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PRODUCTION OF L-ASPARAGINASE BY STRAIN IMPROVEMENT AND WHOLE-CELL IMMOBILIZATION OF *STREPTOMYCES GULBARGENSIS*

AMENA S[1], LINGAPPA K*, PRABHAKAR M AND VISHALAKSHI N
*Department of Microbiology, Gulbarga University, Gulbarga-585106 Karnataka, India.
[1]Department of Biotechnology, Vishweshwariah College of Applied Sciences, Gulbarga-585106 Karnataka, India ***Corressponding Author E-mail**: lingappak_micro@rediffmail.com

ABSTRACT

In the present study, the strain *Streptomyces gulbargensis* was subjected to mutagenesis by physical and chemical agents like UV rays, Ethyl methanesulphonate and Ethidium Bromide in order to isolate strains capable of producing higher yields of L-asparaginase than the prototype. A total of thirty two mutants were obtained, however among all the mutants, *S. gulbargensis* mu24 was found to be the most potent. The maximum L-asparaginase activities recorded with *S. gulbargensis* and its mutant were 30 IU and 44.7 IU respectively at 120 h of fermentation using groundnut cake extract as medium. Hence, L-asparaginase production was enhanced by 1.49 fold by mutation. Also, attempts were made to enhance the L-asparaginase production of the prototype and the mutant strains by whole-cell immobilization in gelatin. The medium with an initial pH 8.5 was inoculated with free and immobilized cells separately and subjected to fermentation at 40^{o}C at 200 rev/min. In an immobilized cell system, the enzyme production was enhanced by 1.10 fold compared to the conventional free-cell fermentation. The immobilized cells retained their ability to produce L-asparaginase over three cycles and the activity remained between 49.4 IU-18.3 IU (mutant strain) and 33.15 IU-12.9 IU (prototype) throughout the three cycles, with the highest activity obtained during the first cycle. Hence, the gelatin-immobilized cells of *S.gulbargensis* mu24 can be explored as an effective biocatalyst which can be repeatedly used for maximum production of L-asparaginase.

KEY WORDS: L-asparaginase, *Streptomyces gulbargensis*, Mutagens, Groundnut cake extract, Immobilization

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 (1). It catalyses the conversion of L-asparagine to L-aspartate and ammonium, and this catalytic reaction is essentially irreversible under physiological conditions (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).This enzyme is produced by a large number of microorganisms that include *E.coli* (4), *Erwinia aroideae* (5), *Pseudomonas stutzeri* (6), *Pseudomonas aeruginosa* (7) and *Enterobacter aerogenes* (8).

Extra-cellular asparaginases are more advantageous than intracellular since they could be produced abundantly in the culture broth under normal conditions and could be purified economically (9). 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.. In addition, improvement of the microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes. Conventionally strain improvement has been achieved through mutation, selection or genetic recombination. The utilization of improved microbes for industrial processes is not new. Today the large scale production of health care products, amino acids, food additives, enzymes and antibiotics serve as testimony to the important role of strain improvement in shaping the pharmaceutical and fermentation industries (10). Stanbury *et al.* (1995) reported that the improvement of the microbial strains offers the greatest opportunity for cost reduction without significant capital investment.

Also, one of the approaches to improve the yield is by long term continuous production of L-asparaginase under cell immobilization. At present, cell immobilization technology is often studied for its potential to improve fermentation processes and bioremediation (12). The immobilization of whole microbial cells and their applications in bioprocessing has been of interest for nearly thirty years (13). Immobilization of whole cells for extra-cellular enzyme production offers several advantages, i.e. the ease to separate cell mass from the bulk liquid for possible reuse, facilitates continuous operation over a prolong period, enhances reactor productivity, ensures higher efficiency of catalysis (13). However, proper selection of immobilization techniques and supporting materials is needed to minimize the disadvantages of immobilization (12). Whole-cell immobilization by entrapment is a widely used and simple technique (14).

In view of the potential applications of L-asparaginase and the need for development of economical methods for improved enzyme production with an overall aim of reducing the cost of industrial process, the strain improvement and whole-cell immobilization can serve as excellent alternatives for increasing enzyme yields. Hence, the present investigation was aimed at enhancing the L-asparaginase production by mutagenesis of *S.gulbargensis* and exploring the practicability of L-asparaginase production from groundnut cake extract by gelatin-immobilized cells of *S.gulbargensis* and *S.gulbargensis* mu24. The reusability of immobilized cells for enzyme production under repeated batch conditions was also investigated. To our knowledge, this is the first report on L-asparaginase production by gelatin entrapped cells of *S. gulbargensis* and its mutant, *S. gulbargensis* mu24.

