Immobilization of *Streptomyces gulbargensis* in polyurethane foam: A promising technique for L-asparaginase production

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Abstract
In the present study, *Streptomyces gulbargensis* and its mutant form, *S.gulbargensis* mu24, immobilized on polyurethane foam were investigated for the production of L-asparaginase using groundnut cake extract as medium. The medium with an initial pH of 8.5 was inoculated with free and immobilized cells separately and then subjected to fermentation by incubation at 40^{O}C and shaking at 200 rev/min. In the immobilized cell system, enzyme production was enhanced by approximately 30% compared to the conventional free-cell fermentation. The immobilized cells were subjected to repeated batch fermentation processes to determine their reusability. These cells retained their ability to produce L-asparaginase over seven cycles and the activities remained between 16.2-41.3 IU/ml and 39-60 IU/ml for *S.gulbargensis* and *S.gulbargensis* mu24, respectively. The maximum enzyme titer was obtained during the third batch by both strains. However, the mutant strain was more potent for L-asparaginase production than the prototype. Therefore, the polyurethane foam immobilized cells of *S.gulbargensis* mu24 can be proposed as an effective biocatalyst, which can be repeatedly used for maximum production of L-asparaginase.
keywords: L-asparaginase; *Streptomyces gulbargensis*; Groundnut cake extract; Immobilization; Polyurethane foam

INTRODUCTION

The enzyme L-asparaginase (L-asparagine aminohydrolase EC 3.5.1.1) has been a clinically acceptable antitumor agent for the effective treatment of acute lymphoblastic leukemia and lymphosarcoma (Verma *et al*., 2007). It catalyses the conversion of L-asparagine to L-aspartate and ammonium, and this catalytic reaction is essentially irreversible under physiological conditions (Prakasham *et al*., 2006). 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 (Prista and Kyriakidis, 2000).This enzyme is produced by a large number of microorganisms that include *Escherichia coli* (Swain *et al*., 1993), *Erwinia aroideae* (Liu and Zajic, 1973) *Pseudomonas stutzeri* (Manna *et al*., 1995), *Pseudomonas aeruginosa* (Abdel-Fatteh and Olama, 2002) and *Enterobacter aerogenes* (Mukherjee *et al*., 2000). In the past few years, significant studies have been carried out on L-asparaginase production and efforts to achieve high productivity under conditions of submerged and solid-state cultivation have also been made. One of the approaches to improve the enzyme yield is by long term continuous production of L-asparaginase under cell immobilization conditions. At present, cell immobilization technology is often studied for its potential to improve fermentation processes and bioremediation (Beshay, 2003). The immobilization of whole microbial cells and their applications to bioprocessing have been of interest for nearly thirty years (Kar and Ray, 2008). Immobilization of whole cells for extra-cellular enzyme production offers several advantages, such as the ease to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolong period, enhancing reactor productivity and ensuring higher efficiency of catalysis (Kar and

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Ray, 2008). However, proper selection of immobilization techniques and supporting materials is needed to minimize the disadvantages of immobilization (Beshay, 2003). The most widely used immobilization method is entrapment, but the adsorption method has two advantages namely its simplicity and better physiological conditions (Pramod and Lingappa, 2008).

Polyurethanes (PU) are one of the most versatile materials in the world today. They are known for being a perfect material for footwear, machinery industry, coatings and paints, rigid insulation, elastic fiber, soft flexible foams and medical devices. Immobilization of cells in polyurethane foam offers several advantages including high mechanical strength, resistance to organic solvents and microbial attack, easy handling, regenerability and cost-effectiveness (Patil *et al*., 2006). It has been found to be applicable in the biochemical and biotechnological fields as a perfect support for enzyme immobilization (Romaskevik *et al*., 2006).

In view of the potential applications of L-asparaginase and the need for the development of economical methods for improved enzyme production with an overall aim of reducing the cost of industrial processes, whole-cell immobilization can serve as an excellent alternative for increasing enzyme yields. Hence, the present investigation was aimed at exploring the practicability of L-asparaginase production from groundnut cake extract by *S. gulbargensis* and *S. gulbargensis* mu24 immobilized on polyurethane foam as a support matrix. The reusability of immobilized cells for enzyme production under repeated batch conditions was also investigated.

MATERIALS AND METHODS

Chemicals: All the chemicals used in the present study were of analytical grade and were purchased from Qualigens Fine Chemicals, India. Polyurethane foam and groundnut cake were obtained from the local market in Gulbarga, Karnataka, India.

