PRODUCTION OF α-GALACTOSIDASE USING PACKED-BED REACTOR

A PROJECT REPORT

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BONAFIDE CERTIFICATE

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ABSTRACT

A packed-bed reactor (PBR) design was used for the purpose of continuous α-galactosidase production with *Aspergillus oryzae*, as a possible alternative to the traditional batch process using fungal cultures. Sodium alginate was used as a carrier to immobilize *A. oryzae* mycelia. Among the carbon sources tested for enzyme production, guar gum supported maximum α-galactosidase synthesis. α-Galactosidase enzyme production was studied at different alginate concentrations and bead diameter. The performance of the immobilized beads was also tested in a PBR at different air flow rates. To improve cell distribution throughout the column, part of the outflow was recycled. As a result continuous production in a PBR showed maximal productivity. The enzyme was optimally active at 50°C for the hydrolysis of p-nitrophenyl-α-D-galactopyranoside (PNPG). Both free and immobilized enzymes showed their optimal activity at pH 4.5. The kinetic properties of α-galactosidase of *A.oryzae* were also investigated using PNPG as substrate. K_m and V_{max} for soluble enzyme was found to be 1.33 mM and 0.70 U, respectively.

Keywords : packed-bed reactor, *A. oryzae,* α-galactosidase, p-nitrophenyl-α-D-galactopyranoside (PNPG).

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ABBREVIALIONS	EXPLANATIONS
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Millilitre
h	Hour
L	Litre
min	Minutes
PNPG	p-Nitrophenyl-α-D-galactopyranoside
M	Molar
mM	Millimolar
nm	Nanometer

1. INTRODUCTION

Enzymes are biocatalysts, which speed up the rates of reactions without undergoing any permanent change. Life on this planet is possible because of the presence of the enzymes, without which many of the reactions would not occur over a period of years. The term enzyme is derived from the Greek meaning 'in yeast' and was first used by Kuhne in 1878 to the extracts or secretions from whole microorganisms. The enzyme activity could be expressed even in the absence of an integrated cellular Buchner demonstrated structure in 1897. Emil Fischer first time proposed the specificity shown by an enzyme for its substrate in 1894. Sumner in 1926 for the first time crystallized the enzyme 'urease' from jack bean extract and has shown that it is protein in nature. X-ray crystallography was used in 1965 to deduce the three-dimensional structure of lysozyme, an enzyme cleaving certain bacterial cell walls. The catalytic activity of certain enzymes is regulated by the binding of small molecules (effectors) in response to changes in physiological conditions. Monod and his colleagues in 1965 to propose their 'allosteric model' to such enzymes.

1.1 α-Galactosidase [α–D-galactoside galactohydrolase, EC 3.2.1.22]

The disaccharide melibiose was hydrolysed by the crude enzyme preparations (melibioses) from bottom fermenting yeast (Fischer and Lindner, 1895). Weidenhagen (1928) who studied the specificity of action of the melibiose using a number of sugars having non-reducing terminal α–D-galactose residues and coined the name α-galactosidase to melibiose.

1.1.1 Specificity

α-Galactosidase exhibited a broad range of specificity and removes the terminal α-D-galactose attached by α-1,2-, α-1,3-, α-1,4-, and α-1,6-linkages (Dey and Pridham, 1972).

α–Galactosidase exhibits two types of activity. They are as follows:

A. Hydrolase activity, and

B. Transgalactosylase activity.

A. **Hydrolase activity**

The configuration of hydrogen and hydroxyl groups on any single carbon atom of a glycosidic substrate plays an important role on the hydrolytic action of particular hydrolases. The hydrolytic action of α-galactosidase on its substrate depends on two main factors, which are as follows:

i) The ring structure of the substrate must be pyranoid, and

ii) The configuration –H and –OH groups on carbon atoms 1, 2, 3, and 4 must be similar to that on α–D-galactose.

B. Transgalactosylase Activity

Blanchard and Albon (1950) for the first time reported the transferase properties α–galactosidase from yeast. They found that galactose from one melibiose was transferred to a second melibiose acceptor molecule leading to the formation of manninotriose. This transgalactosylation property of α-galactosidase was studied extensively with respect to the specificity of galactosyl donor and acceptor specificity, donor and acceptor concentration, pH, temperature and the source of enzyme (Dey and Pridham, 1972).

1.1.2 Polysaccharides

Galactomannans are found as a major component of the endosperm in the seeds of many plants. Galactomannans consists of a backbone of β-(1,4)-linked D-mannosyl residues to which single α–(1,6)-linked D-galactosyl groups are attached. α–D-Galactopyranosyl residues from galactomannan are released during seed germination and serves as energy source for the growing seedling (McCleary *et al*., 1981). The removal of terminal α–D-galactopyranosyl moieties of blood group-B substances by α-galactosidase to type-O has been reported for a limited number of enzymes, including coffee bean α–D-galactosidase (Harpez *et al*., 1975).

1.2 Mechanism of action of α-galactosidase

A plot of log V_{max} versus Hammett constants (σ) results in two sets of straight lines indicating the possibility of substituent in the aromatic ring. Later, Dey and Malhotra (1969) have identified the presence of basic and acidic groups at the active by kinetic studies as carboxyl (deprotonated) and imidazolium (protonated) groups. On the basis of the above results, Dey (1969) has reported two alternative mechanisms for the action of sweet almond α–galactosidase, i.e., two-step mechanism and one-steep mechanism. Mathew and Balasubramaniam (1987) have proposed a new mechanism of action of α-galactosidase from coconut. The effect of pH on K_m and V_{max} values reflects the involvement of two ionizing groups with pKa values of 3.5 and 6.5 in catalysis. Chemical modification studies suggest the presence of two carboxyl groups, a tryptophan and a tyrosine, at or near active site of enzyme.

1.3 Raffinose - family Sugars

The oligosaccharides belong to raffinose-family sugars are raffinose, stachyose and verbascose. These sugars contain one, two or three galactose units joined to sucrose by α–1,6-linkages. The raffinose-family sugars have been identified as one of the contributors of flatus (gas production) in human and experimental animals (Cristafaro *et al*., 1974).

1.4 Flatulence

These carbohydrates include monosaccharides and disaccharides, starch and polysaccharides. Starch is the most abundant legume carbohydrate and total sugars (mono- and oligosaccharides) represent only a small percentage of total carbohydrates of the raffinose-family sugars (raffinose, stachyose, verbascose and ajugose) are predominant in most legumes and account for a significant percentage (31-70%) of total sugars (Hyomowitz *et al*., 1972). Certain legumes such as mung bean, black gram and chickpea contain higher amounts of raffinose-family oligosaccharide oligosaccharides than others. The predominance of a particular oligosaccharide seems to depend on the type of legume. For example, verbascose is the major of oligosaccharide in black gram, Bengal gram (chick pea), red gram and mung beans, whereas stachyose is the

major oligosaccharide in California small white beans, navy beans, soybeans, cowpeas and lupine seeds.

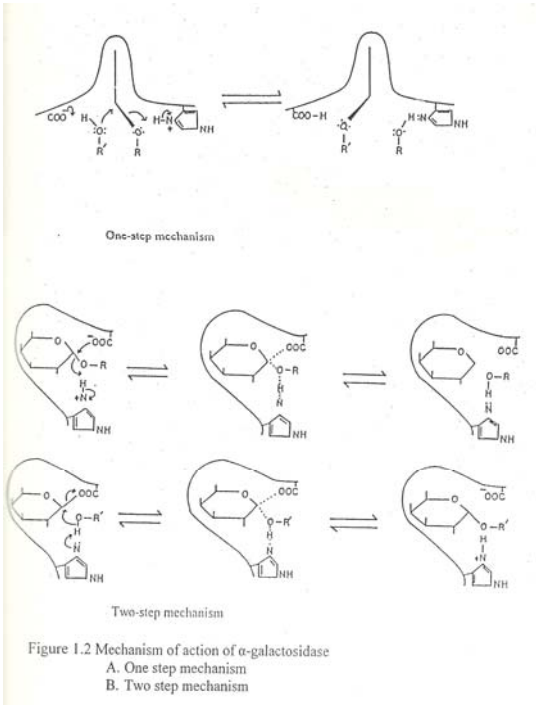

Figure 1.2 Mechanism of action of α-galactosidase
A. One step mechanism
B. Two step mechanism

Fig 1.1: Mechanism of action of α-galactosidase

1.5 Biotechnological Applications of α–galactosidase

α–Galactosidase is widely distributed in nature. α–Galactosidases are a group of exotype carbohydrases, which release α–D-galactose from melibiose, raffinose, stachyose, verbascose, galactomannans glycoproteins, ceramide trihexosides and the higher homologous as well as derivatives (Dey and Pridham, 1972).

