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IMMOBILISATION OF ASPERGILLUS FLAVUS STRAIN S4 SPORES FOR OPTIMAL PRODUCTION AND ACTIVITY OF L-GLUTAMINASE

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Abstract

The ever-increasing applications of enzymes are limited by the relatively poor performance in harsh processing conditions. As a result, there are constant innovations in immobilization protocols for improving biocatalyst activity and stability. Bacterial spores are cheap to generate and highly resistant to environmental stress. The results of the present study showed that calcium alginate is a promising method for the immobilization of spores of *A. flavus* strain S4. L-glutaminase production was maximum at 72 h of incubation. In case of free spores, 48 h of incubation was sufficient for maximum L-glutaminase production. L-glutaminase production was increased even though incubation time was prolonged. Enzyme immobilization study revealed that there was a change in kinetic parameters with marginal increase in enzyme activity at 40°C, pH- 7.0, incubation period- 72 h, inoculum size- 2%, carbon source- dextrose and nitrogen source- combination of ammonium nitrate and yeast extract.

Key Words: L-Glutaminase, Immobilization, *Aspergillus flavus*

Introduction

In bioreactors, the mycelial growth of filamentous fungi results in the development of pellets or in highly viscous mycelial suspension. Growth of the mycelium on the impeller or on the electrodes hampers optimal control process. Immobilization of the fungal cells on a support and particularly the control distribution of the biomass in the bioreactor, offers the possibilities of a better control of the process, easier product separation and continuous operation (Gerin *et al*., 1993). An immobilized cell is defined as the one that by natural or artificial means is prevented from moving separately of its neighbors to other parts of the aqueous phase of the system under study. Attention can be focused on prevention of free movement within the liquid phases of a reactor system.

The application of immobilized microbial cells represents a new, attractive 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 localization in certain defined region of space with retention of viability and with the retention of catalytic activity so that the cells can be used repeatedly or continuously (Klein and Wagner, 1983). The major expected advantage of immobilized cells, in contrast to free cells or immobilized enzymes, is the reduction of the cost of bioprocessing. This helps for the repeated and continuous use of the cells, the maintenance of high cell density, and the provision of a system with minimal cost for cell separation.

When compared with the batch or continuous submerged fermentations, where free cells are utilized for metabolite production, immobilized cells offer several advantages, 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 immobilized systems. Cell metabolism (Galazzo and Bailey, 1990) and cell wall permeability (Fletcher and Marshall, 1982) are 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).

Entrapment is the term of immolization of enzymes by covalent or non-covalent bonds within gels or fibers. Efficient encapsulation has been achieved with alginate–gelatin–calcium hybrid carriers that prevent enzyme leakage and provide increased mechanical stability. Alginate derived from cell walls of brown algae are calcium, magnesium and sodium salts of alginic acid and have been extensively used for immobilization as xanthan–alginate beads, alginate–polyacrylamide gels and calcium alginate beads with enhanced enzyme activity and reusability. Carrageenan, a linear sulfated polysaccharide, has been consistently used for immobilizing a variety of enzymes (Tumturk *et al*., 2007). Gelatin is a hydrocolloid material, high in amino acids and can adsorb up to ten times its weight in water. Its indefinite shelf life has evolved attention for

enzyme immobilization. Agar is gelatin like product made primarily from the algae, *Gelidium* and *Gracilaria* (red seaweeds). Agarose, the predominant component of agar, is a linear polymer, made up of the repeating monomeric units of agarobiose.

Materials and Methods

Immobilization of spores and L-glutaminase of *Aspergillus flavus* strain S4

Immobilization increases the resistance of the microorganisms against destroying factors, stability and catalytic activity of enzymes (Borgio, 2011). The materials used in immobilization of fungal spores and L-glutaminase were agar-agar, agarose, sodium alginate, K- carrangeenan.

The immobilized fungal spores of strain S4 were incubated under optimized conditions to increase the L-glutaminase production with basal media (100 ml) containing - sucrose- 1.0 g, yeast extract- 0.75 g, Glutamine- 4 g, KH_2PO_4- 0.5 g, Na_2HPO_4- 0.5 g, NaCl- 1.0 g, $MgSO_4.7H_2O$- 0.5 g and $CuCl_2$- 0.2 g, incubated for 48 h at 30ºC and pH-7.0 with 160 rpm in orbital shaking incubator.

Agar – Agar

Two grams of agar – agar was dissolved in 100 ml distilled water and sterilized in autoclave for 15 min at 121 lbs. It was cooled to 45ºC and 1 ml of (2%) of strain S4 spores were added to the 10 ml of agar - agar solution and mixed thoroughly for homogenous distribution of spores. Then it was poured in sterile petridish and allowed to solidify. After solidification, the agar – agar was cut with sterile knife to get 1 cm width and length blocks.

Agar-Agar blocks having spores of strain S4 were added to 100 ml of sterile optimized production media and incubated the conical flasks under optimized conditions. After completion of incubation period (24 h intervals) the supernatant was collected by filtering through Whatman filter paper under sterile conditions. The supernatant was used for the assay of L-glutaminase by Imada *et al*., (1973) method. Immobilization of purified enzyme was carried out by taking 1.0 ml (398 U/ml) of purified enzyme, mixed thoroughly with 5 ml of 2% agar – agar solution for homogenous distribution and followed the immobilization procedure as above. The activity of L-glutaminase was determined by using Imada *et al*., (1973) method.

Agarose

The fungal spores were immobilized on agarose following the Nilsson *et al*., (1983) method. Two grams of agarose was dissolved in 100 ml saline water and heated to 55ºC for 2-3 min. It was cooled to 45ºC and 1 ml of spores (1×10^7) of *Aspergillus flavus* strain S4 were added to the 10 ml of agarose solution and mixed thoroughly for homogenous distribution of cells. Then they were poured in sterile petridish and allowed to solidify. After solidification, the agarose was cut with sterile knife to get 1 cm width and length blocks, stored at 4ºC and washed thoroughly with distilled water. Agarose blocks having spores of strain S4 were added to 100 ml of sterile optimized production media and incubated the conical flasks under optimized conditions. After completion of incubation period (24 h intervals) the supernatant was collected by filtering through Whatman filter paper under sterile conditions. The filtrate was used for the assay of L-glutaminase by Imada *et al*., (1973) method.

Immobilization of purified enzyme was carried out by taking 1.0 ml (398 U/ml) of purified enzyme, mixed thoroughly with 5 ml of 2% agarose solution for homogenous distribution and followed the immobilization procedure as above. The activity of L-glutaminase was determined by using Imada *et al*., (1973) method.

Sodium alginate

Immobilization of fungal spores in Ca-alginate was done by the method of Vassileva *et al.,* (1998). Sodium alginate solution (3%) was prepared in 100 ml distilled water and autoclaved for 15 min at 121 lbs. Then it was cooled to 40ºC and 1 ml of (1×10^7) of strain S4 spores were added to 10 ml of sodium alginate, this mixture extruded dropwise through a sterile syringe (syringe size 5 ml and 0.8 mm diameter) into a gently stirred 0.5 M calcium chloride solution. Beads of 2- 3 mm diameter were allowed to form for 20 min at 4ºC. Later the beads were washed thoroughly with distilled water and stored for further analysis.

