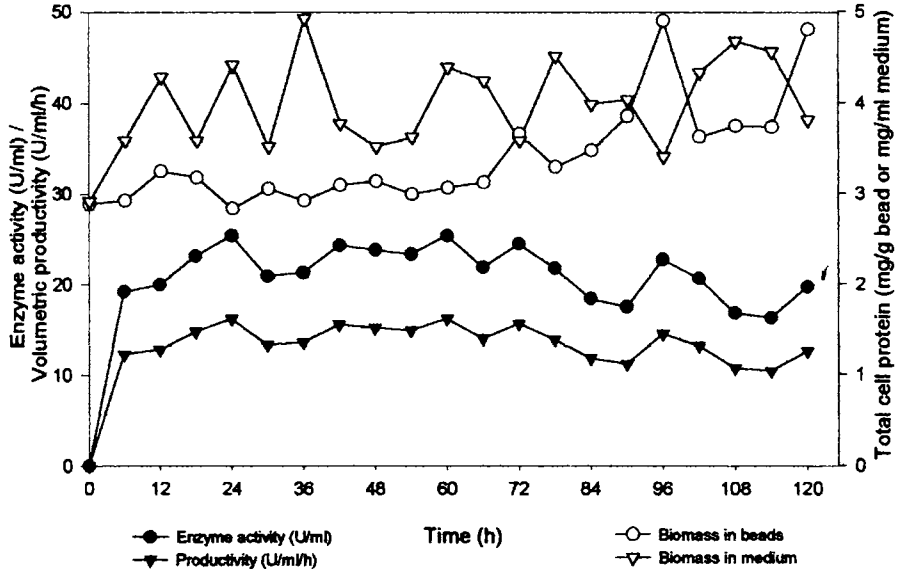


Table 4.3

Performance of a PBR with Ca-alginate immobilized *Pseudomonas* sp. for L-glutaminase production over prolonged continuous operation under conditions that give maximal substrate conversion.

Time (h)	Enzyme yield (U/ml)	Biomass in beads (mg protein/g bead)	Biomass in medium (mg protein/ml)	Productivity (U/ml/h)
0	0.0	2.88	2.91	0.0
6	19.18	2.92	3.58	12.28
12	19.99	3.24	4.28	12.79
18	23.15	3.17	3.58	14.82
24	25.39	2.83	4.41	16.25
30	20.90	3.05	3.51	13.38
36	21.35	2.92	4.92	13.67
42	24.34	3.09	3.77	15.58
48	23.82	3.13	3.51	15.24
54	23.37	2.99	3.62	14.96
60	25.39	3.06	4.39	16.25
66	15.95	3.65	4.24	10.21
72	24.49	2.83	3.58	15.67
78	21.8	3.29	4.51	13.95
84	18.43	3.47	3.98	11.80
90	17.53	3.85	4.03	11.22
96	22.77	4.89	3.4	14.57
102	20.67	3.62	4.33	13.22
108	16.85	3.74	4.67	10.78
114	16.33	3.73	4.56	10.45
120	19.77	4.80	3.81	12.65

4A.4 CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY Ca-ALGINATE IMMOBILIZED *PSEUDOMONAS* SP. IN A CIRCULATING BED REACTOR.

The Circulating bed reactor (CBR) had a working volume of 250cc. The dilution rates calculated for the flow rates 60, 80, 100, 120 and 140 ml/h were 0.24, 0.32, 0.4, 0.48 and 0.56 h^{-1} respectively. Enzyme yield, volumetric productivities, and substrate conversion rate obtained for the different substrate concentrations and dilution rates are shown in Table 4.4. Increase in substrate concentration resulted in an increased enzyme yield and volumetric productivity. Maximal enzyme yield (31.27U/ml) was recorded at 2% substrate concentration, at a dilution rate of 0.24 h^{-1}. High substrate conversion rates were recorded at the lowest dilution rate (0.24 h^{-1}) and the maximal rate of substrate conversion (24.47 %) was obtained in the medium

with 0.5% substrate concentration. In general, at all the substrate concentrations tried, increase in dilution rate led to an increased volumetric productivity, except for a substrate concentration of 0.5%, when there was reduction in enzyme yield at dilution rates above 0.4 h^{-1}. Further, it was observed that substrate conversion was least effective at the higher dilution rates.

Table 4.4

L- Glutaminase production by Ca-alginate immobilized cells of *Pseudomonas* sp. in a circulating bed reactor
Evaluation of performance under different operation conditions

Substrate Conc: (w/v)	F Flow rate (ml/h)	D=F/V Dilution rate (h^{-1})	R = 1/D (V/F) Residence time (h)	Y = Enzyme yield (U/ml)	Std Dev	% substrate conversion	Std Dev	P = YD Productivity (U/ml/h)
	60	0.24	4.17	19.91	2.29	24.47	1.834	4.78
	80	0.32	3.13	16.2	0.38	19.87	0.56	5.18
0.50%	100	0.40	2.50	15.58	0.62	18.9	0.56	6.23
	120	0.48	2.08	10.15	1.06	15.51	0.791	4.87
	140	0.56	1.79	7.75	1.2	13.1	1.856	4.34
	60	0.24	4.17	24.72	0.64	14.78	0.626	5.93
	80	0.32	3.13	19.94	1.7	12.96	0.917	6.38
1.00%	100	0.40	2.50	16.1	1.1	11.75	0.999	6.44
	120	0.48	2.08	13.61	1.31	10.54	0.464	6.53
	140	0.56	1.79	11.72	1.22	9.45	0.28	6.56
	60	0.24	4.17	28.65	1.82	11.22	0.914	6.88
	80	0.32	3.13	24.16	3.34	10.09	0.875	7.73
1.50%	100	0.40	2.50	21.1	2.39	8.8	1.549	8.44
	120	0.48	2.08	18.09	1.47	7.75	0.611	8.68
	140	0.56	1.79	18.01	2.57	7.18	0.485	10.09
	60	0.24	4.17	31.27	0.354	8.36	0.31	7.50
	80	0.32	3.13	26.2	1.058	7.45	0.3	8.38
2.00%	100	0.40	2.50	23.63	1.289	7.09	0.5	9.45
	120	0.48	2.08	20.92	1.232	6.84	0.67	10.04
	140	0.56	1.79	18.46	0.717	6.66	0.14	10.34

4A.4.1 Effect of Operational parameters on reactor performance.

The influence of the operational parameters viz. flow rate and substrate concentration on reactor performance was evaluated based on the enzyme yield, volumetric productivity, and substrate conversion efficiency of the reactor under various combinations of these parameters. The results are presented below.

4A.4.1.1 Effect of dilution rate and substrate concentration on enzyme yield

Increase in dilution rate had a negative impact on enzyme yield. At all the substrate concentrations tried, the enzyme yield decreased linearly along with an increase in dilution (Fig 4.24a). However, a higher substrate concentration in the feed could support an enhanced enzyme yield (Fig 4.24b). Maximal enzyme yield (31.27U/ml) was obtained at the lowest dilution (0.24 h^{-1}) in the medium containing 2% substrate, while lowest yield (7.75U/ml) was obtained with 0.5% substrate and at the highest dilution rate (0.56 h^{-1}) tried.

4A.4.1.2 Effect of dilution rate and substrate concentration on volumetric productivity

In general, the increase in dilution rate resulted in a higher productivity, for media containing 1%, 1.5% and 2% substrate except in the medium containing 0.5% substrate, where the volumetric productivity increased along with an increase in dilution, only up to a dilution rate of 0.4 h^{-1} (Fig 4.25a). At the dilution rates higher than this, the productivity decreased with an increase in dilution. Increase in substrate concentration promoted an increased productivity irrespective of the dilution rates (Fig 4.25b). The maximum and minimum values obtained for volumetric productivity were 10.34 and 4.34 U/ml/h in media containing 2% and 0.5% substrate respectively. Interestingly, the minimal productivity was recorded at the highest dilution rate tried.

4A.4.1.3 Effect of dilution rate and substrate concentration on substrate conversion

The efficiency of the CBR system for substrate conversion was the maximum at lower substrate concentration and dilution. Lower dilution rates supported a better substrate conversion and hence the maximum percentage of substrate conversion (24.47) was obtained at a dilution rate of 0.24 h^{-1} in the medium containing 0.5% substrate (Fig4.26a). Further, at all the substrate concentrations tried, an increase in dilution rate resulted in a reduction in substrate utilisation. It is inferred from the data presented in Figure 4.26b, that the concentration of substrate in the medium affects the efficiency of substrate utilisation and hence a marked reduction in substrate conversion was recorded in response to an increase in substrate concentration.

Fig 4.24 Effect of operational parameters on continuous production of .-glutaminase by Ca-alginate immobilized *Pseudomonas* sp. in a CBR
Dependence of enzyme yield on operational variables

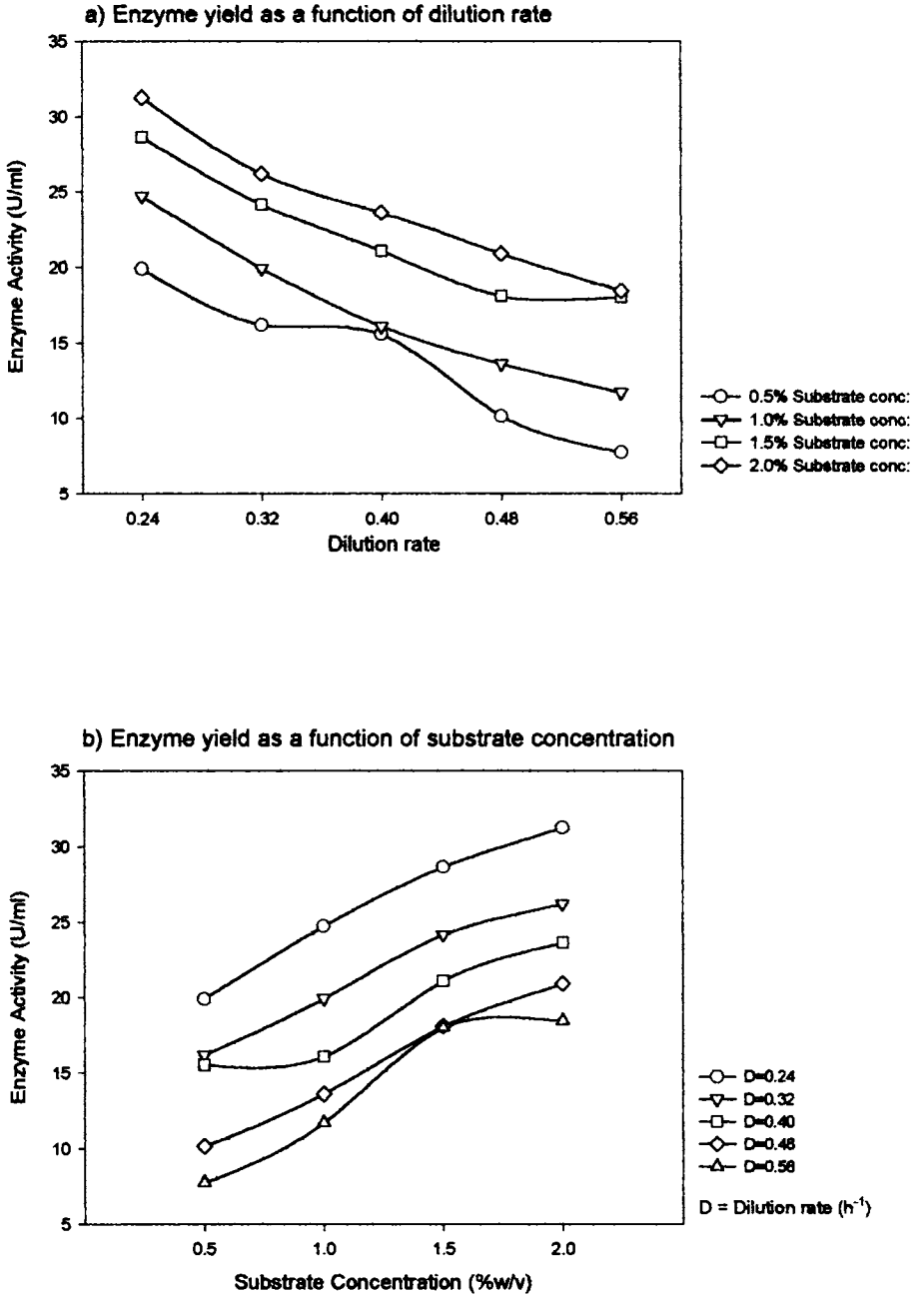
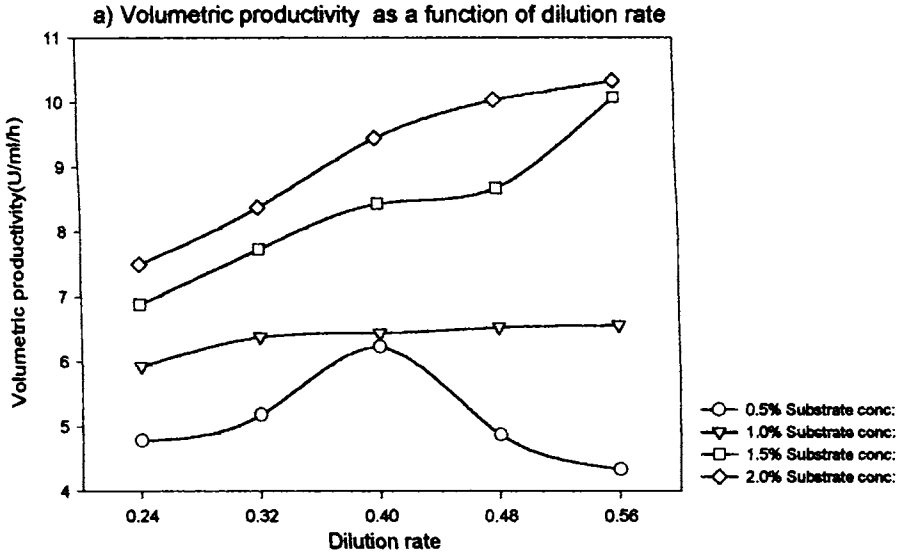


Fig 4.25 Effect of operational parameters on continuous production of L-glutaminase by Ca-alginate immobilized *Pseudomonas* sp. in a CBR
Dependence of productivity on operational variables

a) Volumetric productivity as a function of dilution rate



b) Volumetric productivity as a function of substrate concentration

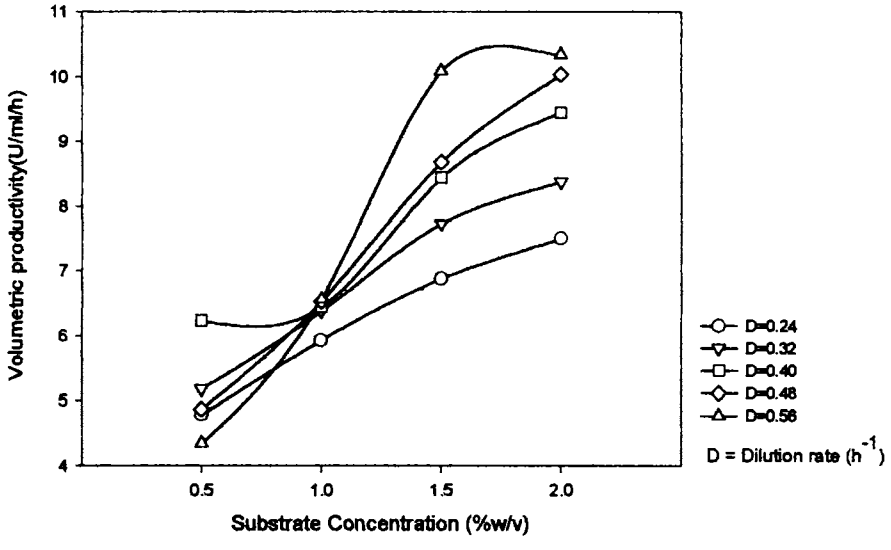
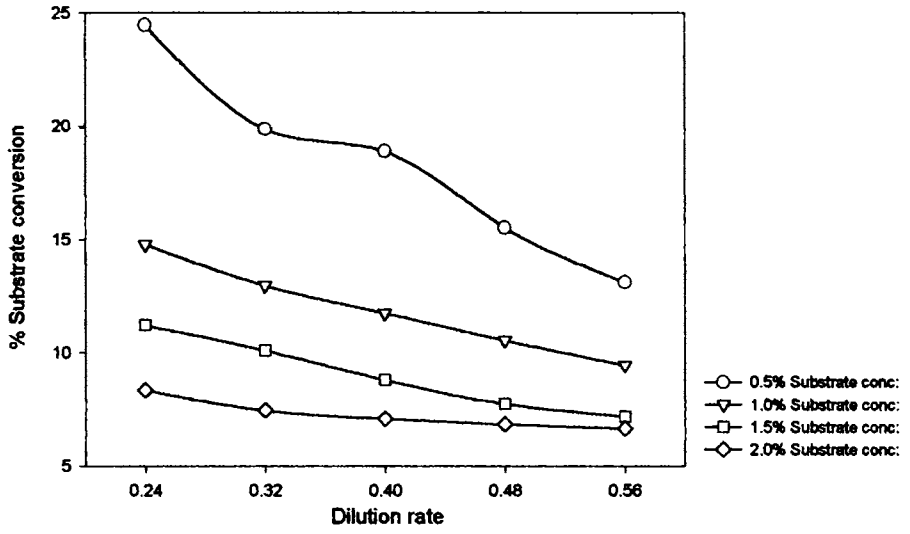
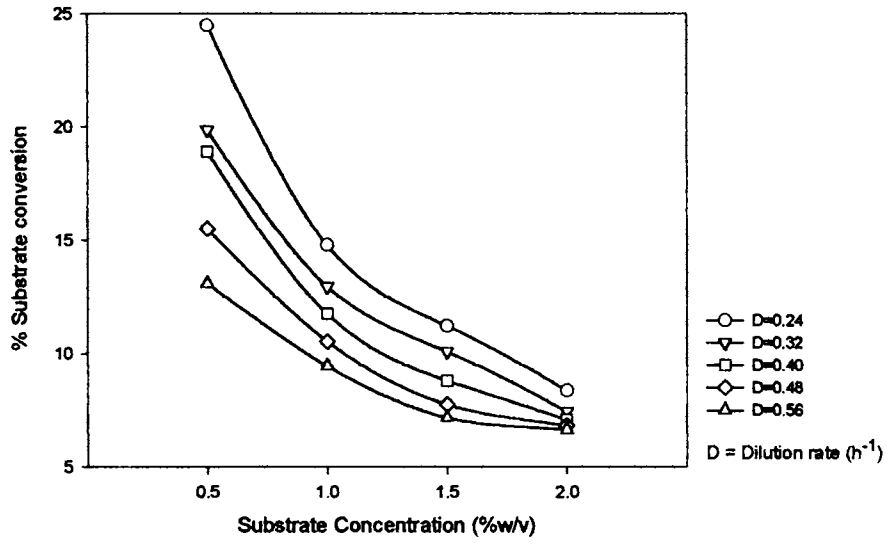


Fig 4.26 Effect of operational parameters on continuous production of _-glutaminase by ca-alginate immobilized *Pseudomonas* sp. in a CBR
Dependence of % substrate conversion on operational variables

a) Substrate conversion as a function of dilution rate



b) Substrate conversion as a function of substrate concentration



4A.4.2 Operational Stability of the reactor

The reactor was operated at the conditions that favoured maximum substrate conversion. The reactor gave a stable enzyme yield that varied between 8.78 and 16.4 U/ml at a substrate concentration of 0.5% and a dilution rate of 0.24 h^{-1} and the mean enzyme yield obtained was 11.71 U/ml (Table 4.5). The productivities were lower compared to the PBR and remained between 2.1 and 3.94 U/ml/h. with the average productivity being 2.811 U/ml/h. Biomass concentration in the beads showed a definite relation with the enzyme yield and productivity. Thus an increased biomass content in beads resulted in an enhanced yield and productivity (Fig 4.27). However, the biomass concentration in the medium showed fluctuating values and did not show any conclusive relation to the enzyme activities obtained.

Fig 4.27 Circulating bed reactor performance over prolonged operation up to 120h at 0.5% substrate concentration and a dilution rate of 0.24 h^{-1}

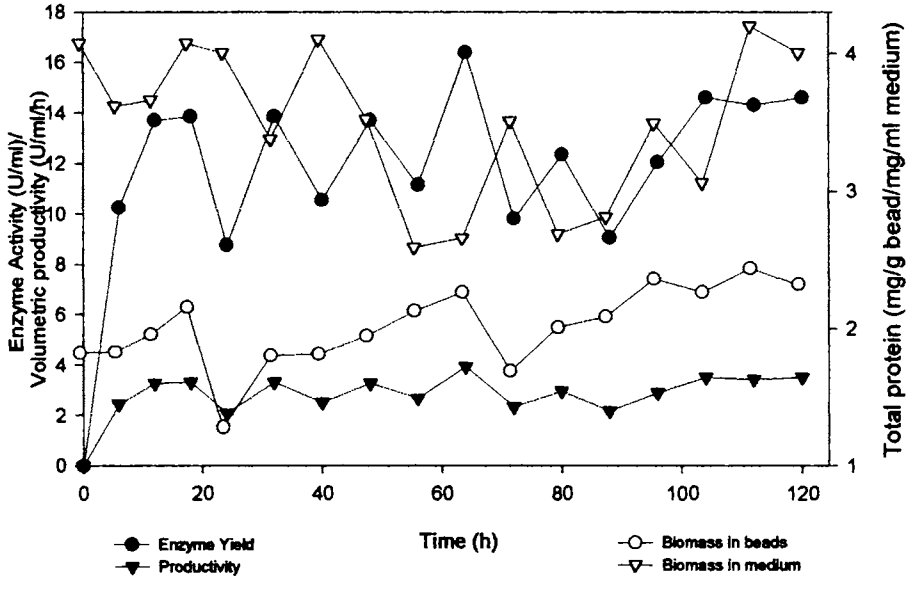


Table 4.5

Performance of a CBR with a fluidised bed of Ca-alginate immobilized *Pseudomonas* sp. for L-glutaminase production over prolonged continuous operation under conditions that give maximal substrate conversion.

Time (h)	Enzyme yield (U/ml)	Biomass in beads (mg protein/g bead)	Biomass in medium (mg protein/ml)	Productivity (U/ml/h)
0	0.0	1.82	4.07	0.0
6	10.26	1.83	3.62	2.46
12	13.71	1.96	3.66	3.29
18	13.86	2.16	4.08	3.33
24	08.76	1.28	4.01	2.10
32	13.86	1.80	3.38	3.33
40	10.56	1.82	4.10	2.53
48	13.71	1.95	3.52	3.29
56	11.16	2.13	2.59	2.68
64	16.40	2.27	2.66	3.94
72	09.81	1.69	3.51	2.35
80	12.36	2.01	2.69	2.97
88	09.06	2.09	2.81	2.18
96	12.06	2.36	3.49	2.89
104	14.61	2.27	3.06	3.51
112	14.31	2.44	4.20	3.43
120	14.61	2.32	4.01	3.51

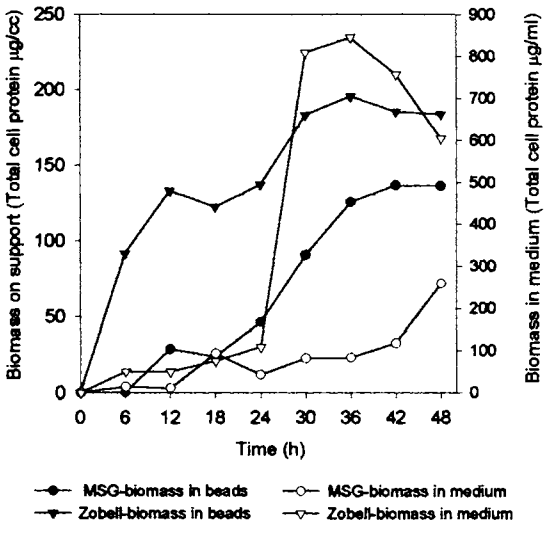
4B. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED BY PHYSICAL ADSORPTION ON POLYSTYRENE BEADS.

4B.1 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON POLYSTYRENE BEADS UNDER BATCH OPERATION

4B.1.1 Selection of immobilization medium and incubation time

With either MSG or Zobell's broth as the growth medium, the cell adsorption on polystyrene beads occurred as a gradual process. Biomass on the support although attained a maximum at 42 h in MSG medium and at 36h in Zobell's broth (Fig 4.28), remained fairly consistent in both the media, after 36 h of incubation. Zobell's broth not only supported a better cell adsorption on the support compared to MSG but also a shorter incubation time for achieving this. Cell growth as free cells in the medium, determined biomass, increased along with incubation time in MSG for the incubation period tried. However, the maximal biomass in medium was attained with 36h of incubation in Zobell's broth and further incubation resulted in a decreased biomass content. Further, cell growth was higher in the Zobell's broth compared to the MSG medium. Based on these results an incubation time for 36h in Zobell's broth was selected as the ideal immobilization condition.

Fig 4.28
Immobilization of *Pseudomonas* cells on polystyrene beads
Selection of growth medium and incubation time

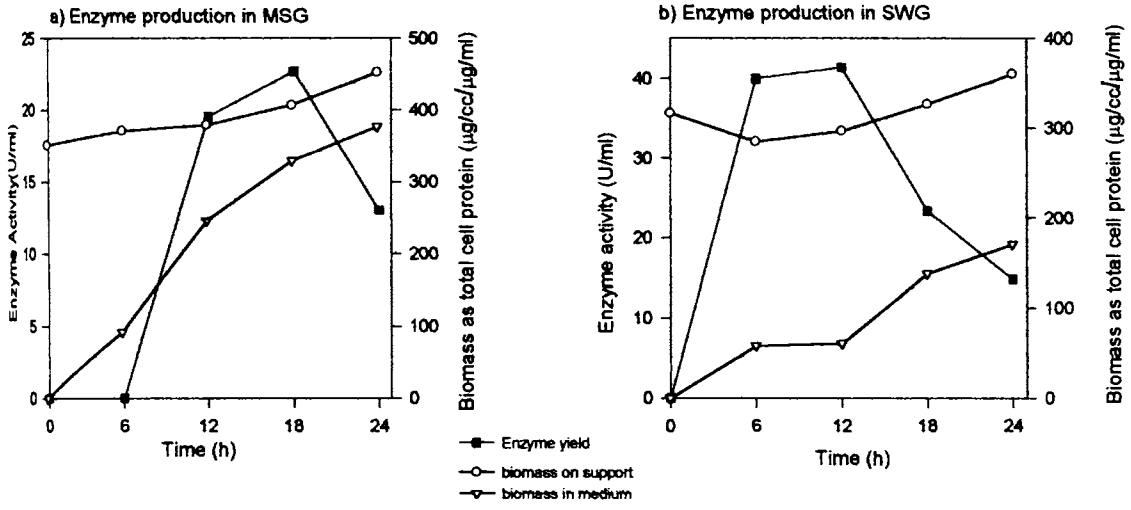


4B.1.2 Selection of enzyme production medium and retention time

Seawater glutamine medium (SWG) was observed to be better than MSG as an enzyme production medium. SWG supported enhanced enzyme yields at all the retention times tested. While the maximum enzyme yield obtained in MSG was 22.7 U/ml at a retention time of 18h (Fig 4.29a), in SWG a maximum enzyme yield of 41.35U/ml was obtained in 12h (Fig 4.29b). SWG was observed to be effective as the

EPM and enzyme production in it commenced as early as the 6th hour of incubation, when a yield of 39.99U was obtained. The cell biomass adhered to the support measured as total protein was 351.4μg/cc for MSG and 317.09 μg/cc for SWG at the start of the production cycle and in both cases it increased with incubation, attaining final values of 452.94μg and 360.76μg/cc respectively. Obviously, cell growth occurred on the support resulting in detachment and growth as free cells in the medium, evidenced by the increase in biomass in both the media along with increase in retention time. 12h retention in SWG was selected as the ideal enzyme production condition.

Fig 4.29 Selection of enzyme production medium and retention time



4B.1.3 Optimisation of parameters for enzyme production

The optimisation of EPM composition and production conditions were carried out based on the Box-Behnken design model with 46 runs in 2 blocks of 23 cases as outlined under section 3D.1.6. The responses obtained for the 46 runs are given in Table 4.6.