MATERIALS AND METHODS:

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

Microorganism The strain *Streptomyces gulbargensis* was obtained from the Department of Microbiology, Gulbarga University, Gulbarga. The isolate was identified as a novel species at Yunnan Institute of Microbiology, China. The 16S rRNA gene sequence of the strain is deposited in the GenBank database under the accession number DQ317411. The type culture/strain is deposited at =CCTCC Ac No 206001; =KCTC Ac No 19179 (15). The strain was maintained on starch casein agar (SCA) slants (pH 7.2) 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 at a temperature of 4^oC. Regular sub culturing of the isolate was performed at an interval of every 4 weeks.

Mutagenesis of *S. gulbargensis*:

S.gulbargensis, a producer of L-asparaginase was subjected to mutation by physical and chemical methods. The mutagens employed in the present study were UV rays, Ethyl methanesulphonate (EMS) and Ethidium Bromide (EtBr).

Treatment with UV rays:

Spore suspension of *S. gulbargensis* was irradiated using a 15W Phillips UV lamp at varying distances (5, 10, 15 and 20 cm) for different exposure times (5, 10, 15 and 20 min). The irradiation was performed in a dark room and the irradiated suspensions were protected from light until they were plated in order to minimize the photo reactivation effects (16).

EMS and EtBr treatment:

Spore suspension of *S. gulbargensis* was treated with EMS and EtBr, each at four different concentrations (2.5, 5.0, 7.5 and 10 mg/ml) for two durations (5 and 10 min). Mutation was carried out by treating 1 ml of spore suspension with 1 ml of EMS and EtBr separately, of each concentration for a period of 5 and 10 min. Following the exposure, the suspensions were plated on SCA medium.

Screening of mutants for L-asparaginase production:

A total of thirty two mutants were obtained by physical and chemical methods and were designated as *S. gulbargensis* mu1 - mu32. The first generation colonies were screened for L-asparaginase production by rapid plate assay method as described by Gulati *et al.* (1997).

Inoculum preparation:

The spore suspension was prepared from 5 d old culture grown on SCA slant by adding 10ml of sterile distilled water containing 0.01% of Tween 80 and suspending the spores with a sterile loop (18). This spore suspension was transferred to 50 ml of starch casein broth. The

flasks were incubated at 40^{o}C in shaker incubator (200 rev/min). The cells were harvested, washed with sterile saline and resuspended in 25 ml of saline solution. This cell suspension 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, Karnataka, India. The extract was prepared using ten grams of the powdered substrate dissolved in 100 ml of distilled water taken in a 250 ml Erlenmeyer flask. The contents of the flasks were heated for about 10 min and 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^{o}C for 15 min and used for fermentation studies.

Immobilization in gelatin
The immobilization was performed as per the modified method of Adinarayana *et al.* (2005). The cell suspensions were added (1:1 v/v) to 15 ml of 20% sterile gelatin, maintained at 45^{0}C, and poured into a sterile Petri dish. The gel was over layered with 10 ml of 5% glutaraldehyde for hardening at 30^{0}C. The resulting blocks were cut into small-sized cubes (4 mm^{3}) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

Fermentation processes:
Batch fermentation with immobilized and free cells:
The batch experiments were performed in 250ml Erlenmeyer flasks each containing 50ml of fermentation medium. Five grams of the above prepared cubes were added to the sterilized production medium**.** The free-cell fermentation was carried out by adding the cell suspension of *S.gulbargensis* and *S.gulbargensis* mu24 separately to the medium. The flasks were incubated at 40^{o}C in a shaker incubator (Remi Orbital Shaker Incubator) maintained at 200rev/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 (19). Therefore the reusability of cells immobilized in gelatin was examined. In case of repeated batch studies, the fermentation medium was aseptically decanted from each flask at every 72 h and the fresh medium was added. The process was continued for the next cycle. Subsequent batches were run at every 72 h intervals. The enzyme activity and cell leakage at every cycle were determined.

Analytical methods:
L-asparaginase assay:
The culture broth was centrifuged at 10,000 rpm (Sigma Refrigerated Centrifuge -3K30) for 8 min (20). The supernatant obtained served as the crude enzyme source. Assay of L-asparaginase was carried out as per Imada *et al.* (1973). One unit of L-asparaginase is defined as the amount of enzyme which liberates 1µmole of ammonia per ml per min (µmole/ml/min).

Cell leakage*:*
The cell leakage from the support matrix was determined as per the method of Adinarayana *et al.* (2005).