Microorganism: The strain *Streptomyces gulbargensis* was obtained from the Department of Microbiology, Gulbarga University, Gulbarga. The isolate was identified as a novel strain at Yunnan Institute of Microbiology, China (Dastager *et al*., 2007). The 16S rRNA gene sequence of the strain was deposited in the GenBank database under the accession number DQ317411 (Dastager *et al*., 2007). The type culture/strain was deposited at =CCTCC Ac No 206001; =KCTC Ac No 19179 (Dastager *et al*., 2007). *S. gulbargensis* was subjected to mutagenesis in order to obtain a higher L-asparaginase producing strain. A total of thirty two mutants (mu1- mu32) were obtained by physical (treatment with UV rays) and chemical methods (treatment with Ethidium bromide and methanesulphonic acid ethyl ester) (Gardener *et al*., 1956). Amongst the thirty two strains, *S. gulbargensis* mu24 appeared as the most potent for L-asparaginase production. The mutant was obtained by treating 1 ml of spore suspension from *S.gulbargensis* with 1 ml of methanesulphonic acid ethyl ester (EMS) at a concentration of 10 mg/ml for 10 min. Following the exposure, spores were suspended in 1ml of saline solution and plated on starch casein agar medium. The strains were maintained on starch casein agar slants containing (g/l) 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 (pH 7.2) at a temperature of 4ºC. Regular subculturing of the isolates was performed at an interval of every 4 weeks.

Inoculum preparation: The spore suspension was prepared from a 5 days old culture grown on starch casein agar slant by adding 10 ml of sterile distilled water containing 0.01% (v/v) Tween 80 and suspending the spores with a sterile loop (Lingappa and Vivek Babu, 2005). This spore suspension (containing 1×10^8 spores/ml) was used as an inoculum for immobilization as well as for free-cell fermentation studies.

Preparation of fermentation medium: The groundnut cake was procured from the local market in Gulbarga city. It has the following composition: moisture (8.5%), total carbohydrates (27.1%), protein (49.1%), fat content (2.0%), ash (4.9%), crude fiber (6.3%), calcium (0.41%), magnesium (0.39%), iron (540 ppm), zinc (60 ppm), copper (22 ppm) and manganese (25.9 ppm). The groundnut cake extract was prepared using 10 g of the powdered substrate dissolved in 100 ml of distilled water in a 250 ml Erlenmeyer flask. The contents of the flasks were heated for about 10 min and cooled to room temperature. It was filtered using the Whatman filter paper No.1. The pH of the medium was adjusted to 8.5 using 0.1 N NaOH/HCl. The extract thus obtained was autoclaved

at 121ºC for 15 min and used for subsequent fermentation studies. All the experiments were performed independently in triplicate and the results were expressed as the mean of three values.

Immobilization on polyurethane foam: The polyurethane foam material used for the immobilization studies had a porosity of 100-500 µm. It was cut into 1cm^2 pieces (Kuhad *et al*., 2004) and washed with distilled water. One gram of the foam pieces was submerged in a 250 ml Erlenmeyer flask containing 50 ml of the fermentation medium and then sterilized at 121ºC for 15 min (Kuhad *et al*., 2004).

Fermentation processes

Batch fermentation with immobilized and free cells: The batch experiments were performed in 250 ml Erlenmeyer flasks each containing 50 ml of fermentation medium. The free-cell fermentation was carried out by adding the spore suspensions of *S. gulbargensis* and *S. gulbargensis* mu24 separately to the medium without supporting matrix. Parallel experiments were conducted with immobilized cells by inoculating the the flask containing polyurethane foam pieces with the spore suspension (Kuhad *et al*., 2004). The flasks were incubated at 40ºC in a shaker incubator maintained at 200 rev/min. Samples were withdrawn at regular intervals of 24 h and assayed for L-asparaginase activity.

Repeated Batch fermentation: One of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously (Reyed, 2007). Therefore, the reusability of cells immobilized on polyurethane foam was examined. In case of repeated batch studies, the fermentation medium was aseptically decanted from each flask every 72 h and fresh medium was then added. The process was continued for the next cycle. Subsequent batches were run at 72 h intervals. The enzyme titers and cell leakage at every cycle were determined.

Analytical methods

L-asparaginase assay: The culture broth was centrifuged at 10,000 rpm for 8 min (Ding *et al*., 2003). The supernatant obtained served as the crude enzyme source. Assay of enzyme was carried out as per Imada *et al*. (1973). One unit of L-asparaginase is defined as the amount of enzyme which liberates 1 µm of ammonia per ml per min (µm/ml/min).

Protease assay: The proteolytic activity was determined as per the modified Anson's method (1938).

Cell growth and cell leakage: The free cells and the cells leaked from the support matrix were collected by centrifugation at 3000 rpm for 10 min and the biomass was determined as per the method of Srinivasalu and Ellaiah, (2005).