Calcium alginate beads having spores of strain S4 were added to 100 ml of sterile optimized production media and incubated the conical flasks under optimized conditions. After completion of incubation period (24 h intervals) the supernatant was collected by filtering through Whatman filter paper under sterile conditions. The supernatant was used for the assay of L-glutaminase by Imada *et al*., (1973) method. mmobilization of purified enzyme in the calcium alginate was done by following the method Dey *et al.,* (2003). Equal volumes of enzyme solution (398 U/ml) and sodium alginate solution were mixed to get 3% (w/v) final concentration of sodium alginate solution in the mixture. The mixture obtained was extruded drop wise through a sterile syringe (syringe size 5 ml and 0.8 mm diameter) into a gently stirred 0.5 M calcium chloride solution. Calcium alginate beads containing enzyme was separated from the $CaCl_2$ solution by filtration. The beads were washed thoroughly with cold distilled water and they were added to 100 ml of sterile optimized production media and incubated the conical flasks under optimized conditions and followed the above procedure. The filtrate was used for the assay of L-glutaminase by Imada *et al*., (1973) method. All preparations were done under sterile conditions in laminar airflow.

K – carrageenan

Immobilization of fungal spores on K-carrageenan was performed by following the method of Wada *et al*., (1979). Three grams of K-carrageenan were dissolved in 1% NaCl solution and was heated to 60ºC. Then it was cooled to 40ºC and 1 ml of (1×10^7) of strain S4 spores was added to 10 ml of K-carrageenan solution. This mixture was extruded dropwise into a freshly prepared 2% KCl solution and placed in refrigerator for curing. Later these beads were thoroughly washed with sterile distilled water and preserved in freshly prepared 2% KCl solution for further use. K-carrageenan blocks having spores of strain S4 was added to 100 ml of sterile optimized production media and incubated the conical flasks under optimized conditions. After completion of incubation period (24 h intervals) the supernatant was collected and was used for the assay of L-glutaminase by Imada *et al*., (1973) method.

Equal volumes of purified enzyme solution (398 U/ml) and carrageenan solution were mixed to get 3% (w/v) final concentration of carrageenan solution. Immobilized enzyme beads were prepared by following the above procedure.

Operational efficiency of immobilized L-glutaminase

Repeated batch fermentation with optimal characteristics was conducted by running the production at 72 h. At the end of each cycle the production medium was recovered, the immobilized fungal spores were washed with sterile saline, fresh production medium was added and the production was continued. The operational efficiency of the immobilized system was determined by the following equation:

$$Operational\ efficiency\ (\%) = 100 \times (Cx/C_1)$$

Where C_1 is the L-glutaminase yield in the 1st operation cycle and Cx is the L-glutaminase yield in the xth operation cycle.

L-glutaminase activity

The enzyme activity was determined by using Imada *et al*., (1973) method. One unit of glutaminase was defined as amount of enzyme that liberates one micromole of ammonia under optimum conditions. The enzyme yield was expressed as units/ml (U/ml).

Protein estimation

Protein estimation was determined according to the method of Lowry *et al*., (1951), using crystalline Bovine serum albumin as standard.

Effect of incubation period on production of glutaminase by alginate immobilized fungal spores

The effect of incubation period for maximum yield of L-glutaminase production was carried out with immobilized fungal spores at different incubation periods by maintaining other optimal parameters constant. For the investigation of optimal incubation time for L-glutaminase production, the immobilized fungal spores were inoculated in the production media and checked the enzyme activities at different incubation periods (24-120 h). Samples were withdrawn aseptically for every 24 h intervals and L-glutaminase activity was determined.

Effect of pH on glutaminase production by alginate immobilized fungal spores

The effect of pH on the production of L-glutaminase was evaluated over a wide pH range 2.0-12.0, using different buffers such as KCl - HCl (pH 2.0), Citrate (pH 3.0 to 6.0), phosphate (pH 7.0), Tris - HCl (pH 8.0 to 9.0), Glycine - NaOH (pH 10.0-12.0) in the reaction mixture by maintaining other parameters constant. The enzyme yield was expressed as units/ml (U/ml).

Effect of temperature on glutaminase production by alginate immobilized fungal spores

The effect of temperature on the production of enzyme was assessed by carrying out the assay at different temperatures ranging from 20-45°C keeping incubation period-72 h, pH-7.0 and other conditions constant. The enzyme yield was expressed as units/ml (U/ml).

Effect of inoculum size on glutaminase production by alginate immobilized fungal spores

The effect of size of inoculum has been studied to check the yield (enzyme) of immobilized fungal spores. Effect of varying concentrations of inoculum size from 1.0% to 5.0% with 0.5% variation on L-glutaminase production was studied by assaying the L-glutaminase activity by maintaining incubation period -72 h, pH-7.0 and temperature-30^{o}C.

Effect of carbon source on glutaminase production by alginate immobilized fungal spores

The fungal spores were tested for the maximum L-glutaminase production by the addition of different carbon sources- sucrose, fructose, maltose, mannose, dextrose and starch (3%) in production medium by keeping the physical parameters constant. The enzyme yield was expressed as units/ml (U/ml).

Effect of nitrogen source on glutaminase production by alginate immobilized fungal spores

Effect of different organic and inorganic nitrogen sources on L-glutaminase production by immobilized fungal spores was studied by the addition of different nitrogen sources- ammonium chloride (0.5%), ammonium nitrate (0.5%), sodium nitrate (0.5%), yeast extract (1%), potassium nitrate (0.5%) and combination of ammonium nitrate and yeast extract (1%+0.5%). The enzyme yield was expressed as units/ml (U/ml).

Purified L-glutaminase immobilization

Immobilization of enzymes has been done to improve catalytic stability of enzymes and can expand the applications of the enzyme in different fields. Immobilized enzymes are widely used in different industries especially in food and pharmaceutical and offer several advantages over free enzymes. Advantages include high productivity, automation, continuous processing, precise control of the extent of reaction, easy product recovery and the enzyme does not contaminate the final product.

The purified L-glutaminase was immobilized by calcium alginate method. The purified enzyme was mixed with 3% (w/v) aqueous solution of sodium alginate to make slurry. The slurry was taken in a syringe and was forced through the needle to form discrete droplets of uniform size with controlled pressure into 0.1 M $CaCl_2$ solution from a height of 10 cm. The beads formed were allowed to harden for 16 h and later beads were checked for leakage by estimating the protein concentration of the bead washings. Protein concentration of the enzyme was determined before conducting immobilization studies by Lowry *et al*., (1951) method. The amount of unbound protein was estimated from the supernatant after immobilization. The total amount of protein immobilized was estimated as follows:

C1 = Amount of protein added for immobilization.

C2 = Amount of protein remained in the supernatant after immobilization.

C1-C2 = Amount of protein that has been immobilized.