α–Galactosidase has the following biotechnological applications:

A. Beet sugar industry,
B. Pulp and paper industry,
C. Food processing industry,
D. Medical application, and
E. Hydraulic fracturing of oil and gas wells.

1.5.1 Applications of α–galactosidase in beet sugar industry

Raffinose is widely distributed in the plant kingdom. In western countries, the table sugar (sucrose) is manufactured from sugar beet. The content of raffinose in the sugar beet is gradually increased during storage and usually it comes to 0.15%. In the beet sugar industry raffinose is known as an obstacle substance for the normal crystallization of beet sugar. When the content of raffinose in beet molasses is gradually increased and it comes to the range of 6% to 10%, the crystallization of bet sugar is abandoned and the molasses is discarded because of the preventive action of raffinose. Especially, at the later half period of the beet sugar manufacture, a large amount of molasses is discarded, because the content of raffinose in the beet is increased during storage.

1.5.2 Use of α-galactosidase in pulp and paper industry

Next to cellulose, hemicelluloses are the most abundant polysaccharides in nature. The major constituents of hemicellulases are hetero-1,4-β-D-xylans and the hetero-1,4- β-D-mannans (galactoglucomannans and glucomannans). Heteroxylans are most abundant in softwoods. In the

case of galactomannans (softwood), enzymatic hydrolysis occurs with the concerted action of the following hydrolytic enzymes: endo-β-1,4-mannases [EC 3.2.1.78], α-galactosidase [3.2.1.22], and β-glucosidase [3.2.1.21].

1.5.3 Application of α-galactosidase in food processing industry

α-Galactosidase is potentially important in the hydrolysis of raffinose-family of oligosaccharides (raffinose, stachyose, verbascose and ajugose) in pulses. Pulses, defined as seeds of leguminous plants provide major portions of the protein requirement of the daily diet in India and for these reason proteins of these seeds and their nutritive value has been the subject of extensive investigation. Habitual Indian diets contain these pulses and average per capital consumption is around 100 g (Reddy *et al*., 1984).

1.5.4 Medical applications of α-galactosidase

Knowledge of human blood groups is essential in clinical medicine for the safe practice of blood transfusion, in addition to its outstanding value in fundamental genetical and anthropological studies, red blood cells have some 100 known blood group determinants (epitopes) that comprise 15 genetically distinct blood group system and rheus (Rh) blood-group have major clinical importance. The A, B and O antigens differ in sugar residues at their non-reducing ends.

1.5.5 Hydraulic fracturing of oil and gas wells

In hydraulic fracturing applications, the polymer solution that is added to the wellbore contains particles (proppants) that are added to hold open crevices generated by applying high levels of hydrostatic pressure in the flooded well. To allow gas or oil to flow to the wellbore viscosity of the fracturing fluid must be subsequently reduced or broken in situ, either by chemical oxidation or enzymatic hydrolysis of the polymer structure.

1.6 PACKED-BED REACTOR

This type of bioreactor is operated under conditions where forced aeration is used, in which air is blown through a sieve, but the substrate bed is not mixed. This mode of operation is appropriate for those SSF processes in which it is not desirable to mix the substrate bed at all during the fermentation due to deleterious effects on either microbial growth or the physical structure of the final product.

– The column may have a cross-section other than circular.
– The column can lie vertically, horizontally or at any angle, depending on the direction of the force effects due to gravity.
– The column can be aerated from any point, and for a vertical column, the air may enter from either top or bottom.
– On the basis of heat removal considerations, the column may be covered with a water jacket that would be called a "traditional packed-bed bioreactor" or use a heat transfer plate inserted into the bed, which is called a "Zymatis packed-bed bioreactor"

The temperature and the O_2 concentration of the air that flows within the bed in a traditional packed-bed bioreactor will change along the bed towards the outlet. The excessive temperature is always the greater problem than the O_2 supply to the microorganism within packed-bed bioreactors. The temperature appears to increase linearly with the increase in bed height, and it also increases linearly as the air flow rate was decreased, except at the lowest bed height. Sangsurasak and Mitchell (1998) studied the validation of the developed model in describing the SSF process and evaluating the temperature gradients in the axial and radial dimensions. It was found that the growth rate was very sensitive to the temperature gradients more than other parameters, including the substrate density. According to the model, increasing the height of the bed will lead to an increase in the rate of aeration to keep the temperature at a certain degree level through the increase in evaporation.

Aloui *et al.,* (2007) used a traditional packed-bed bioreactor. They studied the decolorization of olive mill wastes using four kinds of strains; *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus cinnabarinus* and *Aspergillus niger*; the result showed good prospects for using *P. Chrysosporium* for the decolorization of the olive mill wastes. Sella *et al.,*

(2009) had used a packed-bed bioreactor for spore production of *Bacillus arrophaeus*. The solid state culturing was carried out in glass columns with a 4 cm diameter and a 20 cm length. The maximum spore production was reached (3.3 · 1010 CFU g dry matter–1) at 80 % initial humidity and no aeration. As the *Bacillus* does not prefer aeration during sporulation, a confirmation was done in an Erlenmeyer flask (tray-type bioreactor).

There will be evaporation of the water in the packed-bed as a result of the temperature gradients and air flow through the bed, so it is impossible to prevent evaporation from occurring in packed-bed bioreactor, even if the air supplied to the bed is saturated. To replenish the water lost in the evaporation process, water to the bed can be replenished, so it is possible to use unsaturated air to aerate the bed of the bioreactor. Rojas *et al*., (1996) studied the mechanisms of heat removal (conductive, convective and evaporative) in SSF using a packed-bed reactor. Average temperature gradients obtained during the culture were 1.3 and 0.42°C cm^{-1} in the radial and axial directions, respectively. During maximal metabolic activity of *A. niger* the medium temperature rose from 32 to 48°C, and it was indicated that conductive heat transfer was the least efficient mechanism (8.65 %) when compared with the convective (26.65 %) and evaporative (64.7 %) mechanisms.

The basic feature of this group of bioreactors is that it is "un-mixed", so its operation is static, which means that the hyphae that grow into the inter-particle spaces are not disrupted or squashed onto the particle surface; therefore, it would be an impediment to air flow and increasing the drop in pressure. The pressure drop will affect the bioreactor and decrease the fermentation due to the bed pulling away from the walls, leaving a gap through which the air can pass. Hence, it will lead to heat and mass transfer limitations within the bed and wall of the bioreactor.

Mitchell *et al.,* (1999) have suggested some modifications on the classical packed-bed bioreactor. They suggested dividing the bed into multi-layer trays and put them one above the other to take the classical shape of the packed-bed. This would allow moving the layers continuously at certain time intervals. They used a mathematical model based on the N-tank-in-series theory to evaluate the benefits of this modification upon the classical type. Through the

experiments, and using the mathematical model, they were able to evaluate the heat generation in the modified and classical operation. They found the heat generation in the modified operation was 60% of the value in the classical operation. As a result, the maximum bed temperature in the multi-layer packed bed process is 45°C lower than the classical operation. The advantage of this modification was to reduce the axial temperature gradients that occur in the classical operation, especially near the air outlet point. This was solved by moving the upper trays down in certain time intervals to make sure all the trays underwent the same operational conditions.

In order to reduce the need for a strong aeration, another concept was recently developed, the Zymatis packed-bed bioreactor that was developed by Roussos *et al.,* (1993). In this design, heat removal is prompted by the insertion of closely-spaced internal heat transfer plates into the bed, so the temperature gradients in Zymatis bioreactor is less than the traditional packed-bed bioreactor. Mitchell and Meien (2000) used a mathematical model based on the energy balance for only the Zymatis bioreactor during the growth of *A. niger* on starchy substrate. This developed model provided a useful guidance for optimum design and operation of this bioreactor. According to the experiments and developed model, the key for highest performance and higher productivity was the spacing between the internal cooling plates; 5 cm was the best distance between the internal cooling plates to achieve a faster growth of *A. niger*. Although little quantitative data was provided for the Zymotis bioreactor, enzyme levels achieved with 12 kg of dry matter were comparable to those obtained with column bioreactors of 20 cm height and 2.2 cm diameter.

In traditional packed-bed bioreactors, there is a problem of heat removal on a large scale. This design has been successful on a small scale because the small diameter allows reasonable heat removal through the bioreactor walls. If the substrate bed must remain static, then the best bioreactor design for SSF is the Zymatis packed-bed. To achieve a high productivity on large-scale, the spacing between the internal cooling spaces that are used must be the same as on a laboratory scale, the same as in tray bioreactors.