Table 4.6

Optimisation of enzyme production by *Pseudomonas* sp. cells immobilized on polystyrene Operating variables and levels used, and the responses obtained in the Box-Behnken design

Block	Run #	pH	Temp. (°C) X_1	Glutamine Conc. (%w/v) X_2	Glucose Conc. (%w/v) X_3	Yeast Extract Conc. (%w/v) X_4	Enzyme yield (U/ml) Y
1	1	8	40	1	1	0.25	4.27
1	2	8	30	1	1	0.25	0
1	3	4	40	1	1	0.25	18.43
1	4	4	30	1	1	0.25	6.52
1	5	6	35	1.5	1.5	0.25	29.44
1	6	6	35	1.5	0.5	0.25	14.76
1	7	6	35	0.5	1.5	0.25	13.56
1	8	6	35	0.5	0.5	0.25	11.01
1	9	6	40	1	1	0.5	0
1	10	6	40	1	1	0	10.34
1	11	6	30	1	1	0.5	11.46
1	12	6	30	1	1	0	21.12
1	13	8	35	1.5	1	0.25	0
1	14	8	35	0.5	1	0.25	14.16
1	15	4	35	1.5	1	0.25	18.88
1	16	4	35	0.5	1	0.25	15.06
1	17	6	35	1	1.5	0.5	7.42
1	18	6	35	1	1.5	0	10.34
1	19	6	35	1	0.5	0.5	21.80
1	20	6	35	1	0.5	0	11.91
1	21	6	35	1	1	0.25	16.93
1	22	6	35	1	1	0.25	14.61
1	23	6	35	1	1	0.25	19.33
2	24	6	40	1.5	1	0.25	19.55
2	25	6	40	0.5	1	0.25	14.38
2	26	6	30	1.5	1	0.25	21.57
2	27	6	30	0.5	1	0.25	23.52
2	28	8	35	1	1.5	0.25	6.52
2	29	8	35	1	0.5	0.25	3.60
2	30	4	35	1	1.5	0.25	0
2	30	4	35	1	0.5	0.25	15.06
2	32	6	35	1.5	1	0.5	22.70
2	33	6	35	1.5	1	0	14.83
2	34	6	35	0.5	1	0.5	12.58
2	35	6	35	0.5	1	0	16.48
2	36	8	35	1	1	0.5	27.71
2	37	8	35	1	1	0	22.47
2	38	4	35	1	1	0.5	14.16
2	39	4	35	1	1	0	17.08
2	40	6	40	1	1.5	0.25	11.46
2	40	6	40	1	0.5	0.25	12.13
2	42	6	30	1	1.5	0.25	23.59
2	43	6	30	1	0.5	0.25	23.52
2	44	6	35	1	1	0.25	17.53
2	45	6	35	1	1	0.25	15.51
2	46	6	35	1	1	0.25	28.76

4B.1.3.1 Analysis of the Box-Behnken design data

The analysis of variance for the five process variables indicated that L-glutaminase yield (Y) by the adsorbed cells of *Pseudomonas* sp. can be described by the quadratic polynomial model (Table 4.7)

Table 4.7

Analysis of variance for data pertaining to the enzyme yield (Y)

Source	Sum of Squares	Mean Square	DF	F Value	Prob > F
Block	151.21	1	151		
Model	*1370.51*	*20*	69	*2.63*	*0.0126*
A	43.66	*1*	*44*	*1.68*	*0.2076*
B	290.53	1	291	11.16	0.0027
C	*87.00*	*1*	*87*	3.34	0.0799
D	11.97	1	12	*0.46*	*0.5041*
E	39.13	1	39	1.50	0.2320
A^2	745.21	1	745	28.63	< 0.0001
B^2	96.67	1	97	3.71	0.0659
C^2	7.69	1	8	0.30	0.5916
D^2	32.90	1	33	1.26	0.2720
E^2	10.91	1	11	0.42	0.5235
AB	14.59	1	15	0.56	0.4612
AC	35.52	1	36	1.36	0.2542
AD	3.72	1	4	0.14	0.7085
AE	0.28	1	1	0.011	0.9189
BC	2.59	1	3	0.100	0.7550
BD	0.14	1	1	0.000526	0.9428
BE	4.49	1	4	0.17	0.6814
CD	36.78	1	37	1.41	0.2461
CE	1.02	1	1	0.039	0.8447
DE	0.29	1	1	0.011	0.9173
Residual	624.59	24	26		
Lack of Fit	536.50	20	27	1.22	0.4730
Pure Error	88.09	4	22		
Cor Total	2146.30	45			

The model F value of 2.63 implies that the model is significant and the lack of fit value of 1.22 implies that lack of fit is not significant relative to the pure error. The R^2 value of 0.6869 showed that there was a satisfactory coefficient of determination. The fitted model is given below

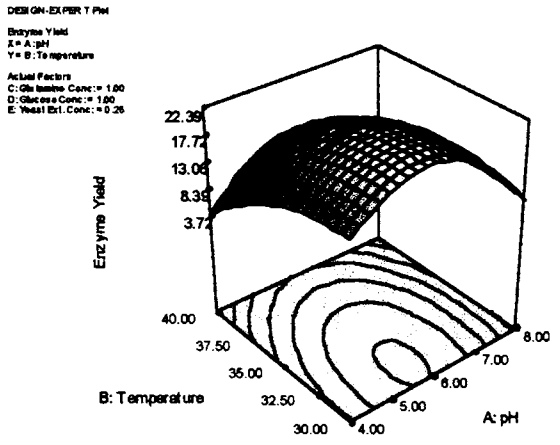
$$Y = 20.81 - 1.65X_1 - 4.26X_2 + 2.33X_3 + 0.87X_4 - 1.56X_5 - 9.24X_1^2 - 3.33X_2^2 - 0.94X_3^2 - 1.94X_4^2 - 1.12X_5^2 + 1.91X_1X_2 + 2.98X_1X_3 + 0.97X_1X_4 - 0.26X_1X_5 + 0.81X_2X_3 - 0.18X_2X_4 + 1.06X_2X_5 + 3.03X_3X_4 + 0.51X_3X_5 - 0.27X_4X_5$$

4B.1.3.2 Interactions between factors

The relationships between operational variables were assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two.

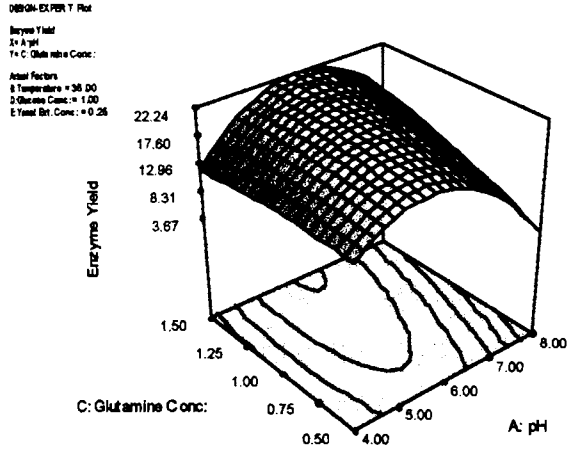
Interaction between pH and temperature

Fig 4.30- Response behaviour of pH and temperature under constant levels of glutamine , glucose and yeast extract



When the concentration of glutamine, glucose, and yeast extract (YE) were held at their middle values, the optimal range of pH was found to be between 5 and 6. At any incubation temperature the dependence of yield on pH showed a parabolic response curve indicating that the yields are negatively influenced by extremes of pH (Fig 4.30). Higher yields were obtained at lower temperatures and drastic reduction in yield was obtained at higher temperature. The optimal operating range was between 30 and 32.5 ^{0}C.

Fig 4.31, Response behaviour of pH and Glutamine concentration under constant levels of temperature, glucose and yeast extract



Interaction between pH and glutamine concentration

The response for pH was parabolic with very low or very high values resulting in a lower yield, irrespective of the temperature of incubation, and the maximal response was obtained in the range 5.5-6.5. The dependency of yield on

glutamine concentration was linear at all the initial pH ranges and the yields increased along with increase in concentration of glutamine (Fig 4.31).

Interaction between pH and glucose concentration

Figure 4.32 shows the dependencies of enzyme yield on pH and glucose concentration. The optimal pH range was between 5.5 and 6.5. The response obtained for glucose concentration also showed a parabolic curve at all the pH ranges. Very high or very low glucose concentration was not favourable for enzyme production. Low glucose concentration may not be able to support sufficiently, the metabolic processes, whereas at high concentrations, glucose might lead the immobilized cells to active growth and cell division instead of enzyme production. The ideal concentration of glucose lied in the range 1-1.25%w/v.

Fig 4.32. Response behaviour of pH and glucose concentration at constant levels of temperature, glutamine and yeast extract

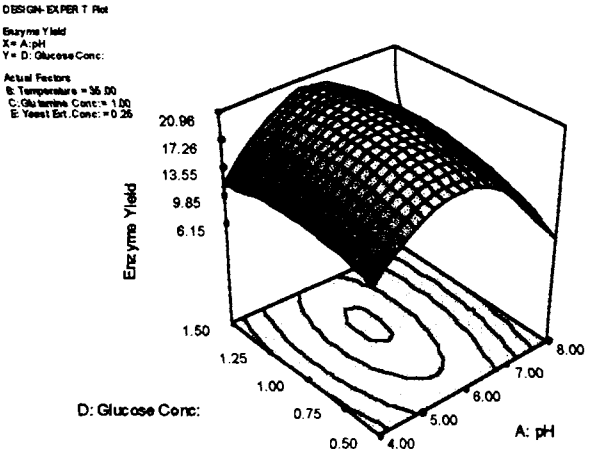
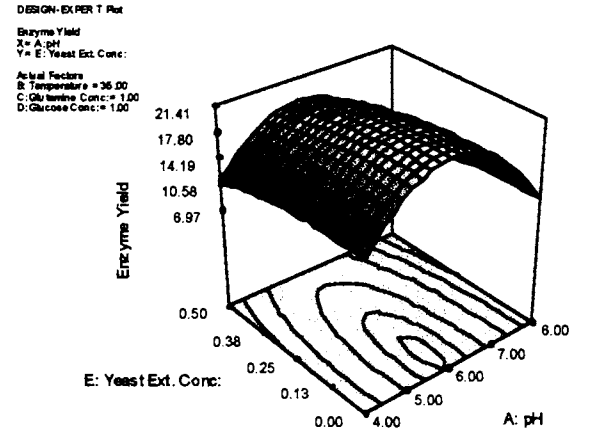


Fig 4.33 Response behaviour of pH and yeast extract conc. at constant levels of temperature, glutamine and glucose



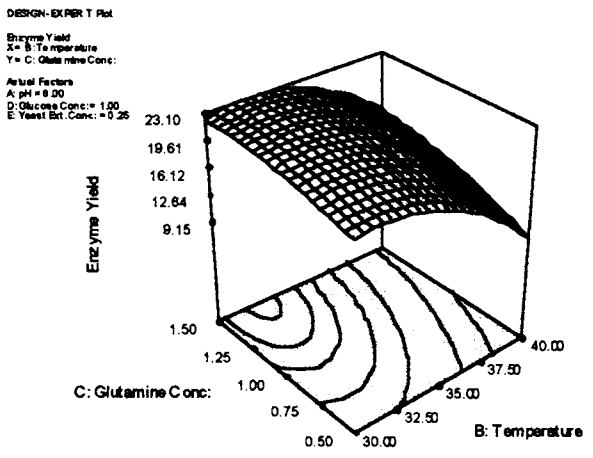
Interaction between pH and yeast extract concentration

The optimal yeast extract concentration was between 0 and 0.13%. Increase in yeast extract concentration though resulted in an enhanced cell growth, led to a reduction in enzyme yield. At the constant values of other parameters the ideal pH was between the range 5.5 and 6 (Fig 4.33). However, at this pH range, a decreased yield was observed at higher yeast extract concentrations.

Interaction between temperature and glutamine concentration

The highest yields were obtained in concentration range between 1.25 and 1.5% glutamine and in the temperature range 32-35 ^{0}C (Fig 4.34). Yields were not only lower at higher temperatures and but also at lower glutamine concentrations. The decrease in yield along with a decrease in glutamine concentration was more evident at higher temperatures.

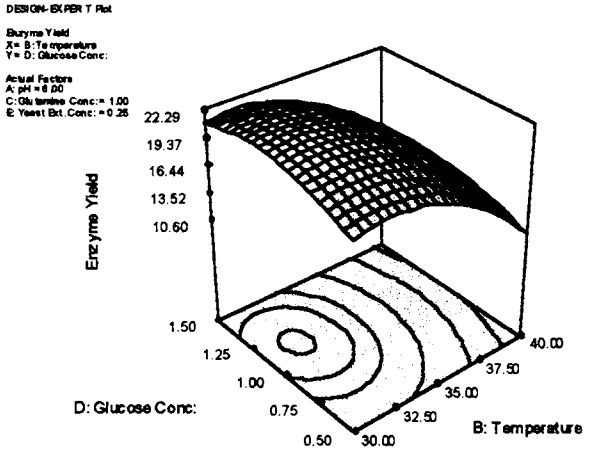
Fig 4.34 Response behaviour of Temperature and glutamine concentration at constant levels of pH, glucose and Yeast extract



Interaction between temperature and glucose concentration

The response obtained for glucose concentration was parabolic giving maximum yields in the concentration range 1-1.25% and decreasing with deviations above or below this range. Temperature range giving maximum response lied between 32-35 ^{0}C (Fig 4.35). The decrease in yield at higher temperature was more pronounced at lower glucose concentrations

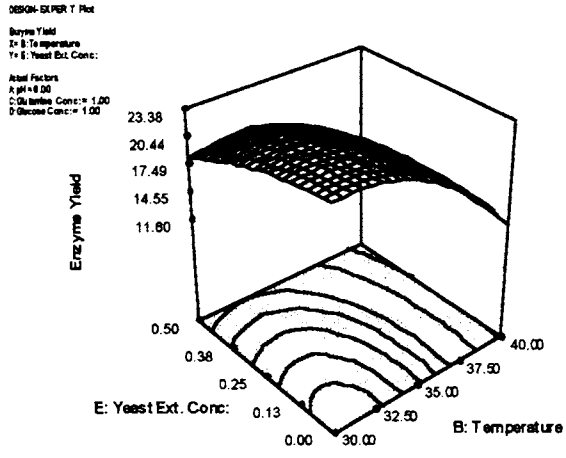
Fig 4.35 Response behaviour of temperature and glucose concentration at constant levels of pH, glutamine and yeast extract



Interaction between temperature and yeast extract concentration

At all the yeast extract concentrations, increase in temperature resulted in a decreased enzyme yield (Fig 4.36). Yeast extract concentration influenced the yield negatively such that the highest yield was obtained at the lowest concentrations. Maximum response was obtained in the concentration range of 0-0.13% yeast extract and within the temperature range 30-32.5 ^{0}C.

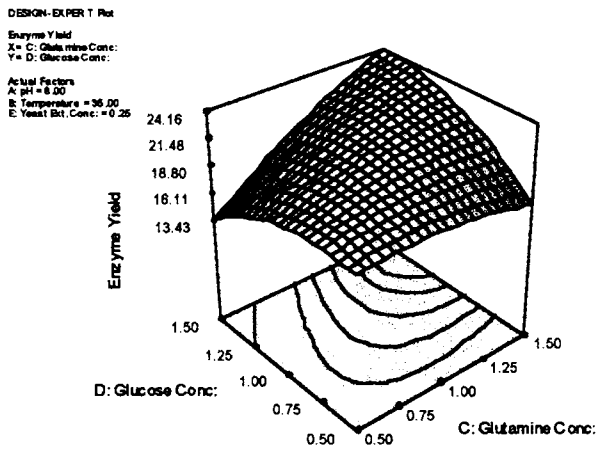
Fig 4.36 Response behaviour of temperature and yeast extract concentration at constant levels of pH, glutamine and glucose



Interaction between glutamine concentration and glucose concentration

There was a prominent interaction between the concentrations of glutamine and glucose. At lower glucose concentrations the enzyme yield was low despite a high glutamine concentration (Fig 4.37). The optimal glutamine concentration was between 1-1.25% at glucose concentrations between 0.5and 0.75%. However, at high glucose concentration the yield increased linearly along with increase in glutamine concentration and the maximal yields were obtained at high concentration of glutamine and glucose concentrations (range 1.25-1.5% for both)

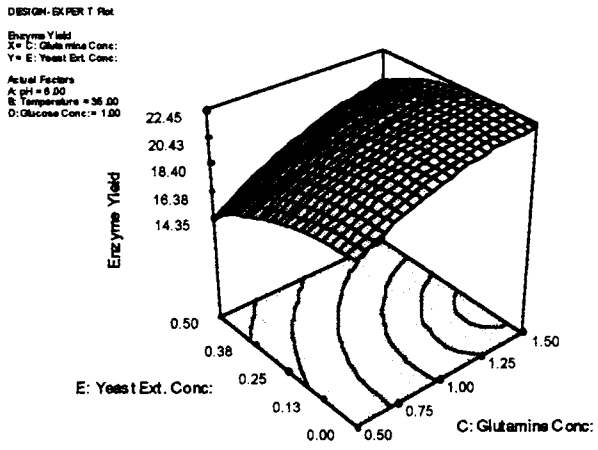
Fig 4.37 Response behaviour of glutamine and glucose concentrations at constant pH, temperature and yeast extract concentration



Interaction between glutamine and yeast extract concentrations

Enzyme yields were higher at higher glutamine concentrations irrespective of yeast extract concentration (Fig 4.38). The maximal enzyme yields were obtained at the highest glutamine concentrations (1.25-1.5%). The ideal yeast extract concentration lied between 0.1and 0.2 %. The operation range for maximum enzyme yield was found close to 1.5% glutamine and near 1.3% yeast extract.

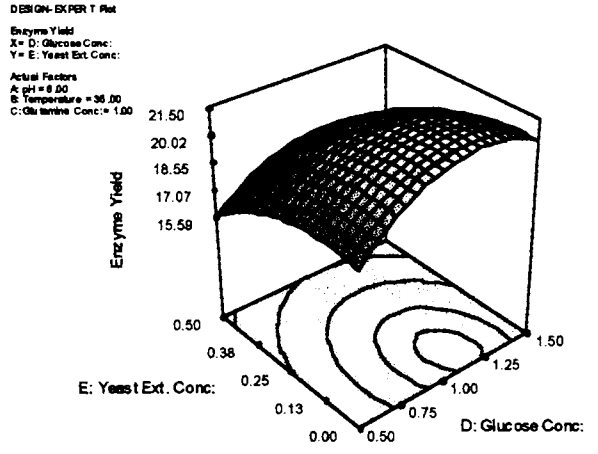
Fig 4.38 Response behaviour of glutamine and yeast extract concentrations at constant pH, temperature and glucose concentration



Interaction between glucose concentration and yeast extract concentration

Glucose concentrations in the range of 1-1.25% promoted the maximal enzyme yield at lower yeast extract concentrations. At high concentrations of yeast extract the yields were lower compared to that obtained at lower concentrations (Fig 4.39). Nevertheless, the yields at these concentrations were maximal in the same glucose concentration range. The operation region for maximal yield lied between a glucose concentration range of 1-1.25% and a yeast extract concentration range of 0-0.13%.

Fig 4.39 Response behaviour of glucose concentration and yeast extract concentration at constant pH temperature and glutamine concentration



B.1.3.3 Optimisation and validation of models

Optimisation of the production conditions for maximal enzyme yield carried out using the numerical optimisation tool in the Design -Expert ® package. Though the temperature range that support maximal enzyme yield was 30-32.5 ^{0}C, growth of the cells was maximum at 35 ^{0}C. Since maximal growth of cells is absolutely essential for maintaining the biomass concentration on polystyrene the temperature was kept constant at 35 ^{0}C for the optimisation. The predicted combination of operating variables for maximal glutaminase production was L-glutamine-1.5%w/v, glucose-1.5%w/v, Yeast Extract-0.11%w/v, initial pH of the medium- 6.33 and 35 ^{0}C-incubation temperature. The predicted enzyme yield was 24.70 U/ml. Under the predicted conditions, the optimal design was validated and the response obtained (15.51U/ml) was 37.21% lower than the predicted yield.

4B.2 PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON POLYSTYRENE BEADS UNDER REPEATED BATCH OPERATION

Pseudomonas sp. cells immobilized by adsorption on polystyrene could synthesise L-glutaminase consistently in the range of 11 - 17U/ml throughout the batches of operation (Table 4.8). The biomass adsorbed on polystyrene increased with every batch of operation till the 13th cycle and thereafter remained fairly constant at above 1.7-mg protein/cc of beads (Fig 4.40). Apparently there was no immediately perceptible direct relationship between the biomass adsorbed on to the beads and enzyme yield, which was also true for the free growing cells. Both the anchored cells and the free growing cells would have equally contributed towards the enzyme production. However it was observed that with the cycles of operation, the enzyme yield had diminished slightly towards the last few cycles which also was true with the biomass adsorbed on support.

Fig 4.40 Repeated batch synthesis of L-glutaminase by *Pseudomonas* sp. cells immobilized on polystyrene beads

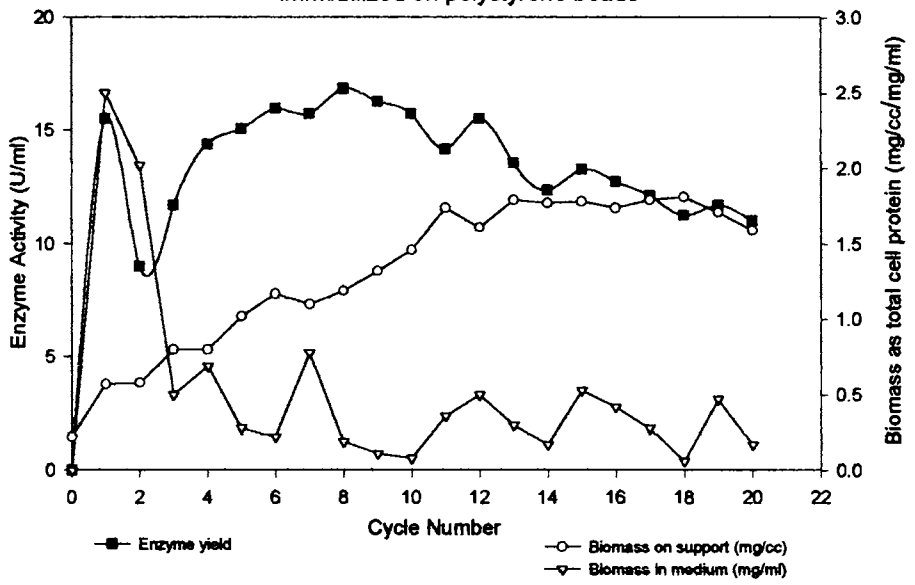


Table 4.8
Repeated batch synthesis of L-glutaminase by *Pseudomonas* sp. adsorbed on polystyrene beads

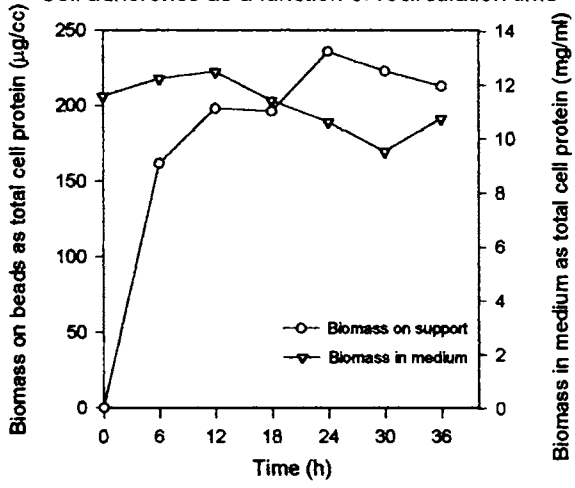
Cycle #	Mean Enzyme yield (U/ml)	Biomass on support (mg protein/cc support)	Biomass in medium (mg protein/ml)
0.000	0.00	0.22	0.00
1	15.51	0.57	2.50
2	8.99	0.58	2.02
3	11.69	0.80	0.50
4	14.38	0.80	0.69
5	15.06	1.02	0.28
6	15.95	1.17	0.22
7	15.73	1.10	0.78
8	16.85	1.19	0.19
9	16.25	1.32	0.11
10	15.73	1.46	0.08
11	14.16	1.74	0.36
12	15.51	1.61	0.50
13	13.56	1.79	0.30
14	12.36	1.77	0.17
15	13.26	1.78	0.53
16	12.73	1.74	0.42
17	12.13	1.79	0.28
18	11.24	1.81	0.06
19	11.69	1.71	0.47
20	11.01	1.59	0.17

4B.3 CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON POLYSTYRENE IN A PACKED BED REACTOR

4B.3.1 Cell immobilization on the polystyrene bed

Cell immobilization on the packed bed of polystyrene beads was effected by re-circulation of an actively growing culture of *Pseudomonas* sp. Cell adsorption on the bed was monitored as total cell protein. Fig 4.41 shows the progress of cell growth and adherence on the bed along with increase in re-circulation time. The biomass adsorbed on polystyrene increased with time up to 24h when it reached the maximum value (235µg/cc). Further increase in re-circulation time did not result in an increase in biomass adsorbed on the bed and the values remained almost constant. Also it was noted that the biomass content in the medium, which increased with re-circulation time till the 18th hour, decreased later during 18-30h and finally increased again after the 30 h

Fig 4.41 Immobilization of *Pseudomonas* sp. cells on polystyrene bed in a PBR.
Cell adherence as a function of recirculation time



4B.3.2 Effect of Operational parameters on Reactor performance.

The void volume of the reactor was determined to be 63.8cc. Dilution rates that calculated for the flow rates 60, 80, 100, 120 and 140 ml/h were 0.94,1.25,1.57,1.88 and 2.19 h^{-1} respectively. Table 4.9 shows the enzyme yield, volumetric productivities and substrate conversion rate obtained at different substrate concentrations and dilution rates for successive continuous runs. Increase in dilution rates resulted in a decreased enzyme yield and a reduction in substrate conversion efficiency. The data presented in Table 4.9 also indicate that the dilution rates, which support maximal volumetric productivity, were dependent on the substrate concentration in the medium used for the experiment. .

Table 4.9

L- Glutaminase production by *Pseudomonas* sp.
immobilized on polystyrene beads in a PBR
Evaluation of performance under different operation conditions

Substrate Conc: (w/v)	F Flow rate (ml/h)	D=F/V Dilution rate (h^{-1})	R = 1/D (V/F) Residence time (h)	Y = Enzyme yield (U/ml)	Std Dev	% substrate conversion	Std Dev	P = YD Productivity (U/ml/h)
	60	0.94	1.06	15.81	1.399	26.49	1.48	14.87
	80	1.25	0.80	14.76	1.578	24.55	3.115	18.51
0.50%	100	1.57	0.64	13.78	3.274	23.91	0.56	21.60
	120	1.88	0.53	10.61	1.709	19.71	3.115	19.96
	140	2.19	0.46	8.26	1.521	19.06	1.48	18.13
	60	0.94	1.06	20.55	0.708	13.73	0.74	19.33
	80	1.25	0.80	18.65	1.348	12.28	1.558	23.39
1.00%	100	1.57	0.64	16.6	0.229	12.11	0.485	26.02
	120	1.88	0.53	13.51	0.769	11.63	0.969	25.41
	140	2.19	0.46	10.07	1.071	11.47	2.39	22.10
	60	0.94	1.06	23.77	1.995	11.31	5.046	22.35
	80	1.25	0.80	20.27	1.978	9.91	1.492	25.42
1.50%	100	1.57	0.64	17.35	1.147	8.94	1.837	27.19
	120	1.88	0.53	15.65	1.238	8.72	0.855	29.44
	140	2.19	0.46	12.66	0.956	7.21	0.673	27.78

4B.3.2.1 Effect of dilution rate and substrate concentration on enzyme yield

Increase in dilution rates resulted in a reduction in enzyme yield irrespective of the substrate concentrations tried (Fig 4.42a). The maximal yield (23.77U/ml) was obtained at the lowest dilution rate (0.94 h^{-1}) in the medium containing the highest substrate concentration (1.5-%) , and the minimal yield (8.26U/ml) was obtained at the highest dilution rate and lowest substrate concentration tried (2.19 h^{-1} and 0.5% respectively). The dependence of enzyme yield on substrate concentration was linear and at any dilution rate, the enzyme yield increased with an increase in substrate concentration (Fig 4.42b).

4B.3.2.2 Effect of dilution rate and substrate concentration on volumetric productivity

The volumetric productivity increased along with an increase in dilution up to a dilution rate of 1.57 h^{-1}, when media with substrate concentrations 0.5% and 1% were used, whereas it increased up to a dilution rate of 1.88 h^{-1} when the media contained 1.5% substrate. Further increase in dilution rate, in either of the above cases, led to a decrease in productivity of the reactor (Fig.4.43a). At each of the dilution rates tested, the volumetric productivity was higher at the higher substrate concentrations, showing a linear relation ship between substrate concentration and productivity (Fig 4.43b)

4B.3.2.3 Effect of dilution rate and substrate concentration on substrate conversion

Substrate conversion efficiency of the PBR system was high at the lower dilution rates and the observed decrease in %substrate conversion was linear with the increase in dilution rates, irrespective of the concentration of substrate in the medium (Fig 4.44a). Increase in substrate concentration had a negative effect on % substrate conversion and a major portion of the substrate supplied through the feed was not utilised by the cells as indicated by the lower percentage of substrate conversion at the higher concentrations tried (Fig 4.44b).