RESULTS

Figure 1 shows the L-asparaginase production pattern by free and immobilized cells of *S. gulbargensis*. The analysis of enzyme production was carried out at 24 h intervals. The standard deviation values in all the experiments were found to be within 10% with respect to the fact that the experiments were conducted in triplicates. In case of free cells, enzyme production reached a maximum activity of 30 IU/ml at 120 h of incubation. On further incubation, the enzyme activity gradually decreased. This may be due to the depletion of essential nutrients required for the growth of the organism. However, the cells immobilized on polyurethane foam showed a significant increase in the production of L-asparaginase from the beginning of the cultivation process. Maximum enzyme titer (40 IU/ml) was observed at 72 h of incubation. Figure 1 also shows that the enzyme production by immobilized cells reached approximately 28 IU/ml at 48 h, which could only be obtained after 96 h with free cells. Similar results were obtained with the mutant strain. Figure 2 shows the L-asparaginase production pattern

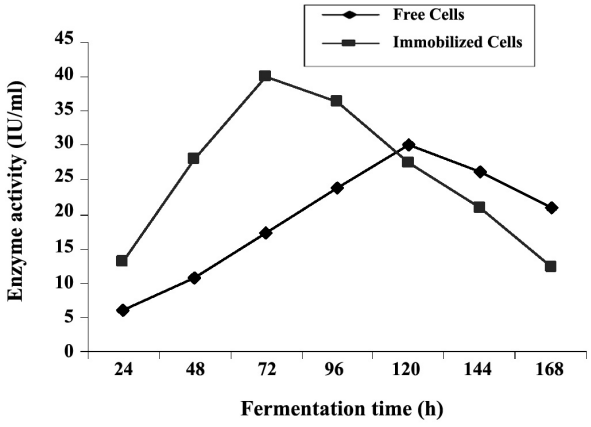

Figure 1. L-asparaginase production by free and immobilized cells of *S. gulbargensis*.

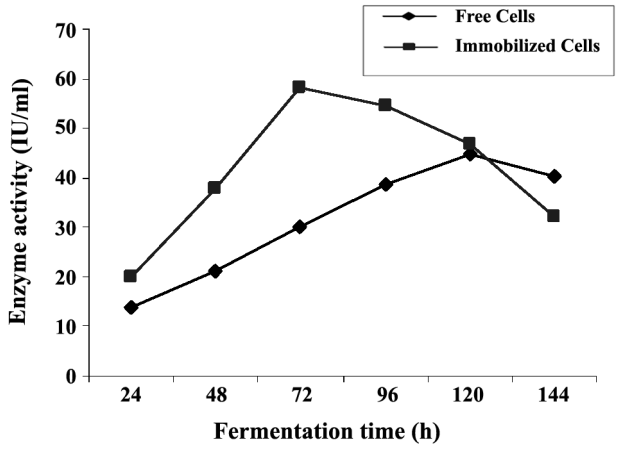

Figure 2. L-asparaginase production by free and immobilized cells of *S. gulbargensis* mu24.

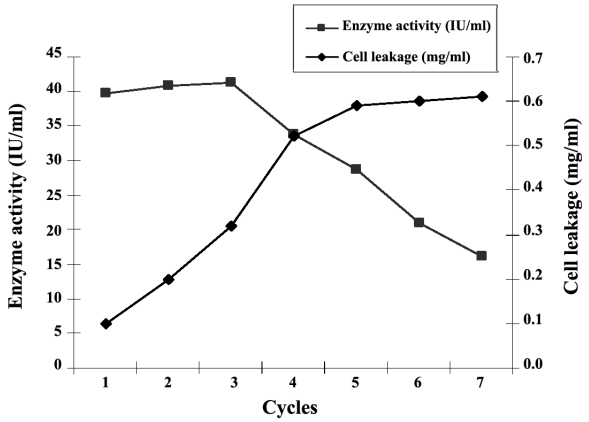

Figure 3. L-asparaginase production by repeated batch culture using immobilized cells of *S. gulbargensis*.