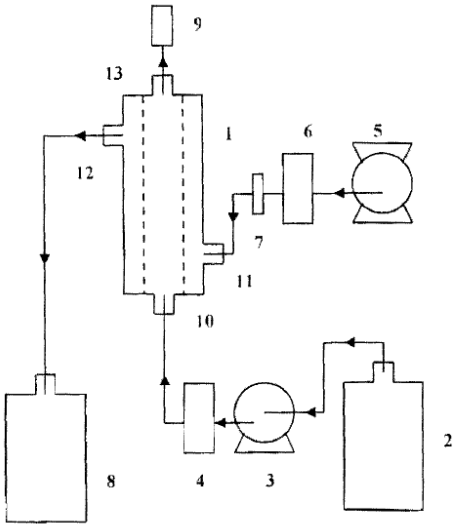

Fig 1.2: Schematic diagram of the continuous fermentation system.
(1) Bioreactor with the immobilized beads, (2) medium feed reservoir, (3) peristaltic pump, (4) flow rate regulator, (5) air pump. (6) rotameter, (7) air filter. (8) product collection vessel, (9) outlet air filter (10) medium inlet, the experiments were carried out in triplicates. (11) air inlet, (12) product outlet. (13) air outlet.

1.7 OBJECTIVES

- Design of Packed-bed reactor
- Optimization of culture conditions for enzyme production
    1. Alginate concentration
    2. Aeration
- Effect of pH and temperature on α-galactosidase activity

2. REVIEW OF LITERATURE

In December 2005, a Japanese consortium, led by The National Institute of Advanced Industrial Science and Technology (AIST) publically released the *A. oryzae* (RIB40) genome. A list of the consortium members can found be on the NITE website.

The genome sequence was obtained by using the whole-genome shotgun (WGS) approach followed by gap closure and directed sequencing of repeats. Mapping of scaffolds to chromosomes was done by hybridisation to electrophorectically separated chromosomes and digested genomic DNA. The assembly and scaffolds were further validated by optical mapping. First-pass automated annotation was carried out using the Cluster of Orthologous Groups (COG) classification.

Dr. Eiji Ichishima of Tohoku University called the koji fungus a "national fungus" ("kokkin") in the journal of the Brewing Society of Japan, because of its importance not only for making the koji for sake brewing but also for making the koji for miso, soy sauce and a range of other traditional Japanese foods. *A. oryzae* RIB40 (= NBRC 100959), or 'Koji-kin' in Japanese, is one of the filamentous fungi most widely used in fermentation industries in Japan. It is exploited in the production of sake, 'miso' (soybean paste), 'shoyu' (soy sauce) etc. and has been safely used in more than 1,000 years. Hence, it is often called 'fungus of the country'. It can be used for large-scale production of enzymes and other proteins and is regarded as an ideal host for the synthesis of active proteins of eukaryotic origins that cannot be achieved with *E. coli.*

The 37-megabase (Mb) genome of *A. oryzae* contains 12,074 genes and is expanded by 7-9 Mb in comparison with the genomes of *Aspergillus nidulans* and *Aspergillus fumigatus*. Comparison of the three *Aspergillus* species revealed the presence of syntenic blocks and *A. oryzae*-specific blocks (lacking synteny with *A. nidulans* and *A. fumigatus*) in a mosaic manner throughout the genome of *A. oryzae*. The blocks of *A. oryzae*-specific sequence are enriched for genes involved in metabolism, particularly those for the synthesis of secondary metabolites. Specific expansion of genes for secretory hydrolytic enzymes, amino acid metabolism and amino acid/sugar uptake transporters supports the idea that *A. oryzae* is an ideal microorganism for fermentation.

2.1 Production of α-galactosidase

Smiley *et al*., (1976) considered Soybean flour contains raffinose and stachyose to be responsible for flatulence often associated with these products. Soybean milk serves as a base for a variety of beverages designed for consumption in developing countries. α-Galactosidase, produced on wheat bran, hydrolyzes the galactooligosaccharides of soybean milk.

Shankar *et al*., (2006) found the Optimum temperature and pH for α-galactosidase activity were 50^{o}C and 4.8 respectively. *A. oryzae* produced α-galactosidase during solid substrate fermentation. Maximum α-galactosidase activity was 5.12 Ug^{-1} with pigeon pea plant waste as solid substrate, when fortified with galactose. α-Galactosidase yield increased, when soybean flour was used as a supplementary carbon source.

Shankar and Mulimani (2007) made Comparisons for α-galactosidase production using red gram plant waste (RGPW) with wheat bran (WB) and other locally available substrates using the fungus *A. oryzae* under solid-state fermentation (SSF). RGPW proved to be potential substrate for α-galactosidase production as it gave higher enzyme titers (3.4 U/g) compared to WB (2.7 U/g) and other substrates tested. Mixing WB with RGPW (1:1, w/w) resulted enhanced α-galactosidase yield. The volume of moistening agent in the ratio of 1:2 (w/v), pH 5.5 and 1 ml of inoculum volume and four days incubation were optimum for α-galactosidase production. Increase in substrate concentration (RGPW + WB) did not decrease enzyme yield in trays.

2.2 Application of α-galactosidase

Prashanth and Mulimani (2005) immobilized the α-galactosidase in calcium alginate in the presence of glutaraldehyde. The optimum pH of the soluble enzyme was 4.8, whereas that of the immobilized enzyme was 4.5. The optimum temperature of the soluble enzyme was 50^{0}C and that of the immobilized enzyme was increased to 57^{0}C. The immobilized enzyme retained its activity for a longer period. The immobilized enzyme exhibited higher K_m and lower V_{max} compared to the soluble enzyme. Immobilized α-galactosidase was used in batch, repeated batch and in continuous mode. After 12 h, soluble and immobilized enzyme resulted in 93 and 81% reduction in raffinose family oligosaccharide content in soymilk. Immobilized calcium alginate

beads were used for repeated hydrolysis at 50^{0}C and showed good operational stability. The performance of immobilized α-galactosidase was also tested in a fluidized bed reactor at different flow rates and 90% reduction of raffinose family oligosaccharides in soymilk was obtained at 40 ml h^{-1} flow rate.

Shankar *et al*., (2011) constructed a fluidized bed reactor for continuous hydrolysis of galactooligosaccharides in soymilk using crosslinked Con A-α-galactosidase complex entrapped calcium alginate. Thermostable α-galactosidase from *A. terreus* was insolubilized using concanavalin A obtained from jack bean extract and in order to maintain the integrity of complex in the presence of its substrate or products, this complex was crosslinked with glutaraldehyde. Soluble α-galactosidase entrapped in calcium alginate retained 82% of enzyme activity whereas, Con A-α-galactosidase complex entrapped in calcium alginate and crosslinked Con A-α-galactosidase complex entrapped calcium alginate retained 74 and 61% activity, respectively. Optimum conditions such as pH (5.0) and temperature (65^{o}C) were the same for all immobilized enzyme preparations and soluble enzyme. Crosslinked Con A-α-galactosidase entrapped complex exhibited enhanced thermostability and showed 62% of activity (38%) after 360 min at 65^{o}C. Entrapped crosslinked Con A-α-galactosidase complex preparation was superior in the continuous hydrolysis of oligosaccharides in soymilk by batch processes compared to the other entrapped preparations. The entrapped crosslinked concanavalin A-α-galactosidase complex retained 95% activity after eight cycles of use.

Naganagouda and Mulimani (2006) studied that that alginate–gelatin fiber entrapped α-galactosidase shows the higher immobilization yield, greater storage stability and better percent hydrolysis of NDO present in soymilk compared to alginate and gelatin entrapped α-galactosidase. α-Galactosidase from *A. oryzae* was physically entrapped in gelatin blended alginate hydrogel fibers hardened with glutaraldehyde immobilization yield resulted in 71.75%. The optimum conditions were not affected by immobilization, and the optimum pH and temperature for free and immobilized enzyme were 4.8 and 50^{o}C, respectively. Immobilized α-galactosidase was more stable at higher pH and temperature. To improve the immobilization system polyols like glycerol (C3) was used with different amounts of Na-alginate and gelatin. The free α-galactosidase activity quickly decreased and the half time of the activity decay was

about 5 days at 4°C. The immobilized enzyme remained very active over along period of time and this enzyme lost about 70% of its original activity over the period of 60 days for storage at 4°C. Immobilized α-galactosidase was used in batch, repeated batch and continuous mode to degrade the non-digestible oligosaccharides present in soymilk. The performance of immobilized α-galactosidase was also tested in a fluidized bed reactor at different flow rates and 93% reduction of NDO in soymilk was obtained at 25 ml h^{-1} flow rate.