Fig 4.42 Effect of operational parameters on continuous production of -glutaminase by *Pseudomonas* sp. immobilized on polystyrene beads in a PBR

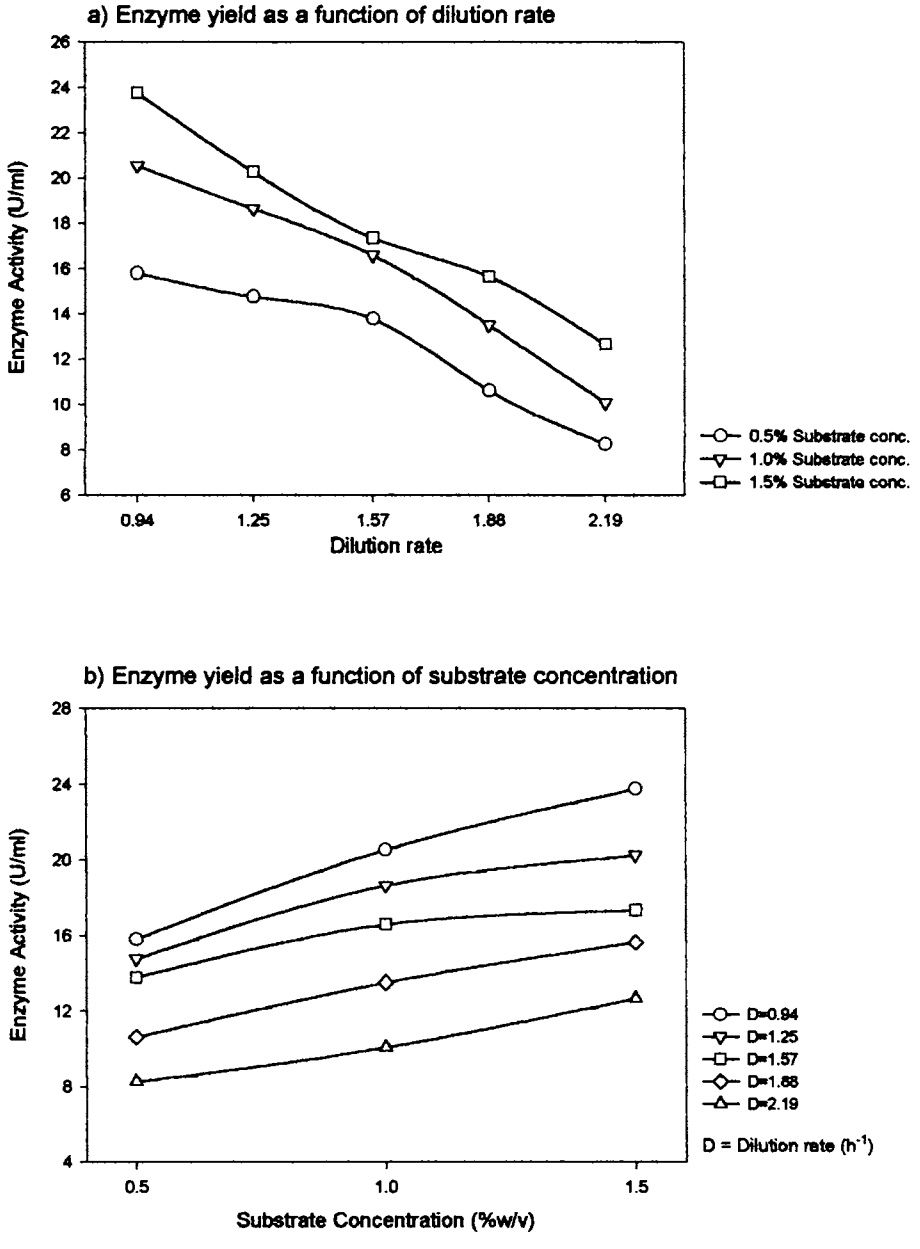


Fig 4.43 Effect of operational parameters on continuous production of -glutaminase by *Pseudomonas* sp. immobilized on polystyrene beads in a PBR

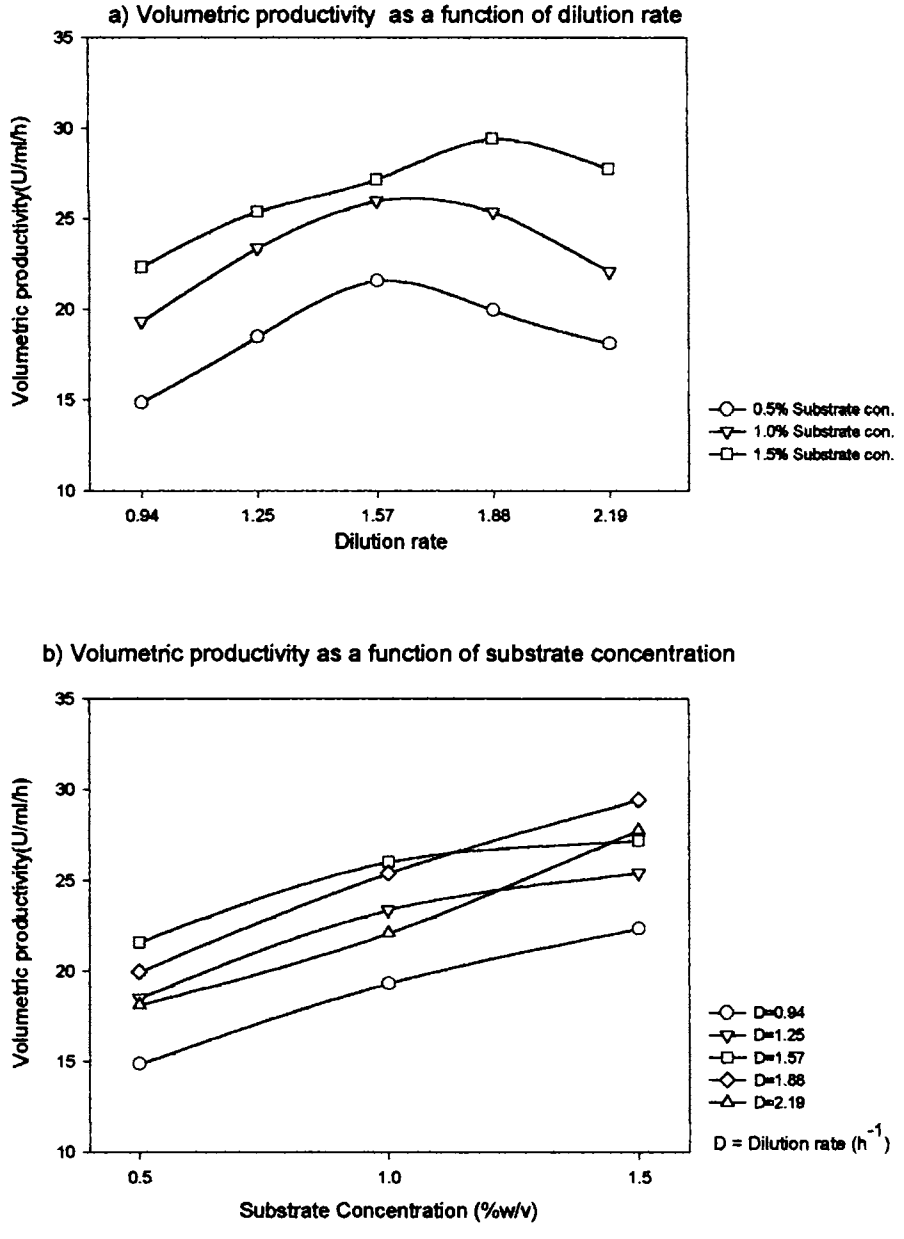
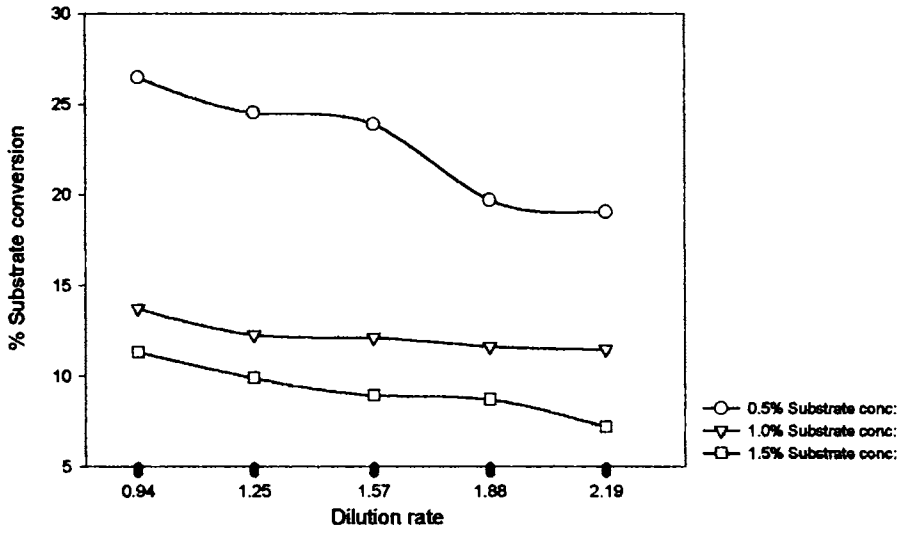
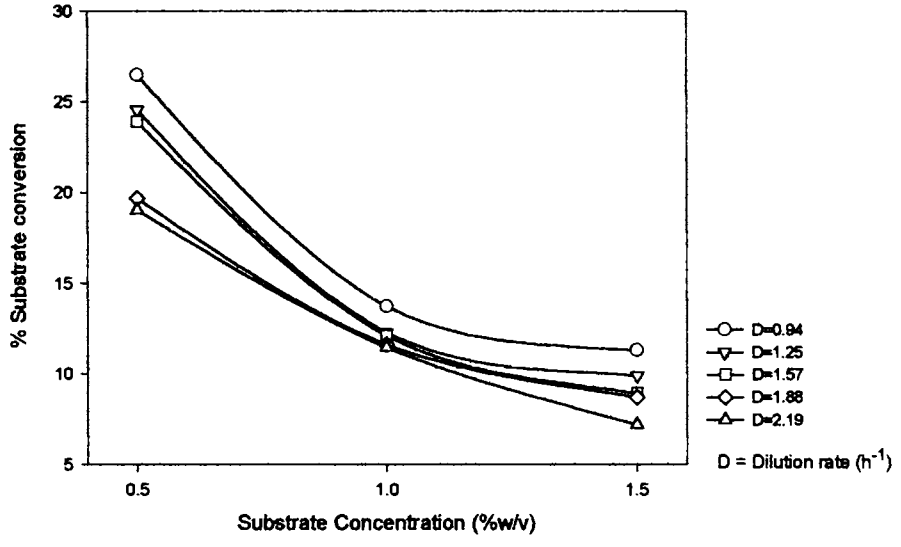


Fig 4.44 Effect of operational parameters on continuous production of L-glutaminase by*Pseudomonas* sp. immobilized on polystyrene beads in a PBR

a) Substrate conversion as a function of dilution rate



b) Substrate conversion as a function of substrate concentration



4B.3.3 Operational stability of the reactor

From the data presented in Figure 4.45 and Table 4.10, obtained for the studies on enzyme production by *Pseudomonas* sp. immobilized on polystyrene in PBR under continuous culture for 72h, operated at the maximum substrate conversion efficiency (0.5% substrate and dilution rate 0.94 h^{-1}), it was noted that the cells could synthesise glutaminase consistently over several hours of continuous operation maintaining an average yield of 12 U/ml. The maximum and minimum enzyme yields recorded were 14.76 and 10.71 U respectively. The biomass in the beads increased from 0.683-mg protein/cc support to 1.106-mg protein/ cc in the first 8h of operation and later remained almost stable between 1.3 and 1.8 mg/cc. The biomass content in the effluent stream varied between 6.349 and 7.291 mg protein/ml. The average volumetric productivity was 11.28 U/ml/h. Enzyme activities and productivities varied in concordance with variations in biomass on the beads and in the medium. It may be assumed that these apparently were dependent on the total cell biomass and not a function of either the concentration of anchored cells or free cells independently.

Fig 4.45 Packed bed reactor performance over prolonged operation up to 72h at 0.94 h^{-1} dilution rate and 0.5% substrate concentration

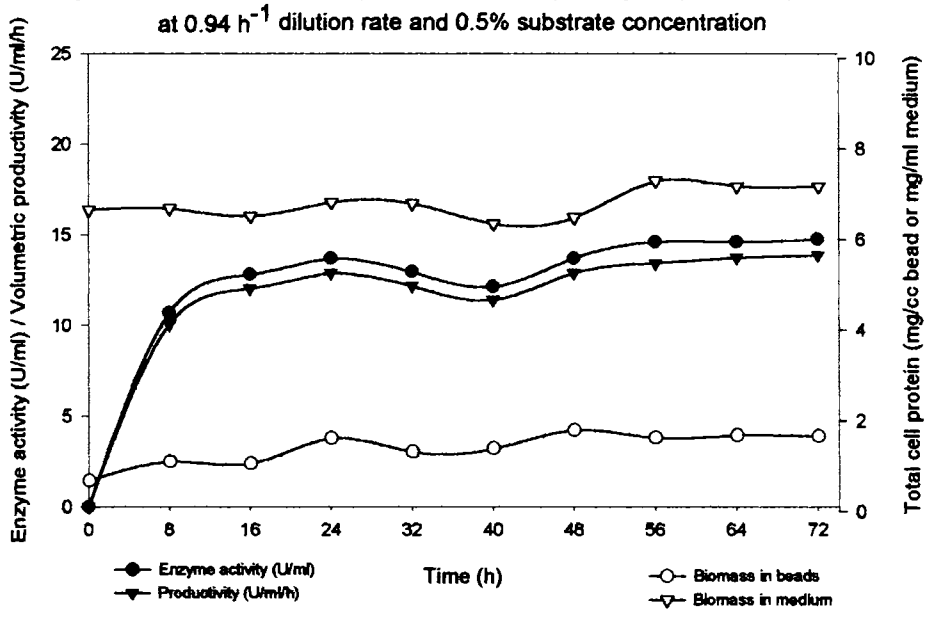


Table 4.10

Performance of a PBR with *Pseudomonas* sp. adsorbed on polystyrene bed for continuous L-glutaminase production over prolonged operation under conditions that give maximal substrate conversion.

Time (h)	Enzyme yield (U/ml)	Biomass in beads (mg protein/cc support)	Biomass in medium (mg protein/ml)	Productivity (U/ml/h)
0.000	0.000	0.683	6.654	0.000
8.000	10.710	1.106	6.681	10.067
16.000	12.810	1.061	6.515	12.040
24.000	13.710	1.625	6.820	12.890
32.000	12.960	1.323	6.790	12.180
40.000	12.130	1.393	6.349	11.400
48.000	13.710	1.802	6.487	12.890
56.000	14.610	1.632	7.291	13.430
64.000	14.610	1.688	7.180	13.730
72.000	14.760	1.667	7.180	13.870

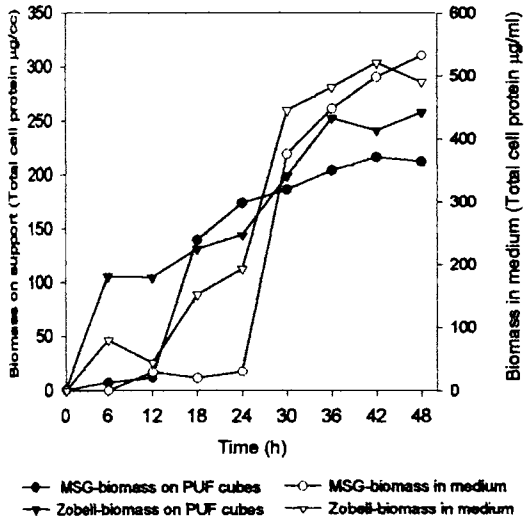
4C. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED BY PHYSICAL ADSORPTION ON POLYURETHANE FOAM CUBES

4C.1 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON PUF CUBES UNDER BATCH MODE

4C.1.1 Selection of immobilization medium and incubation time

Zobell's marine broth was found to be a better medium for growth and adsorption of the bacterial cells on polyurethane foam cubes although MSG supported a higher cell adsorption on the support during the first 24h (Fig 4.46). Though the growth as free cells in the medium increased in both the media, a higher adsorbed cell biomass was observed in Zobell's broth. Maximum cell adsorption was attained with Zobell's broth after 36h of incubation and after 42h in MSG. However, it may be noted that in either of the media, the biomass adsorbed on PUF did not show a considerable difference after 36h. Incubation time of 36h in Zobell's broth favoured the maximum adsorption of cells and hence was selected as the ideal condition for immobilization

Fig 4.46 Immobilization of *Pseudomonas* cells on PUF cubes
Selection of growth medium and incubation time

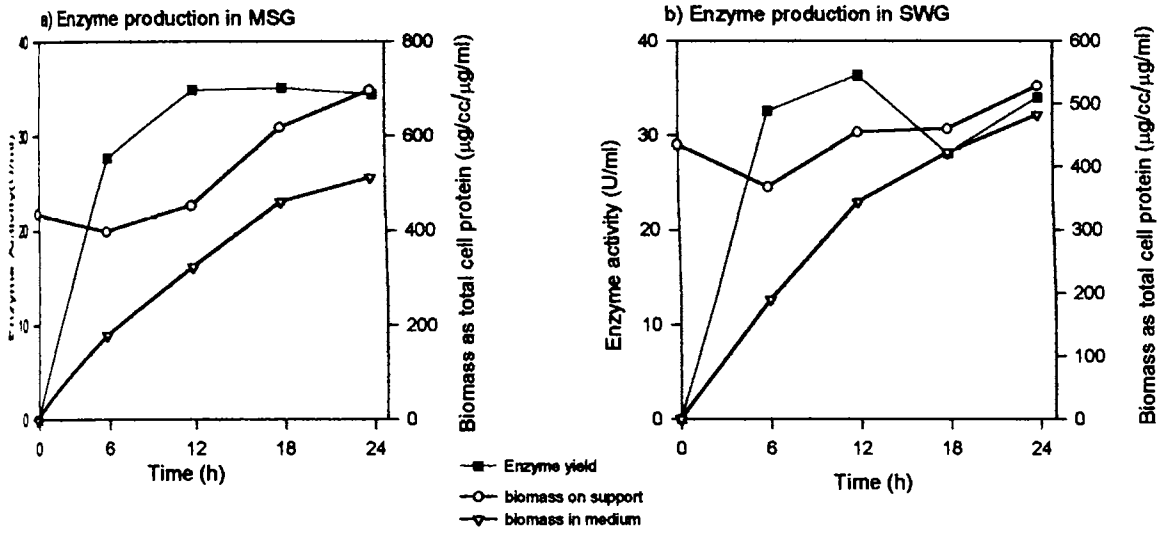


4C1.2 Selection of enzyme production medium and retention time

SWG and MSG were found to be almost equally effective as the enzyme production medium. However, the SWG medium supported higher enzyme yields compared to MSG, when 6h and 12h of the retention times were used in enzyme production. The maximum enzyme yield (36.4 U/ml) in SWG was obtained at 12h (Fig 4.47a) compared to 18h in MSG (35.06 U/ml) -Fig 4.47b. SWG was more effective in the rapid induction of enzyme production by cells immobilized on PUF. The biomass adhered to the support was initially 436.3 µg/cc for MSG and 435.61 µg/cc for SWG, which increased with incubation and reached 695.87µg/cc and

528.49 μg/cc respectively. Biomass due to free cell growth in both the media increased along with retention time. Based on the results, 12h retention time was selected as the ideal enzyme production condition, for use with SWG.

Fig 4.47Selection of enzyme production medium and retention time



4C.1.3 Optimisation of parameters for enzyme production

The various operational variables, their level and combinations tested, and the responses obtained for each combination of variables, are presented in Table 4.11 The observed maximum and minimum responses (Enzyme Yield) were 50.34U/ml and 0 U/ml respectively. The responses obtained for the 46 runs were used for fitting a quadratic model to the Box Behnken design.

Table 4.11

Optimisation of enzyme production by *Pseudomonas* sp. cells immobilized on PUF
Operating variables, levels and the Responses used in the Box-Behnken design

Run #	pH	Temp. (°C) X_1	Glutamine Conc. (%w/v) X_2	Glucose Conc.(%w/v) X_3	Yeast Extract Conc.(%w/v) X_4	Mean Response (Enzyme yield -U/ml) Y
1	8	40	1	1	0.25	0
2	8	30	1	1	0.25	14.08
3	4	40	1	1	0.25	12.81
4	4	30	1	1	0.25	17.08
5	6	35	1.5	1.5	0.25	22.99
6	6	35	1.5	0.5	0.25	37.98
7	6	35	0.5	1.5	0.25	12.36
8	6	35	0.5	0.5	0.25	17.75
9	6	40	1	1	0.5	20.9
10	6	40	1	1	0	16.85
11	6	30	1	1	0.5	22.02
12	6	30	1	1	0	38.43
13	8	35	1.5	1	0.25	14.16
14	8	35	0.5	1	0.25	12.06
15	4	35	1.5	1	0.25	22.47
16	4	35	0.5	1	0.25	18.88
17	6	35	1	1.5	0.5	24.49
18	6	35	1	1.5	0	28.09
19	6	35	1	0.5	0.5	21.57
20	6	35	1	0.5	0	26.52
21	6	35	1	1	0.25	27.86
22	6	35	1	1	0.25	18.88
23	6	35	1	1	0.25	22.70
24	6	40	1.5	1	0.25	14.61
25	6	40	0.5	1	0.25	14.16
26	6	30	1.5	1	0.25	50.34
27	6	30	0.5	1	0.25	19.92
28	8	35	1	1.5	0.25	17.53
29	8	35	1	0.5	0.25	7.34
30	4	35	1	1.5	0.25	18.58
30	4	35	1	0.5	0.25	12.81
32	6	35	1.5	1	0.5	21.12
33	6	35	1.5	1	0	38.20
34	6	35	0.5	1	0.5	14.23
35	6	35	0.5	1	0	10.79
36	8	35	1	1	0.5	12.88
37	8	35	1	1	0	19.33
38	4	35	1	1	0.5	10.56
39	4	35	1	1	0	13.86
40	6	40	1	1.5	0.25	11.46
40	6	40	1	0.5	0.25	11.69
42	6	30	1	1.5	0.25	34.83
43	6	30	1	0.5	0.25	20.67
44	6	35	1	1	0.25	19.55
45	6	35	1	1	0.25	13.71
46	6	35	1	1	0.25	15.06

4C.1.3.1 Analysis of the Box Behnken design data

The design matrix was evaluated for response surface quadratic model using the evaluation tool in Design Expert ® software. Analysis of variance for the five process variables indicated that L-glutaminase yield can be well described by the polynomial model (Table 4.12). Statistical analysis showed that incubation temperature and L-glutamine concentration had significant effects on enzyme production by the immobilized cells.

Table 4.12
Analysis of variance for the response surface quadratic model

Source	Sum of squares	DF	Mean square	F value	Prob >F
Block	49.55	1	49.55		
Model	3004.33	20	150.22	4.86	0.0002
A	55.02	1	55.02	1.78	0.1945
B	824.41	1	824.41	26.69	< 0.0001
C	646.68	1	646.68	20.93	0.0001
D	12.25	1	12.25	0.40	0.5348
E	122.43	1	122.43	3.96	0.0580
A^2	463.38	1	463.38	15.00	0.0007
B^2	3.90	1	3.90	0.13	0.7255
C^2	55.09	1	55.09	1.78	0.1943
D^2	13.26	1	13.26	0.43	0.5186
E^2	47.55	1	47.55	1.54	0.2267
AB	24.06	1	24.06	0.78	0.3863
AC	0.56	1	0.56	0.018	0.8945
AD	4.88	1	4.88	0.16	0.6944
AE	2.48	1	2.48	0.080	0.7793
BC	224.55	1	224.55	7.27	0.0126
BD	51.77	1	51.77	1.68	0.2078
BE	105.06	1	105.06	3.40	0.0775
CD	23.04	1	23.04	0.75	0.3964
CE	105.27	1	105.27	3.41	0.0773
DE	0.46	1	0.46	0.015	0.9043
Residual	741.41	24	30.89		
Lack of Fit	682.09	20	34.10	2.30	0.2181
Pure Error	59.32	4	14.83		
Cor Total	3795.29	45			

The Model F-value of 4.86 implied that the model is significant. The "Lack of Fit F-value" of 2.30 showed that lack of fit was not significant relative to the pure error and there was a satisfacory coefficient of determination ($R^2 = 0.8021$).

The final equation representing the model was

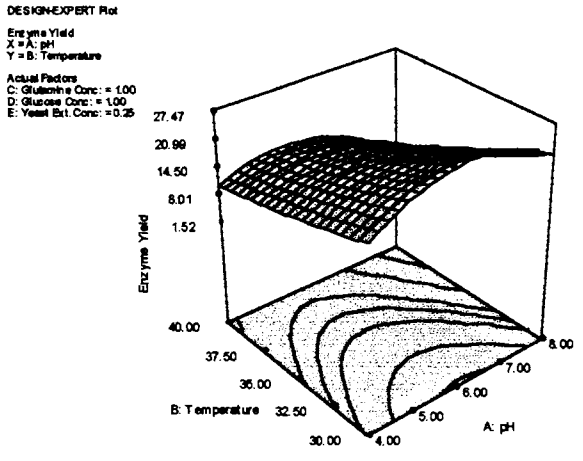
$$Y = 19.63 - 1.85X_1 - 7.18X_2 + 6.36X_3 + 0.87X_4 - 2.77X_5 - 7.29X_1^2 + 0.67X_2^2 + 2.51X_3^2 + 1.23X_4^2 + 2.33X_5^2 - 2.45X_1X_2 - 0.37X_1X_3 + 1.11X_1X_4 - 0.79X_1X_5 - 7.49X_2X_3 - 3.60X_2X_4 + 5.12X_2X_5 - 2.40X_3X_4 - 5.13X_3X_5 + 0.34X_4X_5$$

4C.1.3.2 Interaction between factors

The relationships between reaction factors and response were obtained by generating three dimensional response surfaces by plotting two factors at a time against the response, while the other three were held constant.

Interaction between pH and temperature

Fig 4.48 Response behaviour of pH and temperature under constant levels of glutamine, glucose and yeast extract

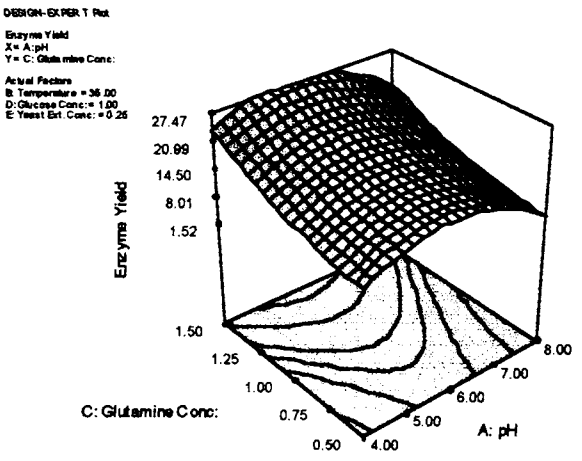


pH showed parabolic response curves at the different temperature ranges of operation with the highest yield being obtained in the 5.5-6.5 range (Fig 4.48). This dependency of yield on pH was true at all the temperature ranges. However, maximal yields were obtained at the lowest range of temperature tested. The increase in yield with reduction of temperature appeared to be linear. Maximal yields were obtained in the pH regime 5.5-6.5 near the lowest temperature (35 ^{0}C)

Interaction between pH and glutamine concentration

Under constant conditions of temperature, glucose and yeast extract concentrations the pH range of 5.5-6.5 supported the maximal yield at all the glutamine concentrations (Fig 4.49). Variations in pH, above or below this, resulted in a decreased yield. Glutamine concentration exhibited a linear relation with yield by promoting enhanced yield at higher concentrations. The highest enzyme yield was recorded near a glutamine concentration of 1.5% and within the pH range of 5.5-6.5

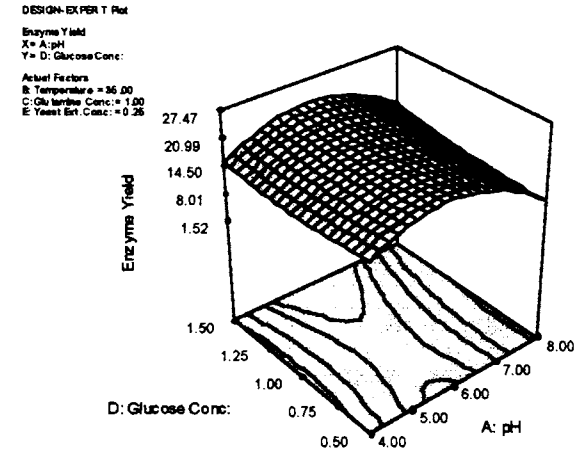
Fig 4.49 Response behaviour of pH and glutamine concentration under constant level of temperature, glucose and yeast extract



Interaction between pH and glucose concentration

Fig 4.50 Response behaviour of pH and glucose concentration at constant temperature, and glutamine and yeast extract concentrations

The response surface plot for pH and glucose interaction shows that the system is saddle with no maximum or minimum points. Higher yields were obtained in glucose concentration range 1.25-1.5% and also near 0.5% in a pH range of 5.5 to 6.5 (Fig 4.50). However, maximal enzyme production occurred in the pH range 5.5-6.5.



Interaction between pH and yeast extract concentration

The response surface (Fig 4.51) obtained for the interaction between pH and yeast extract concentration indicates that this is a saddle system. Maximal yields were obtained in the pH range of 5.5-6.5, at both the higher and lower concentrations of yeast extract. pH dependencies were similar to that obtained under interactions with temperature, or glutamine and glucose concentrations, and the deviations from the optimal range resulted in a reduction of yield.

Fig 4.51 Response behaviour of pH and yeast extract concentration under constant temperature, and concentrations of glutamine and glucose

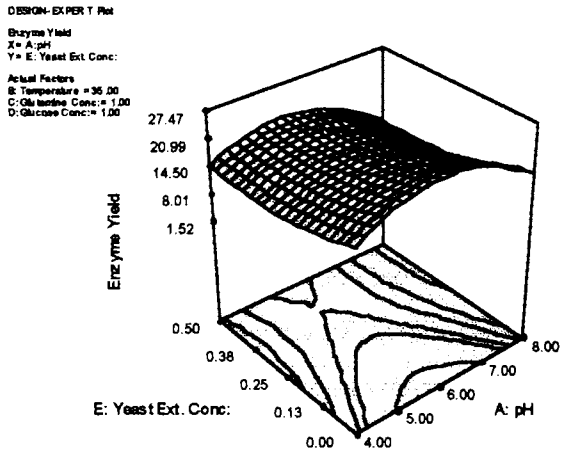
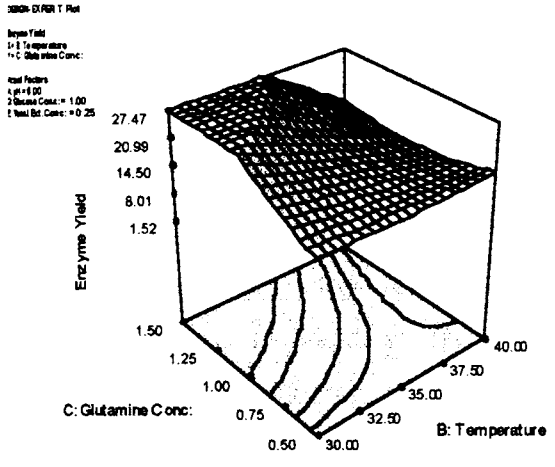


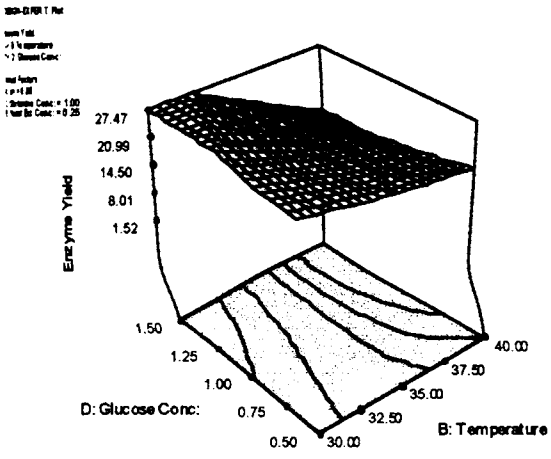
Fig 4.52 Response behaviour of temperature and glutamine concentration under constant pH, and concentrations of glucose and yeast extract



Interaction between temperature and glutamine concentration

At lower concentrations of glutamine, increase in enzyme yield was linear with the reduction of temperature, and maximal yields were obtained in the temperature range 30-32.5 ^{0}C. However, at higher glutamine concentrations there was a steep increase in yield with reduction of incubation temperature up to a temperature near 35 ^{0}C, after which there seemed to be no effect of temperature reduction on enzyme yield (Fig 4.52). The enzyme yields remained fairly consistent at temperatures below 35 ^{0}C, in this concentration regime of glutamine (1-1.5%). The ideal operating region for maximal enzyme production lied between a glutamine concentration of 1% and 1.5% and in a temperature range of 30-35^{0}C.

Fig 4.53 Response behaviour of temperature and glucose concentration at constant levels of pH and concentrations of glutamine and yeast extract

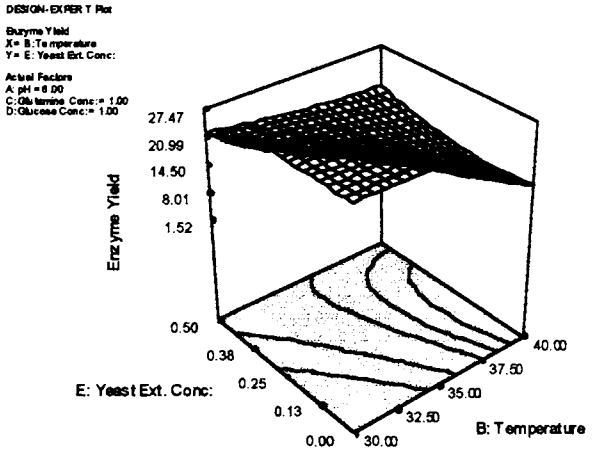


Interaction between temperature and glucose concentration

The interaction of temperature with glucose concentration was similar to that obtained with glutamine. At lower glucose concentrations, the yield increased with lowering of temperature and reached the maximal values at the lowest temperature (Fig 4.53). Lowering of temperature below 32.5 ^{0}C, at concentrations of glucose between 1 and 1.5%, did not had any positive effect on enzyme yield and the yield remained steady in this temperature regime. The highest enzyme yields were recorded in the temperature range 30-32.5 ^{0}C and at a glucose concentration range of 1-1.5%

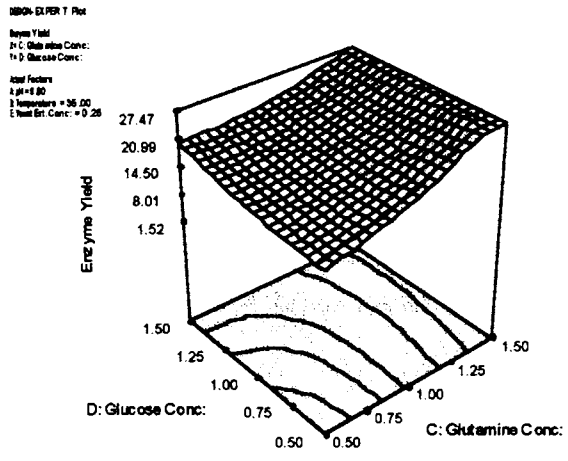
Interaction between temperature and yeast extract concentration

Fig 4.54 Response behaviour of temperature and yeast extract concentration under constant levels of pH, glutamine and glucose



Increase in yeast extract concentration while resulted in a decrease in enzyme yield at lower temperature ranges, supported a marginal increase in yield at the higher ranges of temperature (Fig 4.54). However, at the lower temperatures, yeast extract had a negative impact on enzyme yield and the yields reduced proportionately along with an increase in yeast extract concentration. In the region 30-35 ^{0}C and yeast extract concentration of 0-0.25%, the yield remained almost stable and unaffected by changes in either of the two factors.