Effect of pH on activity of immobilized enzyme

The effect of pH on immobilized L-glutaminase activity was evaluated over a wide pH range 2.0-10.0, using different buffers such as KCl - HCl (pH 2.0), Citrate (pH 3.0 to 6.0),

phosphate (pH 7.0), Tris - HCl (pH 8.0 to 9.0), Glycine - NaOH (pH 10.0) in the reaction mixture under optimal conditions. The enzyme activity was expressed as units/ml (U/ml).

Effect of temperature on activity of immobilized enzyme

The effect of temperature on the immobilized enzyme activity was assessed by carrying out the assay at different temperatures ranging from 20-70°C under optimal conditions. The enzyme activity was expressed as units/ml (U/ml).

Determination reusable efficiency of immobilized L-glutaminase

To determine reusable efficiency, the immobilized alginate beads having L-glutaminase were washed with 0.1 M phosphate buffer (pH 7.6) and enzyme activity was assayed at 40ºC for 72 h of incubation for several cycles till the activity decreased to about 10% of the original. Enzyme activity is expressed in terms of relative activity (%).

Determination of kinetic parameters

The kinetic parameters for immobilized enzyme were calculated by using various concentrations of glutamine (1-5%) under standard assay conditions. The kinetic rate constants, K_M and V_{max} were determined.

Results and Discussion

Immobilizations of spores were carried out in different matrices. Key production parameters such as incubation time, temperature, pH, inoculum size, carbon and nitrogen sources were studied using optimized production media (as in chapter IV) for L-glutaminase production. Un immobilized spores (free) were taken as control.

Effect of incubation time on L-glutaminase production by immobilized spores of *Aspergillus flavus* strain S4 in different matrices

When the immobilized spores of *Aspergillus flavus* strain S4 in optimized media were treated at different incubation periods (24-120 h), the maximum L-glutaminase production (360±0.5 U/ml) was obtained with sodium alginate, followed by agarose (280±0.3 U/ml), agar-agar (255±0.6 U/ml) and K-carrageenan (245±0.3 U/ml) at 72 h of incubation period. On the other hand, unimmobilized (free) spores showed maximum enzyme production (305±0.8 U/ml) at 48 h of incubation under the same standard optimized conditions. The results were depicted in Fig.1.

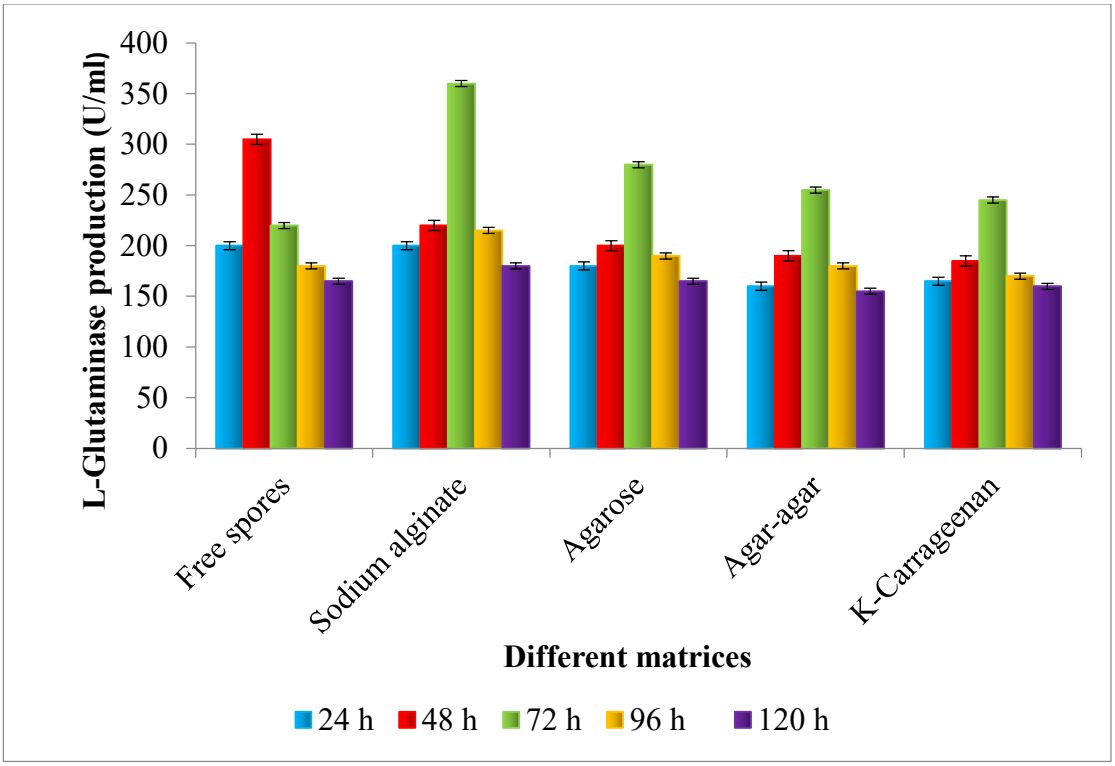

Fig. 1 Effect of different incubation periods on L-glutaminase production by immobilized *Aspergillus flavus* strain S4 spores on different matrices
The bars indicate the standard deviation of three replicates analyzed.

Mahesh *et al*., (2015) studied L-glutaminase production pattern by free and immobilized cells of *Pseudomonas* KLM9 in different matrices and sodium alginate supported better enzyme yield at 3.5% concentration and was reported to be the better supporting matrix than the others at 36 h of incubation. There are several reports suggest that sodium alginate is the suitable matrix for immobilizing microbes for the production of biomolecules including enzymes.

Effect of temperature on production of L-glutaminase by immobilized spores of *Aspergillus flavus* strain S4 in sodium alginate

L-glutaminase enzyme production was checked with immobilized spores at different temperatures (20, 25, 30, 35, 40 and 45ºC) and maximum enzyme production (365±0.8 U/ml) was observed at 40ºC for 72 h of incubation. A marked increase in enzyme production (19.6%) was observed when compared with the production of free spores (305±1.5 U/ml) at 30ºC for 48 h of incubation. The results were depicted in Fig. 2. A loss of 49.3% enzyme production was observed at 45ºC in the case of immobilized spores.

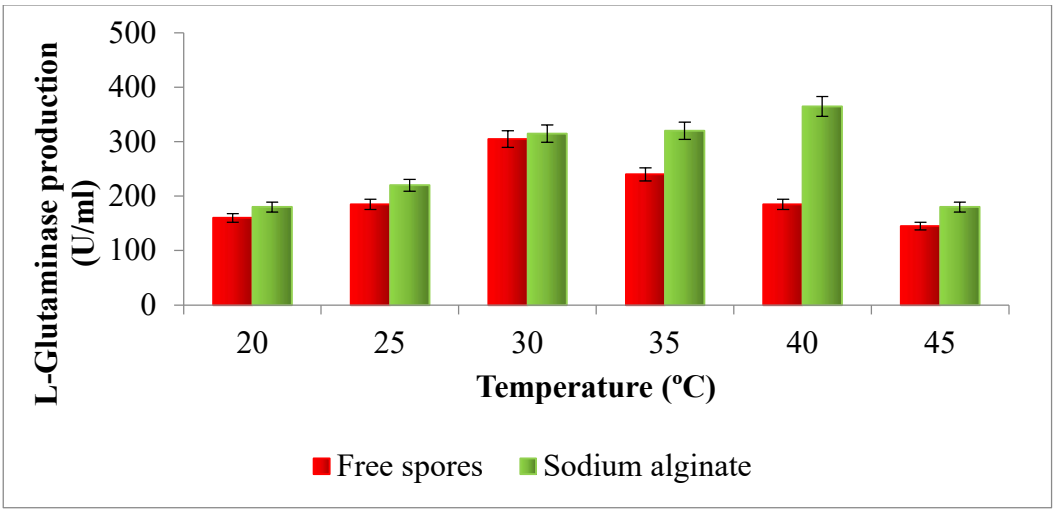

Fig. 2 Effect of temperature on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate
The bars indicate the standard deviation of three replicates analyzed.

