JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2010. Vol. **4**(2), p. 623-628

Immobilization and Characterization of L-Asparaginase from *Streptomyces gulbargensis*

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(Received: 28 December 2009; accepted: 02 February 2010)

The enzyme L-asparaginase has been a clinically acceptable anti-tumor agent for the effective treatment of acute lymphoblastic leukemia. In the present study, the purified L-asparaginase from *Streptomyces gulbargensis* was immobilized in three different matrices such as calcium alginate, gelatin and alginate-gelatin fibers. The activity yields obtained with calcium alginate, gelatin and alginate-gelatin fibers was 39%, 44% and 69% respectively. Hence, entrapment in alginate-gelatin fibers was found to be comparatively superior to the other two matrices. Characterization of the alginate-gelatin entrapped L-asparaginase revealed that the immobilized enzyme showed a shift in optimum pH value from pH 9.0 (optimum pH value of free enzyme) to 9.5. However, there was no change in the optimum temperature for the immobilized enzyme compared to the free enzyme, being 40°C for both forms of enzymes. Further, there was a significant increase in the pH and temperature stability of the immobilized L-asparaginase compared to the free enzyme.

Key words: *Streptomyces gulbargensis,* Groundnut cake extract, Immobilization, L-asparaginase.

The application of enzyme technologies to pharmaceutical research, development and manufacturing is a growing field and is the subject of interest. The concept of therapeutic enzymes has been around for at least 40 years[1]. Therapeutic enzymes are gaining importance mainly because of their broad variety of specific uses such as oncolytics, thrombolytics or anticoagulants and as replacements for metabolic deficiencies[2].

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L-asparaginase (L-asparagine aminohydrolase EC 3.5.1.1), the enzyme which converts L-asparagine to L-aspartic acid and ammonia has been used as a chemotherapeutic agent[3]. It has received increased attention in recent years for its anti-carcinogenic potential[2]. The clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumor cells unable to synthesize this amino acid are selectively killed by L-asparagine deprivation[3]. The enzyme is produced by a large number of micro organisms that include *Enterobacter cloacae*[4], *Serratia marcescens*[5] and *Thermus thermophilus*[3]. The enzymes isolated from *Escherichia coli* and *Erwinia carotovora* are now being used in the treatment of acute lymphoblastic leukemia[6].

The enzyme L-asparaginase also finds a potential application in food industry as an acrylamide degrading enzyme as asparagine appears to be a key participant in acrylamide formation in foods. In principle, L-asparaginase-

catalyzed hydrolysis of asparagine to aspartic acid and ammonia in food could be a useful approach to reduce the extent of heat induced acrylamide formation[7].

In view of the potential application of L-asparaginase, attempts were made to immobilize it on different supports, which could be a useful approach for the construction of asparagine-based biosensors for the detection of asparagine levels in leukemia blood samples and also its application as an acrylamide-degrading enzyme in food industry. For industrial use, enzymes must be produced at low cost and should be reused and reproduce results with consistent efficiency. To achieve this, many techniques of immobilization of enzymes on different types of supports have been developed. For successful development and application of an immobilized biocatalyst, enzyme support is generally considered as the most important component[8]. Immobilization of enzyme in gel may offer several advantages such as repeated use of the enzyme, ease of product separation and improvement in enzyme stability.

The present study focused on the entrapment of *Streptomyces gulbargensis* L-asparaginase in calcium alginate, gelatin and alginate-gelatin fibers. There are very few reports on the immobilization of L-asparaginase in the above mentioned matrices. Also, attempts were made to compare the kinetic properties of immobilized L-asparaginase with those of the free enzyme. To our knowledge, this is the first report on the immobilization and characterization of *S. gulbargensis* L-asparaginase.

MATERIAL AND METHODS

Source of chemicals

All the chemicals used were of analytical grade. Chemicals used in this study were purchased from Qualigens and SD Fine Chemicals, India.

Microorganism

The strain *S.gulbargensis* was isolated from soil collected from Gulbarga region of Karnataka province, southern India. It was identified as a novel species at Yunnan Institute of Microbiology, China. The type culture/strain is deposited at China (=CCTCC Ac No 206001) and Korea (=KCTC Ac No 19179). The 16S rRNA gene sequence of the strain is deposited in the GenBank database under the accession number DQ317411[9]. It was maintained on starch casein agar (pH 7.2) containing (gL^{-1}) Starch 10, K_2HPO_4 2.0, KNO_3 2.0, NaCl 2.0, Casein 0.3, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and agar 20 at a temperature of 4°C. Regular sub culturing of the isolate was performed at an interval of every 4 weeks.