Fig 4.55 Response behaviour of glutamine concentration and glucose concentration under constant levels of pH, temperature and yeast extract concentration



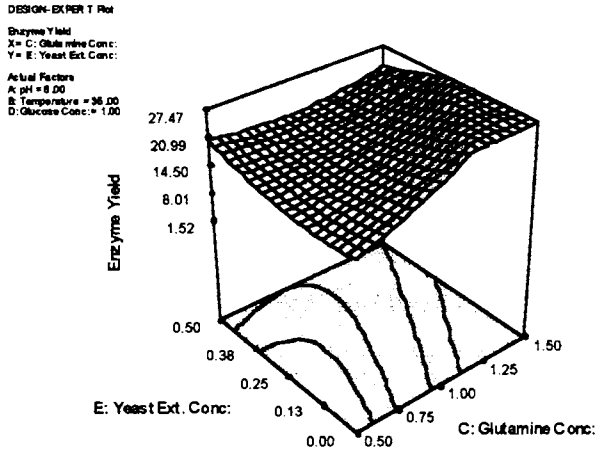
Interaction between glutamine concentration and glucose concentration

Increase in glutamine concentration resulted in higher yields at all the concentrations of glucose tried and this increase in yield was more at lower concentrations of glucose (Fig 4.55). Similarly, at lower concentrations of glutamine the yield increased with increase in glucose concentration. But at higher glutamine concentrations increase in glucose concentration led to a marginal reduction in yield. Higher yields were obtained at glutamine concentrations above 1.25% and irrespective of the glucose concentration in the medium

Interaction between glutamine concentration and yeast extract concentration

At the concentrations of yeast extract up to 0.25%, an increase in glutamine concentration led to an increase in yield (Fig 4.56). However, it was observed that at lower glutamine concentrations there was a marginal increase in yield along with increase in yeast extract concentration, which might be attributed to enzyme production by the free growing cells that are more under these conditions. Maximal yield was obtained under a concentration range of 1-1.5% glutamine and 0-0.13% yeast extract.

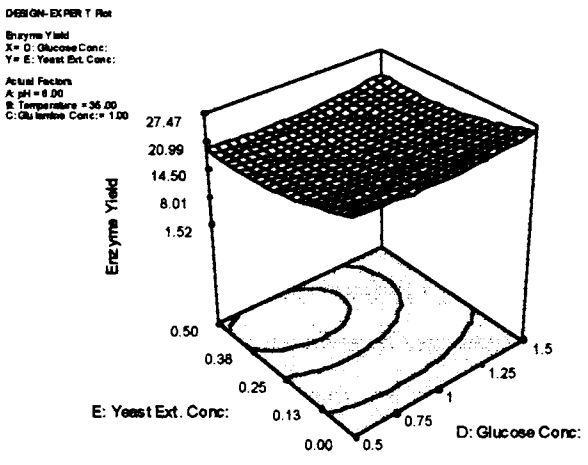
Fig 4.56 Response behaviour of glutamine and yeast extract concentrations at constant levels of pH, temperature and glucose



Interaction between glucose concentration and yeast extract concentration

Lower concentrations of glucose and yeast extract resulted in a high yield, although the difference in yield at other concentrations were marginal (Fig 4.57). In general, at all the concentration range of glucose, decrease in yeast extract concentration could bring about an increase in yield.

Fig 4.57 Response behaviour of glucose concentration and yeast extract concentration under constant levels of pH temperature and glutamine



4.1.3.3 Optimisation and validation of Model

Optimisation of the operational parameters were performed using the optimisation function in Design Expert® package. Because the long term viability and growth of *Pseudomonas* cells while adsorbed onto the support is equally important as the enzyme yield, which in turn is related to the biomass content itself, the temperature was kept constant at 35 ^{0}C, the ambient temperature for growth of *Pseudomonas* sp. The levels of other parameters were optimised to get the maximum response. The Optimal conditions employed were L-glutamine concentration -1.5 %w/v, glucose concentration - 0.5%w/v, and Yeast Extract concentration - 0 % w/v and a medium pH of 6.01. The predicted enzyme yield was 41.82 U/ml. Batch studies were conducted under the optimised conditions for validation of the predicted model and the enzyme yield obtained (22.47U/ml) was found to be 46.27% lower than the predicted value.

4C.2 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. CELLS IMMOBILIZED ON PUF CUBES UNDER REPEATED BATCH OPERATION

Immobilized cells of *Pseudomonas* sp. could synthesise L-glutaminase consistently over 20 cycles of repeated batch operation, and gave an average yield of 18.79 U/ml (Table 4.13 and Fig 4.58). The maximum and minimum yields obtained were 22.47 U/ml and 15.95 U/ml respectively. The biomass on support also remained fairly consistent, after an initial drop, which might be due to detachment of cells from PUF. This was further evidenced by the fact that the cell concentration in the medium increased after the first cycle and remained high in the second cycle, when there was reduction in the adsorbed biomass. In some of the batches, it was observed that the enzyme yields increased along with free cell growth in the medium indicating that besides the anchored cells, the free cells might also contribute to the overall enzyme production by the system. Nevertheless the system as a whole had maintained its efficiency for repeated use in enzyme production.

Fig 4.58 Repeated batch synthesis of L-glutaminase by *Pseudomonas* sp. cells immobilized on PUF cubes

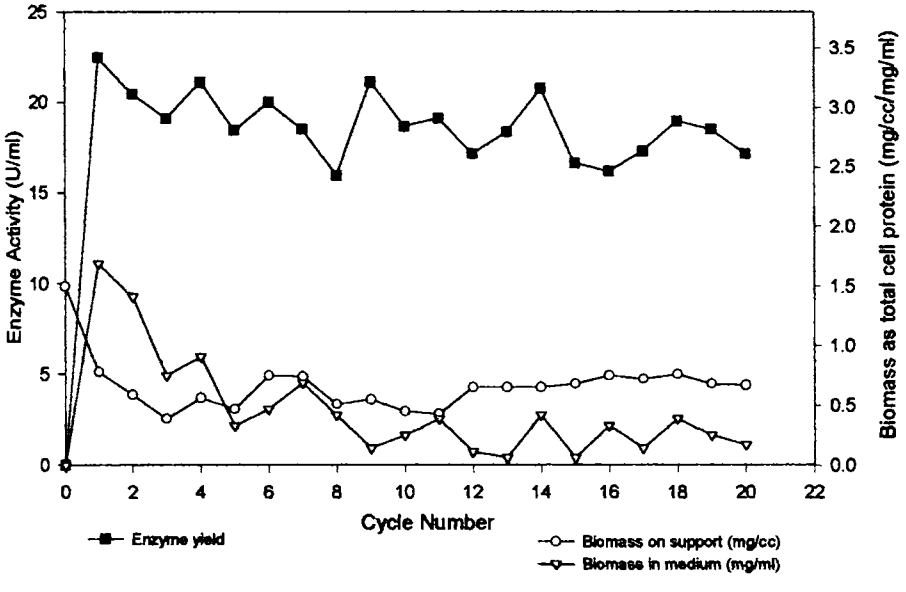


Table 4.13
Repeated batch synthesis of L-glutaminase by *Pseudomonas* sp. adsorbed on PUF cubes

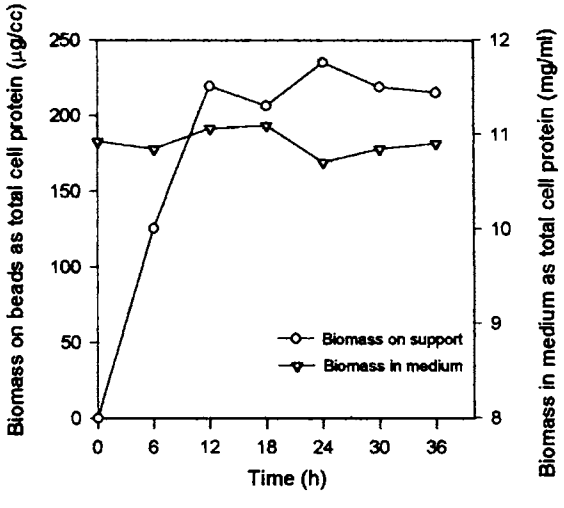
Cycle #	Mean Enzyme yield (U/ml)	Biomass on support (mg protein/cc support)	Biomass in medium (mg protein/ml)
0	0.00	1.50	0.00
1	22.47	0.78	1.69
2	20.45	0.59	1.41
3	19.10	0.39	0.75
4	21.12	0.56	0.91
5	18.43	0.47	0.33
6	19.99	0.75	0.47
7	18.50	0.74	0.69
8	15.95	0.51	0.42
9	21.12	0.55	0.14
10	18.65	0.45	0.25
11	19.10	0.43	0.39
12	17.15	0.65	0.11
13	18.35	0.65	0.06
14	20.75	0.65	0.42
15	16.63	0.68	0.06
16	16.18	0.75	0.33
17	17.30	0.72	0.14
18	18.95	0.76	0.39
19	18.50	0.68	0.25
20	17.15	0.67	0.17

4C.3 CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON PUF IN A PACKED BED REACTOR

4C.3.1 Cell immobilization on the PUF bed

Continuous re-circulation of the actively growing culture of *Pseudomonas* sp. through PUF bed in the PBR resulted in cell adsorption on the bed and the maximal adsorption was attained at 24h (Fig 4.59). With further circulation, the adsorbed biomass remained almost constant above 200 µg protein /cc of

Fig 4.59 Immobilization of *Pseudomonas* sp. cells on PUF bed in a PBR.
Cell adherence as a function of recirculation time



PUF. Cell biomass in the medium due to free cell growth remained between 10.7 and 11.1 mg protein /ml. Presumably, the loss of cells from medium due to adsorption on PUF bed was made up by an enhanced growth rate of cells within the medium. By 36h of re-circulation, an equilibrium value for the attached biomass was attained.

4C.3.2 Effect of Operational parameters on Reactor performance.

The reactor void volume was 78.6cc and the dilution rates tested were 0.76,1.02, 1.27, 1.53, and 1.78 h^{-1} respectively for the flow rates 60, 80,100,120 and 140 ml/h. The optimised SWG medium and operation temperature was kept at 35 ^{0}C by using pre warmed medium for circulation. Substrate concentration was varied to the desired level. Enzyme yield and substrate conversion efficiency was found to decrease along with an increase in dilution rate. An increase in substrate concentration led to increase in enzyme activity, whereas this resulted in a decreased percentage of substrate conversion. The volumetric productivities increased with dilution rate and substrate concentration. The reactor performance under different operation conditions is given in Table 4.14

Table 4.14

L- Glutaminase production in a Packed bed reactor with PUF as packing material - Evaluation of performance under different operation conditions

Substrate conc. (w/v)	F Flow rate (ml/h)	D =F/V Dilution rate h^{-1}	R = 1/D Residence time (h)	Y= Enzyme yield (U/ml)	Std Dev.	% Substrate Conversion	Std Dev.	P = YD Productivity (U/ml/h)
	60	0.76	1.31	16.35	0.458	26.17	3.494	12.48
	80	1.02	0.98	14.08	0.722	23.91	0.560	14.33
0.50%	100	1.27	0.79	13.31	1.978	23.58	0.560	16.93
	120	1.53	0.66	12.06	1.297	21.32	0.970	18.41
	140	1.78	0.56	10.66	1.137	20.35	1.679	18.99
	60	0.76	1.31	22.22	3.76	12.6	1.282	16.96
	80	1.02	0.98	20.47	0.78	12.28	0.560	20.83
1.00%	100	1.27	0.79	17.03	1.554	11.47	0.280	21.67
	120	1.53	0.66	14.53	0.259	11.31	0.280	22.18
	140	1.78	0.56	12.88	2.403	10.82	1.009	22.94
	60	0.76	1.31	28.61	3.067	9.48	0.373	21.84
	80	1.02	0.98	24.99	0.931	8.72	1.165	25.44
1.50%	100	1.27	0.79	22.67	0.487	7.86	0.746	28.84
	120	1.53	0.66	19.57	1.359	7.54	0.493	29.88
	140	1.78	0.56	17.45	2.136	7.43	0.323	31.08

4C.3.2.1 Effect of dilution rate and substrate concentration on enzyme yield

Higher dilution rates resulted in lower yield of enzyme irrespective of the substrate concentration in the media (Fig 4.60a). At all the substrate concentrations tried, this decrease in enzyme yield was linear with the increase in flow (dilution) rate indicating the requirement of a minimum residence time for efficient enzyme production by immobilized cells. Increase in substrate concentration had resulted in an increase in enzyme yield at all the dilution rates tried (Fig 4.60b). The maximum enzyme yield obtained was 28.61 U/ml at a dilution of 0.76 h^{-1} in the medium containing 1.5% L-glutamine and the minimum yield was obtained in the medium containing 0.5% substrate at a dilution of 1.78 h^{-1}.

4C.3.2.2 Effect of dilution rate and substrate concentration on volumetric productivity

The volumetric productivity of the reactor increased linearly with dilution rates and the maximum productivity was observed at the highest dilution, irrespective of the substrate concentration used (Fig 4.61a). The substrate concentration also influenced the volumetric productivity positively and at all the dilution rates. The maximal productivity was obtained in the medium containing the highest L-glutamine concentration (Fig 4.61b).

4C.3.2.3 Effect of dilution rate and substrate concentration on substrate conversion

Increase in dilution rates resulted in a decreased efficiency of substrate conversion (Fig 4.62a). Utilisation of substrate by the immobilized cells reduced with the increase in rate of flow (dilution) indicating the need for an optimal contact of the cells with substrate molecules to effect maximal substrate conversion. Nevertheless, at increased substrate concentrations also the percentage of conversion was less efficient (Fig 4.62b).

Fig 4.60 Effect of operational parameters on continuous
-glutaminase production by *Pseudomonas* sp immobilized on PUF in a PBR

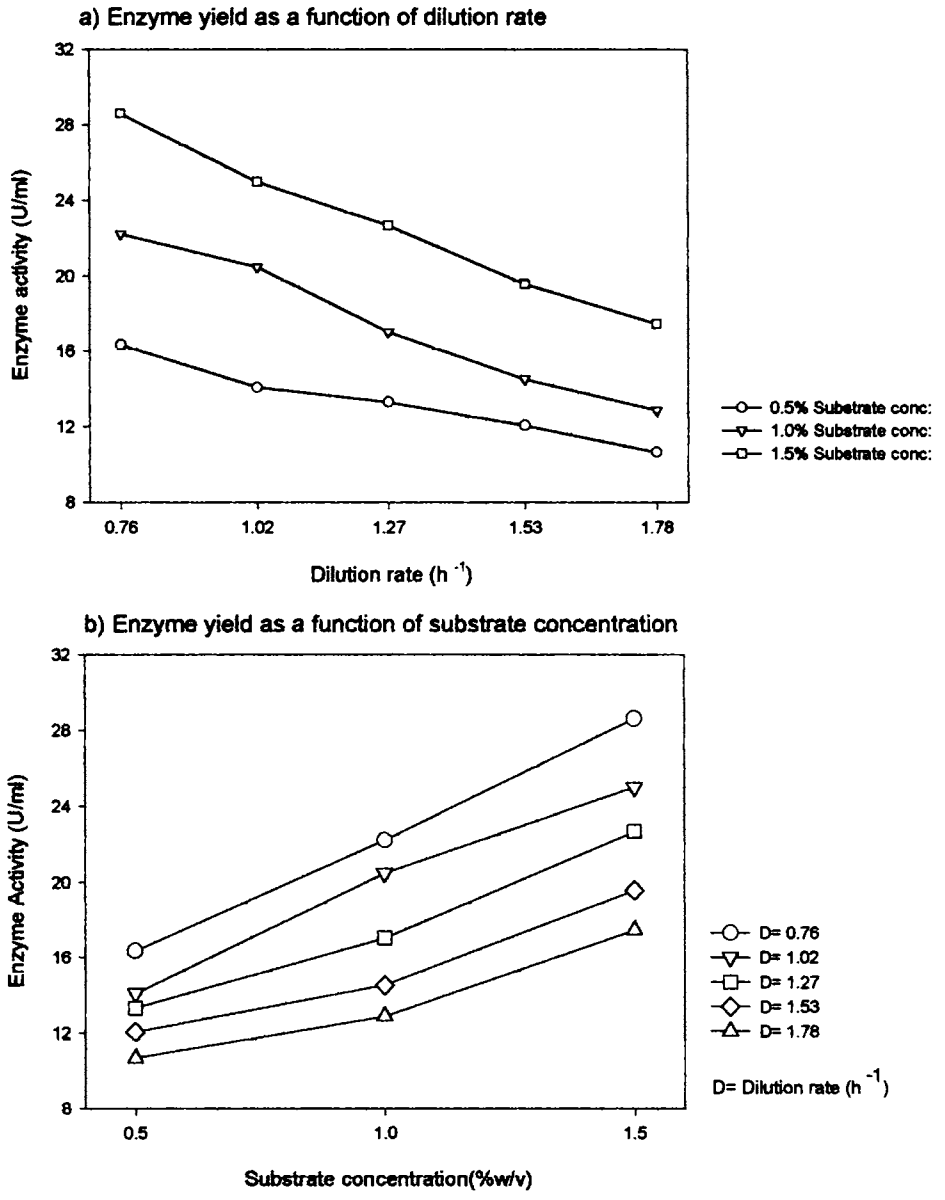


Fig 4.61 Effect of operational parameters on continuous production of L-glutaminase by *Pseudomonas* sp. immobilized on PUF cubes in a PBR

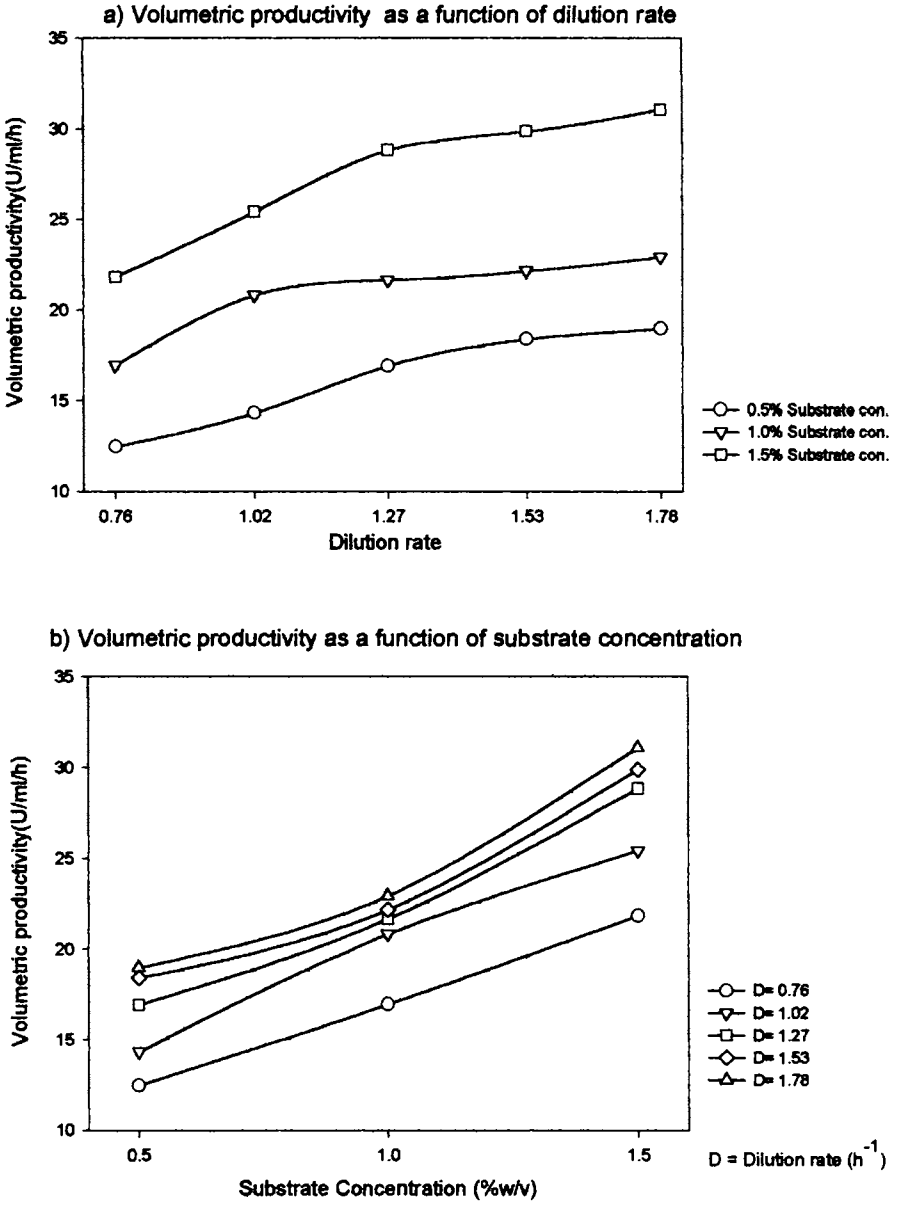
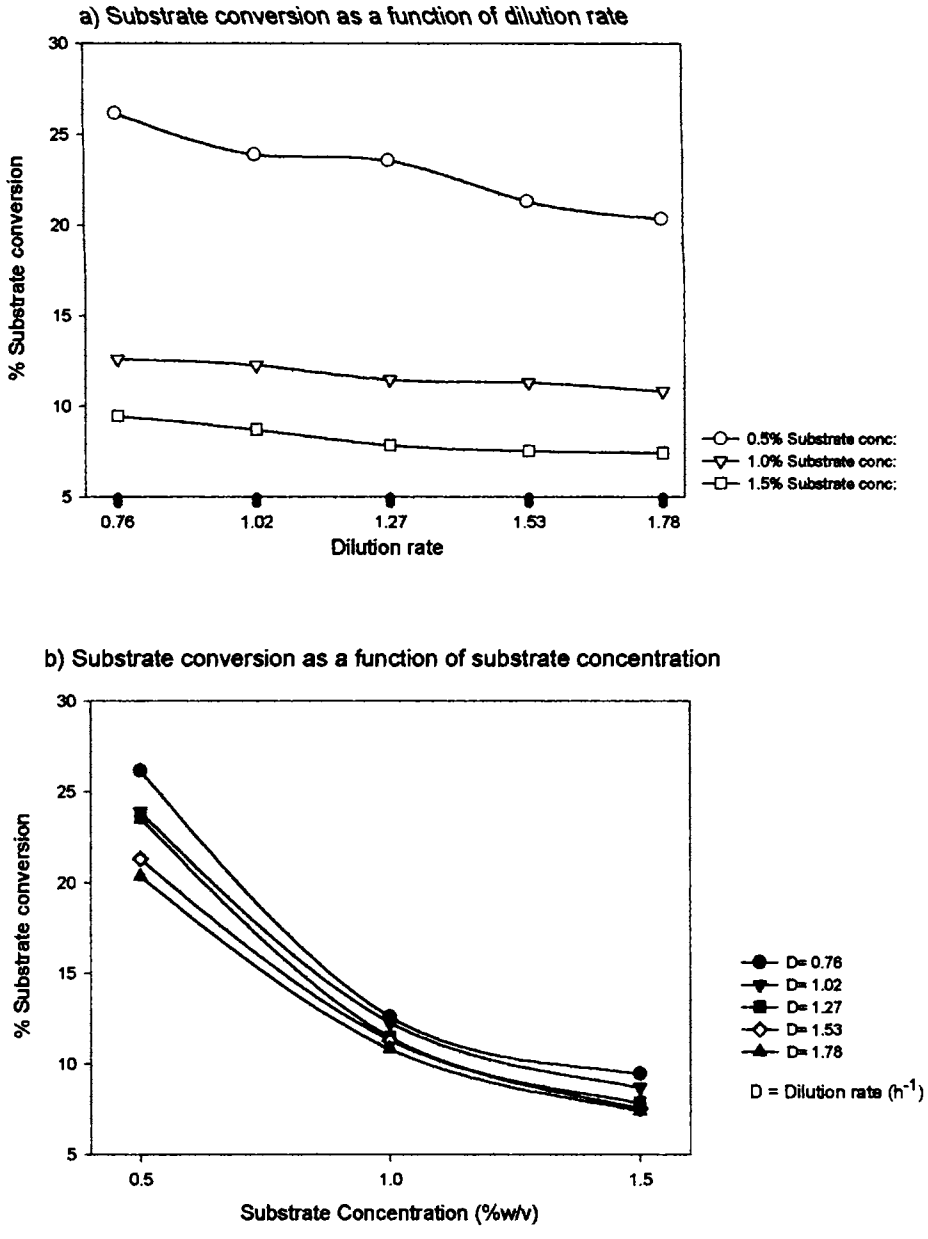


Fig 4.62 Effect of operational parameters on continuous production of L-glutaminase by*Pseudomonas* sp. immobilized on PUF cubes in a PBR



4C.3.3 Operational stability of the reactor

The PBR was operated at the conditions giving maximum substrate conversion (substrate concentration 0.5% w/v and dilution rate 0.76). Throughout the 72h of continuous operation, the cells immobilized on PUF discs could synthesise L-glutaminase consistently between 12 and 18U/ml (Fig 4. 63). The average enzyme yield was 13.725 U/ml (Table 4.15). Volumetric productivity of the reactor showed an absolute dependence on enzyme production as expected in continuous operation at fixed dilution rate. The average volumetric productivity was 10.431U/ml/h. Biomass adsorbed on support almost doubled after 8h of operation and thereafter maintained a fairly consistent concentration (average protein content -0.416 mg/cc). Biomass in medium due to free cell growth also remained stable between 6.5 and 7.5 mg/ml. In general, the PBR with cells immobilized on PUF discs could maintain a stable enzyme yield and productivity, and the adsorbed biomass on PUF remained almost constant indicating the attainment of an equilibrium between cell growth and detachment. However, it was observed that with prolonged operation, there is a gradual deterioration in the texture of PUF and fibres were detached from the main body of the discs which remained entrapped in the bed.

Fig 4.63 Packed bed reactor performance over prolonged operation up to 72h at 0.76 h^{-1} dilution rate and 0.5% substrate concentration

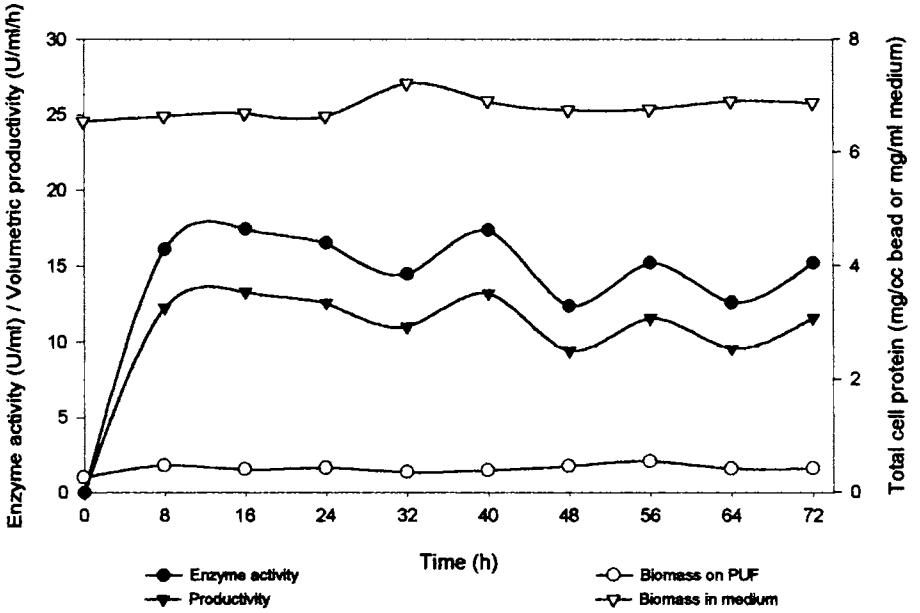


Table 4.15

Performance of a PBR with Pseudomonas sp. adsorbed on PUF bed for continuous L-glutaminase production over prolonged operation under conditions that give maximal substrate conversion.

Time (h)	Enzyme yield (U/ml)	Biomass in beads (mg protein/cc support)	Biomass in medium (mg protein/ml)	Productivity (U/ml/h)
0	0.00	0.26	6.54	0.00
8	16.10	0.47	6.63	12.24
16	17.45	0.40	6.68	13.26
24	16.52	0.43	6.63	12.56
32	14.46	0.35	7.21	10.99
40	17.36	0.39	6.90	13.19
48	12.36	0.46	6.74	09.39
56	15.21	0.55	6.76	11.56
64	12.58	0.42	6.90	09.56
72	15.21	0.42	6.87	11.56

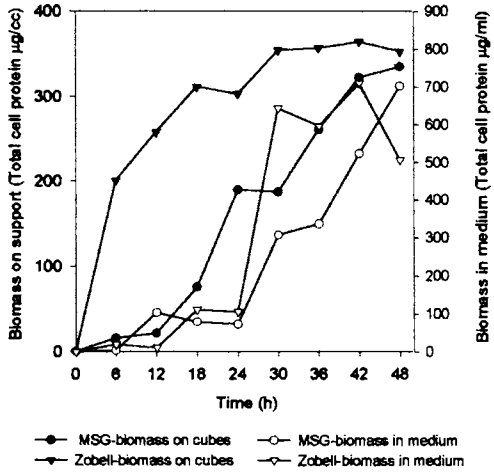
4D. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED BY PHYSICAL ADSORPTION ON NYLON WEB

4D.1 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON NYLON WEB UNDER BATCH MODE

4D.1.1 Selection of immobilization medium and incubation time.