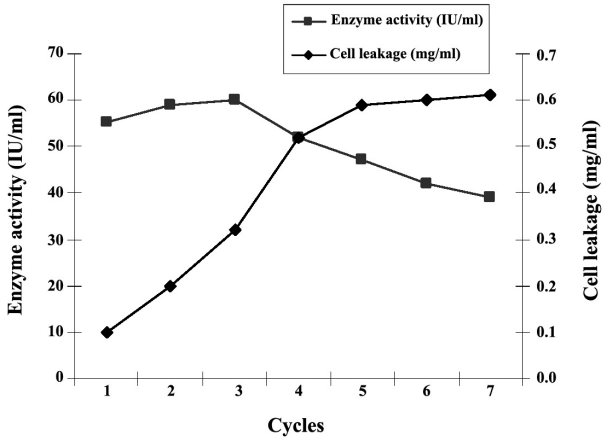

Figure 4. L-asparaginase production by repeated batch culture using immobilized cells of *S. gulbargensis* mu24.

by free and immobilized cells of *S. gulbargensis* mu24. In case of free cells, enzyme production reached a maximum activity of 44.7 IU/ml at 120 h of incubation. In case of immobilized cells maximum enzyme titer (58.2 IU/ml) was observed at only 72 h of incubation. The data obtained in the present study revealed that high amounts of L-asparaginase can be obtained in a shorter period with immobilized cells when compared to the free cells.

The possible reuse of *S. gulbargensis* and *S. gulbargensis* mu24 cells immobilized onto polyurethane foam for enzyme production in the semi-continuous mode is shown in Figure 3 and Figure 4, respectively. The repeated batch fermentation process could be successfully conducted for seven batches. The maximum enzyme titer was reached during the third cycle of the repeated batch fermentation with both the wild type and the mutant strains. The highest activities of 60 IU/ml and 41.3 IU/ml were obtained during the third cycle of the repeated batch fermentation with *S. gulbargensis* mu24 and *S. gulbargensis*, respectively. Further replacement of the medium had no positive effect on enzyme productivity. Enzyme activities remained between 16.2-41.3 IU/ml (*S. gulbargensis*) and 39-60 IU/ml (*S. gulbargensis* mu24) throughout the seven cycles (Figs. 3 and 4). At the same time, gradual cell leakage from the matrix was observed from the first to the seventh cycle. With each successive cycle, the cell leakage was found to increase. Determination of proteolytic activities of free and immobilized cells of *S. gulbargensis* mu24 revealed that the activity was higher in case of immobilized cells (158.2 IU/ml) than that of the free cells (127 IU/ml) after 120 h of incubation.

The enzyme production profiles reveal a clear difference between the activities of free and immobilized fermentation processes. The maximum L-asparaginase production rate in case of *S. gulbargensis* mu24 was found to be 3492 IU/ml h and 2682 IU/ml h with respect to immobilized and free cells.

DISCUSSION

In this study, the sterilized groundnut cake extract was inoculated with free and immobilized cells. Cell immobilization is one of the common techniques for increasing the overall cell concentration and produc-

tivity. The separation of products from immobilized cells is easier compared to suspended cell systems (Beshey, 2003). From the results obtained, it is evident that the production of L-asparaginase by immobilized cells is higher than that of free cells. The higher production in case of immobilized cells could be due to the minimal growth of cells in the matrix and also due to enzyme activities being retained at higher levels than that of the free cells (Kar and Ray., 2008). Immobilization studies using the polyurethane foam as a support material for *S. gulbargensis* mu24 revealed that L-asparaginase production was enhanced by 30.2% as compared to free cells. Kuhad *et al*. (2004) have reported a 32% increase in pectinase production by *Streptomyces* sp. RCK-SC after employing a polyurethane foam as an inert support matrix. Similarly, Kapoor *et al*. (2000) have reported a 1.5 fold increase in polygalactouronase production by *Bacillus* sp. MG-cp-2 using the polyurethane foam as support matrix.

The stability of the biocatalysts and their ability to produce L-asparaginase under repeated batch cultivation was also investigated. L-asparaginase was produced in repeated batch shake cultures, with the time for each batch being 72 h. Srinivasalu and Ellaiah (2005) studied neomycin production by *Streptomyces marinensis* NUV-5 immobilized on polyurethane foam and obtained significant productivity of neomycin, under repeated batch fermentation (up to seven batches; with each batch lasting 96 h).

The present study has revealed that L-asparaginase production by immobilized cells of *S. gulbargensis* and *S. gulbargensis* mu24 is higher compared to the free cells. The immobilized-cell system leads to higher enzyme activity than that of the free-cell system during the same period of fermentation. Furthermore, the long term stability and reusability of the matrix also add to the specific advantages. However, enzyme activity is comparatively higher in the case of *S. gulbargensis* mu24 than *S. gulbargensis*. To date, the present work is the first study investigating the potential use of polyurethane foam as a support for the production of L-asparaginase by *S. gulbargensis* and its mutant strain. Polyurethane foam was the most successful matrix in comparison to other matrices employed in previous studies (data not shown). Thus, it can be concluded that immobilization of *S. gulbargensis* and *S. gulbargensis* mu24 on polyurethane foam is a promising method for the production of L-asparaginase, using groundnut cake extract as substrate.

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