Mahesh *et al*., (2015) reported optimum temperature of 37°C for good yield of L-glutaminase by immobilized *Pseudomonas* sp. KLM9. Mahmod (2016) also carried out immobilization studies of L-glutaminase from *Bacillus subtilis* using chitin and reported that enzyme retained 22% of its original activity at 37°C and pH-8.0.

Effect of pH on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate

L-glutaminase enzyme production was studied with immobilized spores at different pH (2 to 12). Maximum enzyme production was observed with free spores (310±0.15 U/ml) and immobilized spores (375±0.25 U/ml) at the same pH (7.0) at 48 h and 72 h of incubation respectively. An increase of enzyme production (21%) was observed with immobilized spores when compared to free spores. The results were depicted in Fig. 3.

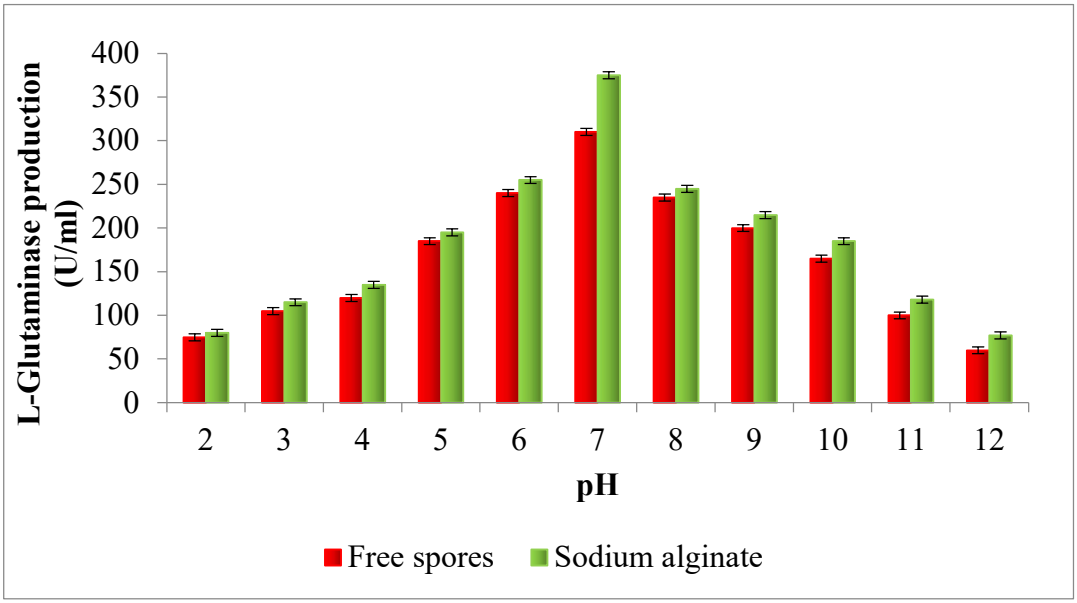

Fig. 3 Effect of pH on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate

The bars indicate the standard deviation of three replicates analyzed.

Effect of Carbon sources on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate

L-glutaminase enzyme production was determined with immobilized spores using different carbon sources maltose, mannose, dextrose, fructose and starch. The maximum enzyme production (385±0.33 U/ml) was observed with glucose (3%) in the case of immobilized spores at 72 h of incubation whereas free spores showed maximum enzyme production (312±0.25) with 2% glucose at 48 h of incubation. An increase of enzyme production (23%) was observed with immobilized spores when compared to free spores. The results were depicted in Fig. 4.

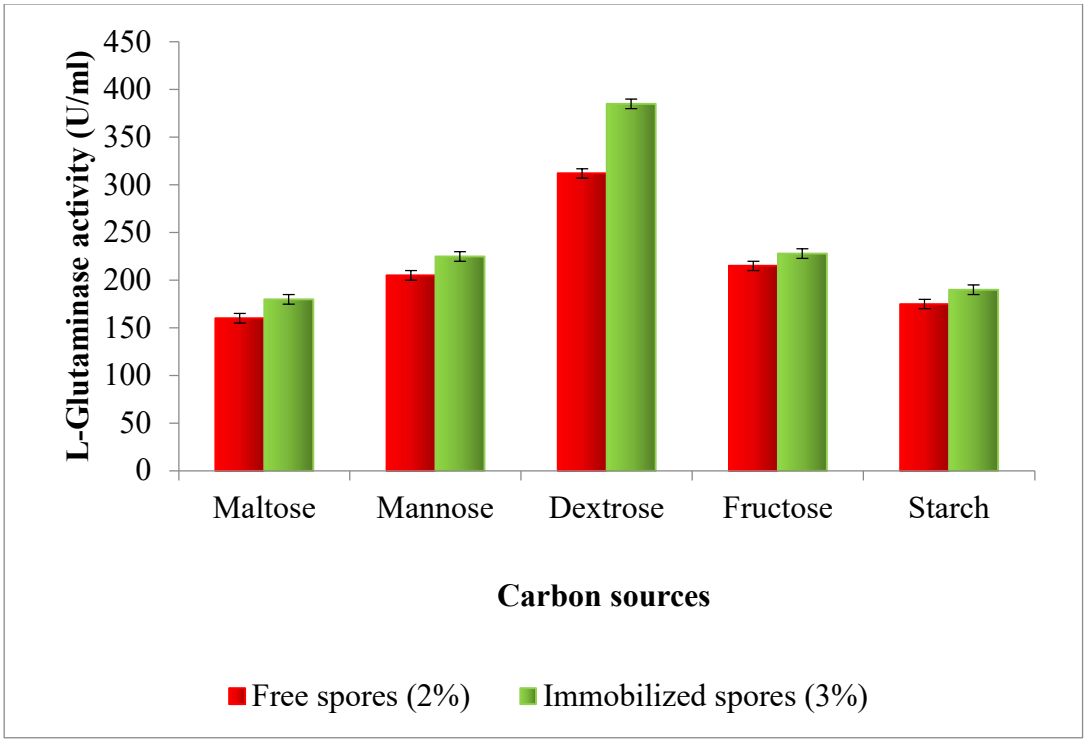

Fig. 4 Effect of Carbon sources on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate
The bars indicate the standard deviation of three replicates analyzed.