When either MSG or Zobell's broth was used as the immobilization medium, cell growth on the support increased with incubation time. While the attached biomass increased to 200-mg protein/cc of beads in 6h when Zobell's broth was used it took 24h in MSG to attain the same cell concentration on support (Fig 4.64). The cell biomass on support attained stable concentration in Zobell's broth after 30h of incubation while in MSG medium the maximal biomass was attained only after 48h of incubation, which however was less than the equilibrium adsorbed biomass concentration in Zobell's broth. Biomass in medium increased with incubation in Zobell's broth and the maximal free cell concentration was observed after 40h, which however decreased after 48h of incubation. Whereas, in MSG medium the cell concentration in medium increased linearly with incubation time and never achieved a plateau. Despite the fact that the cell concentration on support attained a stable range after 30h of incubation in Zobell's broth, a 36h-incubation period in Zobell's broth was selected as the ideal immobilization condition.

Fig 4.64
Immobilization of *Pseudomonas* sp. cells on Nylon cubes
Selection of growth medium and incubation time

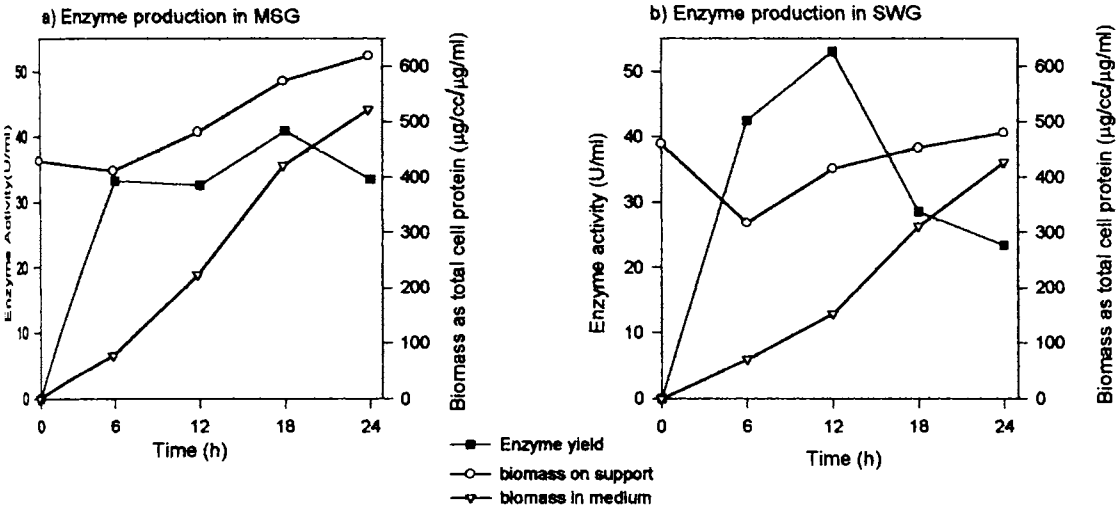


4D1.2 Selection of enzyme production medium and retention time

Seawater glutamine (SWG) medium was observed to be superior to MSG, as the enzyme production medium. In MSG medium the maximal enzyme yield of 40.9

U/ml was obtained after 18h of retention (Fig 4.65a), whereas in SWG a high enzyme yield (42.47U/ml) was obtained with a retention time of only 6h (Fig 4.65b). The maximum enzyme yield in SWG (53.03 U/ml), however was obtained after 12h of retention. The cell biomass attached to support decreased after 6h of retention in both the media and increased with further retention. Growth as free cells in MSG and SWG increased linearly with increase in retention time. SWG was selected as the ideal enzyme production medium and 12h as the ideal retention time.

Fig 4.65 Selection of enzyme production medium and retention time



4D.1.3 Optimisation of parameters for enzyme production

The operational variables, their levels and combinations tested, and the responses, obtained are presented in Table 4.16. The maximum and minimum enzyme yields obtained were 0 U/ml and 26.52 U/ml respectively. The responses obtained for the 46 runs were used for fitting a quadratic model to the Box Behnken design.

Table 4.16

Optimisation of enzyme production by *Pseudomonas* sp. immobilized on Nylon web
Operating variables, levels and the Responses used in the Box-Behnken design

Run #	pH	Temp. (°C) X_1	Glutamine Conc. (%w/v) X_2	Glucose Conc. (%w/v) X_3	Yeast Extract Conc.(%w/v) X_4	Mean Response (Enzyme yield -U/ml) Y
1	8	40	1	1	0.25	0
2	8	30	1	1	0.25	12.13
3	4	40	1	1	0.25	9.21
4	4	30	1	1	0.25	11.98
5	6	35	1.5	1.5	0.25	24.72
6	6	35	1.5	0.5	0.25	23.37
7	6	35	0.5	1.5	0.25	20.67
8	6	35	0.5	0.5	0.25	16.85
9	6	40	1	1	0.5	2.02
10	6	40	1	1	0	13.71
11	6	30	1	1	0.5	19.18
12	6	30	1	1	0	15.88
13	8	35	1.5	1	0.25	12.13
14	8	35	0.5	1	0.25	11.09
15	4	35	1.5	1	0.25	9.59
16	4	35	0.5	1	0.25	14.16
17	6	35	1	1.5	0.5	19.33
18	6	35	1	1.5	0	24.94
19	6	35	1	0.5	0.5	24.72
20	6	35	1	0.5	0	16.40
21	6	35	1	1	0.25	23.22
22	6	35	1	1	0.25	18.73
23	6	35	1	1	0.25	17.53
24	6	40	1.5	1	0.25	15.06
25	6	40	0.5	1	0.25	13.78
26	6	30	1.5	1	0.25	15.95
27	6	30	0.5	1	0.25	15.06
28	8	35	1	1.5	0.25	10.49
29	8	35	1	0.5	0.25	7.64
30	4	35	1	1.5	0.25	6.29
30	4	35	1	0.5	0.25	14.83
32	6	35	1.5	1	0.5	18.88
33	6	35	1.5	1	0	26.52
34	6	35	0.5	1	0.5	18.43
35	6	35	0.5	1	0	14.98
36	8	35	1	1	0.5	12.13
37	8	35	1	1	0	13.48
38	4	35	1	1	0.5	1.05
39	4	35	1	1	0	6.59
40	6	40	1	1.5	0.25	13.93
40	6	40	1	0.5	0.25	9.89
42	6	30	1	1.5	0.25	17.53
43	6	30	1	0.5	0.25	20.07
44	6	35	1	1	0.25	19.18
45	6	35	1	1	0.25	19.33
46	6	35	1	1	0.25	18.57

4D.1.3.1 Analysis of the Box Behnken design data.

The design matrix was evaluated for response surface quadratic model using the evaluation tool in Design Expert ® software. Analysis of variance (Table 4.17) for the five process variables indicated that L-glutaminase yield could well be described by the polynomial models. Statistical analysis showed that incubation temperature had significant effects on enzyme production by the immobilized cells.

Table 4.17
Analysis of variance for the response surface quadratic model

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Block	18.16	1	18.16		
Model	1396.79	20	69.84	5.54	< 0.0001
A	1.82	1	1.82	0.14	0.7077
B	139.12	1	139.12	11.03	0.0029
C	20.70	1	20.70	1.64	0.2123
D	1.07	1	1.07	0.085	0.7737
E	17.56	1	17.56	1.39	0.2496
A^2	709.33	1	709.33	56.25	< 0.0001
B^2	167.65	1	167.65	13.30	0.0013
C^2	10.66	1	10.66	0.85	0.3669
D^2	6.93	1	6.93	0.55	0.4656
E^2	9.83	1	9.83	0.78	0.3861
AB	21.90	1	21.90	1.74	0.2000
AC	7.87	1	7.87	0.62	0.4373
AD	32.43	1	32.43	2.57	0.1218
AE	4.39	1	4.39	0.35	0.5607
BC	1.70	1	1.70	0.14	0.7165
BD	10.82	1	10.82	0.86	0.3634
BE	56.18	1	56.18	4.45	0.0454
CD	1.53	1	1.53	0.12	0.7310
CE	30.75	1	30.75	2.44	0.1315
DE	48.51	1	48.51	3.85	0.0615
Residual	302.64	24	12.61		
Lack of Fit	284.32	20	14.22	3.10	0.1402
Pure Error	18.32	4	4.58		
Cor Total	1717.58	45			

The Model F-value of 5.54 indicates that the the model is significant. The lack of fit value was 3.10 implying that the lack of fit is not significant. The coefficient of determination was satisfactory ($R^2 = 0.8219$)

The final equation representing the model was

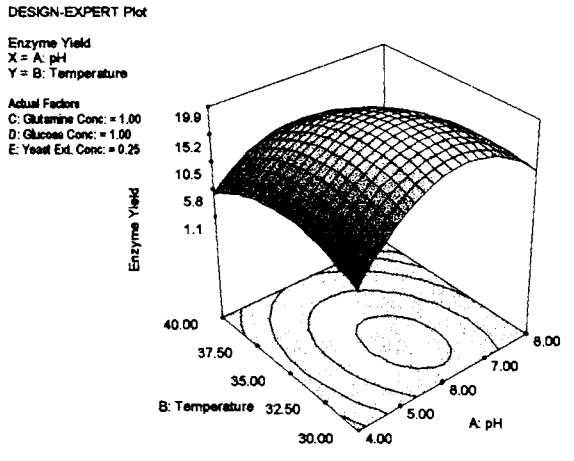
$$Y = 19.43 + 0.34X_1 - 2.95X_2 + 1.14X_3 + 0.26X_4 - 1.05X_5 - 9.02X_1^2 - 4.38X_2^2 + 1.11X_3^2 + 0.89X_4^2 - 1.06X_5^2 - 2.34X_1X_2 + 1.40X_1X_3 + 2.85X_1X_4 + 1.05X_1X_5 - 0.65X_2X_3 + 1.64X_2X_4 - 3.75X_2X_5 - 0.62X_3X_4 - 2.77X_3X_5 - 3.48X_4X_5$$

4D.1.3.2 Interaction between factors

The relationships between the operational variables and response were obtained by generating three dimensional response surfaces by plotting response against two factors at a time while the other three were held constant

Interaction between pH and temperature

Fig 4.66 Response behaviour of pH and temperature under constant levels of glutamine , glucose and yeast extract

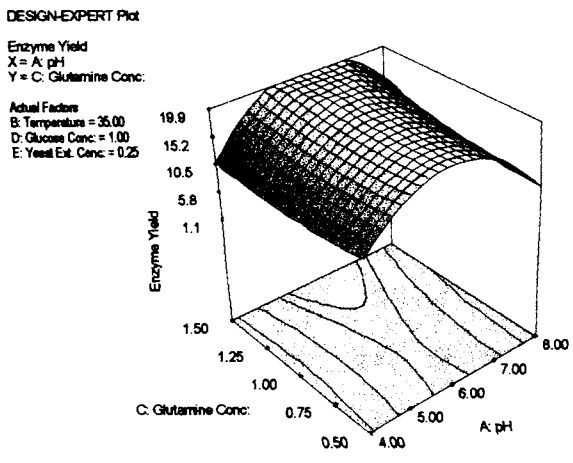


Response curves for pH was parabolic at any incubation temperature tried. Enzyme yields increased with initial pH peaking around a pH range of 5.5-6.5 and decreased with further increase in pH (Fig 4.66). The response obtained for temperature also showed a similar pattern with the response attaining peak values in the range of 32.5-35 ^{0}C. The maximum yield was obtained at a pH range of 5.5-6.5 at temperatures between 32.5 and 35 ^{0}C

Interaction between pH and glutamine concentration

Maximal enzyme activities were recorded in the pH range of 5.5-6.5 and was unaffected by the concentration of glutamine (Fig 4.67). At all the pH ranges, however, the yields increased linearly with increase in glutamine concentration. The increase in yield at higher glutamine concentration was more evident in the pH range of 5.5 to 6.5 where the maximum yields were obtained.

Fig 4.67 Response behaviour of pH and glutamine concentration at constant values of temperature and concentrations of glucose and yeast extract



Interaction between pH and glucose concentration

Though the yield changed parabolically with pH, at low concentrations of glucose, the pH range 5-6 gave the maximal yield. Whereas, the yield was higher in the pH range 5.5-7 at glucose concentrations above 1.25% (Fig 4.68). When pH was below 5 or above 7, the yields decreased with increase in glucose concentration, and in the pH range 5-7 higher yields were obtained at both extremes of glucose concentration indicating a saddle system. The maximal response region for the data was obtained at glucose concentration between 1.25and 1.5% and in the pH range 5.5-7

Fig 4.68 Response behaviour of pH and glucose concentration at constant temperature and concentrations of glutamine and yeast extract

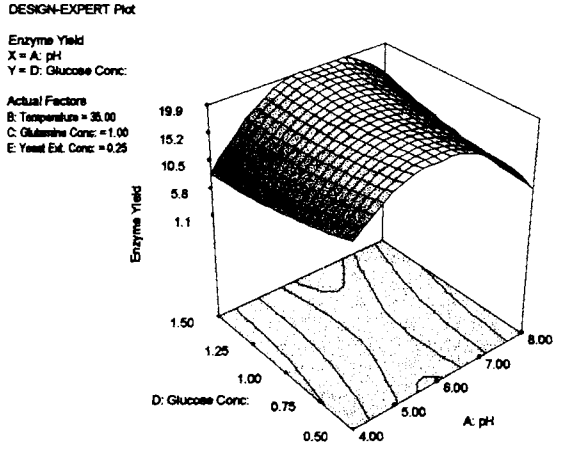
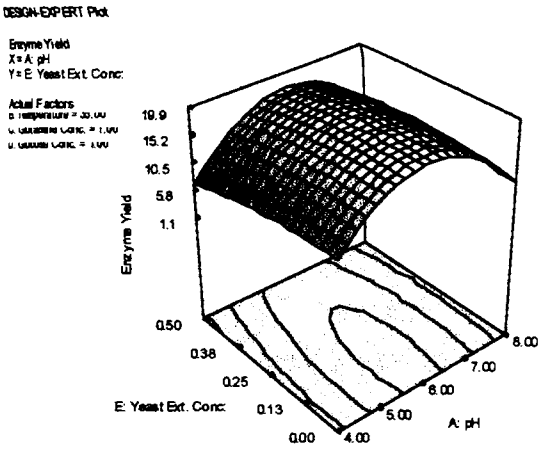


Fig 4.69 Response behaviour of pH and yeast extract concentration under constant temperature and concentrations of glutamine and glucose



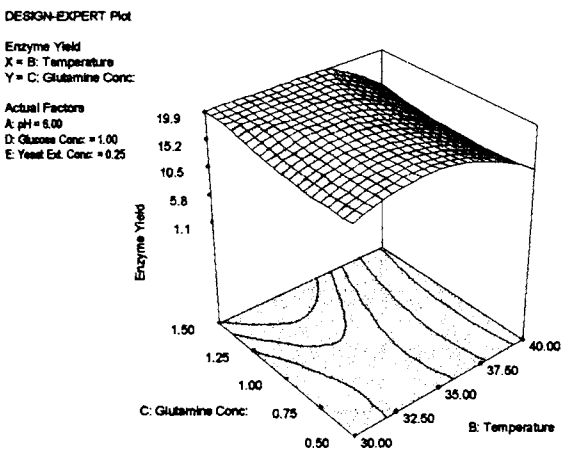
Interaction between pH and yeast extract concentration

The response pattern on variation of pH was parabolic and similar to that obtained in interaction plots with temperature, glutamine concentration and glucose concentration. Yeast extract concentration also showed an almost parabolic curve at least in the 5.5-6.5 pH range (Fig 4.69). The yields were lower at higher concentrations of yeast extract and increased gradually with reduction in concentration. The peak response was obtained in the concentration range 0 -0.25% and at a pH between 5.5 and 6.5.

Interaction between temperature and glutamine concentration

At low concentrations of glutamine, the temperature dependence of yield showed a parabolic response curve with the maximal yield occurring between 32.5-35 ^{0}C. At high glutamine concentrations, glutamine had a strong influence on the yield and in the temperature region 30-35 ^{0}C, enzyme yields obtained were consistently higher (Fig 4.70). The yields obtained were similar and the response surface showed a plateau in this regime. The operation region for maximum yield may be deduced to lie in the temperature range of 30-35 ^{0}C and at glutamine concentrations above 1.25%.

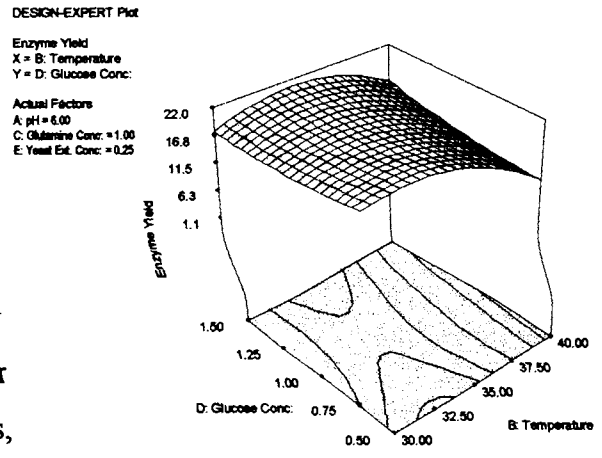
Fig 4.70 Response behaviour of temperature and glutamine concentration at constant pH and concentrations of glucose and yeast extract



Interaction between temperature and glucose concentration

The dependence of yield on temperature showed parabolic response curves at all the concentrations of glucose (Fig 4.71). However, the peaks obtained were at different temperature ranges for the lower and higher glucose concentrations (30-32.5 ^{0}C and 32.5-35 ^{0}C respectively) Low glucose concentrations gave better yield at lower temperatures, whereas at higher temperatures increase in glucose concentration favoured a slight increase in the enzyme yield

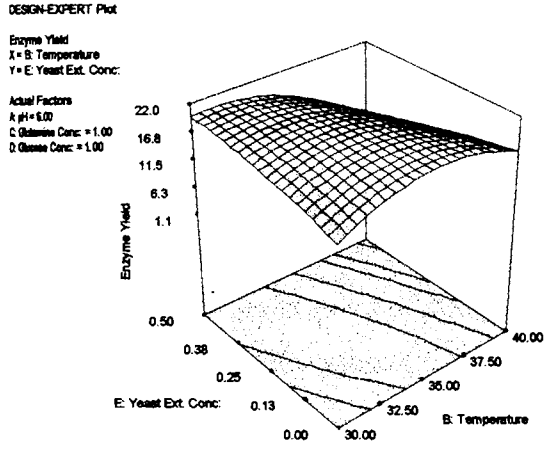
Fig 4.71 Response behaviour of temperature and glucose concentration under constant levels of pH, glutamine and yeast extract



Interaction between temperature and yeast extract concentration

At low concentrations of yeast extract the yield changed parabolically with incubation temperature. The maximal yield was obtained in the temperature range 32.5-37.5 ^{0} when yeast extract concentration was below 0.13%. At yeast extract concentrations above 0.4%, the temperature range for maximal yield was lower (30-35 ^{0}C). There was a reduction in yield at higher temperatures, which became amplified when there was an increase in yeast extract concentration (Fig 4.72). The lowest yields were recorded in this region and the drop in production was very drastic

Fig 4.72 Response behaviour of temperature and yeast-extract concentration at constant levels of pH, glutamine and glucose



Interaction between glutamine concentration and glucose concentration

The difference in yields obtained at various combinations of glutamine and glucose was not remarkable compared to the other interactions. However, minor differences did occur and it was observed that glutamine played a dominant role in bringing about these differences. At the different concentrations of glucose a better yield was incurred with increase in glutamine concentration (Fig 4.73). The enzyme yield also increased with increase in glucose concentration

Fig 4.73 Response behaviour of glutamine concentration and glucose concentration at constant pH , temperature and yeast extract concentration

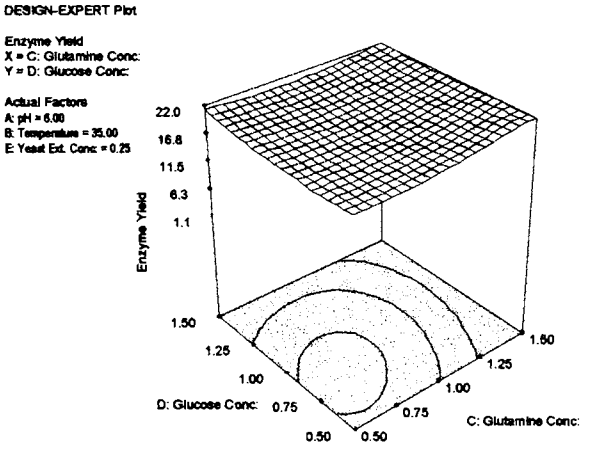
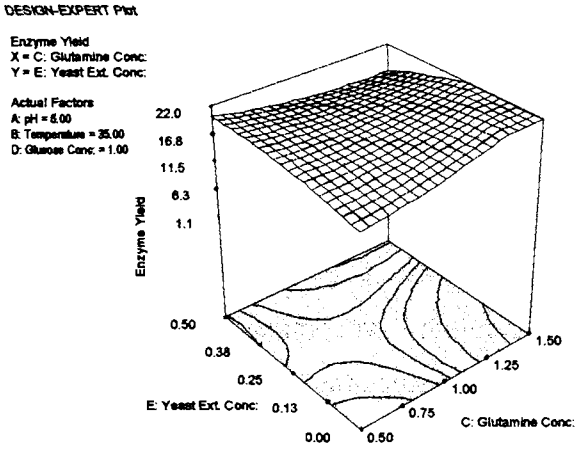


Fig 4.74 Response behaviour of glutamine concentration and yeast extract concentration at constant levels of pH temperature and glucose



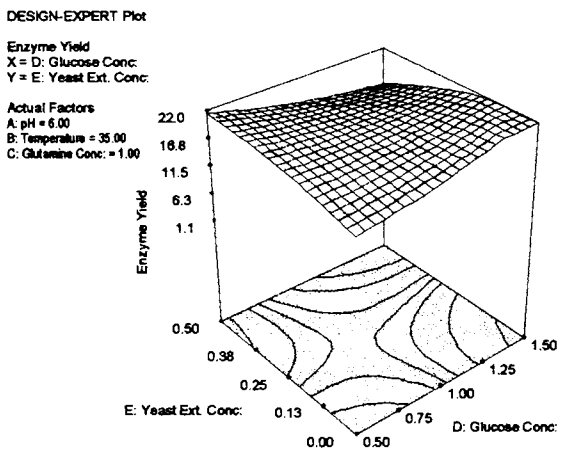
Interaction between glutamine concentration and yeast extract concentration

Enzyme yields were higher at lower yeast extract concentration for higher concentrations of glutamine. At lower glutamine levels, increase in yield was observed with yeast extract concentration in the range of 0.2-0.4%. When glutamine concentrations were above 1%, increase in yeast extract concentration led to a reduction in yield. Maximum yield was obtained with 1.25-1.5% glutamine and with yeast extract concentration below 0.13%. The centre point of the system was neither a maximum nor a minimum point indicating a saddle system (Fig 4.74)

Interaction between glucose concentration and yeast extract concentration

The response data for interaction between glucose and yeast extract concentrations indicated a saddle system (Fig 4.75). High responses were obtained at high glucose concentrations when the yeast extract concentrations were low, and at low glucose concentrations when yeast extract concentration was high. Lowest yields were recorded in the region of high concentration of both factors. The availability of these nutrients at high concentrations favours cell growth, and under these conditions the entire machinery of the bacteria is dedicated to cell division and biomass production sidelining enzyme synthesis. Maximal enzyme yields were obtained at the concentration range of 1.25-1.5% glucose with yeast extract concentration remaining below 0.13%

Fig 4.75 Response behaviour of glucose concentration and yeast extract concentration under constant pH, temperature and glutamine concentration



4D.1.3.3 Optimisation and validation of model

Optimisation of the operational parameters was carried out using the optimisation function in the Design Expert® package. As the long term viability and growth of *Pseudomonas* cells, while adsorbed onto the support are important for stability of the immobilized cell system, the temperature was kept constant at 35 ^{0}C (the ambient temperature for growth of *Pseudomonas* sp.). The levels of other parameters were optimised to give the maximum response. The Optimal conditions included L-glutamine concentration - 1.5 %w/v, glucose concentration - 1.42 %w/v, Yeast Extract concentration - 0.02 %w/v and a medium pH of 6.06. The predicted enzyme yield was 27.4 U/ml. Batch studies were conducted under the optimised conditions for validation of the predicted model and an enzyme yield of 22.92 U/ml was obtained. The actual yield was found to be 16.35% lower than the predicted value.

4D.2 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED ON NYLON CUBES UNDER REPEATED BATCH OPERATION

The immobilized cells of *Pseudomonas* sp. could repeatedly synthesise the enzyme throughout the batches with an average yield of 17.991U/ml (Table 4.18 and Fig 4.76). A maximum enzyme yield of 22.92U/ml and the minimum yield of 12.81 U/ml was obtained. There was no reduction in efficiency of the cells towards the last batches, and in fact the enzyme yield showed higher values for the last four batches of reuse. The adsorbed biomass increased from batch to batch and remained above 1mg-protein/cc after the tenth cycle. However, the biomass in medium due to growth as free cells exhibited fluctuations. No definite correlation was observed between enzyme yield and either adsorbed or free cell concentration. Presumably, environmental factors also would have influenced the enzyme production by immobilized cells other than the biomass concentration. From the results it may be observed that there is no loss in efficiency of the immobilized cells with repeated use and that the cell loss due to detachment is practically not at all a problem since the lost cells are rapidly being replaced either by the growth of adsorbed cells or by re-adsorption from medium.

Fig 4.76 Repeated batch synthesis of L-glutaminase by *Pseudomonas* sp. cells immobilized on Nylon cubes

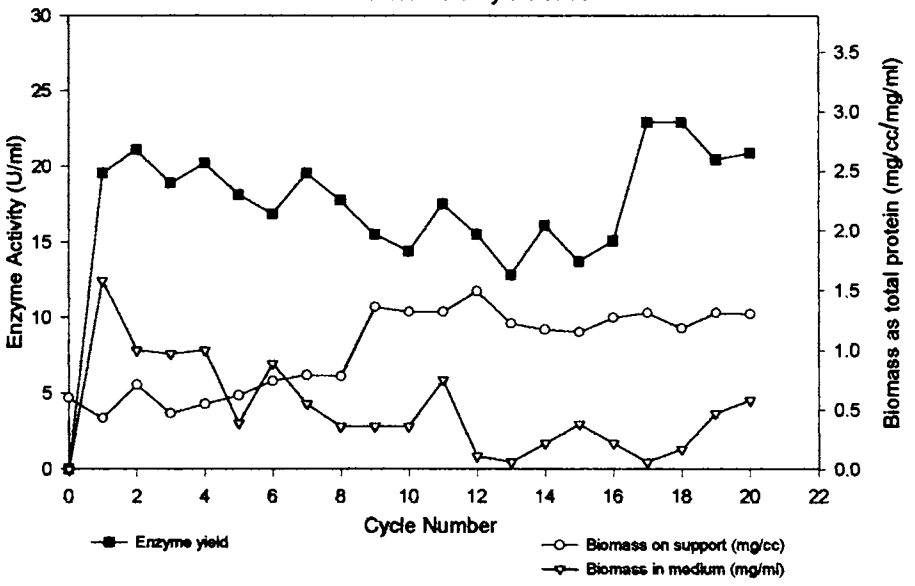


Table 4.18
Repeated batch synthesis of L-glutaminase by *Pseudomonas* sp. adsorbed on Nylon cubes

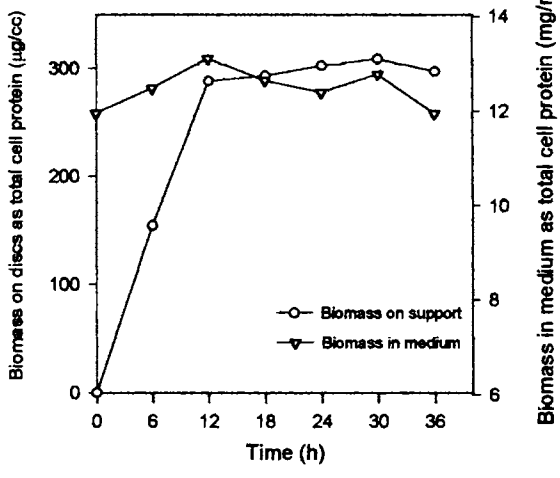
Cycle #	Mean Enzyme yield (U/ml)	Biomass on support (mg protein/cc support)	Biomass in medium (mg protein/ml)
0	0.00	0.60	0.00
1	19.55	0.43	1.58
2	21.12	0.71	1.00
3	18.88	0.47	0.97
4	20.22	0.55	1.00
5	18.12	0.62	0.39
6	16.85	0.74	0.89
7	19.55	0.79	0.55
8	17.75	0.78	0.36
9	15.51	1.36	0.36
10	14.38	1.32	0.36
11	17.52	1.32	0.75
12	15.51	1.49	0.11
13	12.81	1.22	0.06
14	16.10	1.17	0.22
15	13.71	1.15	0.38
16	15.06	1.27	0.22
17	22.92	1.31	0.06
18	22.92	1.18	0.17
19	20.45	1.31	0.47
20	20.89	1.30	0.58

4D.3 CONTINUOUS PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON NYLON WEB IN A PACKED BED REACTOR

4D.3.1 Cell immobilization on the Nylon bed

Re-circulation of the actively growing culture resulted in cell growth and adsorption on the medium evidenced by the increase in biomass on the Nylon discs and in the medium. Figure 4.77 shows the progress of cell growth and adherence on the bed along with

Fig 4.77 Immobilization of *Pseudomonas* sp. cells on Nylon bed in a PBR
Cell adherence as a function of recirculation time



increase in re-circulation time. The biomass adsorbed onto the support increased with circulation time and attained an equilibrium value in the range of 290-310 μg/cc after 12h of re-circulation. The maximum biomass, however, was recorded after a circulation time of 30h (308.81μg/cc). Biomass content in the medium also increased with re-circulation time up to a re-circulation time of 12h after which it also remained almost constant between 11 and 13 μg/cc.

4D.3.2 Effect of Operational parameters on Reactor performance.

Void volume of the reactor was determined to be 118.8cc and the dilution rates corresponding to flow rates 60, 80, 100, 120 and 140ml/h were calculated to be 0.51, 0.67, 0.84, 1.01 and 1.18 h^{-1} respectively. Operation temperature was maintained at 35 ^{0}C by using pre warmed medium and the substrate concentrations were varied to desired levels for the experiment. As observed in other systems of continuous production, dilution rate showed a negative relation with enzyme yield and substrate conversion, and a positive relation with volumetric productivity. Increase in substrate concentration resulted in an increased enzyme yield and productivity, where as it also resulted in decreased efficiency for substrate conversion (Table 4.19).