Inoculum preparation

Spore suspension was prepared from 5 days old culture grown on starch casein agar slant by adding 10ml of sterile distilled water containing 0.01% (v/v) of Tween 80 and suspending the spores with a sterile loop[10]. One ml of this spore suspension was used as an inoculum for fermentation studies.

Production of L-asparaginase

Production of L-asparaginase was carried out in groundnut cake extract. The groundnut cake was procured from the local market in Gulbarga, Karnataka, India. The extract was prepared using ten grams of the powdered substrate dissolved in 100 ml of distilled water, taken in 250 ml Erlenmeyer flask. The contents of the flask were heated for about ten minutes, cooled to room temperature and then filtered using Whatman filter paper No.1. The pH of the medium was adjusted to 8.5 using 0.1N NaOH/HCl. The extract thus obtained was autoclaved at 121°C for 15 min and used for subsequent fermentation studies. The flasks were incubated at 40°C for 5 days in a shaker incubator maintained at 200 rev/min (Remi Orbital Shaker Incubator).

Purification of L-asparaginase

The L-asparaginase from *S.gulbargensis* was purified by four steps, with a final yield of 32% and a purification fold of 82.12[11].

Immobilization of L-asparaginase and Its Characterization

Entrapment in Calcium alginate

The enzyme was immobilized in calcium alginate as per the method of Naganagouda and Mulimani[12]. 1 ml of purified enzyme solution was added to 4 ml of 3% sodium alginate solution The mixture was extruded drop wise using a sterile hypodermic syringe needle into 0.2 M $CaCl_2$ solution maintained at 4°C to form beads. The beads were allowed to harden in the $CaCl_2$ solution

for 2 h. The resulting spherical beads were washed with distilled water and stored in 50 mM Tris-HCl buffer (pH 9.0) at 4°C until further use.

Entrapment in Gelatin

The immobilization was performed as per the modified method of Adinarayana[13] *et al*. 5 ml of purified L-asparaginase solution was added to 15 ml of 10% sterile gelatin, maintained at 45°C, and poured into a sterile Petri dish. The gel was over layered with 10 ml of 5% glutaraldehyde for hardening at 30°C. The resulting blocks were cut into small-sized cubes (4 mm^3) and the cubes were washed thoroughly with distilled water for complete removal of excess glutaraldehyde. The blocks were then stored in 50 mM Tris- HCl buffer (pH 9.0) at 4°C until further use.

Entrapment in Alginate-gelatin fibers

Immobilization of L-asparaginase in calcium alginate-gelatin composites was performed as per the method of Youssef and Al-Omair[14]. Combinations of gelatin-alginate mixtures were prepared by adding gelatin (3%) to sodium alginate solution (5%) in water and then cross linking with glutaraldehyde. Sodium alginate (500 mg) and gelatin (300 mg) were added to distilled water (8 ml) in a conical flask which was autoclaved for 15 min at 121°C. The hot solution was allowed to cool to room temperature. The purified L-asparaginase solution (3 ml) was then added and the mixture was stirred for 15 min. Gluteraldehyde (0.3 ml of 25% solution in water) was added and the contents were stirred for an additional 15 min. The slurry was taken into a syringe, added drop-wise into 0.2 M $CaCl_2$ solution from 5-cm height, and kept for curing at 4°C for 2 h. The fibers were washed with 50 mM Tris- HCl buffer (pH 9.0) and then with distilled water, cut into 3 mm long pieces and stored in the same buffer until used.

L-asparaginase assay

Assay of L-asparaginase was carried out as per Imada[15] *et al.* 0.5 ml of 0.04 M L-asparagine was taken in a test tube, to which 0.5 ml of 0.05 M buffer (Tris-HCl buffer pH 7.2), 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2 ml and the reaction mixture was incubated for 30 min at 37°C. After the incubation period, the reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid. 0.1 ml was taken from the above reaction mixture and added to 3.7 ml of distilled water and to that 0.2 ml of Nessler's reagent was added and incubated for 15-20 min at 20°C. The O.D was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of trichloroacetic acid. The enzyme activity was expressed in IU. One IU of L-asparaginase is the amount of enzyme which liberates 1µmole of ammonia per ml per min (µmole/ml/min).

Activity Yield

Activity yield represents the percent of the ratio of the enzyme activity of the immobilized enzyme to the total units of soluble/ free enzyme used for immobilization. The activity yield (%) was defined as:

$$\text{Activity Yield (\%)} = A / B \times 100$$

A =activity of immobilized enzyme

B = activity of soluble/free enzyme

Characterization of immobilized L-asparaginase

Effect of pH and temperature on immobilized L-asparaginase activity

The activity of immobilized L-asparaginase was evaluated at different pH values. The immobilized enzyme was incubated with 0.04 M L-asparagine and 0.05 M buffers of pH 4-10, with an increment of pH 0.5, under assay conditions and the amount of ammonia liberated was determined. Buffers used were potassium phosphate (pH 4.0-7.0), Tris- HCl (pH 8.0-9.0) and glycine- NaOH (pH 10). To test the effect of temperature on immobilized L-asparaginase activity, the reactions were performed over the range of temperatures ranging from 10-70°C with an increment of 10°C.