Effect of nitrogen sources on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate

L-glutaminase enzyme production was determined with immobilized spores using different organic and inorganic nitrogen sources such as ammonium chloride (0.5%), ammonium nitrate (0.5%), sodium nitrate (0.5%), yeast extract (1%), potassium nitrate (0.5%) and combination of ammonium nitrate (0.5%) and yeast extract (1%) . The maximum enzyme production was observed with ammonium nitrate (375±0.33 U/ml), yeast extract (388±0.24 U/ml) and combination of ammonium nitrate and yeast extract (398±0.28 U/ml) for immobilized spores. In case of free spores, maximum enzyme production was also observed with ammonium nitrate (315±0.13 U/ml), yeast extract (320±0.28 U/ml) and combination of ammonium nitrate and yeast extract (335±0.14 U/ml). A marked increase in enzyme activity (18.8%) was observed with combination of ammonium nitrate (0.5%) and yeast extract (1%) for enzyme production with free spores and immobilized spores, when incubated for 72 h, when compared to free spores at 48 h of incubation. The results were depicted in Fig.5. Farag *et al.,* (2015) studied the effect of nitrogen sources on L-asparaginase, an aminohydrolase like L-glutaminase and reported that ammonium sulfate was the best nitrogen source for the maximum production of L-asparaginase from *Aspergillus terreus*.

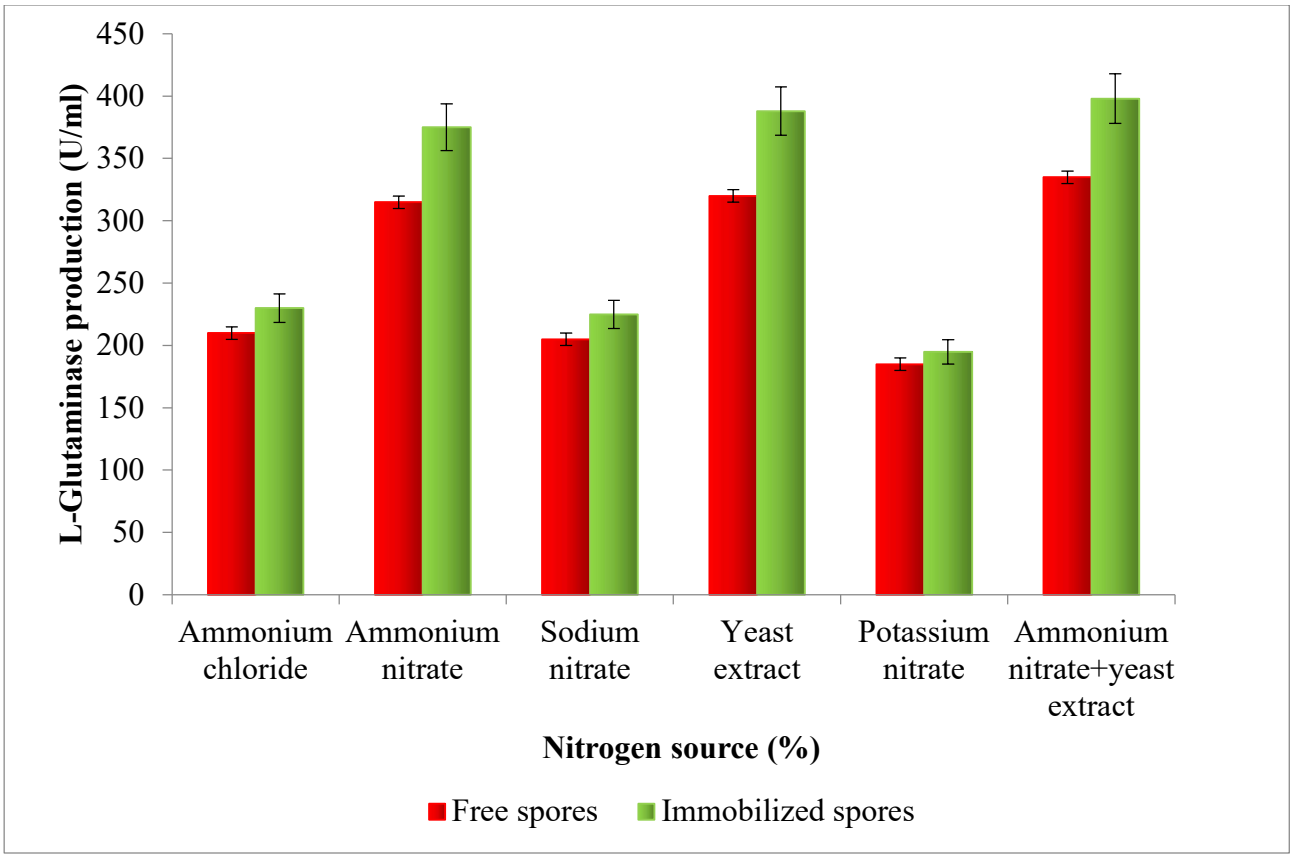

Fig. 5 Effect of nitrogen sources on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate

The bars indicate the standard deviation of three replicates analyzed.

Effect of inoculum size on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate

Effect of inoculum size on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium alginate was studied at different concentrations (1-5%) to check production of L-glutaminase. Maximum enzyme production was observed with 2% inoculum size for both free spores (340±0.33 U/ml) and immobilized spores (405±0.31 U/ml) (Fig. 6). A marked increase (19.1%) in enzyme production was observed with 2% inoculum for immobilized spores when compared to free spores.

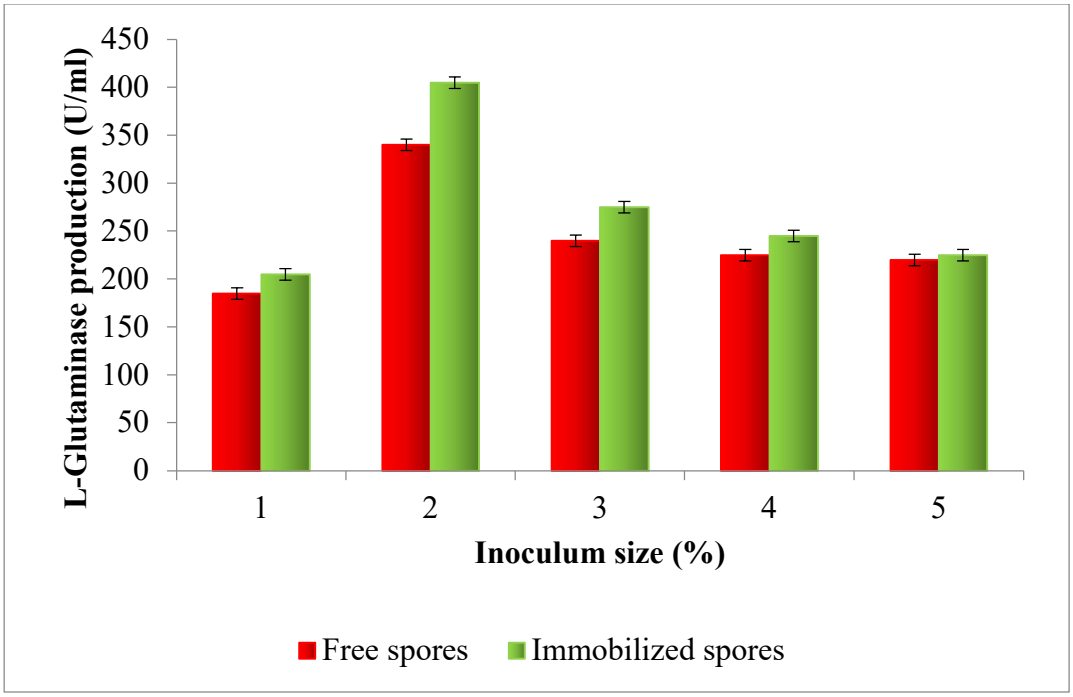

Fig. 6 Effect of inoculum size on production of L-glutaminase by immobilized *Aspergillus flavus* strain S4 spores in sodium aliginate.
The bars indicate the standard deviation of three replicates analyzed.