Kulkarni *et al*., (2006) determined the general properties of free and immobilized enzymes. α-Galactosidase from *A. oryzae* was immobilized on chitosan beads using glutaraldehyde as a crosslinking agent. The optimum pH for the free and immobilized enzymes was 4.8 and 4.6 respectively. The optimum temperature for the free enzyme was 50°C, whereas that of immobilized enzyme was increased to 56°C. Kinetic parameters were determined with synthetic substrate (p-nitrophenyl α-D-galactopyranoside) and raffinose. Immobilized enzyme showed a higher K_m and a lower V_{max} than the free enzyme. The immobilized enzymes were used in batch, repeated and continuous mode. A level of 92% hydrolysis was observed at a flow rate of 60 ml/h. The immobilized enzyme was used repeatedly ten times without any change in the performance of the immobilized enzyme in fluidized-bed reactor. The results obtained are of considerable interest for industrial purposes.

2.3 Packed-bed reactor

Elisa d'Avila Costa Cavalcanti *et al*., (2005) employed in the packed bed bioreactors with the aim of increasing productivity and scaling up of lipase production using *Penicillium simplicissimum* in solid-state fermentation. The influence of temperature and air flow rate on enzyme production was evaluated employing statistical experimental design, and an empirical model was adjusted to the experimental data. It was shown that higher lipase activities could be achieved at lower temperatures and higher air flow rates. The maximum lipase activity (26.4 U/g) was obtained at the temperature of 27°C and air flow rate of 0.8 L/min.

Ahmet R. Ozdural *et al*., (2001) determined the apparent kinetic parameters of immobilized glucose oxidase on weak base ion exchanger resin (Duolite A 568) for different substrate flow rates in a recirculation system and compared with those for soluble glucose

oxidase. In this study, a simple and effective technique for characterizing Michaelis-Menten apparent kinetic parameters in packed-bed immobilized enzyme reactors is presented. It was observed that, for the experimental conditions, the immobilized enzyme K_m values in a packed-bed reactor were less than the soluble enzyme K_m value and were flow dependent. The value of K'_m decreased with increasing flow rate.

Muthukaruppan *et al*., (2012) implemented Homotopy perturbation method to give approximated and analytical expression of concentration of substrate and effectiveness factor for all values of the parameters. A mathematical model of packed-bed immobilized enzyme (IME) reactor is presented. The model is based on system of reaction-diffusion equations containing a non-linear term related to Michaelis-Menten kinetics of the enzymatic reaction. A simple and closed approximate analytical expression of the steady-state concentrations and effectiveness factor in a Packed-bed immobilized enzyme reactor are derived for all values of the reaction diffusion parameters.

Ali and Zulkali (2011) reviews different types of bioreactors that have been used for various purposes and the recent process developments in solid-state fermentation. Solid-state fermentation has gained renewed attention, not only from researchers but also from industries, due to several advantages over submerged fermentations. This is partly because solid-state fermentation has lower energy requirements, higher yields, produces less wastewater with less risk of bacterial contamination, and partly because of environmental concerns regarding the disposal of solid wastes.

2.4 Production of enzymes using packed-bed reactor

Nakkharat and Haltrich (2006) immobilized the β-galactosidase from *Talaromyces thermophilus* CBS 236.58 onto Eupergit C produced galacto-oligosaccharides (GalOS) in batchwise and continuous packed-bed mode of operation. A maximum yield of GalOS of 12, 39 and 80 g l^{-1} was obtained for initial lactose concentrations of 50, 100 and 200 g l^{-1}, respectively, for batch conversion experiments. The immobilized enzyme could be re-used for several cycles for lactose hydrolysis and transformation. The maximum GalOS concentration of approximately 50 g l^{-1} was obtained with the dilution rate of 0.375 h^{-1} in a packed-bed reactor, when using an

initial lactose concentration of 200 g l^{-1}. Continuous conversion of lactose in the packed-bed reactor resulted in the formation of relatively more trisaccharides than when employing the immobilized enzyme in discontinuous mode of operation.

Marie-Pierre Bousquet *et al.,* (2000) compared two immobilized lipases from *Candida antarctica* (Chirazyme L2, c.-f., C2) and *Rhizomucormiehei* (Chirazyme L9, c.-f.) in stirred batch and packed bed configurations. The enzymatic synthesis of a mixture of unsaturated fatty acid α-butylglucoside esters, containing more than 60% α -butylglucoside linoleate, was achieved through lipase catalyzed esterification. The continuous evaporation under reduced pressure of the water produced enabled substrate conversions greater than 95% to be reached. When the synthesis was carried out in stirred batch mode, *C. Antarctica* lipase appeared to be of greater interest than the *R. miehei* enzyme in terms of stability and regioselectivity. Surprisingly, a change in the process design to a packed bed configuration enabled the stability of *R. miehei* lipase to be significantly improved, while the *C. antarctica* lipase efficiency to synthesize unsaturated fatty acid α-butylglucoside esters was slightly decreased. Water content in the microenvironment of the biocatalyst was assumed to be responsible for such changes. When the process is run in stirred batch mode, the conditions used promote the evaporation of the essential water surrounding the enzyme, which probably leads to *R. miehei* lipase dehydration. In contrast, the packed bed design enabled such water evaporation in the microenvironment of the biocatalyst to be avoided, which resulted in a tremendous improvement of *R. miehei* lipase stability. However, *C. antarctica* lipase led to the formation of 3% diesters, whereas the final percentage of diesters reached 21% when *R. miehei* enzyme was used as biocatalyst. A low content of diesters is of greater interest in terms of α-butylglucoside linoleate application as linoleic acid carrier, and therefore the enzyme choice will have to be made depending on the properties expected for the final product.

Samia and Ahmed (2010) investigated Alkaline protease production and the stability of biocatalyst in both free and immobilized cells. *Bacillus licheniformis* ATCC 21415 cells were immobilized on different carriers using different methods of immobilization including physical adsorption, covalent binding, ionic binding and entrapment. The immobilized cells were prepared by covalent binding on wool (as a new carrier) through 1% glutaraldehyde had the

highest enzyme activity (9.0 U/mL) with the highest specific productivity (6.17 U/g wet cells/h). The results showed that the immobilized cells were more efficient for enzyme production by repeated batch fermentation (5 cycles, 480 h) with 57% residual activity whereas the free cells retained 35% after 2 cycles. In continuous production the highest enzyme activity (9.9 U/mL) was obtained at a dilution rate of 0.1/h while the highest enzyme yield (763.6 U/h) and the highest reactor productivity (3.32 U/mL/h) were attained at a dilution rate of 0.4/h. Packed-bed bioreactor was a successful method for continuous production of alkaline protease for a long time (168 h) with 53% relative activity. The bioreactor affected the highest specific productivity (118.2 U/g wet cells/h) which was 12-24 times higher than other systems of enzyme production.

Mohamed *et al*., (2000) investigated the immobilized cells of five bacterial cultures on different carriers for the production of cyclodextrin glucosyltransferase (CGTase) .The entrapped cells of *Bacillus amyloliquefaciens* in calcium alginate showed the highest enzyme activity (70.8 U ml^{-1}). The enzyme production with respect to alginate concentration, bead diameter, and maximal cell loading in the immobilization matrix was optimized. In repeated batch fermentation, the immobilized cells retained their ability to produce CGTase consistently over 14 cycles and the activity remains between 70 and 88 U ml^{-1} throughout the cycles. Continuous culture was investigated in packed-bed and fluidized-bed reactors. In packed-bed reactor, maximal productivity (23 KU l^{-1} h^{-1}) with enzyme concentration of 48 U $ml^{-1}$and specific productivity of 141.8 U g wet $cells^{-1}$ h^{-1} was attained at a dilution of 0.48 h^{-1}. Continuous production in fluidized bed reactor showed maximal productivity (30.4 KU l^{-1} h^{-1}) with enzyme concentration of 53.0 U ml^{-1} and specific productivity of 230.9 U g wet $cells^{-1}$ h^{-1} at a relatively high dilution rate of 0.57 h^{-1}.