Table 4.19

L- Glutaminase production in a packed bed reactor with Nylon web as packing material-Evaluation of performance under different operation conditions

Substrate conc. (%w/v)	F Flow rate (ml/h)	D = F/V Dilution rate (h^{-1})	R = 1/D Residence time (h)	Y= Enzyme yield (U/ml)	Std Dev	% Substrate conversion	Std Dev	P = YD Productivity (U/ml/h)
	60	0.51	1.98	13.03	0.979	22.61	1.119	6.58
	80	0.67	1.49	12.63	1.161	17.12	2.017	8.51
0.50%	100	0.84	1.19	11.06	1.134	16.8	2.439	9.31
	120	1.01	0.99	9.26	1.8	11.31	1.48	9.35
	140	1.18	0.85	7.86	0.396	8.4	2.238	9.26
	60	0.51	1.98	17.85	2.067	14.21	0.28	9.02
	80	0.67	1.49	16.45	0.975	12.44	0.74	11.08
1.00%	100	0.84	1.19	15.48	2.777	10.18	1.454	13.03
	120	1.01	0.99	13.71	1.429	9.53	1.958	13.85
	140	1.18	0.85	11.31	2.625	9.37	1.558	13.33
	60	0.51	1.98	22.7	1.761	10.23	1.038	11.46
	80	0.67	1.49	19.7	1.423	9.69	1.408	13.27
1.50%	100	0.84	1.19	16.2	0.487	9.48	0.813	13.64
	120	1.01	0.99	13.86	0.749	9.37	1.615	14.00
	140	1.18	0.85	12.56	0.624	9.15	0.493	14.80

4D.3.2.1 Effect of dilution rate and substrate concentration on enzyme yield

The enzyme yield decreased along with an increase in dilution rates at all the substrate concentrations tried (4.78a). The decrease was more drastic at the highest substrate concentration tried (1.5% w/v) and the yield obtained at this concentration for 1.01 h^{-1} dilution was very comparable to that obtained at substrate concentration 1% for the same dilution rate. However, upon operation at each individual dilution rate, the yield was more at higher substrate concentration (Fig 4.78b). The maximum and minimum enzyme yields obtained were 22.7 U/ml and 7.86U/ml respectively.

4D.3.2.2 Effect of dilution rate and substrate concentration on volumetric productivity

In general, the volumetric productivity was higher at the higher dilution rates. In media with substrate concentrations 0.5% and 1%, the productivity increased with increase in dilution rate up to a dilution of 1.01 $h^{-1}$and decreased with further increase in dilution rate. At the highest substrate concentration tried, the increase was linear and the productivity increased with dilution till the highest dilution rate attempted (4.79a). Substrate concentration influenced the productivity such that a higher substrate concentration resulted in higher productivity at any dilution rate (Fig 4.79b).

4D.3.2.3 Effect of dilution rate and substrate concentration on substrate conversion

It was observed that an increase in either dilution rate or substrate concentration resulted in a decreased percentage of substrate conversion (Fig. 4.80 a & b). While the maximum substrate conversion efficiency (22.61%) was obtained at a dilution of 0.51 h^{-1} with the lowest substrate concentration (0.5%w/v) attempted, the minimum was recorded with 2% (w/v) substrate and at the highest dilution (1.18 h^{-1}).

Fig 4.78 Effect of operational parameters on continuous production of L-glutaminase by *Pseudomonas* sp. immobilized on Nylon discs in a PBR

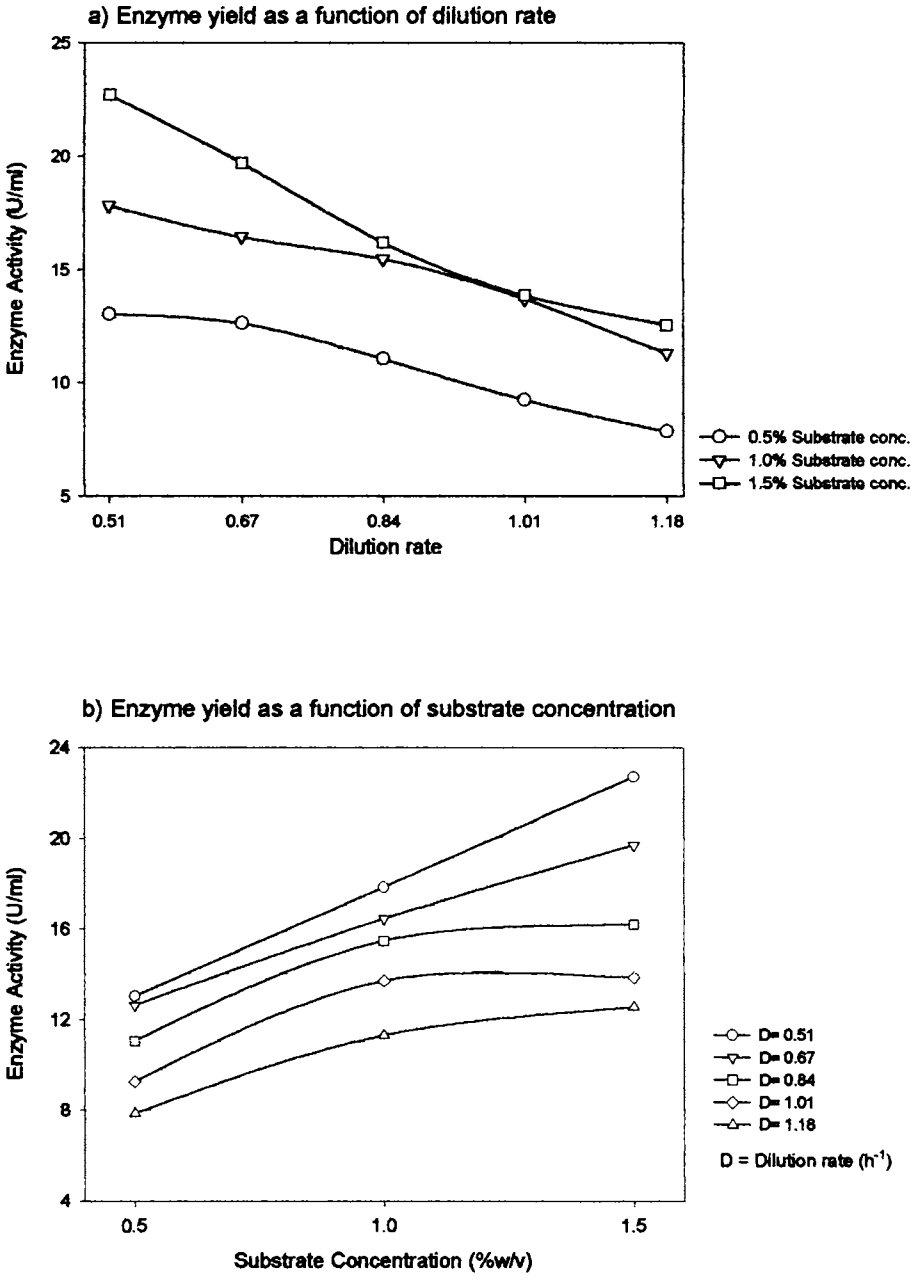
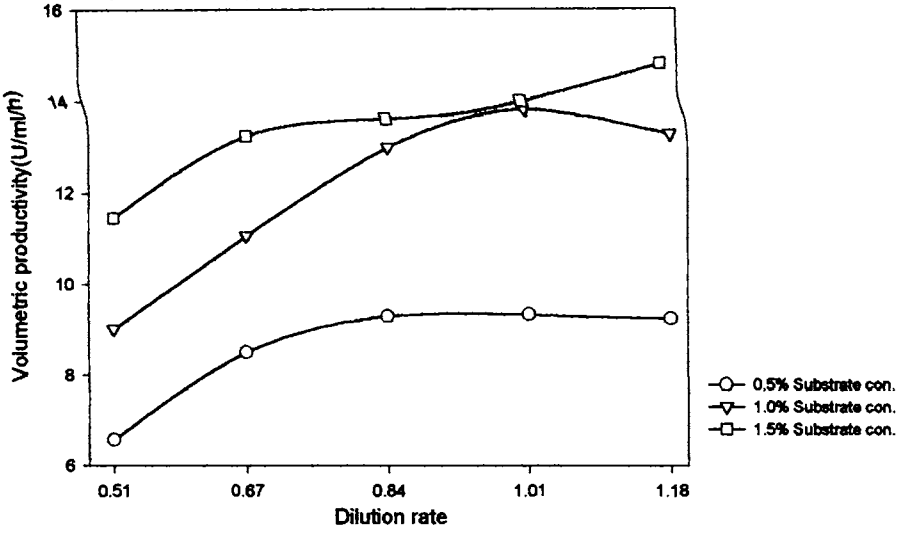


Fig 4.79 Effect of operational parameters on continuous production of L-glutaminase by *Pseudomonas* sp. immobilized on Nylonweb discs in a PBR

a) Volumetric productivity as a function of dilution rate



b) Volumetric productivity as a function of substrate concentration

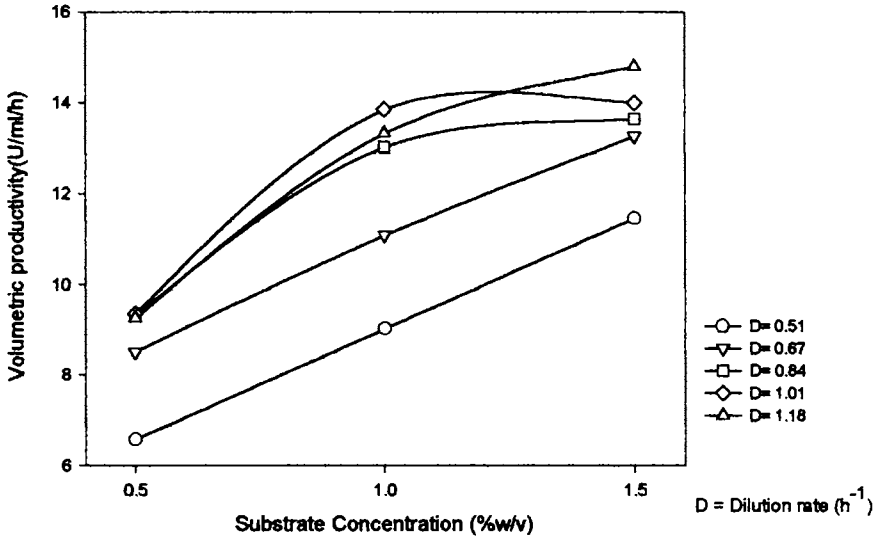
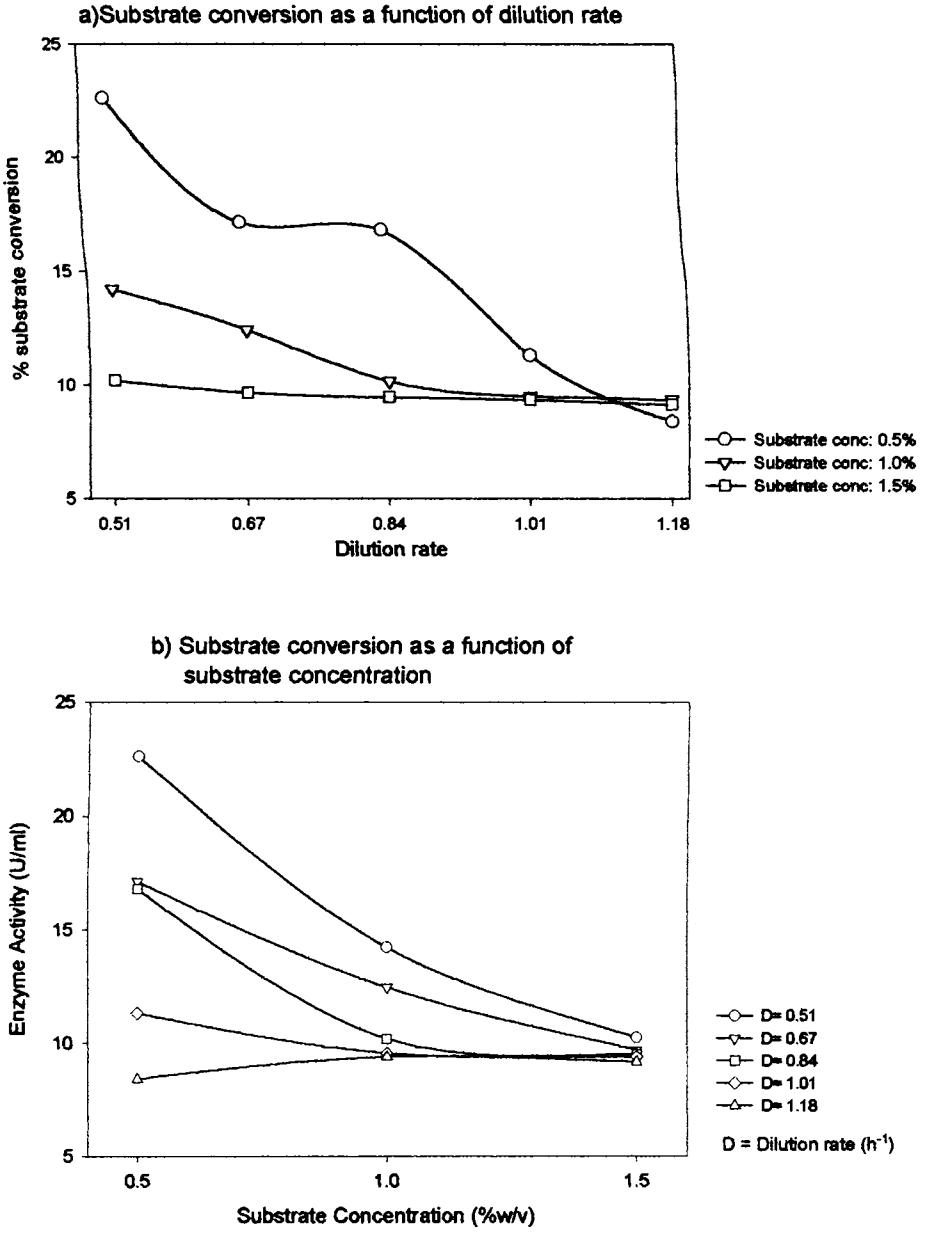


Fig 4.80 Effect of operational parameters on continuous production of L-glutaminase by *Pseudomonas* sp. immobilized on Nylon discs in a PBR



4D.3.3 Operational stability of the reactor

The performance of the PBR on prolonged operation was assessed employing the conditions that supported maximum substrate conversion efficiency (0.5% substrate and 0.51h $^{-1}$ dilution rate), and for a total duration of 72h after equilibration. The cells immobilized on nylon discs were able to synthesise L-glutaminase continuously through out the period of study with a average yield of 13.179 U/ml.

After 16h the enzyme production remained stable between 13 U/ml and 16U/ml (Table 4.20 and Fig 4.81). The average volumetric productivity recorded was 6.721U/ml/h. The adsorbed biomass remained stable between 0.65 and 0.75 mg protein/cc after the 16th hour of operation. The minor variations in enzyme yield were related to the variations in biomass in the medium indicating the contribution of free cells to the enzyme yield.

Fig 4.81 Packed bed reactor performance over prolonged operation up to 72h at 0.51 h^{-1} dilution rate and 0.5% substrate concentration

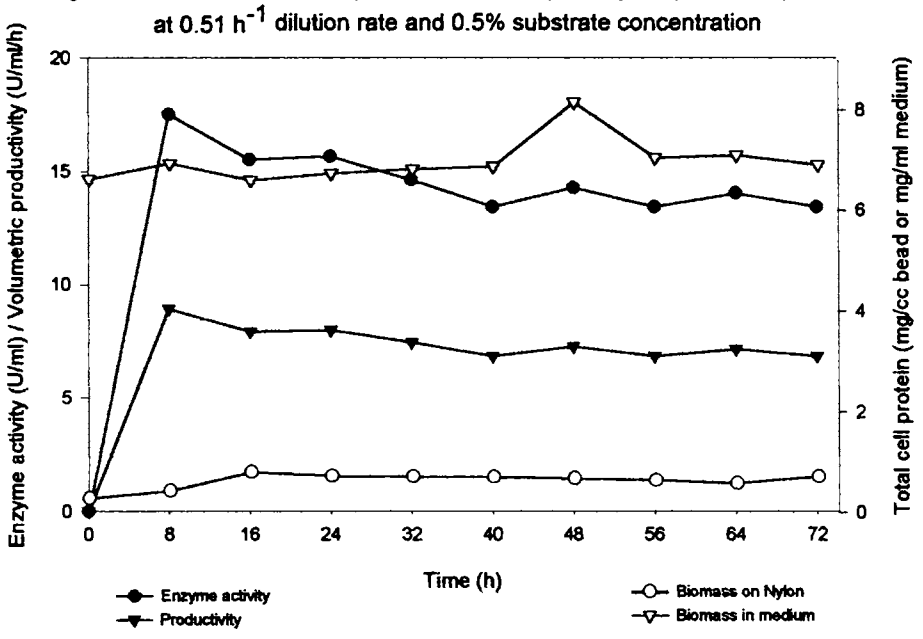


Table 4.20

Performance of a PBR with *Pseudomonas* sp. adsorbed on Nylon bed for continuous L-glutaminase production over prolonged operation under conditions that give maximal substrate conversion.

Time (h)	Enzyme yield (U/ml)	Biomass in beads (mg protein/cc support)	Biomass in medium (mg protein/ml)	Productivity (U/ml/h)
0	0.00	0.28	6.63	0.00
8	17.51	0.43	6.93	8.93
16	15.51	0.80	6.60	7.91
24	15.65	0.72	6.74	7.98
32	14.61	0.72	6.82	7.45
40	13.41	0.70	6.88	6.84
48	14.26	0.68	8.15	7.27
56	13.41	0.64	7.04	6.84
64	14.02	0.58	7.10	7.15
72	13.41	0.72	6.90	6.84

5.DISCUSSION

5A. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED BY Ca-ALGINATE ENTRAPMENT

5A.1 *L-glutaminase production under batch mode operation*

Viable cell immobilization by entrapment in Ca-alginate beads is accomplished by mixing of cells with sodium alginate under sterile conditions followed by gelation resulting in beads of a predetermined diameter range-usually 1-4mm (Groboillot *et al*, 1994). The resulting beads are then exposed to the growth medium or used as such after activation for production of enzyme. During the production phase in a nutrient rich medium, cell growth may result along with enzyme production, and both enzyme production and growth depend on the diffusional limitations imposed by the porosity of the gel matrix, and later by the accumulating biomass. With extended use of the cell containing beads, the structural and mechanical properties of the beads are affected by the pressure build up due to cell growth, and the ability to synthesise enzyme decreases due to limited diffusion of substrate and nutrients in and out of the bead matrix. Ca-alginate entrapment techniques are also sensitive to the presence of chelating agents such as citrate and phosphate (Gerbsh & Buchholz, 1995). The selection of ideal immobilization conditions is mandatory for the production of beads with structural integrity and prolonged ability for enzyme synthesis.

The effectiveness of Ca-alginate immobilization of marine *Pseudomonas* sp. BTMS -51 for L-glutaminase production was tested in terms of enzyme production and bead stability.

The strength of the soluble gelling agent or support (sodium alginate) affects the strength of the beads due to its effect on the crosslinking of gel. Increasing the concentration of alginate solution results in a tighter crosslinking (Kierstan and Coughlan, 1985). In the present study also, it was observed that the bead stability increased with support (alginate) concentration and the most stable beads were obtained at the highest concentration of alginate tried confirming better crosslinking of gel at high alginate concentrations. However, very high concentrations of alginate are difficult to work with and the increased crosslinking may impose diffusional limitations above a particular concentration. This was evident by the decrease in

enzyme yield from the beads prepared with solutions containing alginate at above 3% level.

The cell concentration within the beads is an important determinant of the enzyme yield and as expected higher yields were obtained at higher cell concentrations. The decrease in yield at the highest concentration of cells tried might be attributed to the reduction in availability of substrate probably due to the limitations in diffusion of the substrate into the inner layers of the beads where the cells are starved gradually by clogging of the pores. The decreased enzyme yield at high cell loading of the beads may be attributed to the decrease in effective diffusion coefficient with increased cell concentration (Hahn-Hagerdal, 1990). Westrin & Axelsson (1991) demonstrated that the effective diffusion coefficient decreases with presence of cells in the gel beads, and the diffusion coefficients have been correlated with yeast cell concentration in a Ca-alginate gel (Axelsson and Persson, 1988). L-Glutaminase is produced by *Pseudomonas* sp. BTMS -51 during its log phase of growth under submerged fermentation (Renu & Chandrasekaran, 1992) and with use of cells at its mid log phase of growth (18h) for immobilization, the maximal enzyme yields were recorded. Aged cells were not efficient in enzyme production compared to the cells at their log phase. The efficiency of the biocatalyst produced by immobilization is affected by the physiological state of the cell prior to immobilization (Flint, 1987) which is evident from the lower yields obtained with older cells.

Increase in concentration of the $CaCl_2$ solution used for immobilization resulted in relatively stable beads and the longest bead dissolution time was recorded when beads prepared in 0.3M $CaCl_2$ were used. Kierstan and Coughlan (1985) have indicated that the gel crosslinking is affected by concentration of $CaCl_2$ and a higher concentration promotes a better crosslinking. However, the process of gelation induces shrinking in Ca-alginate gel (Axelsson and Persson, 1988) which can cause diffusional limitations with increased crosslinking. This might be the probable reason for the observed decrease in enzyme synthesis when beads were prepared in solutions with $CaCl_2$ concentrations above 0.15M.

Curing of the beads is necessary for the hardening of the gel beads and resultant stability. A minimum curing time of 20 minutes was proposed by Kirsten and Bucke (1977) and varying curing times were used by several others (eg: 2h by Jamuna &

Ramakrishna 1992 & Sabu *et al*, 2001., 5h by Fukushima & Motai, 1990). The ideal curing time in the present study was evaluated in terms of the enzyme yield from the immobilized cell beads and was observed to be 2h. The stability of beads against dissolution in citrate remained almost same with curing over this time. However, increase in curing time beyond 2h led to a reduction in enzyme synthesis, which again might be attributed to gel-shrinkage.

Though it is accepted that the porosity of an immobilization matrix has limited influence on the diffusion limitations, it is also known that the cell content have a direct influence on diffusion coefficient. It is suggested that immobilization matrices may structure water (Mattiason & Hahn-Hagerdal, 1982), and the close vicinity of the immobilized cell would thus constitute an environment with reduced water activity.

The performance of the entrapped cells is favoured by a porous and elastic immobilization matrix (Nagashima *et al*, 1984., Johansen & Flink, 1986, Pundle *et al*, 1988) often interpreted as a mass transfer effect. It has been observed that the space available for cell growth is more important for metabolic activity (Fujimura & Kaetsu, 1985) and this probably can account for the differences in enzyme production with variation in levels of alginate, and $CaCl_2$ and curing time, which apparently affects the porosity of the matrix.

Storage of immobilized viable cell beads at lower temperatures slows down the metabolic activities of the cells and an activation step is often necessary to bring the cells back to the production phase. The medium and conditions used for activation plays an important role in the rejuvenation of the immobilized cells. Activation in a medium where the nutrient supplied is the substrate/inducer of the enzyme to be synthesised will also help in the acclimatisation of the cells to the production conditions (Kumar *et al*, 2001). 1% glutamine in seawater was the best among the activation media tested and media without glutamine or prepared without seawater was least effective. It has been observed that marine microorganisms show an absolute preference to the media with seawater as base for growth and enzyme production (Nagendraprabhu & Chandrasekaran, 1996, Sabu *et al*, 2000). The optimal concentration of glutamine required for activation was determined to be 1%. Interestingly, it was observed that the immobilized cells were better activated at an acidic pH of 5 compared to other initial medium pH tried. The temperature optimum

for activation did not correlate with that required for enzyme production by the immobilized cells. Highest enzyme yield was obtained with beads activated at room temperature though the temperature preferred for enzyme production was 35 ^{0}C. The immobilized cell beads required an activation of 18h for active enzyme synthesis. Lee *et al* (1983) followed the time course of activation of Ca-alginate entrapped yeast cells used in ethanol fermentation for 35h and observed that the activity of cells is correlated to cell growth in gels during activation.

The initial pH of the enzyme production medium and incubation temperature for maximal enzyme production by the immobilized cells were similar to that obtained under submerged fermentation and solid state fermentation for the same organism (Keerthi, 1999). These environmental parameters are independent in exerting their influence on growth and performance of bacteria (Chandrasekaran *et al*, 1991). It may be presumed that even under immobilized conditions the optimal pH and incubation temperature for enzyme production remained unchanged. The reduction in yield at higher temperatures might be attributed to an enhanced cell growth and secretion of other proteins into the medium, since it was observed that the protein content of the medium remained almost consistent at temperatures above 35 ^{0}C.

Enzyme production by the immobilized cells remained above 30 U/ml over a considerable range (0.75-3%) of substrate concentrations. The yield increased with increase in substrate concentration and highest yield was recorded at 2% substrate concentration. However, further increase in concentration of glutamine did not favour an enhanced enzyme production. Although glutamine might be having an inducing effect on enzyme synthesis (Nagendraprabhu, 1996) an increase in concentration beyond the optimum, probably do not further enhance the enzyme production, for the cells would already have reached their maximum ability to synthesise glutaminase.

Medium composition affects operational stability by its influence on cell growth and *de novo* protein synthesis as well as creating environmental conditions for prolonged product synthesis (Freeman & Lilly, 1998). Optimisation of the concentrations of medium components affecting enzyme production, eg., carbon source (Feijoo *et al*, 1995), nitrogen source (Jager *et al*, 1985) have significantly improved enzyme yields in immobilized whole cell operations.

In the present study it was observed that incorporation of any carbon source resulted in an enhanced yield compared to the control. Glucose was the best among the carbon sources tested followed by sucrose, sorbitol and mannitol. Availability of carbon sources along with glutamine might probably result in an enhanced cell growth replacing the dead cells, which eventually lead to the presence of a metabolically hyperactive group of cells within the bead matrix. Higher activities obtained with mannitol and sorbitol, added to the medium might be due to the stabilising effect of these polyols (Galinsky & Tyndall, 1992). The optimal concentration of glucose for enzyme synthesis was 1% (w/v). Addition of nitrogen sources into the medium on the other hand had a negative impact on glutaminase production and the yields obtained were lower than the control. In the presence of the additional nitrogen sources along with glutamine, bacteria supposedly prefer them to glutamine for their growth requirements. Similar observations were reported for glutaminase production under solid state fermentation on inert supports by *Vibrio costicola* (Nagendraprabhu & Chandrasekaran, 1997) and *Pseudomonas* sp. (Keerthi, 1999). The possibility of a repression mechanism operative under presence of easily available nitrogen sources may not be ruled out, and only further studies could confirm such a mechanism. However, it was noted that in the presence of additional nitrogen sources, the cells enter an active growth phase resulting in rapid cell growth and leaching of cells from beads indicated by the increase in turbidity of the medium. Presence of easily available nitrogen sources probably eliminates the necessity for glutamine breakdown using glutaminase, which consequently results in a decreased enzyme synthesis.

The retention time or the incubation time required for maximal enzyme synthesis by the immobilized cells under batch operation could be significantly lower than that of the free cells under similar conditions. Immobilized whole cells have an enhanced reaction rate due to an increased cell density (Kolot, 1980., Furusaki, 1988). Since the cells are present at their optimum concentrations in beads right from the beginning of the operation unlike in submerged or solid state fermentations, it may be expected that the activated cells directly enter the production phase and start synthesising the enzyme. The optimal retention time of 12h for maximal enzyme yield (30.34U/ml) obtained in the present study testifies this argument as this was half the incubation time required for the same organism for glutaminase production under solid

state fermentation or submerged fermentation (Keerthi, 1999). The enzyme activities remained above 25U/ml with retention up to 24h and decreased with further retention probably due to enzyme inactivation.

5A.2 *L-glutaminase production under repeated batch operation*

The mode of operation dictates different environmental conditions for the immobilized cells and during batch operation, medium composition is subject to continuous change with depletion of nutrients, substrates and precursors, and accumulation of products by products and cell fragments (Freeman and Lilly, 1988). This may affect both maintenance of high cell loading (Markl *et al*, 1993; Bunch, 1994) and high productivity (Freeman *et al* , 1993). Repeated batch operation was reported to result in sharp declines in productivity for amylase production (Clementi & Cobbetti, 1987), whereas consistent productivities over several cycles have been reported for amylase (Jamuna & Ramakrishna, 1992), cellulase (Tamada *et al*, 1986), and lignin peroxidase (Linko, 1988). In the present study, it was observed that without any reactivation step between the cycles of operation, the production of glutaminase was fairly consistent throughout the cycles of operation. The possible reduction effect in cell loading was not conspicuous at least for the 20 cycles of operation. Switching between a growth medium and the production medium at periodic cycles, may be done to eliminate possible cell loss and consequent decrease in productivity, if at all it occurs with operation after the tested number of cycles. The biomass content of the beads showed a clear correlation with the yield obtained indicating that the enzyme production was a function of the cell concentration under the conditions of operation. Substrate depletion or product accumulation did not exert a dominant influence on productivity within the short time of retention employed in the cycles.

5A.3 *Continuous production of L-glutaminase by the immobilized cells*

Continuous operation, providing simultaneously a fresh supply of nutrients as well as continuous removal of products, by-products, free cells, and cell fragments has the potential for steady state operation in which the immobilized cells are in equilibrium with medium composition optimal for attaining maximal productivity (Freeman &

Lilly, 1998). The dependence of yield on substrate has to be determined to calculate the concentration of limiting substrate to be used in the feed medium. The availability of substrate depends on the concentration of the substrate in the feed as well as the dilution rate. As the yields obtained from batch experiments can be quite different in continuous culture, determination of yields from batch operations can give only a rough approximation of the ideal operation conditions (Goldberg and Er-el, 1981). Hence, in the present study different combinations of substrate concentrations and dilution rates were tried for continuous production of L- glutaminase in a packed bed reactor and a circulating bed reactor. The optimum substrate concentration and dilution rate that give maximum enzyme yield, volumetric productivity, and substrate conversion was determined so that the reactor may be operated at conditions that give maximum productivity or substrate conversion.

In either of the reactor configurations an increase in substrate concentration resulted in an increased yield and productivity, whereas the substrate conversion efficiency was lower at high substrate concentrations. Increase in dilution rate in general resulted in decreased yield and substrate conversion but an increase in productivity.