According to Sabu *et al.,* (2000) inoculum concentration plays a significant role in immobilized whole-cell reactors. Of the different concentrations of spore inoculum tested, a concentration of 12 × 10^8 spores/g of beads gave the maximal enzyme yield of 13.1 U/ml by Immobilized Marine *Beauveria bassiana* BTMF S-10 in Packed-bed reactor. The enzyme yield increased with an increase in spore inoculum concentration, characteristic of the immobilized-cell fermentations. However, there was a decrease in enzyme yield with the higher spore concentration of 16 × 10^8 spores/g of beads, which possibly is owing to substrate limitation at that spore concentration.

Operational efficiency of immobilized spores of *A. flavus* strain S4 in alginate beads

The stability of immobilized *A. flavus* strain S4 cells in repeated batch production was investigated in order to assess their ability for long term growth and synthesis of L-glutaminase. Fig. 6.7 illustrates the L-glutaminase production by *A. flavus* strain S4 spores. As a control, parallel experiments with a suspension of free spores were carried out. Free spores were not reusable for second cycle due to their mycelia growth pattern (results not shown). On the contrary, the immobilized spores in sodium alginate beads were reusable as they were easy to separate after each cycle with no significant loss in L-glutaminase productivity. Spores entrapped in calcium alginate beads retained about 90% of their initial activity during the first four batches when each cycle continued for 72 h. At the end of the 7th cycle, the immobilized spores lost 49% of their initial productivity. The operational efficiency for each operational cycle was shown in Fig.7.

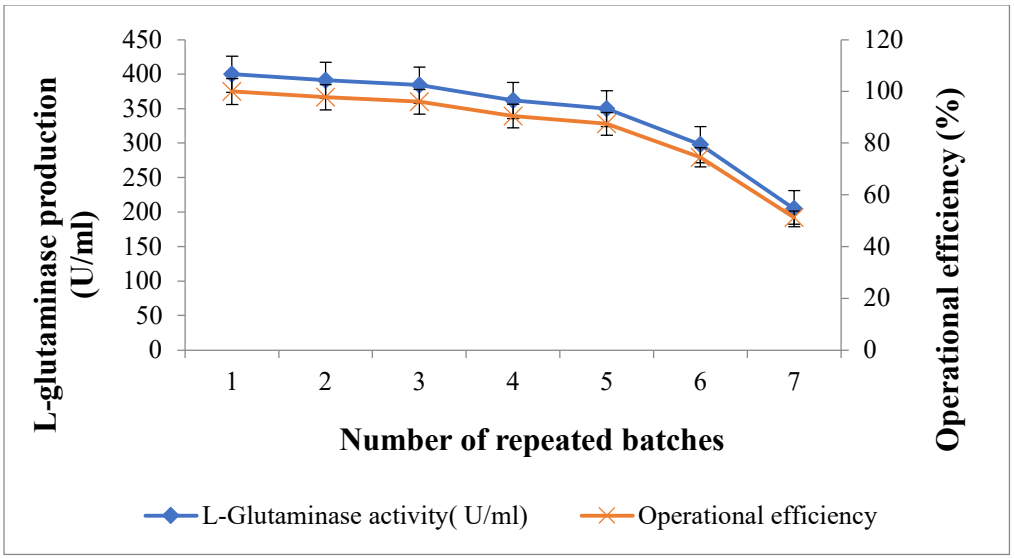

Fig. 7 L-glutaminase production on repeated cycles of immobilized spores of *A. flavus* strain S4 on sodium alginate

The bars indicate the standard deviation of three replicates analyzed.

Mahesh *et a*l., (2015) studied the possible reuse of immobilized *Pseudomonas* sp. KLM9 in sodium alginate for the production of L-glutaminase. In repeated batch fermentation the enzyme titer was remain constant for initial four cycles with 163 IU thereafter the enzyme productivity was slightly decreased and this was continued to another three cycles further there was declivity reported. A total of seven cycles were reported to be suitable for reusability of immobilized cells of *Pseudomonas* sp. KLM9 for L-glutaminase production.

Immobilization of L-glutaminase

The purified enzyme from *Aspergillus flavus* strain S4 was immobilized by calcium alginate method. Entrapment is the most common technique used for immobilization of enzymes. The most important feature of entrapment is the integration of enzyme in the matrix with retention of its biological functionality. So this calcium alginate approach seems to be good for entrapping of the enzyme without reducing its accessibility to substrate. Immobilization is considered a promising approach for enhancing the fermentation processes, enzymes production (Beshay, 2003; Kar and Ray, 2008).

Effect of pH on the activity of immobilized L-glutaminase

The pH profile of the immobilized L-glutaminase of *A. flavus* strain S4 was determined using different buffers of varying pH values by keeping other parameters constant. It was observed that there was no change in optimum pH upon immobilization but the activity was increased (11.2%) when compared with free enzyme (Fig. 8).

Immobilized L-glutaminase of *A. flavus* strain S4 was tested at different pH range (2-10). Optimum enzyme activity (442±0.22 U/ml) was recorded at pH-7.0. On the other hand free enzyme showed maximum enzyme activity (398±0.12 U/ml) at pH-7.0.