Rodriguez-Durán *et al*., (2011) was to improve the tannase production by a locally isolated *A. niger* strain in an SSF system. Tannin acyl hydrolase, also known as tannase, is an enzyme with important applications in the food, feed, pharmaceutical, and chemical industries. However, despite a growing interest in the catalytic properties of tannase, its practical use is very limited owing to high production costs. Several studies have already demonstrated the advantages of solid-state fermentation (SSF) for the production of fungal tannase, yet the optimal conditions for enzyme production strongly depend on the microbial strain utilized. The SSF was

carried out in packed-bed bioreactors using polyurethane foam as an inert support impregnated with defined culture media. The process parameters influencing the enzyme production were identified using a Plackett–Burman design, where the substrate concentration, initial pH, and incubation temperature were determined as the most significant. These parameters were then further optimized using a Box-Behnken design. The maximum tannase production was obtained with a high tannic acid concentration (50 g/l), relatively low incubation temperature (30^0C), and unique low initial pH (4.0). The statistical strategy aided in increasing the enzyme activity nearly 1.97-fold, from 4,030 to 7,955 U/l. Consequently, these findings can lead to the development of a fermentation system that is able to produce large amounts of tannase in economical, compact, and scalable reactors.

Catarina Almeida *et al*., (2005) tested a packed bed reactor (PBR) design for the purpose of continuous pectinase production with yeasts, as a possible alternative to the traditional batch process using fungal cultures. Two different carriers – a porous glass (Siran) and a cellulosic carrier obtained from spent grains (barley) – were used to immobilize *Kluyveromyces marxianus* CCT 3172, a yeast strain secreting endopolygalacturonase. To improve cell distribution throughout the column, part of the outflow was recycled. Cell loads of 0.204 and 0.247 $g_{biomass}/g_{carrier}$ were obtained at the top and bottom of the PBR with spent grains, respectively. Using the PBR with Siran as the immobilization support, 0.071 $g_{biomass}/g_{carrier}$ was the biomass load at the top of the column while at the bottom a value of 0.147 $g_{biomass}/g_{carrier}$ was found. The highest value for pectinase volumetric productivity (PV = 1.68 U/ml h) was achieved in the PBR with Siran for a D = 0.260 h^{-1} and a glucose concentration on the inlet of S_{in} = 40 g/l. Both carriers were suitable for pectinase production. The best results were obtained with a high and uniform biomass concentration in the column, together with high dilution rates and total glucose consumption.

Jarun Chutmanop *et al*., (2008) investigates the production of food-grade proteases by solid-state fermentation using readily available Thai rice bran. An inexpensive and readily available agroindustrial substrate such as rice bran can be used to produce cheap commercial enzymes by solid-state fermentation. A local strain of *A. oryzae* (Ozykat-1) was used to produce proteases. Rice bran used alone proved to have poor substrate morphology (insufficient porosity)

for satisfactory solid-state fermentation. A certain amount of wheat bran was necessary to improve the morphology of the substrate. The following variables affected protease production: substrate composition, initial moisture content and initial pH. A high protease activity (~1200 U g^{-1} dry solids) was obtained on a substrate that had a wheat bran to rice bran ratio of 0.33 by dry weight, a moisture content of 50%, initial pH of 7.5, and incubation temperature of 30^0C. Nutritionally, rice bran used alone was as good a substrate as mixed bran for producing protease, but rice bran had poor morphological characteristics for consistent fermentation. A substrate that had a wheat bran to rice bran ratio of 0.33 by dry weight was best for producing protease.

2.5 Application of packed-bed reactor

Chia-Hua Hsu and Martin Lo (2003) calculated Metabolic fluxes through the carbon pathways for xanthan gum production by Xanthomonas campestris. Xanthan gum biosynthesis in a centrifugal, packed-bed reactor (CPBR) was characterized using metabolic flux analysis. Use of 5.0% instead of 2.5% glucose resulted in an enhanced glucose uptake rate (ns) in all bioreactors. The ns values in CPBR were significantly higher than those in STR, as were the assembling rate (n1) of sugar nucleotides transformed from Glc-6-P. However, the highest ratio of n1/n2 was found in CPBR-LC with 5.0% glucose, indicating that most glucose was utilized for xanthan synthesis with the minimum maintenance requirements in the CPBR-LC operation. Despite the high xanthan productivity and glucose uptake achieved in CPBR-GC, CPBR-LC with 5.0% initial glucose concentration was the most energy-efficient operation among all systems studied.

Fernanda *et al*., (2011) compared the properties of free and immobilized α-galactosidase (*A. oryzae*), entrapped in alginate–gelatin beads and cross-linked with glutaraldehyde. The free and immobilized forms of the enzyme showed no decrease in enzyme activity when incubated in buffer solutions in pH ranges of 4.5–7.0. The kinetics of lactose hydrolysis by the free and immobilized enzymes were studied at maximum substrate concentrations of 90 g/L and 140 g/L, respectively, a temperature of 35^oC and a pH of 4.5. The Michaelis–Menten model with competitive inhibition by galactose fit the experimental results for both forms. The Km and Vm values of the free enzyme were 52.13±2.8 mM and 2.56±0.3 glucose/L min mg enzyme, respectively, and were 60.30±3.3 mM and 1032.07±51.6 galactose/ L min mg catalyst, respectively, for the immobilized form. The maximum enzymatic activity of the soluble form of

α-galactosidase was obtained at pH 4.5 and 55^oC. Alternatively, the immobilized form was most active at pH 5.0 at 60^oC. The free and immobilized enzymes presented activation energies of 6.90±0.5 kcal/mol and 7.7±0.7 kcal/mol, respectively, which suggested that the immobilized enzyme possessed a lower resistance to substrate transfer.

Mitra Dadvar and Muhammad Sahimi (2002) studied deactivation of the mesoporous particles using a three-dimensional pore network model of the pore space with distributed pore sizes and interconnectivities, and investigate several plausible mechanisms of deactivation of the porous particles. Immobilized glucose isomerase is widely used for converting glucose to fructose by enzymatic isomerization. The process takes place in a packed-bed reactor consisting of mesoporous particles with distributed pore sizes and interconnectivities. Its efficiency is however, significantly affected by deactivation of the mesoporous particles. The results of the present study, which will be used as the input for simulation of the phenomenon at the reactor level, demonstrates the strong effect of the particles' morphology on the deactivation process.

Sanjay and Sugunan (2005) characterized the immobilized enzymes using XRD, surface area measurements and At MAS NMR and the activity of the immobilized enzymes for starch hydrolysis was tested in a fixed bed reactor (FBR) Glucoamylase from *A. niger* was immobilized on montmorillonite clay (K-l0) by two procedures, adsorption and covalent binding. XRD shows that enzyme intercalates into the inter-lamellar space of the day matrix with a layer expansion up to 2.25 nm. Covalently bound glucoarnylase demonstrates a sharp decrease in surface area and pore volume that suggests binding of the enzyme at the pore entrance. NMR studies reveal the involvement of octahedral and tetrahedral Al during immobilization. The performance characteristics in FBR were evaluated. Effectiveness factor (η) for FBR is greater than unity demonstrating that activity of enzyme is more than that of the free enzyme. The Michaelis constant (K_m) for covalently bound glucoamylase was lower than that for free enzyme. i.e.. the affinity for substrate improves upon immobilization. This shows that diffusional effects are completely eliminated in the FBR. Both immobilized systems showed almost l00% initial activity after 96 h of continuous operation. Covalent binding demonstrated better operational stability.

Molinari *et al*., (2000) evaluated the growth conditions to obtain high activity of mycelium-bound enzymes. Dry mycelium of a strain of *A. oryzae* efficiently catalyzed the esterification between free acetic acid and primary alcohols (geraniol and ethanol) in organic solvent. A medium containing Tween 80 as carbon source furnished mycelium with the highest activity in the hydrolysis of α-naphthyl esters (α -N-acetate, butyrate, caprylate). Dry mycelium was employed to select suited conditions for an efficient acetylation of ethanol and geraniol in heptane. Maximum productions were obtained using 30 g l^{-1} of lyophilized cells: 12.4 g l^{-1} of geranyl acetate were produced at 80°C starting from 75 mM geraniol and acetic acid (84% molar conversion) and 4.1 g l^{-1} of ethyl acetate at 50°C from 50 mM ethanol and acetic acid (94% molar conversion) after 24 h. The stability of the mycelium-bound carboxylesterases are notable since only 10 –30% loss of activity was observed after 14 days at temperatures between 30 and 50°C.