In the PBR it was observed that at 0.5% substrate concentration the productivity increased with dilution rate only up to a dilution rate of 1.06 h^{-1}, and in CBR the dilution rate (0.4 h^{-1}) that gave maximum productivity at this substrate concentration was still lower. However, at higher substrate concentrations both the reactors gave maximum productivity at the highest dilution rates tried (1.48 h^{-1} and 0.56 h^{-1} for PBR and CBR respectively). This indicates that the availability of substrate is the key factor in enzyme production by the immobilized cells. In media with low substrate concentrations, increase in residence time (decrease in dilution rate) resulted in a better contact between the cells and the substrate and consequently more enzyme is produced. At higher dilution rates the enzyme output from the reactor increases although the activity is lower due to the high flow rate (Jamuna & Ramakrishna, 1992). However, after a particular dilution rate, the output of enzyme decreases because of very high flow rates and low yield of the enzyme. With increase in concentration of the substrate, at higher dilution rates, though the flow rates are higher the concentration of substrate per unit volume is also higher and the enzyme

synthesis can proceed at higher rates even with a higher dilution. This effect was observed in the case of CBR operation where higher productivities were observed at higher dilution rates when the substrate concentrations were above 1% (w/v). In PBR, however, even an increase in substrate concentration could not enable the system to overcome the reduction in productivity at increased dilutions (beyond 1.27 h^{-1}). A higher cell concentration and consequent increase in demand for substrate might be the reason for this observation.

The substrate conversion efficiency of either of the reactors decreased with increase in dilution and substrate concentration. With increase in dilution rates the contact time of substrate with cells is reduced resulting in a decreased conversion of substrate and enzyme synthesis. At higher substrate concentrations the concentration of substrate per unit volume though is higher and can account for an increased enzyme synthesis, for a given immobilized cell reactor system under continuous operation, the biomass is at a constant equilibrium at steady state, and the substrate utilised is also constant. This means that at substrate concentrations above a limiting concentration there is no increase in substrate consumption along with increase in concentration. Hence, the calculations of substrate conversion efficiency will give lower values at higher substrate concentrations although the amount of substrate consumed might be same or near it.

A comparison of the PBR and CBR in terms of the performance under the given operating conditions may not be justified as the reactor systems had different cell loading and operating volumes. This implies that though the flow rates experimented were same, due to the peculiarities of the reactors, the dilution rates are different. Nevertheless, it may be observed that a better yield, productivity, and substrate conversion was obtained in the PBR. This is probably due to the high cell loading in the PBR compared to the CBR. The operating conditions that gave maximum productivity were a substrate concentration of 2% for both the reactors and a dilution of 1.27 h^{-1} and 0.56 h^{-1} for PBR and CBR respectively.

In many fermentations, the cost of the substrate is an important economical parameter as is the case with this system. Maximum substrate conversion efficiency becomes a primary concern in such operation and some investigators (e.g. Sanchez *et al*, 1996) have preferred to operate at conditions that give maximum substrate

utilisation despite the fact that a higher productivity may not be achieved under such conditions. The extended operational stability of the PBR and CBR was hence tested under conditions that gave maximal substrate conversion efficiency.

Operation under a low substrate concentration did not affect the operational stability for the tested duration of operation. A consistent average value of around 20 U/ml of glutaminase was obtained through out the operation in the case of PBR, whereas this value was around 12 U/ml for CBR. Further, it was noted that there was no significant reduction in biomass content of the beads in either of the reactors. Stable continuous production of cellulase for 200h (Webb *et al*, 1986) and ribonuclease for 960h (Manolov, 1992) have been reported. Though in some cases the prolonged operation under continuous mode may result in a sharp decay in productivity, this could be moderated by regeneration treatments with growth media (Freeman & Lilly, 1998). Whereas in the present study, extended operation of PBR or CBR with immobilized *Pseudomonas* cells for glutaminase production neither showed any signs of decay for the 120h operation, nor warranted a regeneration treatment atleast for 120h. This probably implies an attainment of equilibrium between growth of the cells within the beads, and their death and possible detachment from it. Such a steady state is very much desirable as the beads contain cells at a metabolically active and viable state eliminating the need for incorporation of a regeneration phase. Under such conditions the only limitations for prolonged continuous operation would be the stability of the beads.

It may be concluded from the studies on Ca-alginate immobilized marine *Pseudomonas* sp. that the immobilized cell system can successfully be exploited in the production of L-glutaminase. Yields form the immobilized cells were comparable to that obtained for the same organism under SSF (Keerthi, 1999) and higher than that obtained under SmF (Renu & Chandrasekaran, 1992). Further advantage of the reusability of biomass coupled with the reduction in production and down time, and suitability for continuous production are definite improvements over the other modes of fermentation previously attempted for glutaminase production using the same organism. The immobilized cells could be repeatedly used for several cycles of batch operation without loss of activity, and could successfully and consistently produce the enzyme under continuous mode in a packed bed reactor or circulating bed reactor.

Considering the enzyme yield given by the free cells, very high productivities were obtained in the PBR.

5B. L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* SP. IMMOBILIZED BY PHYSICAL ADSORPTION ON INERT SUPPORTS.

Practical application of the most widely studied method of entrapping cells in polymer gels such as alginate or carrageenan is limited by the problems of gel stability (Ogbonna *et al*, 1989) as well as mass transfer limitations within the gel beads (Tanaka *et al*, 1984., Ogbonna *et al*, 1991). Also the complexity of the process and requirement of sophisticated equipment limit the large-scale production of gel beads for industrial applications. From the viewpoint of mass transfer and ease with which immobilization can be achieved, immobilization by passive cell adhesion to surfaces is preferred to cell entrapment (Ogbonna *et al*, 1994). Immobilization via adsorption begins generally with a sterilised support inoculated with cell or spore suspension. The biofilm subsequently developed upon exposure to growth medium is comprised of multiple cell layers located either in contact with the bulk medium or on the surface of inner channels in a macroporous structure. Unlike gel entrapment, the adsorption technique allows cell detachment and relocation with potential establishment of equilibrium between adsorbed and freely suspended cell populations even at early stages of cell culture (Freeman & Lilly, 1998).

In the present study three inert supports namely Polystyrene (PS), polyurethane foam (PUF) and Nylon web (NW) were evaluated as carriers for immobilization of marine *Pseudomonas* sp. BTMS 51 and subsequent production of L-glutaminase.

5B.1 L-GLUTAMINASE PRODUCTION BY *PSEUDOMONAS* IMMOBILIZED ON INERT SUPPORTS UNDER BATCH MODE

5B.1.1 Immobilization of cells on the carriers

The majority of microorganisms are capable of adhering to surfaces and the relevance of substratum properties, bacterial surface properties, and molecular conditioning films in adhesion processes has become increasingly evident over the past several years (Marshall, 1994). When clean solid surfaces come in contact with the

aqueous phase, the physicochemical properties of the surface are immediately modified by molecular adsorption at the surface to give a surface conditioning film. The film can alter the surface properties and also can act as a concentrated energy source for the microorganisms initially colonising the surface. Complex sequences of events are involved in the adhesion of microorganisms to surfaces and this is dependent on the characteristics of the adsorbent, characteristics of the microorganisms, and characteristics of the immediate environment (Durand & Navarro, 1978). It is often difficult to determine the correlation between physicochemical properties of the bacteria and substrata with the retention of bacteria on surfaces Dagostino *et al*,(1991).

Since the medium used for growth of the organism is important in development of a productive adhered biomass on the inert supports, two media were tested as the "immobilization medium". Mineral salts glutamine (MSG) medium had previously been employed for inoculum build up and as an enzyme production medium for glutaminase production by marine bacteria (Keerthi, 1999., Renu & Chandrasekaran, 1992). Zobell's marine broth (ZMB) was included in the experiment since this is the most common nutrient rich medium reported for cultivation of marine bacteria. As the final intention was development of maximum adsorbed biomass capable of glutaminase production, the biofilm developed on the treated carriers were tested for their ability to synthesise glutaminase.

It was observed that Zobell's broth was a better medium for growth and adsorption of *Pseudomonas* cells on any of the supports (Table 5.1). A comparison of the efficiency of the supports in terms of adsorbed biomass showed that Nylon web followed by PUF, was the preferred substratum for attachment. Least adsorption was observed on polystyrene. This could be attributed to the presence of large internal surface areas and water channels in NW and PUF whereas PS has a very compact matrix structure preventing a free circulation of water and nutrients through the internal surfaces. Further, polystyrene has a low water absorbency of 2g/100cc (Brydson, 1982). Studies on adsorption on polystyrene have shown that the polymer was not a good support for immobilization of fungal spores(Lele *et al*, 1996). On the contrary, NW and PUF had been successfully employed in the production of endoglucanse and xylanase (Haapala *et al*, 1995). Evidently, under given conditions,

the equilibrium concentration of absorbed cells is determined by the nature of the substratum.

Another interesting observation was that the incubation time required for maximal cell adherence on the supports was independent of the nature of the support used, and was closely related to the type of growth medium (Table 5.1). Thus the incubation time was 36h in Zobell's broth while it was 42h in the MSG medium. Moreover, the maximal observed cell adsorption was lower in the MSG medium for any support. This indicate that the time required to achieve the equilibrium concentration is governed by the nature of growth medium. Zobell's broth, being richer in nutrients compared to MSG, supported an enhanced growth rate of the cells and the equilibrium is attained in a shorter duration. In MSG, however, the cells take longer to achieve the equilibrium which was attained at a lower biomass concentration apparently due to limitations imposed by nutrient scarcity of the medium on the specific growth rate of the bacterium.

It may be concluded that the quantity of cells adhering to the surface is determined primarily by the nature of the support matrix when the conditions of growth are not limiting, and for a given support these limits are dictated by the nutrient availability and nature of immediate environment (ie. nature of the growth medium).

Table 5.1
Comparison of cell adherence on different supports while growing *Pseudomonas* in MSG and Zobell's broth

Incubation time (h)	Cell growth on support- Biomass expressed as total protein (µg/cc of support material)					
	Polystyrene		PUF		Nylon web	
	MSG	Zobell	MSG	Zobell	MSG	Zobell
0	0	0	0	0	0	0
6	0	91.49	6.93	105.55	15.59	200.65
12	28.42	133.07	11.44	104.36	21.49	257.49
18	23.57	122.33	139.31	131.00	75.55	310.85
24	46.09	137.23	173.62	144.16	189.22	302.54
30	90.45	183.32	186.10	198.74	186.79	353.83
36	125.45	195.45	203.77	252.29	260.26	356.28
42	136.54	185.40	216.25	241.10	321.94	363.88
48	136.19	183.67	212.09	257.83	334.42	352.44

5B.1.2 Enzyme production by the immobilized cells

Carriers, after the immobilization cycle, were transferred to enzyme production medium to study their efficiency for L-glutaminase production. Mineral salts glutamine medium (MSG) and seawater glutamine medium (SWG) were used as the enzyme production medium and incubations were carried out for a total period of 24h with periodic sampling and assay for enzyme activity. Comparison of the L-glutaminase production by cells immobilized on the three inert supports show that Nylon web was superior to the other supports used as carriers irrespective of the type of enzyme production medium used. With either MSG or SWG as the enzyme production medium, the highest enzyme yield was obtained with Nylon web used as carrier followed by polyurethane foam and polystyrene (Table 5.2). Although same volume of the support was used in the fermentation, Nylon and PUF had a macroporous structure allowing free circulation of water and nutrients consequently allowing better cell growth and probable increase in enzyme production. On the contrary, polystyrene system had the least pore size and gave the lowest enzyme yield and the adsorbed biomass content was low. This, as discussed earlier, could be due to diffusional limitations imposed by the structure of the support itself.

The specific surface area available for cell adsorption is inversely proportional to the average pore radius (Messing *et al*, 1979) and consequently polystyrene has the maximum surface area followed by polyurethane and Nylon web. A carrier with more surface area should theoretically perform better than those with a lesser surface area. Nevertheless, polystyrene, which has the largest specific surface area among the three carriers, did not perform well in comparison with the other supports. The size of the pores is an important determinant of the efficiency of cell adsorption and maintenance. With a smaller size, the pores in polystyrene are clogged easily by cell growth preventing diffusion resulting in cell death in the inner layers and the available viable biomass is considerably low. This is not reflected in the biomass assays as the assays determined only total cell protein. Therefore, though it may seem that the differences in biomass between the different systems may not account for the differences in enzyme yield, it actually may, as the yields are determined by quantity of viable cells and not the total biomass.

However, this may not explain the differences in enzyme yield between the PUF and Nylon systems. The pore size in PUF was not limiting to bring about a reduction in cell growth due to nutrient limitation, and the system supported a marginally higher adsorbed biomass compared to Nylon web. Nevertheless, NW system yielded an enhanced enzyme production indicating that the higher yields obtained with NW system might be due to factors other than cell concentration on support. It is known that the surface charges and free chemical groups on the surface of carriers might interact with the immobilized microbes (Haapala *et al*, 1995) possibly influencing the metabolism. Numerous reports have indicated that certain physiological characteristics of bacteria are altered when the organisms are adhered to a surface (Marshall, 1994). The physicochemical conditions at surfaces might induce altered gene expression, and consequent genetic responses and the possible mechanisms leading to these has been reviewed recently (Goodman & Marshall, 1994). The increase in enzyme yield with the Nylon system, despite a lesser adsorbed-biomass, may be a possible consequence of such interactions. Nylon web was reported to be a better carrier in comparison to polyurethane foam in the production of endoglucanase and xylanase by immobilized *Trichoderma reesei* (Haapala *et al*, 1995).

It may be assumed that for the given medium, enzyme production is determined by the total active biomass of the system up to a critical concentration which in turn is closely related to the nature of support used, through parameters which impose diffusional limitations. The interaction between the carrier surface and bacteria is also a prime determinant of the performance of immobilized cells in terms of L-glutaminase production.

Table 5.2

Comparison of L-glutaminase production by *Pseudomonas* sp adsorbed on inert supports with MSG and SWG as the enzyme production medium

Incubation Time (h)	MSG			SWG		
	PS	PUF	NW	PS	PUF	NW
0	0.00	0	0	0	0	0
6	0.00	27.63	33.26	39.99	32.58	42.47
12	19.55	34.83	32.58	41.35	36.4	53.03
18	22.7	35.06	40.9	23.37	28.09	28.54
24	13.03	34.38	33.48	14.83	33.93	23.37

Table 5.2 shows that for a given support system, production of the enzyme was dependent on the type of medium used. With PS, PUF or NW as the carrier, more of the enzyme was produced in the SWG medium although a better cell growth and adsorption was obtained when MSG was used as the enzyme production medium. This indicate that beyond a critical biomass concentration enzyme production becomes growth dissociated, and is more dependent on the medium composition and the prevailing environmental conditions. The conditions required for optimum growth and maximal synthesis of cellulase by *Trichoderma* sp. are reported to be different (Mandels, *et al*, 1975) and a growth rate close to zero was preferred for maximal synthesis of cellulase (Ryu *et al*, 1979). In this study, as the cells at a maximum attainable concentration in any of the support was employed, the important factors which influenced enzyme production were the porosity of the support through its effect on the diffusional limitations and the nature of the medium used. Being a marine organism, *Pseudomonas* sp. would be expected to perform better in a seawater based medium and the results obtained are in agreement with earlier reports on enzyme production employing marine *Vibrio costicola* (Nagendraprabhu, 1996., Nagendraprabhu & Chandrasekaran, 1995). Composition of the seawater is often different with the different sources and is largely variable. Undefined components in the seawater may be acting as nutrient supplements and /or as inducers keeping the bacterial cells at an active synthetic phase. The promotional effect of seawater when used as a base for media in enzyme production under SSF by marine bacteria (Nagendraprabhu & Chandrasekaran, 1995) and fungi (Suresh & Chandrasekaran, 1999, Sabu *et al*, 2000) has been recognised.

The time required for maximal enzyme synthesis by the immobilized cells was found to be independent of the nature of the carrier. This was 18h when MSG was used as enzyme production medium while in SWG medium the cells required only 12h for maximal enzyme production. Cells entrapped in alginate also gave the maximal yield with 12h retention in SWG. It is evident from the foregoing discussions that MSG was a better medium than SWG when cell growth is considered, and apparently a faster growth rate was not responsible for the lower incubation time required for enzyme production in SWG, because, if this were the case, a lower incubation time would have

been obtained with MSG as enzyme production medium. The substrate and carbon source was present at equal concentrations in both the media, and the pH of the media and incubation conditions were identical. The shorter incubation time required for enzyme production in seawater confirms the probable effect of seawater in guiding the cells to an active phase quickly and efficiently. Requirements of marine bacteria in terms of carbon and nitrogen sources, and micronutrients can be quite different from their terrestrial counterparts and is largely understudied. It may be possible that seawater satisfies this needs better, if not completely, which could be the reason for the observed reduction in incubation time.

5B.1.3 Optimisation of medium and process conditions for enzyme production

Medium formulation is an essential stage in the design of successful laboratory experiments, pilot scale development, and manufacturing processes. The constituents of the medium must satisfy the elemental requirements for cell biomass and metabolite production, and there must be an adequate supply of energy for biosynthesis and cell maintenance (Stanbury *et al*, 1995). The control of pH and temperature may also be extremely important for achieving optimal productivity. The composition of the medium affects operational stability by its influence on cell growth and *de novo* protein synthesis as well as creating environmental conditions for prolonged product synthesis. It may also affect substrate and oxygen solubility, and cell matrix interactions via swelling of gels or weakening of cell-carrier interactions (Freeman & Lilly, 1998).

A whole growth medium is usually employed in the generation of primary immobilized cell population, which is followed by switching to a production medium with composition designed to sustain prolonged productivity. Systematic studies on optimisation of medium components eg carbon source (Tsay & To, 1987) sugar, phosphate, and trace elements (Honecker *et al*, 1989., Rymowicz *et al*, 1993) have resulted in significant increases in product concentration, reduction in by-product formation, and extended operational stability of immobilized cell systems. In continuous mode of operation, maintenance of a stable level of nutrients required change in the dilution rate due to observed gradual change in cell load (Arcuri *et al*, 1986., Keshavarz *et al*, 1990). Studies on the optimisation of concentrations of medium

components affecting enzyme production by immobilized cells resulted in significantly improved enzyme yields (Jager *et al*, 1985., Feijoo *et al*, 1995.,)

Similarly the operating temperature is found to influence the reaction rate and stability of the immobilized cells. Studies on short-term effects of temperature on cellulase production by immobilized fungi (Tamada *et al*, 1986), and short term pH dependence of several processes were characterised (Adlercrentz *et al*, 1985., Tsay and To, 1987., De Jong *et al*, 1990)

Optimisation of a medium for fermentation must take into consideration the possible effects of the medium on the process such that it meets the criteria of maximum yield of product or biomass, the maximum rate of product synthesis, minimum yield of undesirable products, minimum problems during media making and sterilisation, and other aspects of the production process (Stanbury *et al*, 1995). It should be recognised that efficiently grown biomass produced by an optimised high productivity growth phase is not necessarily the best suited for synthesising the desired product. Different combinations and sequences of process conditions need to be investigated to determine the growth conditions, which produce the biomass with the physiological state best constituted for product formation.

In the present study, as fully-grown cells immobilized on the inert carriers were employed, the desired medium composition and conditions were that ideal for L-glutaminase production. From prior experience with the production of L-glutaminase by Ca-alginate immobilized cells, the seawater glutamine medium, which gave maximum product yield was used as the starting medium and the medium composition and production conditions were optimised. It was recognised from the fermentation with Ca-alginate immobilized cells, that though incorporation of nitrogen sources other than the substrate (glutamine) in the medium results in a decreased enzyme production, can also lead to an increase in cell growth. As it is necessary for maintaining an equilibrium concentration of adsorbed biomass for prolonged operational stability, it becomes mandatory that a certain level of cell growth do occur on the carriers to make up for cell loss due to detachment. Yeast extract, which supported cell growth in the Ca-alginate system along with enzyme production comparable to the control medium (lacking additional nitrogen sources), was therefore incorporated into the medium so

that a critical biomass concentration was maintained on the carriers throughout the production phase.

The classical method of optimisation by changing one independent variable while fixing all others at a certain level was not followed in this study. Such an effort is often time consuming and expensive for a large number of variables (Stanbury *et al*, 1995). Statistically designed experiments for optimisation of process variables which involve simultaneously varying several factors are increasingly being employed in the identification of important nutrients and environmental parameters, the interactions between them, and the optimal levels of each for the desired final objective (product yield). These methods have been discussed by Stowe & Mayer (1966), Hendrix (1980), Nelson (1982), Greasham & Inamine (1986), Bull *et al* (1990) Hicks (1993) and Myers & Montgomery (1995).

Fractional factorial designs are usually used for screening and determining the medium components, in an already formulated medium, which have significant effect and could be further optimised (Zhu *et al*., 1996.,Ooijkaas *et al*, 1999) with the Plackett and Burman (1946) design being the most common and efficient design for screening purposes, if it is not known which components should be present in the medium (Srinivas *et al*, 1994., Murthy *et al*, 1999). Further optimisation of these variables are often carried out by response surface methods like Box-Behnken (Box & Behnken, 1960) designs or central composite designs. As the important process variables affecting L-glutaminase production by marine bacteria was known from the previous studies with the submerged fermentation system (Renu & Chandrasekaran, 1992), SSF on inert support (Nagendraprabhu & Chandrasekaran, 1995) and also from the studies with same organism immobilized in Ca-alginate gels (Kumar *et al*, 2001), a screening process was considered not necessary. The parameters considered important (pH, operating temperature, glutamine concentration and yeast extract concentration) were optimised directly by the Box-Behnken method of response surface design.

Statistical design of experiments has rarely been applied with immobilized cell systems for optimisation of process variables. A central composite rotatable design was recently employed for optimisation of caspaicinoid production (Suvarnalatha *et al*, 1993) by immobilized *Capsicum* cells and an orthogonal experimental design was used for optimisation of gibberilic acid production by immobilized *Gibberella fujikuori*

Escamilla *et al* (2000). However, there are no reports on the optimisation of enzyme production by immobilized bacteria employing a response surface method though the interactions between variables might be extremely important in fermentations employing immobilized bacteria.

The levels of parameters were selected based on the results obtained with Ca-alginate immobilized cells of *Pseudomonas* sp. Nevertheless, the maximum level of the substrate was limited to 1.5% (w/v) because, at concentrations above this, it is evident from the earlier discussions (section 5A.3) that the efficiency for substrate utilisation reduces drastically even though there is some increase in enzyme production. Optimisations were performed for the three support systems independently as it is known that the adsorbed quantity of the microorganism varies with different supports (Navarro *et al*, 1976), and also the physiological state of the organism might be different upon adsorption on to different supports (Novarro & Durand, 1977., Fletcher & Marshall, 1982). Physicochemical conditions at the surfaces are known to induce altered genetic responses in immobilized bacteria (Goodman & Marshall, 1994) and these conditions are presumably different for the different supports used in the experiment. Hence, it was considered important to optimise the process variables for the three different carrier systems though all of them contained the same *Pseudomonas* cells adhered to the surface. The experiments were designed according to Box and Behnken (1960) with five parameters at three levels and the results obtained were used to fit a quadratic polynomial model using Design -Expert® package. The response was subjected to analysis of variance to determine the parameters with significant effects and the interactions between them were determined by analysis of three-dimensional response surfaces. Ideal levels of the variables that could give maximum product yield were determined using computer aided optimisation.

The parameters with significant effects on L-glutaminase production were found to be pH of the medium and incubation temperature for the polystyrene system, whereas, for the PUF and Nylon web systems, glutamine concentration and the interaction between yeast extract concentration and temperature had significant effects respectively besides temperature.

pH is reported to have a dominant effect on the adsorption of microbes on supports (Navarro, 1975) and with most support -microbe combinations there exist an

ideal pH range for efficient cell attachment on the carrier surface. It is also known that the organism synthesises more glutaminase while adsorbed to polystyrene than in the free suspension (Keerthi, 1999). One of the possible reasons for the observed decrease in enzyme activity at pH extremes is cell desorption from the supports at these pH ranges. With all the three support systems, it was also noted that the interactions with other operational variables did not affect the nature of pH dependence for enzyme production by immobilized cells. Irrespective of the different levels of operation temperature, and concentrations of glutamine, glucose and yeast extract, the optimum pH range for maximal enzyme production was found to be 5.5-6.5. A medium pH of 6 was optimal for L-glutaminase production by the organism under submerged and solid state fermentation (Keerthi, 1999) and was also found to be the optimum for salt tolerant glutaminase production by yeast (Sato *et al*, 1999). It is evident from the observations that the interaction between process variables and the cell-matrix interactions did not affect the nature of pH dependence of cells for enzyme production, which remained the same as that for free cells. Such a similarity in optimal pH for product formation by free and immobilized cells is not uncommon and was reported for citrulline production by *Pseudomonas putida* (Yamamoto *et al*, 1974) and malic acid production by *Gluconobacter melnogenus* (Divies, PT-Fr. 2320349,1975)

Unlike pH, the optimal temperature range obtained was different for the different support systems. Nevertheless it remained between 30-35 ^{0}C for all the carrier systems tried. For the polystyrene and polyurethane foam systems, the optimal temperature range was 30-32.5 ^{0}C, whereas a wider range (30-35 ^{0}C) was recorded for the nylon web system. Apart from this difference due to cell-matrix interactions, the dependence on temperature for enzyme production by the immobilized cells remained unaffected. For a given support system, the interaction of operating temperature with other process variables had least impact on the optimal temperature range. The optimal operating temperature was lower than that obtained for free cells under submerged fermentation or for the cells adsorbed on polystyrene under solid state fermentation (Keerthi, 1999), which in either of these cases was found to be 35 ^{0}C. An incubation temperature of 35 ^{0}C was also found to be ideal for glutaminase production by marine *Vibrio costicola* under SSF on polystyrene as support (Nagendraprabhu & Chandrasekaran, 1997). However, in both these studies the temperature range tested

had a 5 ^{0}C difference between adjacent levels and the real optimum would have been masked by the lack of data on enzyme production at temperatures between the tested levels.

The existence of wider temperature optima for glutaminase production by cells immobilized on nylon web might be due to the influence of process temperature on cell growth. It may be noted from the earlier discussions (5B.1.2) that the adsorbed biomass was comparatively lower for the NW system in the EPM, and this probably is due to cell desorption from the matrix. Keerthi (1999) has reported 35 ^{0}C as the optimal growth temperature for *Pseudomonas* sp BTMS-51. At higher temperature the metabolic activities of bacteria increase resulting in enhanced protein synthesis and cell division resulting in an enhanced growth rate. Increase in temperature, below a critical level, therefore can bring about rapid growth probably replacing the desorbed cells and maintaining a high cell load. This coupled with the cell-matrix interactions might probably account for the slight increase in optimal temperature range observed for the NW system. Cell matrix interactions might be the dominant factor in this effect and the specific reasons, and physiological changes due to such interactions, which can bring about differences in behaviour of immobilized cells, could not be determined from the present understanding of the process.

In general, pH and temperature exerted their influence on enzyme production by immobilized cells independent of the other process variables for a given system of carrier matrix, and the optimal values of each were almost similar if not same as that for the free cell systems. Chandrasekaran *et al* (1991) has reported that these factors are largely characteristic of the organism and is independent of the nature of fermentation system or medium constituents. Unlike the influence of medium constituents, on a few metabolic pathways and probable indirect effects on growth through a cascade of cellular events, the effect of environmental parameters are more complete and affect the whole of cell metabolism equally. Each species or strain might be evolved for life in a particular environment with the whole cellular metabolism streamlined to fit that environment and the adaptations are built into them at the genetic level, which probably is the reasons for the consistency in dependence of an organism to these factors. It is often observed that such adaptations to a particular

environment are so complete that the organism fails to thrive in a different environment as is the case with many extremophiles.

The cell-matrix interactions in the different support systems probably affected the dependence of immobilized cells on substrate concentration. In general, increase in glutamine concentration resulted in a higher enzyme yield in all the systems and interactions with pH of the medium or incubation temperature could not alter this dependence. At all the pH and temperature ranges tested, the enzyme production increased with increase in glutamine concentration irrespective of the carrier type. However, the interactions with glucose and yeast extract concentration gave different results in the different support systems. In the PUF and NW systems, the effect of glutamine was dominant to glucose, and at all the concentrations of glucose, an increase in yield linear with increase in glutamine concentration was obtained. This implies that the minimal glucose concentration tried was sufficient to meet the energy requirement of the immobilized cells and the concentration of glutamine became the limiting factor in enzyme production employing these carrier systems. With the PS system, it may be assumed that the concentrations of both glucose and glutamine become limiting factors and therefore a higher enzyme production was obtained at high concentrations of both. Dependence of immobilized cells for glutaminase production on substrate concentration at varying levels of yeast extract showed that in the NW system while the effects of glutamine dominate at low yeast extract concentrations, at high yeast extract concentrations, it had lesser effects. The observed increase in yield with increase in glutamine concentration at lower yeast extract concentrations might be due to an increase in synthesis of the enzyme while the increase in yield at higher yeast extract concentration is probably because of an increase in the biomass. At high yeast extract concentrations the cell growth is enhanced resulting in a higher cell load and a consequent increase in enzyme yield though the specific productivity is probably the same or even lower. Also under these conditions an increase in glutamine concentration has probably no effect on enzyme synthesis as the cells preferentially utilises yeast extract for growth. But it was also noted that at high yeast extract concentrations, the yield decreases with increase in glutamine concentration, which could not be explained, by the increase in biomass. The possible existence of two independent glutaminases in *Pseudomonas* one of which is probably a constitutive

enzyme is not ruled out as it was observed that a basal level of glutaminase activity was noted in medium even without addition of glutamine (Keerthi, 1999). Also two activity peaks were obtained for the enzyme upon purification studies using gel filtration which was confirmed by native polyacrylamide gel electrophoresis (Keerthi, 1999). Hence, it may be assumed that at high yeast extract concentrations, the former type is expressed, and the increased yield is due to the presence of higher biomass, though the specific productivity may be low. Whereas, at low yeast extract concentration, with a high concentration of glutamine, the inducible glutaminase might be expressed at a higher level, despite the system having a lesser biomass.

The effects of glucose and yeast extract concentrations were also different for the different systems and the interactions of either of these with pH, temperature and glutamine concentrations have been discussed under interactions of the above said parameters with them. The interaction between concentrations of glucose and yeast extract was very different for the three support systems. With the PS system, it was observed that irrespective of the concentration of yeast extract, the optimal glucose concentration was between 1 and 1.25% (w/v), and yeast extract concentrations below 0.13 % (w/v) was best suited for enzyme production regardless of glucose concentration. This implies that the energy source and nitrogen source was required at an optimal concentration to effect maximal enzyme synthesis. Probably the effect was exercised at the level of cell maintenance requirements on these nutrients. In the PUF system, the cell adsorption was intense, and also, the highest cell loading was recorded when PUF was used as the support matrix. Cells immobilized on PUF showed an absolute dependence on glutamine for enzyme synthesis and the increase in glucose or yeast extract concentrations resulted in a decreased enzyme production, though the decrease was marginal. Presumably, higher concentration of these nutrients shifted the equilibrium from the enzyme production phase because of an easy availability of the nitrogen and energy sources. NW system showed the highest cell desorption and this implied that cell growth was essential along with enzyme production to maintain a high cell load on support. While the increase in the availability of either of the nutrients resulted in a better yield, the yields were lower when both the nutrients were available in plenty. Probably under such a situation, the requirements of both carbon and nitrogen sources are met and there do not exist a need to utilise glutamine, and hence

synthesise the enzyme. When either carbon or nitrogen source is limiting, the cells might use glutamine instead resulting in a higher yield.