Effect of pH and temperature on stability of immobilized L-asparaginase

For pH stability check, the immobilized enzyme was incubated at different pH (4-10) with an increment of pH 0.5, in the absence of substrate. The pre-incubation was carried out for 60 min and then the residual activity was measured. Thermostability studies of immobilized L-asparaginase were carried out by pre-incubating the enzyme (without substrate) at temperatures 10-70°C in 10°C increment for 60 min. The residual activity was then determined as described earlier.

RESULTS AND DISCUSSION

The purified L-asparaginase was entrapped in three different matrices such as calcium alginate, gelatin and alginate-gelatin fibers. The activity yields obtained with calcium alginate, gelatin and alginate-gelatin fibers was 39%, 44% and 69% respectively. Hence, the results obtained in the present study revealed that entrapment in alginate-gelatin fibers was comparatively superior to the other two matrices. In alginate-gelatin fibers, the advantages of alginate and gelatin have been combined. In general, calcium alginate beads are not normally used for enzyme entrapment as large pore size of these beads result in enzyme leakage out of such beads[12]. Immobilization of enzyme in gelatin requires additional process and the procedure involves freezing and thawing which denatures the enzyme and lowers the immobilization efficiency. The immobilization in alginate-gelatin fibers is simple and its subsequent use is comparatively cheap and safe with durable enzyme activity. In the present study, the kinetic properties of the alginate-gelatin entrapped L-asparaginase were compared to those of the free enzyme.

Characterization of immobilized L-asparaginase

Effect of pH and temperature on immobilized L-asparaginase activity

Fig. 1 shows the effect of pH on immobilized and free enzyme. There was a shift in the optimum pH value of the immobilized L-asparaginase from pH 9.0 (optimum pH value of free enzyme) to 9.5. Youssef and Al-Omair[14] observed a similar shift in the pH optimum of alginate-gelatin entrapped L-asparaginase II from *E.coli* W3110. The optimum pH value shifted from pH 7.5 to 8.5 after immobilization. This shift could be due to increased proton concentration in the enzyme microenvironment which would require a more alkaline assay medium for the maximum activity of the enzyme[16]. Changes in pH activity have been explained on the basis of an unequal distribution of hydrogen and hydroxyl ions between the polyelectrolyte phase on which the enzyme is immobilized and the external solution[14].

Fig. 2 shows the effect of temperature on immobilized and free enzyme. It was observed that there was no change in the temperature optima for the immobilized enzyme compared to the free enzyme. The maximum activity was recorded at 40°C for both forms of enzymes. Naganagouda and Mulimani,[12] made a similar observation that the optimum temperature of alginate-gelatin entrapped á-galactosidase from *Aspergillus oryzae* was not affected, being 50°C for both forms of enzymes. On the contrary, Youssef and Al-Omair[14] reported that the temperature optimum of immobilized L-asparaginase II from *E.coli* W3110 was raised to 50^{0o}C from 40°C. Our results are in good

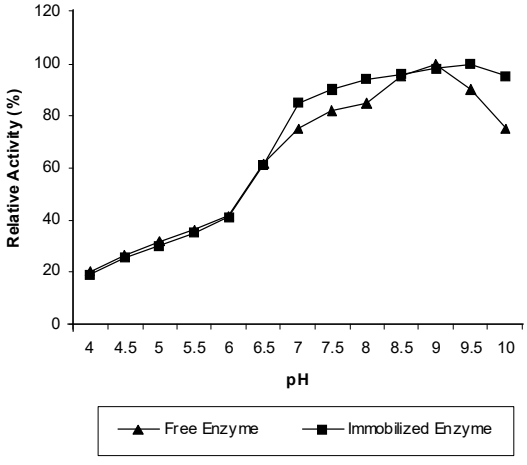

Fig. 1. Effect of pH on Free and Immobilized L-asparaginase

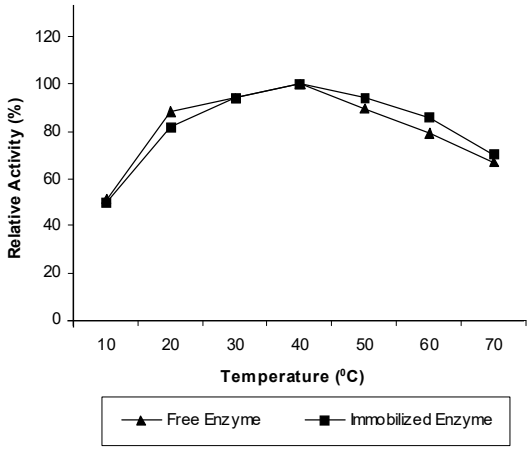

Fig. 2. Effect of Temperature on Free and Immobilized L-asparaginase

agreement with those of Naganagouda and Mulimani[12].