Kunamneni Adinarayana *et al*., (2005) investigated the effect of *B. subtilis* PE-11 cells immobilized in various matrices, such as calcium alginate, k-Carrageenan, polyacrylamide, agar-agar, and gelatin, for the production of alkaline protease. Calcium alginate was found to be an effective and suitable matrix for higher alkaline protease productivity compared to the other matrices studied. All the matrices were selected for repeated batch fermentation. The average specific volumetric productivity with calcium alginate was 15.11 U/mL/hour, which was 79.03% higher production over the conventional free-cell fermentation. Similarly, the specific volumetric productivity by repeated batch fermentation was 13.68 U/mL/hour with k-Carrageenan, 12.44 U/mL/hour with agar-agar, 11.71 U/mL/hour with polyacrylamide, and 10.32 U/mL/hour with gelatin. In the repeated batch fermentations of the shake flasks, an optimum level of enzyme was maintained for 9 days using calcium alginate immobilized cells. From the results, it is concluded that the immobilized cells of *B. subtilis* PE-11 in calcium alginate are more efficient for the production of alkaline protease with repeated batch fermentation. The alginate immobilized cells of *B. subtilis* PE-11 can be proposed as an effective biocatalyst for repeated usage for maximum production of alkaline protease.

Mielgo *et al*., (2001) established a correlation between residual MnP activity in the effluent and decolourisation. The degradation of an azo dye, Orange II, by immobilised

Phanerochaete chrysosporium in a continuous packed bed bioreactor for periods longer than 30 days has been carried out. Nearly complete decolourisation (>95%) was achieved when working at a high dye load rate of 0.2 g l^{-1} d^{-1}, a temperature of 37°C, a hydraulic retention time (HRT) of 24 h and applying oxygen gas in a pulsed flow. These conditions allowed Manganese peroxidase (MnP) production and the subsequently Orange II decolourisation. Apparently, for decolourisation to be effective, a minimum MnP activity was required, no substantial increase in efficiency at MnP activities higher than 10 U 1^{-1} was observed. The treatment caused, the breakdown of the chromophoric group as well as the cleavage of the aromatic ring.

Mohammadi *et al*., (2008) developed Immobilized cell reactor (ICR) as a novel bioreactor to convert hydrolyzed sugars to organic acids. Sugar fermentation by *Propionibacterium acid-propionici* entraped by calcium alginate was carried out in continuous mode to produce propionic and acetic acids. In continuous fermentation, more than 90 percent of glucose conversion and 60 percent conversion of xylose were obtained at a retention time of 28 h. The present research has demonstrated that the microorganism preferred glucose as carbon source over other carbon sources. The highest sugar concentration (120 g/L) in the ICR column was successfully converted to propionic acid. The achieved results in ICR with high substrate concentration are promising for scale up operation.

Abdel-Naby *et al*., (1999) compared the catalytic properties and stability of the immobilized tannase with the corresponding free enzyme. Tannase enzyme from *A. oryzae* was immobilized on various carriers by different methods. The immobilized enzyme on chitosan with a bifunctional agent (glutaraldehyde) had the highest activity. The bound enzyme retained 20·3% of the original specific activity exhibited by the free enzyme. The optimum pH of the immobilized enzyme was shifted to a more acidic range compared with the free enzyme. The optimum temperature of the reaction was determined to be 40°C for the free enzyme and 55°C for the immobilized form. The stability at low pH, as well as thermal stability, were significantly improved by the immobilization process. The immobilized enzyme exhibited mass transfer limitation as reflected by a higher apparent Km value and a lower energy of activation. The immobilized enzyme retained about 85% of the init\al catalytic activity, even after being used 17 times.

Alexandros Ch. Pappas *et al*., (2002) described an enzymatic method for the individual or simultaneous determination of pyruvic acid and acetaldehyde . Alcohol dehydrogenase (ADH) was immobilized onto aminopropyl-modified controlled pore glass, which was then used for the construction of packed-bed (PB) reactors. ADH catalyses the reduction of acetaldehyde to ethanol, in the presence of the coenzyme NADH, which is oxidized to NAD^+. Photometric measurements in a fully automated flow injection (FI) manifold are used to monitor the decrease of NADH absorbance at 340 nm. The possibility of pyruvate measurements, by combining the above mentioned system with soluble pyruvate decarboxylase (PyDC) is also demonstrated. PyDC catalyses the decarboxylation of pyruvate to acetaldehyde. Analytical parameters such as the buffering system, working pH, flow rate, sample size, and NADH concentration were studied. The interference of various compounds present in real samples was also investigated. Linear calibration graphs over the ranges 0.08–1.25 and 0.04–0.4mM acetaldehyde were constructed in the presence of 50mM succinate pH 7.5 and 50mM phosphate pH 7.0 buffering systems, respectively. A linear calibration graph over the range 0.08–1.25mM pyruvate was also constructed in the presence of 50mM succinate pH 7.5 buffer solution. The reactors remain active for more than 6 months under specified storage conditions. The maximal sample throughput is 30 h^{-1} and the R.S.D. of the method is 0.9% for 0.2mM acetaldehyde (n = 6). The suitability of the proposed method for real samples was tested by recovery studies.

Gergo Mezohegyi *et al.,* (2007) investigated the anaerobic reduction of azo dye Acid Orange 7 (AO7) in a continuous upflow packed bed reactor (UPBR) containing biological activated carbon (BAC). Preliminary batch experiments using graphite proved the catalytic effect of using a solid electron mediator in the reactor. Before the start of continuous experiments, AO7 adsorption studies were done to control adsorption effects on initial decolorization rates. In a continuous UPBR-BAC system, high azo dye conversion rates were achieved during very short space times (τ) up to 99% in 2.0 min. In order to know which are the crucial and most influencing properties of BAC in AO7 reduction, other materials-graphite and alumina-with different properties were also tested in UPBRs. The results show that both electron-mediating capability and specific surface area of activated carbon contribute to higher reduction rates.

Compared to other continuous and biological processes treating azo dyes, UPBR-BAC seems to be a very effective and promising system for anaerobic azo dye degradation.

Amaya-Delgado *et al*., (2006) tested the immobilized biocatalyst in a tubular fixed-bed reactor to investigate its possible application for continuous sucrose hydrolysis. A commercial extracellular invertase (EC 3.2.1.26) from *Saccharomyces cerevisiae* has been inmobilized by covalent bonding on novel microbeads of nylon-6 using glutaraldehyde. The enzyme was strongly bound on the support, immobilized with an efficiency factor of 0.93. The biocatalyst showed a maximum enzyme activity when immobilized at pH 5.0, but optimum pH activity for both immobilized and free invertases was 5.5. The optimum temperatures for immobilized and free enzymes were 60 and 65^{0}C, respectively. Kinetic parameters were determined for immobilized and free invertases: Vmax values were 1.37 and 1.06 mmol min^{-1} mg^{-1}, respectively. The K_m and K_i values were 0.029 and 0.71M for immobilized invertase and 0.024 and 0.69 M for free invertase. It was found that the thermal stability of the immobilized invertase with regard to the free one increased by 25% at 50 ^{0}C, 38% at 60^{0}C and 750% at 70^{0}C. The effects of two different sugar concentrations and three flow rates on the productivity of the reactor and on the specific productivity of the biocatalyst were studied. The system demonstrated a very good productivity up to 2.0 M sugar concentration, with conversion factors of 0.95 and 0.97, depending on sucrose concentration in the feeding. This approach may serve as a simple technique and can be a feasible alternative to continuous sucrose hydrolysis in a fixed bed reactor for the preparation of fructose-rich syrup.

Camacho Paez *et al.,* (2002) synthesised Structured triglycerides (ST) enriched in n-3 polyunsaturated fatty acids (PUFAs) (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) in position 2 of the triglyceride backbone by acidolysis of cod liver oil (CLO) and caprylic acid (CA) catalysed by the 1, 3-specific immobilised lipase Lipozyme IM. The reaction was carried out in three ways: (1) in a batch reactor (where the infuence of temperature on the incorporation of CA into the CLO triglyceride was studied); (2) in an immobilised lipase packed-bed reactor (PBR) by recirculating the reaction mixture from the exit of the bed to the substrate reservoir (product recirculation) to determine the equilibrium composition; and (3) in a PBR without recirculation. A "lag" period of duration inversely proportional to the initial water

amount of the lipase, was observed when new lipase was used. Apparently, during this "lag" period the hydro-enzymatic layer that surrounds the lipase surface reaches its water equilibrium content. A reaction scheme, where only the fatty acids in the positions 1 and 3 of the glycerol backbone were exchanged by CA, was proposed. The exchange equilibrium constants between CA and the native fatty acids of CLO were determined. The n-3 PUFAs (EPA and DHA) were the most resistant native fatty acids to exchange with exchange equilibrium constants of 1.32 and 0.28, respectively. Also, average reaction rates and kinetic constants of exchange of CA and native fatty acid of CLO were calculated. Low kinetic constants were observed for EPA, DHA and palmitic acid. For acidolysis reaction in the continuous mode PBR, the lipase amount/(flow rate × substrate concentration) ratio ($m_L/q[TG]_0$) could be considered as the intensive variable of the process for use in scale up of the PBR. A simple equation was proposed for the prediction of the fatty acid composition of the ST at the exit of the PBR as a function of the intensive variable $m_L/q[TG]_0$. At equilibrium, the ST produced had the following composition: CA 57%, EPA 5.1%, DHA 10.0% and palmitic acid 6.3% (only considering the major fatty acids). In addition, the proportion of EPA and DHA that esterified the position 2 of the ST was 13.5%, which represented 44% of the total fatty acids in the position 2 of the resultant ST.