The optimal conditions for maximal enzyme production by *Pseudomonas* cells immobilized on the three supports were calculated using computer aided optimisation method (Design-Expert® package). Though the ideal temperature range of operation was found to be between 30-35 ^{0}C, the optimisation was carried out by fixing the temperature at 35 ^{0}C which was the temperature optimum for maximal growth of the organism. This was considered important, so as to maintain the growth of the organism at higher levels to make up for the loss of cells from the support matrix due to desorption. The optimised production conditions are presented in Table 5.3

Table 5.3

Optimised conditions for glutaminase production by *Pseudomonas* sp. Immobilized on polystyrene, polyurethane foam and nylon web

Support matrix	pH	Temperature (^{0}C)	Glutamine Conc: (% w/v)	Glucose Conc: (% w/v)	Yeast extract Conc: (% w/v)
PS	6.33	35	1.5	1.5	0.11
PUF	6.01	35	1.5	0.5	0
NW	6.06	35	1.5	1.42	0.02

Validation of the optimised production conditions under batch fermentation studies in shake-flask cultures revealed that the enzyme yield was considerably lower than the predicted values for the polystyrene and PUF systems. The predicted yields were 24.7 U/ml and 41.82 U/ml respectively, whereas the observed yields in the optimised conditions were 15.51 U/ml and 22.47U/ml. However, the decrease in yield from the predicted values for NW system was low (16.35%). Park and Reardon (1996) have also observed such differences in the predicted and observed yields for recombinant protein production by *Bacillus subtilis*. In this case the large differences might be due to the fixing of operation temperature at 35 ^{0}C, though the ideal operating temperature was found to be between 30 and 32.5 ^{0}C for the PS and PUF systems. The differences in these values for the NW system was lower, probably because in the nylon system the ideal operating temperature range was wider (30-35 ^{0}C). However, the selection of the operating temperature, which suited maximal cell growth did had

its advantages in terms of operational stability, since it was found that upon repeated batch operations and continuous operation employing the cells immobilized on these supports, there occurred no reduction in cell load. Thus, though the constraint of operating temperature fixation may seem to be disadvantageous in terms of the quantity of enzyme produced, it actually may not be the case, since it can increase the operational stability and consequent reduction in the number of reactivation treatments. This implies a reduced down time, and a probable reduction in the cost of production due to a lesser demand for frequent reactivation cycles.

Much of these studies form primary data on the effect of interaction of medium constituents and the interactions between cells and the surface of carrier matrices on extracellular enzyme production by an immobilized marine bacterium. Even with the widely studied systems, the exact nature of such interactions is largely uncharacterised. The alterations in physiology of the adsorbed organism and alterations in surface properties of adsorbent upon microbial adhesion is widely agreed upon (Hahn-Hagerdal, 1989., Dagostino *et al*, 1991., Marshall, 1994.,) and is different for the different microorganisms and support matrices. The foregoing discussions were largely based on the current knowledge of the subject and an extensive understanding of the observations and interpretations of the results at the cellular level requires further studies on the interactions at the molecular level. The altered image of biofilm structure has enormous implications in the assessment of mass transport to and from biofilms, especially in terms of the optimisation of biotechnological processes based on biofilm activity.

5B.2 PRODUCTION OF L-GLUTAMINASE BY *PSEUDOMONAS* SP. IMMOBILIZED ON INERT SUPPORTS UNDER REPEATED BATCH OPERATION

Repeated batch operations have resulted in a sharp decrease in activity of the immobilized cells following initial few cycles, in several operations (Ohlson *et al*, 1980., Sonomoto *et al*, 1983). Moderation of this decline could be effected partially by introducing interim reactivation treatments in richer medium (Sonomoto *et al.*, 1982, 1983., Dalili & Chau, 1988). Such decline in activity of the immobilized cells is probably due to the limitations imposed by the production medium and operation

conditions. The medium used for production of metabolites may not essentially be the one which support cell growth (Stanbury *et al*, 1995). The production media are often designed so as to obtain a maximum product yield and in many cases lacks nutrients and growth factors compared to the growth medium used for cultivation of the organism. Though this is advantageous in terms of limiting the quantum of unwanted by products and free cell growth in immobilized systems, it may also result in a rapid or gradual decline in the cell load eventually reducing the product yield and operational stability. A selection of medium components and operation conditions that simultaneously satisfies requirements of a critical level of cell growth along with enzyme production have facilitated extended operation for several cycles in repeated batch operation without reduction in cell load or enzyme yield. Yeast extract was included in the production medium for PS and NW systems with the intention of supplementing growth requirements and an operation temperature of 35 ^{0}C was selected despite the optimum for enzyme production being different, for supporting cell growth so as to make up for cell loss from the supports.

By the use of a medium, which could support the cell growth as well as enzyme production, the three support systems, showed extended operational stabilities. The enzyme production in all the cases remained fairly consistent and did show any decay in productivity with increase in the cycles of operation. Though there was cell desorption from the supports, the biomass concentration on PS was found to increase after each cycle of operation and attained an equilibrium value higher than the initial concentration after few cycles of operation. In PUF system, the cell load dropped initially and then increased and maintained a consistent concentration for the rest of the cycles though this concentration was lower than the initial. With the NW system also there was an initial drop in cell load, which was not as steep as in PUF. However, cell growth upon the support allowed a rapid regaining of the lost cells with subsequent cycles of operation and after few cycles there was an increase in adsorbed cell mass from the initial values which probably attained an equilibrium so that it remained stable afterwards. This when related to the yeast extract concentration in the medium can explain the observed pattern of cell loading in the different batches of operation for each support system. It may be noted that PS system received a medium with maximum yeast extract concentration and this would have resulted in the increase in

adsorbed biomass with the initial few batches of operation, until it reached an equilibrium. PUF system on the contrary received no yeast extract at all and hence the biomass declined to a value, which could be supported by the production medium. Finally for the NW system, the concentration of yeast extract was not as high as that for PS system and therefore the difference between equilibrium biomass concentration and initial values of it were not large compared to PS. In any of the carrier systems tried, the enzyme yields were consistent over the 20 cycles of operation attempted and the biomass concentrations remained above a critical concentration suited for each combination of medium and carrier system. However, it was observed that the PUF and Nylon systems were efficient in terms of a consistent higher yield (18.79 and 17.99 U/ml respectively) compared to the polystyrene system. Among these PUF was marginally better in terms of activity and efficiency. Nevertheless, the adsorbed biomass on PUF was reduced from the initial concentration after few cycles and though any effect of this is not evident in the data for the 20 cycles of operation, in a long term consideration this might result in a further decease due to cell loss unless regeneration steps are incorporated in between. For NW system due to a higher cell loading at equilibrium than the initial, the frequency of regeneration step can be reduced if not eliminated completely. Operational stability over several cycles with operation time over 200h were reported for repeated batch production of cellulase (Linko *et al*, 1996) and lignin peroxidase (Linko, 1988) with nylon web as carrier.

5B.3 CONTINUOUS PRODUCTION OF GLUTAMINASE BY *PSEUDOMONAS* SP CELLS IMMOBILIZED ON INERT SUPPORTS

5B.3.1 Immobilization of cells on the bed of support matrix packed inside the reactor.

The adsorption approach of immobilization allows cell detachment and relocation with potential establishment of equilibrium between adsorbed and freely suspended cell populations even at early stages of cell culture. This is often highly advantageous as this dynamic equilibrium maintains an active cell population throughout the operation, which increase the efficiency and operational stability of the system. Recirculation of an inoculated growth medium through a packed bed of support matrix is commonly employed in the immobilization of microorganisms by

adsorption on these matrices. The widely known examples are reactors used in vinegar production and the trickling bed filters used for wastewater treatment. The method was employed for immobilization of yeast on a bed of sugarcane bagasse in a packed bed reactor (Cheung *et al*, 1986). Hollo *et al*, (1990) incubated a culture of *Pseudomonas aeruginosa* with PVC sheets in a column reactor for effecting immobilization by adorsption on the plastic which was employed in removal of heavy metals from waste water.

It was observed that in any of the three support systems used in the PBR, the cell concentration adsorbed on the carrier bed increased with increase in circulation time and attained their equilibrium values around 18h of circulation, which corresponds to the mid log phase of the culture upon free growth. This implies that irrespective of the carrier type, 18h is the time required for attaining equilibrium between adsorption and desorption of cells from the matrix in the given medium. Beyond 18h fluctuations did occur in the observed biomass on support (Table 5.4) and highest cell loading was not always obtained at exactly 18h. Nevertheless, the values did not deviate much from the mean values in each individual system and the equilibrium concentration of cells was maintained through out the time of study (36h). However, the equilibrium cell concentrations were different for the different support systems and were in the order PS<PUF<NW.

Table 5.4

Comparison of cell adherence on beds of polystyrene, polyurethane foam and nylon in a packed bed reactor upon circulation of inoculated growth medium

Circulation time	Biomass on support (μg/cc)		
(h)	PS system	PUF system	NW system
0	0.00	0.00	0.00
6	161.72	125.70	154.53
12	198.03	219.54	288.19
18	196.38	206.66	293.28
24	235.98	235.25	302.44
30	222.78	218.91	308.81
36	212.88	215.39	297.10

A number of factors are known to interact in the adsorption of the microorganisms, which involves characteristics of adsorbent, microorganism, and environment. Since the same medium and conditions were used in all the three support systems the quantity of adsorbed cells is apparently decided by the nature of support system. Navarro *et al* (1976) has observed that the adsorbed quantity of a kind of microorganism varied for different supports. The higher equilibrium cell concentration obtained with PUF and NW is accomplished due to the macroporous nature of these carriers. Recirculation of an inoculated medium with air supply and mixing effected in an external loop was thus found to be an efficient method of immobilizing *Pseudomonas* sp. cells on these carrier matrices. Though the minimal circulation time required for achieving the equilibrium adsorbed cell concentration was 18h, for further studies on enzyme production employing the packed bed reactors a circulation time of 36h was used so that a stable equilibrium of adsorbed cell population develops on the matrix surfaces.

5B.3.2 Enzyme production in PBR - effect of operational parameters on reactor performance

The steady state achievable in continuous processes is often advantageous in increased biomass and metabolite productivity (Hospodka, 1966., Trilli, 1990). Cell concentration, substrate, product, and by-product concentration should remain constant throughout the fermentation and therefore the demands of the fermentation in terms of process control are also constant. Theoretically the fermentation to produce a metabolite should be more productive in continuous culture than in batch because a continuous culture may be operated at the dilution rate which maintains product output at its maximum, whereas in batch culture product formation may be a transient phenomenon during fermentation (Stanbury *et al*, 1995).

Under steady state conditions, the specific growth rate is controlled by the dilution rate, which is an experimental variable. Though the increase in dilution rates results in an increase in productivity, it shall also be noted that a continuous culture may be operated only at dilution rates below the maximum specific growth rate of the organism, since further increase in dilution rates would result in a total cell washout from the reactor. Thus within certain limits, dilution rates may be used to control the

growth rate of the culture. The cell growth in a continuous culture of this type is controlled by the availability of a limiting chemical component of the medium and the system is described as a chemostat. The control effect of dilution rates on chemostat culture is essentially described by the Monod (1942) equation. Immobilization of the microorganisms is an effective way to overcome the limitations in the use of higher dilution rates because under the immobilized conditions cell wash out is almost completely eliminated. Adsorbed cells when used in continuous culture presents a system with the advantage of self-regulation, characteristic of the chemostat together with that of high cell loading and reduced problem of cell washout. Freeman & Lilly (1998) observed that unlike gel entrapment the adsorption approach allows cell detachment and relocation and an equilibrium between adsorbed and freely suspended cells is established even during the early stages of operation.

If, in a continuous process operating as a chemostat, the specific rate of product formation is strictly related to the specific growth rate, then as the dilution rate increases so will the specific rate of product formation (Stanbury *et al*, 1995). If the specific rate of product formation is independent of the specific rate of growth, then it will be unaffected by dilution and thus concentration of product will decrease with increasing dilution but output (Productivity) will remain constant. If product formation occurs only within a certain range of growth rates then a more complex relation ship is produced. From the above considerations, a chemostat process for the production of a metabolite can be designed to optimise either output (productivity) or product concentration. Heijnen *et al* (1992), pointed out that when specific rate of product formation is growth related, the advantage of high productivity obtained at high dilution rates must be balanced against the disadvantage of low product concentration resulting in increased downstream processing costs. Another important factor in selection of the operation conditions is the concentration of limiting substrate in the medium especially when the substrate is costly, and a few workers (Sanchez *et al*, 1996) have preferred to operate under conditions of maximum substrate conversion though it may not always be that which give high output or product concentration.

Since a study on continuous production of an extracellular enzyme by marine bacteria immobilized by adsorption on inert carriers has never been reported, the conditions which gave maximal product concentration, productivity and substrate

conversion were evaluated in order that the system may be operated so as to satisfy any of these objectives. Back-flow of the cells from the reactor can result in cell growth within the medium reservoir and several measures have been suggested to prevent growth of microorganisms into the feed line. Stafford (1986) suggested the introduction of drip tube into the medium feed line to break the flow path for this purpose but in several instances this may result in plugging of the flow path by insoluble medium constituents and also the uniformity in flow is disrupted. Further, such an arrangement is possible only when the medium reservoir is at an elevated position. A down flow reactor configuration was deliberately used in the studies to prevent the back flow of medium from reactor and to maintain uniformity in flow rates. The medium optimised for batch production of glutaminase cells adsorbed on to polystyrene, polyurethane foam and nylon web was used for the continuous production studies with the variations in substrate concentrations.

5B.3.2.1 Effect of dilution rates on reactor performance

In reactor with polystyrene or nylon web as the packing material, an increase in flow rates could bring about an increase in volumetric productivity up to a flow rate of 120 ml/h (dilution rates 1.88 h^{-1} and 1.01 h^{-1} respectively) at any concentration of substrate in the medium. Though the flow rates tried were same, it should be noted that the dilution rates were different due to differences in voidage of the two systems, and PS system gave better productivities and yield at higher dilution rates compared to the NW system. At identical substrate concentrations the reactor with PS bed also gave better enzyme yields at all the dilution rates tried. This is quite an unexpected deviation as the Nylon web system supported a better cell adsorption and higher cell load compared to the PS under batch experiments. The observed deviation could have been a result of cell growth within the interstitial spaces of the polystyrene beads when used as the packed bed. The decrease in volumetric productivities at the highest dilution rates tried (2.19 h^{-1} and 1.18-h^{-1} respectively for PS and NW) for the two systems might be the consequence of the reduction in enzyme production due to a lesser contact between the substrate and immobilized cells at higher dilution. Nevertheless this effect was not observed with the PUF system which had a greater porosity compared to NW

and larger pore size compared to PS. Due to the occurrence of a relatively increased area of contact surface, the contact would have been more effective in the PUF system. Moreover, compared to the PS system, the PUF had a lower dilution rate (1.78-h^{-1}) at the highest flow rate tried. The highest product concentrations were also recorded for the PUF system, at all the flow rates, which also might be a consequence of increased porosity and resultant reduction in diffusional limitations. For a given system of carrier as bed material, the product concentration (enzyme yield) was found to decrease with increase in dilution rates at any given substrate concentration, and the lowest yield was observed at the highest dilution rate. This is a general characteristic of the chemostat operation and it is suggested that the specific rate of product formation and product output will behave in the same way as biomass concentration within the system when specific rate of product formation is related to specific growth rate (Stanbury *et al*, 1995). The mechanism underlying the controlling effect of the dilution rate is essentially the dependence of the system on substrate availability and the relationship is evident from the Monod equation ($\mu = \mu max\ S/ Ks + S$, where μ = specific growth rate, μmax is maximum specific growth rate, S is the residual substrate concentration and Ks is the substrate utilisation constant numerically equal to substrate concentration when μ is half μmax) for specific growth rate (Monod, 1942). It follows that at steady state, as specific growth rate is equal to dilution rate, the substrate concentration in the medium and hence the availability of substrate for the cells is determined by the dilution rates. Although an uncoupling of the production from growth, is generally a characteristic of the immobilized cell fermentations (Kuek, 1986., Ramakrishna & Prakasham, 1999), it may not essentially be true, at least in the case of physically adsorbed cells. Cell detachment and relocation frequently occurs on the adsorbent (Freeman & Lilly, 1998), and it is proposed that the appearance of free cells in the medium is not due to desorption alone, but is also a consequence of synchronous growth of immobilized cells along with release of one offspring following cell division (Hattori *et al*, 1972., Navarro and Durand, 1980). Hence, it may be proposed that the specific rate of product formation in these systems is related to the specific growth rate, and the behaviour of the system follows the general kinetics of product formation in a chemostat. Substrate conversion efficiency was reduced with reduction in contact times (increase in dilution rate) in all the support systems. Decrease of the residence

time was found to reduce substrate utilisation in many continuous operation experiments (Lee *et al*, 1983., Ramakrishna *et al*, 1990). The lower product yields at higher dilution are directly correlated to the lower substrate utilisation, and this in turn is due to the close relation of it with the specific growth rate.

5B.3.2.2 Effect of substrate concentration on reactor performance

The growth and metabolite production in a chemostat is controlled by the availability of limiting nutrient in the medium (Stanbury *et al*, 1995). The dynamic equilibrium established in a chemostat culture is a consequence of the interplay of the cell concentration and substrate availability dictated by the dilution rate at a given substrate concentration. Higher substrate concentrations often increases the range of operable dilution rates which can have positive effects on reactor productivity.

It was observed that in all the carrier systems studied, an increase in substrate concentration resulted in increased enzyme yield (product concentration) and volumetric productivity (reactor output). The increase in product concentration and productivity was most evident in the PUF system, probably due to a better porosity and the highest value for productivity was recorded at the highest substrate concentration and dilution rate for this system. In both the PS and NW systems, the highest dilution rate could not support the highest productivity even at the highest substrate concentration. It follows that, though further increase in dilution rates might result in a better output for the two systems, if the concentration of substrate is increased beyond the attempted levels, it can also lead to a very low percentage of substrate utilisation. This is evidenced by the fact that there was a considerable reduction in percentage of substrate conversion at the high substrate concentrations. Higher productivities under conditions of high substrate concentration and dilution rates are achieved at the cost of substrate remaining unutilised, which may not be desirable, at least in the production of glutaminase, as the limiting substrate used in the present study (L-glutamine) was costly compared to the usual media constituents.

The conditions for maximum yield were a high substrate concentration and low dilution rate, those for maximal productivity were a high substrate concentration and dilution rate, and for maximal percentage of substrate conversion were a low substrate

concentration and low dilution rate. Ultimately, it depends upon the operator to decide whether to operate at the maximum product concentration, productivity, or substrate conversion. However a balance between the advantage of high productivity at high dilution rates and the disadvantage of low product concentration have to be often made (Heijnen, 1992).

A comparison between the three packed bed reactor configurations would not be judicious as the dilution rates were different for the different bed materials notwithstanding the fact that the same bed heights and flow rates were used for the three. Nevertheless, it may be perceived that under the given set of operational conditions the reactor with PUF as bed material was the better performer in terms of product concentration and productivity, whereas the PS system was best in terms of substrate conversion. Polystyrene system though may seen to be better than nylon, in the light of these observations, may not actually be so because upon prolonged operation clogging of the interstitial spaces with dead cells and cell debris can occur. Washing out of cell debris and desorbed cells maintains an active cell population in the PUF and Nylon systems for an extended time.Whereas, in PS this may not happen as the dead cells accumulate in the spaces between the beads which is also serving as channels for medium flow. It may be expected that the PS system continue to support higher cell growth and resulting increase in productivity until space limitations begin to occur and this was found to be true upon extended operation of the three systems.

5B.3.3. Operational stability of the reactors

The extended operational stability of the three reactor systems were evaluated under conditions that gave maximal substrate conversion efficiency for a total period of 72h in each case. As for the Ca-alginate immobilized cells, operation under a low substrate concentration did not affect the operational stability for the tested duration of operation. Consistent average yield of 12 U/ml, 13.72U/ml, and 13.18U/ml were obtained respectively for the PS, PUF and NW systems, and there was no decay in product yield after the 72h of operation. The average volumetric productivities for the systems were 11.28, 10.43 and 6.72 U/ml/h respectively.

The pores in PS are very small and easily clogged which will impose diffusional limitations eventually resulting in cell death in the inner surfaces. Channelling is a possible occurrence and consequently a lesser contact time may be expected in the PS system. Despite such possibilities the system performed better than NW and the enzyme yield and productivity was found to increase gradually with the duration of operation confirming the argument that cell growth does occur in the interstitial spaces of the beads. However, the 72h-operation period was not sufficient to observe the clogging effect. Prolonged use of immobilized viable cells often involves potential continuous cell growth along with parallel cell death and lysis (Hamer, 1990., Karel *et al*, 1990). Cell supporting matrices should therefore be permeable to exchange of whole cells and cell debris with the surrounding medium. PS could not be considered a better support than NW in view of these discussions. In fact some of the longest stable operation ranges for extracellular enzyme production by adsorbed cells were reported with Nylon web as the carrier (Linko, 1988., Linko *et al*, 1996). Higher cell loading could be achieved in Nylon web by chemical treatments, which would pose it as a better carrier.

It may be concluded that any of these carrier matrices could effectively be used as packing material in a column reactor for continuous production of glutaminase with extended operational stability. Although the yields obtained were not high compared to that obtained under continuous operation with Ca-alginate immobilized cells, stability is a major constraint with the Ca-alginate beads and unless given interim stabilisation treatments, bead expansion and weakening is inevitable. Hence, immobilization by adsorption on inert supports could be a better choice for prolonged continuous operation and production of glutaminase by the marine *Pseudomonas* sp.

6. SUMMARY AND CONCLUSIONS

Pseudomonas sp. BTMS-51 isolated from the marine sediments of Cochin coast and available in the culture collection of the Department of Biotechnology, Cochin University of Science and Technology was used in the present study. The bacterial cells were immobilized by gel entrapment in Ca-alginate and by physical adsorption on three nutritionally inert supports - polystyrene, polyurethane foam and nylon web. The conditions, which favour maximum enzyme production by immobilized cells, were optimised under batch mode, and the efficiency for enzyme production was determined under the optimised conditions. Repeated batch operations were performed to evaluate the potential for reuse of the immobilized cells. The operational conditions which supported maximal enzyme yield, productivity and substrate conversion were standardised for continuous operation in plug flow reactors and operational stability was determined by extended operation for a minimum of 72h.

The optimal conditions for production of stable Ca-alginate immobilized beads were standardised initially. 3% (w/v) sodium alginate concentration, a 1% (w/v) concentration of 18h old cells, 0.15M $CaCl_2$, and 2h curing time were ideal for production of stable beads with maximal enzyme yield. The ideal medium for bead activation was 1% glutamine in aged seawater, and conditions optimal for bead activation included pH 5, 28 $\pm$ 2 ^{0}C, and 12h incubation. Enzyme production by the Ca-alginate immobilized beads was most effective in seawater glutamine medium containing 2% (w/v) glutamine and 1% (w/v) glucose in aged seawater. A medium pH of 6, incubation temperature of 35 ^{0}C, and retention time of 12h supported the maximum enzyme yield. Incorporation of nitrogen sources other than glutamine in the medium led to a decrease in the enzyme production. Seawater with the minimal added nutrients could serve as an efficient enzyme production medium by the Ca-alginate entrapped cells.

Consistent enzyme yield (21-33U/ml) was obtained over the 20cycles of repeated batch operation and there was no decay in enzyme production indicating the suitability of entrapped cells for repeated use.

Upon continuous operation in a packed bed reactor or circulating bed reactor, increase in dilution rates generally resulted in a reduction in the yield and percent

substrate conversion but at the same time, an increased volumetric productivity. Increase in substrate concentration could bring about an increase in yield and productivity and a reduced rate of substrate conversion. The operation conditions for maximal product yield were 2% (w/v) substrate concentration and a dilution rate of 0.64-h^{-1} for the PBR, while it was 2% (w/v) substrate concentration and a dilution of 0.24-h^{-1} for the CBR. The conditions that supported maximal productivity were a dilution rate of 1.27-h^{-1} and 0.56-h^{-1} respectively for PBR and CBR, at a substrate concentration of 2% (w/v) for both. Maximal rate of substrate conversion was at a substrate concentration of 0.5 % (w/v), and at dilution rates 0.64-h^{-1} and 0.24-h^{-1}, respectively for PBR and CBR. Both the reactors showed an extended operational stability with out decay in productivity.

Physical adsorption of the cells on inert supports was achieved through growth of the cells in an immobilization medium containing the support particles. The medium that supported maximal adsorption of cells was Zobell's broth irrespective of the type of support. Incubation time required for maximal cell adsorption was identical (36h) for the three supports. Initially, mineral salts glutamine medium (MSG) and seawater glutamine medium (SWG) was tested as the basal enzyme production medium and the ideal retention time for maximal enzyme production was determined. SWG was the better medium in terms of enzyme yield and 12h was the ideal retention time.

The medium composition and conditions for maximal enzyme production by cells immobilized on the three supports were optimised using a response surface Box-Behnken experimental design. The interactions between the various operational parameters were determined from computer generated three dimensional response graphs.

When polystyrene (PS) beads were used as the support system for immobilization, temperature had a statistically significant effect on enzyme yield. pH of the medium and operation temperature had effect independent of other parameters and the optimal range was found to be pH 5.5-6.5 and 30-32.5 ^{0}C for maximal enzyme yield. Interactions between other parameters produced differences in enzyme yield by the immobilized cells. The optimal levels of the operational parameters were determined, while fixing the operation temperature at 35 ^{0}C, using a software package and was pH 6.33, 1.5 % (w/v) glutamine n, 1.5% (w/v) glucose and 0.11%

(w/v) yeast extract. The observed enzyme yield 15.51 (U/ml) was 37.21% lower than the predicted yield (24.7U/ml) as validated by batch operation in shake flasks.

Temperature and glutamine concentration had significant effects on enzyme production by cells immobilized on polyurethane foam (PUF). The dependence of enzyme production by cells on temperature was independent of the interactions and the optimal operating range was 30-32.5 ^{0}C. The effect of glutamine concentration on enzyme production was different upon interaction with the different parameters. The optimal pH range was 5.5-6.5. Interactions between other parameters also affected the enzyme production by PUF immobilized cells. The predicated optimal conditions for maximal enzyme production were 1.5% (w/v) glutamine, 0.5% (w/v), and a pH of 6.01 when the operating temperature was fixed at 35^{0}C. Maximal enzyme production conditions required that yeast extract be absent form the medium. The production conditions were validated by batch studies and the observed enzyme yield was 46.27 % lower than that predicated.

Temperature, both alone and upon interaction with yeast extract concentration, had significant effects on enzyme production when nylon web (NW) was the adsorbent, for cell immobilization. pH dependence for enzyme production was unaffected by interactions with other parameters, and similar to polystyrene or PUF systems, the ideal range was 5.5-6.5. The optimal range of operation temperature was 30-35 ^{0}C. Enzyme production by cells immobilized on nylon web was influenced by interactions between other parameters. The predicted levels of operational parameters were pH 6.06, 1.5% (w/v) glutamine, 1.42% (w/v) glucose and 0.02% (w/v) yeast extract, for maximum enzyme yield, when the operation temperature was fixed at 35 ^{0}C. The observed enzyme yield of 22.92 (U/ml) was 16.35% lower than the predicted value (27.4U/ml).

Reusability of the cells immobilized by adsorption on the three supports was evaluated by repeated batch operation for 20cycles in each case. All the three systems supported consistent enzyme yield over the several cycles of operation and no decay in productivity or cell loading on the supports was observed upon prolonged operation. The mean enzyme yield obtained were 13.88, 18.79 and 17.99 U/ml respectively for the PS, PUF and NW systems.

Suitability of the cells immobilized on beds of PS, PUF and NW for continuous production of the enzyme was evaluated in a packed bed reactor. Bacteria

were immobilized on the bed surface by circulation of an actively growing culture aerated in an external loop, through the reactor column for 36h and enzyme production was achieved by circulation of production medium through the bed. The conditions that favoured maximum yield, productivity and rate of substrate conversion were determined subsequently.

Irrespective of the nature of support matrix, lower yield and percent substrate conversion rate were observed at higher dilution rates. Higher dilutions in general, resulted in increased volumetric productivities, which was also dependent on substrate concentration and availability. Concentration of substrate positively affected the yield and productivity, and showed a negative influence on the rate of substrate conversion.

Irrespective of the nature of support matrix, the conditions required for maximal enzyme yield under continuous culture were the lowest dilution rate and highest substrate concentration. Whereas, the conditions for maximal productivity were the highest substrate concentration and highest dilution rate for PUF and NW systems. For the PS system, the productivity decreased marginally at the highest dilution rate tried. Highest percentage of substrate conversion was obtained at the lowest substrate concentration and dilution rates in all the three systems.

Continuous operation of the reactors with any of the three bed materials was stable for an extended operation period of 72h and did not show any signs of decay in terms of enzyme yield or cell loading, indicating the potential for continuous production of glutaminase by cells immobilized on these support matrices.

Conclusions

Based on the data obtained in the present study, it is concluded that marine bacteria are ideal candidates for immobilization using either Ca-alginate entrapment or physical adsorption on to synthetic inert supports and the process of immobilization does not negatively influence them. Thus, Ca-alginate entrapment of the bacteria was found to be well suited for reuse of the biomass and extended operational stability during continuous operation. Adherence of the bacterium to inert supports was observed to be strong and it imparted minimal stress on the

immobilized bacterium and allowed detachment and relocation on the supports which enabled the formation of a dynamic equilibrium maintaining a stable cell loading. This is particularly desirable in the industry for extended operational stability and maintenance of consistently higher outputs.

Response surface analyses of the enzyme production data obtained with various combinations of process variables, throws more light on the nature of interactions of various operational parameters among themselves, and on extracellular enzyme production by immobilized marine bacteria. Further, it also enabled the investigation process to evolve a statistically sound optimisation, which consequently facilitated a near accurate recognition of optimal conditions for maximal enzyme production by immobilized marine bacteria.

Information generated on the continuous production of glutaminase by bacteria adsorbed on inert supports, in a packed bed reactor, indicate scope for further scale up and application in industrial production. Moreover, the data obtained on parameter interactions suggest a scope for further development of an ideal model for industrial production of extracellular enzyme employing marine bacteria.

It is proposed that marine *Pseudomonas* sp. BTMS-51 is ideal for industrial production of extracellular L-glutaminase and immobilization on to synthetic inert support such as polyurethane foam could be an efficient technique, employing packed bed reactor for continuous production of the enzyme.

May be the present study is the first of its kind in making several pioneering attempts with respect to marine bacteria in employing different immobilization techniques, synthetic carriers, reactor systems, use of sea water as fermentation medium, and response surface methodology for optimisation of operational parameters for production of extracellular enzymes.

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