Effect of pH and temperature on stability of immobilized L-asparaginase

Figure 3 depicts the effect of pH on stability of free and immobilized L-asparaginase. The immobilized L-asparaginase showed a stability of 70-98% in the pH range of 6.0-10. At pH 6.0, the free enzyme showed a residual activity of 41% compared to the entrapped enzyme which retained 70% of the activity. The residual activity at pH 10.0 was found to be 91% and 98% with free and immobilized L-asparaginase respectively.

Thermal stability was increased after entrapment of L-asparaginase in alginate-gelatin fibers as shown in Fig. 4. At 50°C, the free enzyme displayed a residual activity of 88% compared to the immobilized form which could display a residual activity of 98%. This probably reflects the fact that the entrapped enzyme is not chemically modified but remains in its native form in gel matrix. The free L-asparaginase II of *E.coli* W3110 retained 22.4% of initial activity after heat treatment at 60°C for 30 min, while the alginate-gelatin immobilized enzyme could display a residual activity of 66.8%[14]. Thermal stability of immobilized enzyme increases because the immobilization process will make the enzyme more rigid and that the process will change the microenvironment of the enzyme[16].

Verma[17] *et al.,* have reported the immobilization of *E.coli* K-12 L-asparaginase for the development of novel, diagnostic biosensor for monitoring asparagine levels in leukemia

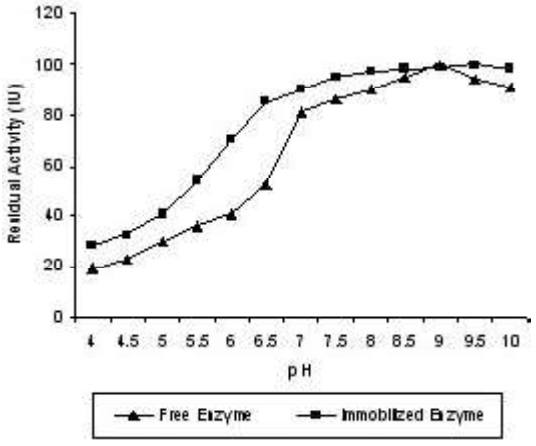

Fig. 3. Effect of pH on Stability of Free and Immobilized L-asparaginase

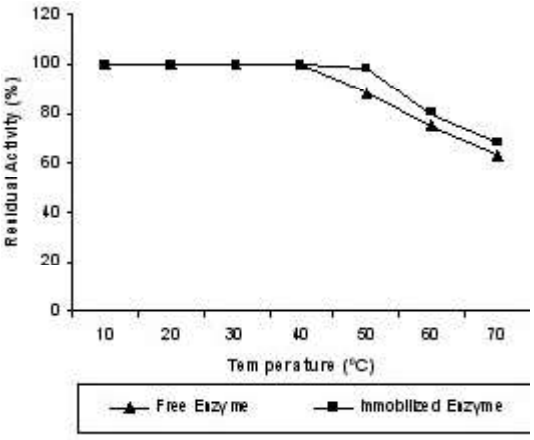

Fig. 4. Effect of Temperature on Stability of Free and Immobilized L-asparaginase

samples. Three different matrices have been used in their studies i.e., nitrocellulose membrane, silicon gel and calcium alginate. The detection limit of asparagine achieved with nitrocellulose membrane is 10^{-1} M, with silicon gel is 10^{-10}–10^{-1} M, and with calcium alginate beads was 10^{-9}–10^{-1} M. Furthermore, the calcium alginate bead system of immobilization has been applied for the asparagine range detection in normal and leukemia serum samples.

The present study concluded that entrapment of L-asparaginase in alginate-gelatin is a better choice over the other two matrices employed in the study and such immobilized enzyme can serve as a promising biocatalyst. Further, the significant increase in the stability of immobilized L-asparaginase at high temperature as well as at wide pH range increases possibility of its use in the construction of asparagine-based biosensors for the detection of asparagine levels in leukemia blood samples and also its application as an acrylamide-degrading enzyme in food industry.

ACKNOWLEDGEMENTS

The first author is thankful to Dr. Dastager S. G. for providing the strain *Streptomyces gulbargensis*.

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