3. MATERIALS AND METHODS

3.1 Materials

Glucose, galactose, guar gum, soyabean meal, and p-nitrophenyl-α-D-galactopyranoside (PNPG) was obtained from Sigma Chemical Co., USA. Sodium carbonate, sodium alginate, calcium chloride and other reagents used were of analytical grade.

A. oryzae capable of producing extracellular α-galactosidase was obtained from the Department of Biochemistry, Gulbarga University, Gulbarga.

3.2 Methods

3.2.1 Preparation of buffer

The following buffers were used in our experiments and prepared according to the book Biochemical methods by Dr.S.Sadasivam and Dr.A.Manickam (2004). All preparations were carried out using distilled water.

(i).Acetate buffer

Stock solution of A: 0.2M solution of acetic acid (11.55 ml of acetic acid in 1000 ml) and stock solution of B: 0.2M solution of sodium acetate (16.4 g of sodium acetate in 1000 ml) was prepared. Acetate buffer (pH from 3.6 to 5.5) was prepared by mixing appropriate proportion of A and B, diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.

(ii). Sodium phosphate buffer

Stock solution of A: 0.2M monobasic sodium phosphate solution (27.8 g in 1000 ml) and stock solution of B: 0.2M solution of dibasic sodium phosphate (53.65 g in 1000 ml) was prepared. Phosphate buffer (pH from 6.0 to 7.5) was prepared by mixing appropriate proportions of A and B, diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.

(iii).Tris-HCl buffer

Stock Solution of A: 0.2M solution of Tris (hydroxymethyl) aminomethane (24.2g in 1000 ml) and stock solution of B: 0.2N HCl was prepared. Tris-HCl buffer (pH from 7.0 to 10.0)

was prepared by mixing appropriate proportions of A and B, diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.

(iv). Preparation of PNPG

30 mg of p-nitrophenol-α-D-galactopyranoside was dissolved in distilled water and the volume was made upto 10 ml. This was used as substrate for α-galactosidase assay.

(v). 0.2 M Na_2CO_3

5.3g of Na_2CO_3 was dissolved in distilled water and the volume was made upto 250 ml. This was used to arrest the reaction.

3.2.2 Production of α-galactosidase from *A. oryzae*

The Czapek-dox medium used in the production studies composed of :

KH_2PO_4	1.0 g
Kcl	0.5 g
$NaNO_3$	1.0 g
$MgSO_4$	0.5 g
$ZnSO_4.7H2O$	0.05 g
$(NH_4)_2MoO_4$	0.05 g
$FeSO_4.7H2O$	0.01 g
$CuSO_4$	0.005 g
Distilled water	1 L
pH	5.5 (before autoclaving)

For production studies, galactose at 2% (w/v) was added to the above medium before autoclaving. Batch, submerged fermentations were carried out on an orbital shaker at 110 rpm and 28-30°C) for 5 to 7 days. Then the fermented broth was filtered through Whatmann filter paper no.1 and the filtrate thus collected was centrifuged at 10,000 rpm for 20 min and the clear supernatant was used as enzyme source.

3.2.3 Assay of α-galactosidase activity

α-Galactosidase activity was assayed by spectrophotometric measurement of the release of p-nitrophenol from p-nitrophenol-α-D-galactopyranoside (PNPG) at 405 nm by the modified method of Dey and Pridham (1969). The reaction mixture consisted of 100 µl of 10 mM PNPG in water, 800 µl of acetate buffer (0.2 M, pH 4.5), and 100 µl of appropriately diluted enzyme. After incubation at 37ºC for 15 min, the reaction was stopped by the addition of 3 ml of 0.2 M sodium carbonate. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per min under assay conditions.

3.3 Biochemical characterization of α-galactosidase

3.3.1 Determination of optimum pH and temperature

The effect of pH on the enzyme activity was measured at 60ºC in the pH range from 3.6 to 7.0. The following buffers were used: 100 mM acetate buffer (pH 3.6 to 5.5), 100 mM phosphate buffer (pH 6.0 to 7.5). For the determination of the temperature optimum, enzyme activity was assayed in the range of 20-70ºC using acetate buffer (100 mM, pH 5.0).

3.3.2 Kinetic studies (K_m and V_{max})

α-Galactosidase assay was performed by varying the concentrations of PNPG from 10 µl to 100 µl. The absorbance of the p-nitrophenol released was measured at 405 nm and readings were tabulated.

3.4 Immobilization in sodium-alginate

Sodium alginate solutions (1, 2, 3, and 4%, w/v) were prepared. Both alginate and cell suspension (*A. oryzae* mycelia) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added dropwise into 0.2 M CaCl2 solution from 5-cm height. The resulting spherical beads were washed with sterile distilled water. The beads were stored in 0.2M acetate buffer (pH 4.8) at 4ºC. When the beads were not being used, they

were preserved in 0.9% sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar flow unit.

Table 3.1: Effect of alginate concentrations for the immobilization of *A. oryzae*

Sodium alginate (%)	Sodium alginate (g) used
1	0.25
2	0.50
3	0.75
4	1.00

3.5 Batch experiments

The batch experiments were performed for both soluble and immobilized enzymes at different incubation periods. The experiments were carried out in a 250 ml Erlenmeyer flasks each containing 100 ml of Czapek-dox medium. The flasks were charged with immobilized beads that are obtained from 10 ml gel with the calculated amounts of the immobilized cells. Parallel experiments were carried out with equal amounts of free cells. The cultures were incubated for 72 h in a rotary shaker (110 rpm) at 30°C. All the experiments were carried out in triplicates.

3.6 Repeated batch experiments

Repeated batch experiments was carried out in 250 ml Erlenmeyer flasks each containing 100 ml of Czapek-dox medium. Each flask was inoculated with the beads obtained from 10 ml alginate gel with the calculated amounts of the immobilized cells. Parallel experiments were carried out with equal amounts of free cells. Fermentation was conducted at 30°C for 24 h under shaking conditions (110 rpm). At the end of each run, immobilized beads were collected by filtration and the beads were washed with 25 ml of 0.05 M $CaCl_2$ and distilled water. Distilled water washed beads were charged in to 250 ml capacity Erlenmeyer flasks containing 50 ml of fresh medium. All the experiments were carried out in triplicates.

3.7 Continuous fermentation

3.7.1 Design of packed-bed reactor for production of α-galactosidase from *A. oryzae*

A glass column (90 cm in length and 2.5 cm in diameter) was used for continuous production of α-galactosidase. The bioreactor was incubated at 37°C. The column was packed with 560 g of cell-immobilized beads (average diameter 3 mm) comprising about 200.6 g of wet weight cells. The alginate beads containing *A. oryzae* were packed in the column. Different flow rates of 30, 60, 90 and 120 ml h^{-1} were used for the production of α-galactosidase. The medium was fed through the bottom by a peristaltic pump with a flow rate regulator. Aeration was provided by means of an air filter. The air flow was optimized at 0.3 vvm. The effluent was collected in a holding tank.

4. RESULT & DISCUSSION

4.1 Effect of pH on soluble and immobilized α-galactosidase

The effect of pH of α-galactosidase activity was checked between the range 3-7. Maximum enzyme activity (0.227 U) was obtained at pH 4.5 for both soluble and immobilized enzyme (Fig 4.1 and 4.2). Optimum pH for the production of α-galactosidase from *Bacillus circulans* is over the range (6.5 – 7.0). (EI-Shebawy *et al*., 2007). Optimum pH for for production of α-galactosidase from white-rot fungus *Pleurotus florida* is (4.6 – 5.0) (Ramalingam *et al*., 2007).