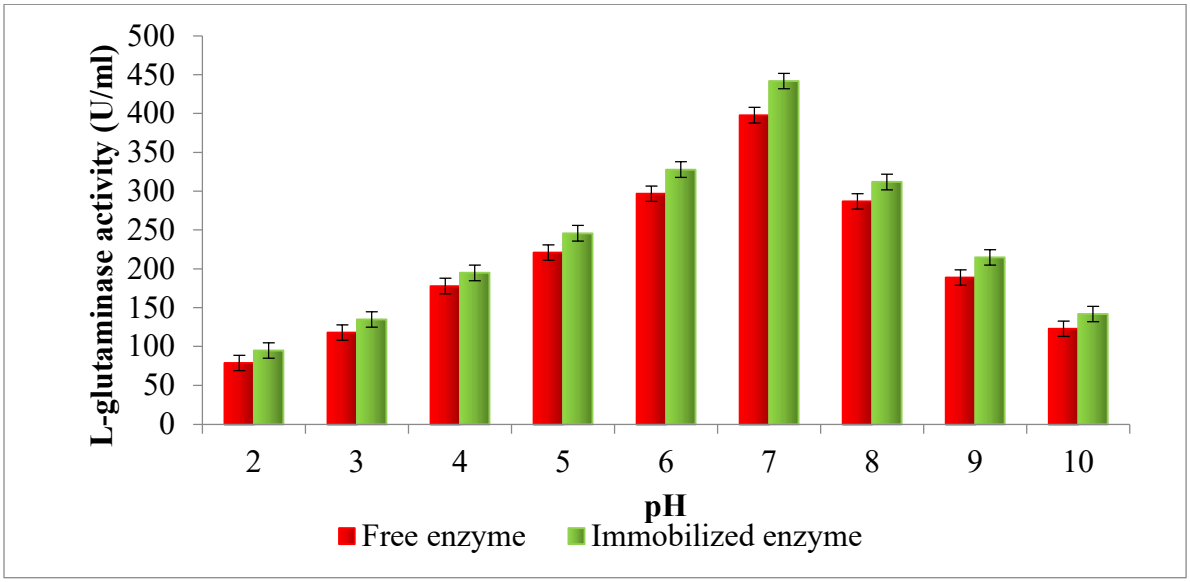

Fig. 8 Effect of pH on the activity of L-glutaminase (free and immobilized enzyme) The bars indicate the standard deviation of three replicates analyzed.

Effect of temperature on the activity of the immobilized enzyme

From the data presented in Fig. 9 it was clear that the immobilized L-glutaminase of *A. flavus* strain S4 was active at all the temperatures (20-70ºC) tested, with optimum enzyme activity (455±0.22 U/ml) recorded at 40ºC. On the other hand free enzyme showed maximum enzyme activity (398±0.12 U/ml) at 30ºC. An increase of enzyme was observed with immobilized enzyme (14.3%) when compared with free enzyme.

The temperature optima of L-glutaminase showed a shift of 10°C on entrapment in calcium alginate beads. The higher temperature profile on entrapment in calcium alginate beads may be due to some conformational effects on enzyme entrapment, which protect the enzyme against heat denaturation.

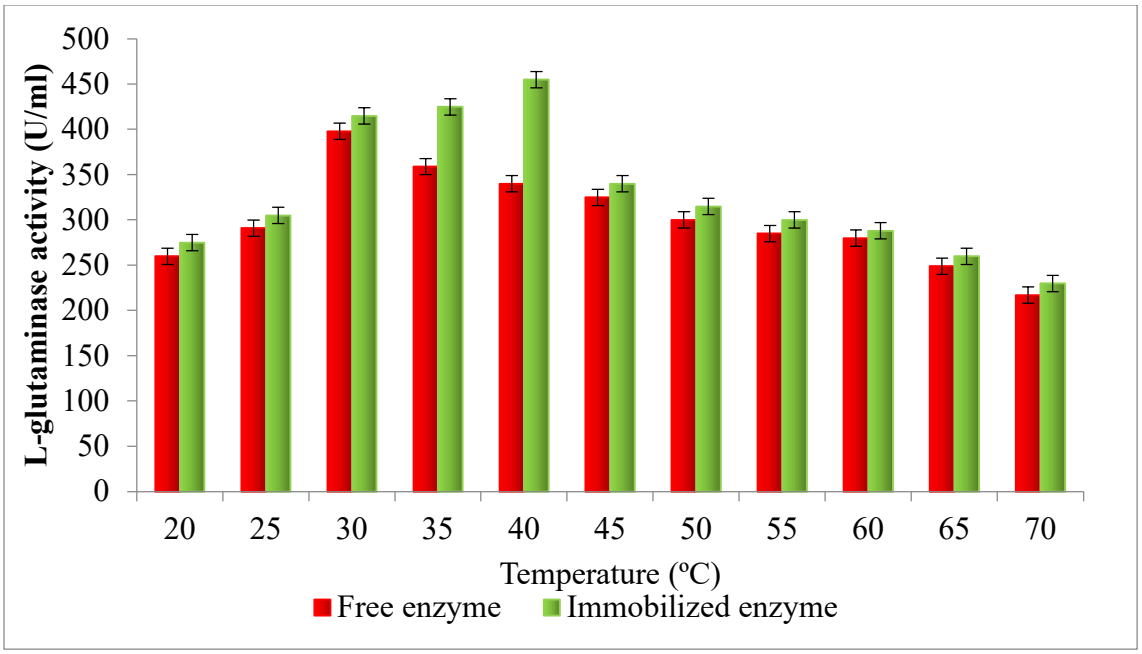

Fig. 9 Effect of temperature on the activity of L-glutaminase (free and immobilized) The bars indicate the standard deviation of three replicates analyzed.

Immobilized enzyme showed same optimum pH and temperature as that of free enzyme, indicating that immobilization of the enzyme did not alter the pH or temperature optima of the enzyme.

Karahan *et al*., (2014) studied temperature effect on the activities of free and immobilized L-glutaminase enzyme of *Hypocria jecorina*. The enzyme was immobilized on polyacrylic acid. The maximum catalytic activity was obtained at 50°C for free and immobilized enzymes. Mahmod (2016) studied the influence of temperature on *Bacillus subtilis* glutaminase immobilized with DEAE cellulose, chitin powder and charcoal as supporting matrices. DEAE-cellulose immobilized glutaminase retained 87% of the original activity when incubated at 37°C for 120 min. The activity of glutaminase decreased at 55°C and 65°C with three kinds of supports.

Reusable efficiency of immobilized L-glutaminase

Immobilized enzymes have many advantages over their free state counterparts. Most important of those are reusability and operational stability. These factors have economic implications like cost effectiveness for the process. Hence attempts have been made to immobilize the purified enzyme produced by *A. flavus* strain S4 and its reusability. The enzyme beads were assayed for L-glutaminase activity by the standard assay procedure with glutamine as substrate. There has been a meagre increase in the L-glutaminase activity with immobilization. Reusability was checked by repeating L-glutaminase assay for seven cycles. The immobilized enzyme has retained L-glutaminase activity for four cycles and later the enzyme activity decreased drastically (Fig.10).

With repeated use, the strength of binding between the matrix and enzyme was weakened, leading to leaching of enzyme from the matrix and loss in activity.

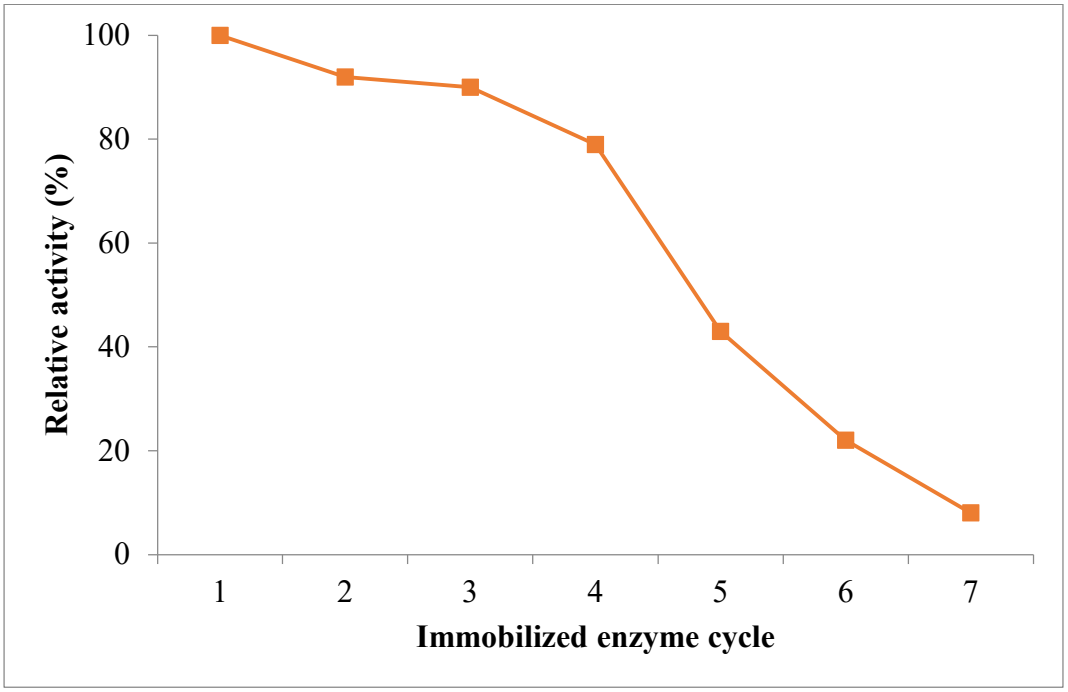

Fig. 10 Relative activities of immobilized enzyme at different operational cycles

Operational stability over several cycles with operation time over 200 h were reported for repeated batch production of cellulase (Linko *et al*., 1996) and lignin peroxidase (Linko, 1988) with nylon web as carrier.

Advantages of using immobilization process have been reported in such a way that facilitate continuous operation over a prolonged period, offer possible recycling of immobilized beads and simple way for harvesting the products, reactor productivity, ensures higher efficiency of catalysis (Kar and Ray, 2008) and development of economical methods focusing on lowering the cost of industrial process.

Kinetic studies of immobilized enzyme

Studies on the kinetic parameters of immobilized enzyme revealed that the L-glutaminase activity was increased with increase in substrate concentration and maximum L-glutaminase activity was observed at 4.0% glutamine beyond which there was slight inhibition in activity was recorded.

K_M and V_{max} values of both free and immobilized enzymes were calculated from the intercepts on x and y axis of the Lineweaver–Burk plots. For the free enzyme, K_M was found to be

5.28 mM (Chapter-V, Fig-5.13) and the K_M value of immobilized L-glutaminase enzyme was 6.24 mM (Fig. 11). K_M value was found to be slightly higher in the case of immobilized enzyme when compared to free enzyme. V_{max} also slightly varied in both the cases (Table 6.1). The slight increase in K_M value when compared with that of the free enzyme may be due to the lower accessibility of the substrate to the active site of the immobilized enzyme.

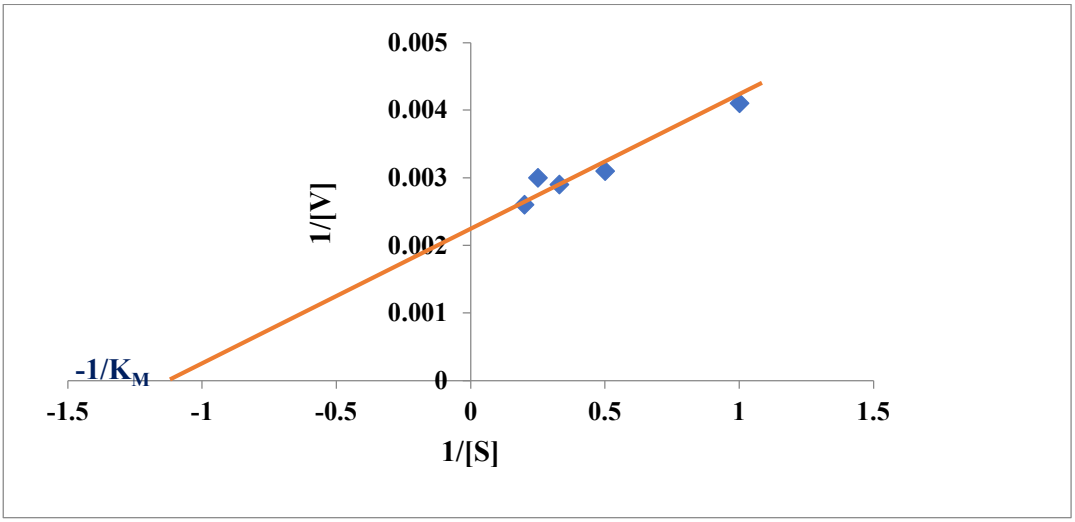

Fig. 6.11 Lineweaver-Burk plot for immobilized L-glutaminase

Table 6.1 The kinetic properties of free and immobilized L-glutaminase

Enzyme	K_M (mg/ml)	Vmax (U/l)
Free	5.28 mM	454.5
Immobilized	6.24 mM	476.2

Immobilized L-glutaminase exhibited K_M value slightly higher than the free enzyme. The maximum rate of the reaction catalyzed by the immobilized enzyme was lower than the free enzyme. This increase in K_M clearly indicates an apparent low affinity of the enzyme towards its substrate compared to the free enzyme. The higher values of K_M as compared to pure enzymes are probably due to the rigid immobilization of the enzyme complex which restricts its interaction with the substrate. Karahan *et al*., (2014) reported the kinetic parameters of the immobilized L-

glutaminase (K_M and V_{max} values) as 0.38 mM and 10.9 U/l respectively. No drastic change was observed in the K_M and V_{max} values upon immobilization.

Conclusion

The results of the present study showed that calcium alginate is a promising method for the immobilization of spores of *A. flavus* strain S4. L-glutaminase production was maximum at 72 h of incubation. In case of free spores, 48 h of incubation was sufficient for maximum L-glutaminase production. L-glutaminase production was increased even though incubation time was prolonged. Enzyme immobilization study revealed that there was a change in kinetic parameters with marginal increase in enzyme activity at 40ºC, pH- 7.0, incubation period- 72 h, inoculum size- 2%, carbon source- dextrose and nitrogen source- combination of ammonium nitrate and yeast extract). However the beneficial aspect of immobilized enzyme is its operational stability and reusability. This approach could be utilized for enzyme recycling without affecting the integrity of the catalytic sites of the enzyme. Since immobilized enzymes can be recovered from reaction mixture and can be made available for reuse again, so, they are preferred to free enzymes. At last, it can be concluded that enzyme immobilization is the best method for retention of enzymes activity for longer periods. The results of the present study show that immobilized L-glutaminase may be used more successfully than free L-glutaminase in the breakdown of glutamine. Although this immobilization technique using alginate beads improved some properties of L-glutaminase, experiments need to be carried out to study thermal stability, operational stability and other parameters in order to achieve better L-glutaminase production/ activity and to recommend its usage in industries.

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