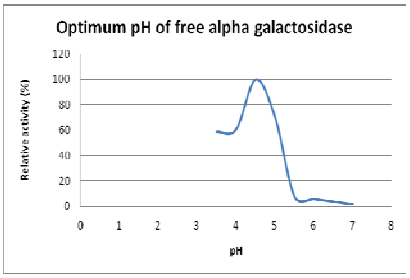

Fig 4.1: Effect of pH on activity of free α-galactosidase from *A.oryzae*

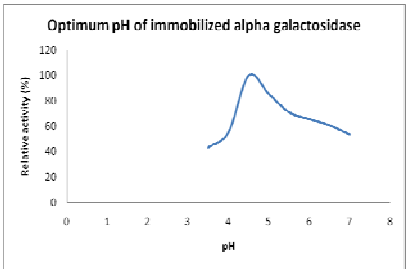

Fig 4.2 : Effect of pH on immobilized α-galactosidase from *A.oryzae*

4.2 Effect of temperature on soluble and immobilized α-galactosidase

The effect of temperature of α-galactosidase activity was checked over temperature range between 20-70. Maximum enzyme activity (0.434 U) was obtained at 50°C for both soluble and immobilized enzyme (Fig 4.3 and 4.4). Optimum temperature for production of α-galactosidase from *Bacillus circulans* is 40° C (EI-Shebawy *et al*., 2007). Optimum temperature for production of α-galactosidase from white-rot fungus *Pleurotus florida* is 55° C (Ramalingam *et al*., 2007).

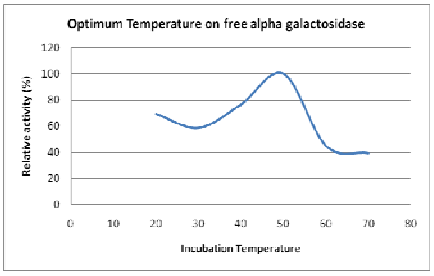

Fig 4.3: Effect of temperature on activity of free α-galactosidase from *A.oryzae*

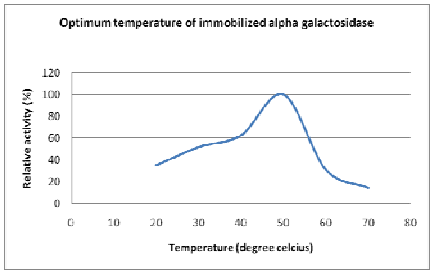

Fig 4.4: Effect of temperature on activity of immobilized α-galactosidase from *A.oryzae*

4.3 Determination of K_m and V_{max} for α-galactosidase

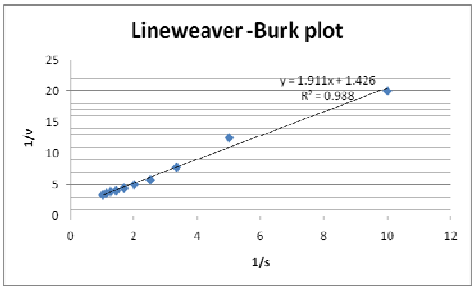

Fig 4.5: Determination of K_m and V_{max} for α-galactosidase using PNPG as substrate

4.4 Immobilized *A.oryzae* for production of α-galactosidase

The suitability for production of α-galactosidase activity by *A. oryzae* in the immobilized matrix (sodium alginate) was investigated in submerged fermentation. In another set of experiment, an equal quantity of *A. oryzae* mycelia was used to study the production of enzyme. The amount of enzyme produced by the immobilized cells was lower than the corresponding amount of free cells. The effectiveness factor of the immobilized cells, which is the ratio of the enzyme activity of the immobilized cells to that of the same amount of free cells under identical conditions, was in the order of 0.3-0.5. The effectiveness factor of the immobilized cells would always be less than one because the immobilized cells represent a heterogeneous catalysis fermentation in which the activity, or rather synthesis, of primary or secondary metabolites is dependent upon the external and internal mass transport and adequate oxygen supply.

Kunamneni Adinarayana *et al*., (2005) studied the effect of *B. subtilis* PE-11 cells immobilized in various matrices, such as calcium alginate, k-carrageenan, polyacrylamide, agar-agar, and gelatin, for the production of alkaline protease. Calcium alginate was found to be effective and suitable matrix for higher alkaline protease productivity compared to the other insoluble matrices. The results showed that calcium alginate was a promising method for immobilizing *B. subtilis* PE-11 for alkaline protease production. Advantages of this technique such as long life-term stability, reusablility, and possibility of regeneration can easily be used for scale-up studies. Based on the aforesaid reasons we have used the alginate for immobilization.

4.5 Optimum condition for immobilization of *A. oryzae* in sodium alginate

Different concentration of sodium alginate (1-4 %, w/v) were investigated for the immobilization process. In all cases, a constant amount of cells were used.The beads prepared from 1% (w/v) alginate concentration were much softer and showed the highest number of leaked cells,so they were excluded. 2% (w/v) alginate concentration showed the medium strength and there is no leakage of cells. The alginate concentration with 3% (w/v) and 4% (w/v) is more viscous, so we rejected. The recommended concentration for the production of α-galactosidase is 2% (w/v).

Table 4.1: Effect of bead diameter on α-Galactosidase activity

Bead diameter (mm)	α–Galactosidase activity (U)
2	0.038
3-4	0.029
5-6	0.026

The effect of bead diameter was investigated using 2% (w/v) alginate concentration. The results indicated that the beads of smaller diameter (2 mm) showed the highest number of leaked cells (11.3%). In fact, the bacterial cells grown preferentially near the bead surface, are continuously released out in the culture medium. It is worthy to note that the surface area of the beads resulted from certain volume of alginate gel increased with the decrease of the beads diameter. Therefore, the number of the leaked cells increased with decrease of bead diameter

(Abdel-Naby *et al*., 2000). Similar results were observed for the present studies. The alginate solution was made into beads of different diameters (2-6 mm).The results indicated that the beads of small diameter showed the highest number of leaked cells. In fact the fungal cells grown preferentially near the bead surface are continuously released out in the culture medium. Therefore the number of leaked cells increased with the decrease of bead diameter. On the other hand, the beads of diameter higher than 5-6 mm showed a lowest α-galactosidase activity (0.026 U). The highest enzyme activity was (0.029 U) obtained with bead diameter of 3-4 mm.

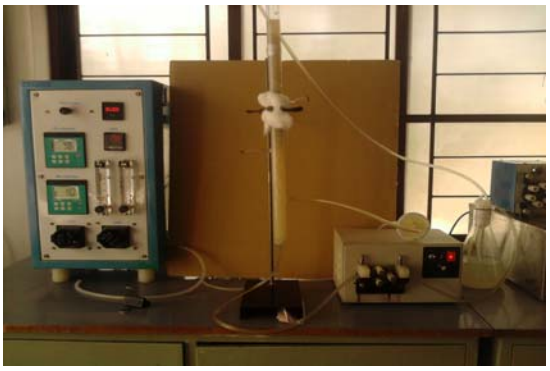

Fig 4.6: packed-bed reactor set-up

4.6 Continuous production of α-galactosidase in a packed-bed reactor

The continuous production of α-galactosidase was accomplished in a packed bed reactor, using the optimized medium. The fermentation was carried out in a batch operation initially for 24 h, then continuous operation was started the flow rate was varied between 6.25 and 90.0 ml per hour.

The aeration rate was investigated and maximal enzyme activity was obtained at a rate of 0.33 vvm. higher rates, however, did not significantly affect the enzyme yield. (Abdel-Naby *et al*., 2005).

Of the various air flow rates (0.2-0.4) used in the present, at 0.2 vvm air didn't diffuse uniformly beads in the column. Because of this we varied the flow rate from 0.3 to 0.4 vvm. At 0.4 vvm air flow rate bed material goes upwards, due to high aeration. Air flow rate at 0.32 vvm resulted in maximum enzyme production.

The system was considered to be in a steady state only after atleast five replacement volumes (residence time).

5. CONCLUSION

The activity of free and immobilized cells of *A. oryzae* was performed in batch and continuous cultures. Packed-bed reactor supported the highest activity (0.104 U) of immobilized cells in continuous operation. In addition, the long term viability and continued metabolic activity is one of the most important advantages with the immobilized system, this is particularly so with the continuous fermentation.

APPENDIX

The Czapek-dox medium used in the production studies composed of :

KH_2PO_4	1.0 g
Kcl	0.5 g
$NaNO_3$	1.0 g
$MgSO_4$	0.5 g
$ZnSO_4.7H2O$	0.05 g
$(NH_4)_2MoO_4$	0.05 g
$FeSO_4. 7H2O$	0.01 g
$CuSO_4$	0.005 g
Distilled water	1 L
pH	5.5 (before autoclaving)

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