RESULTS AND DISCUSSION:
Classical strain development has typically relied on mutation and random screening of improved strains. This empirical approach has a long history of success, best exemplified by the improvements achieved for various antibiotic productions by fungal or actinomycete cultures. Thus application of strain improvement to new fermentation processes continues to be documented in the literature, despite the age of the technology (10). In the present study, a total of thirty two mutants were obtained by physical and chemical methods and were designated as *S. gulbargensis* mu1 - mu32. Mutagenesis by UV rays and EMS was found to be beneficial, since mutants capable of producing higher yields of L-asparaginase than the prototype were obtained, whereas EtBr treatment did not yield any productive mutants. However, among all the thirty two mutants, *S. gulbargensis* mu24 was found to be the most potent based on its performance to rapid plate screening for L-asparaginase production, and hence was selected for further studies. *S. gulbargensis* mu24 was obtained by treating 1 ml of spore suspension of *S. gulbargensis* with 1 ml of EMS at a concentration of 10 mg/ml for 10 min. Results depicted in Fig. 1 reveal that *S. gulbargensis* mu24 yielded 44.7 IU of L-asparaginase compared to the prototype which produced 30 IU of enzyme at 120 h of

fermentation. Hence the present study clearly indicates that L-asparaginase production was enhanced by 1.49 fold by mutation

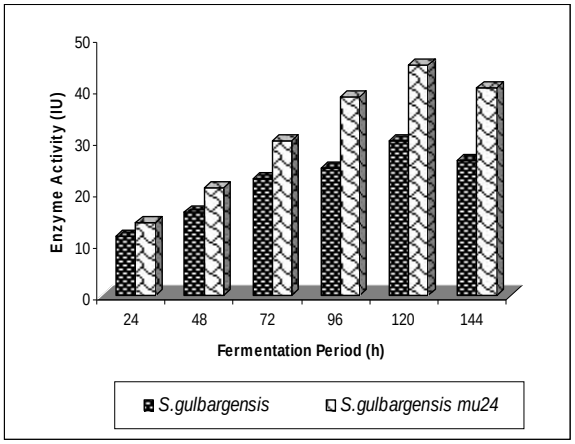

Fig. 1: L-asparaginase production by *S. gulbargensis* and its mutant, *S. gulbargensis* mu24 in groundnut cake extract

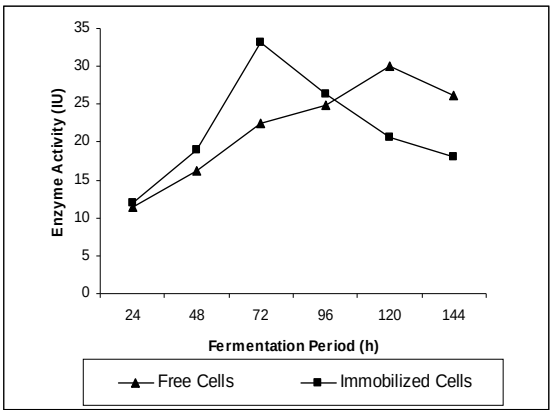

Fig. 2: L-asparaginase production by Free and Gelatin-immobilized cells of *S. gulbargensis*

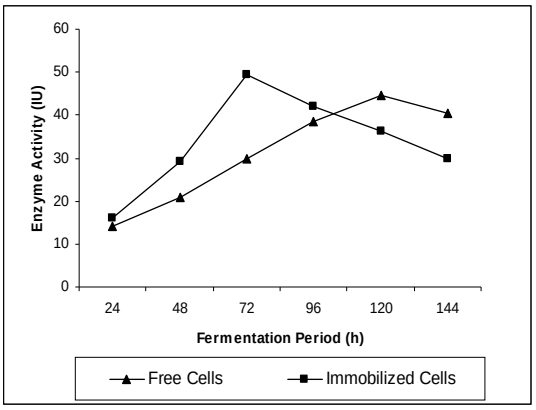

Fig. 3: L-asparaginase production by Free and Gelatin-immobilized cells of *S. gulbargensis* mu24

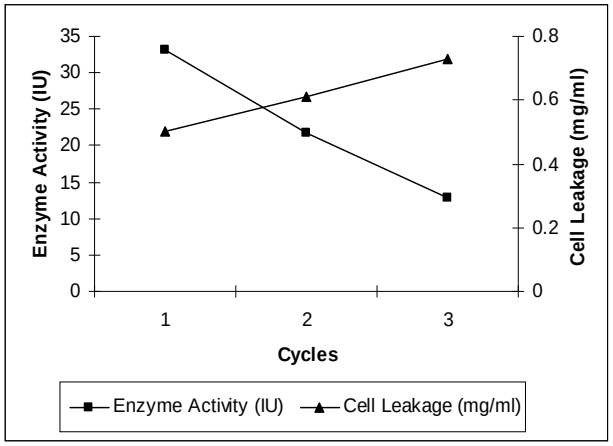

Fig. 4: L-asparaginase production by repeated batch fermentation using Gelatin-immobilized cells of S. gulbargensis

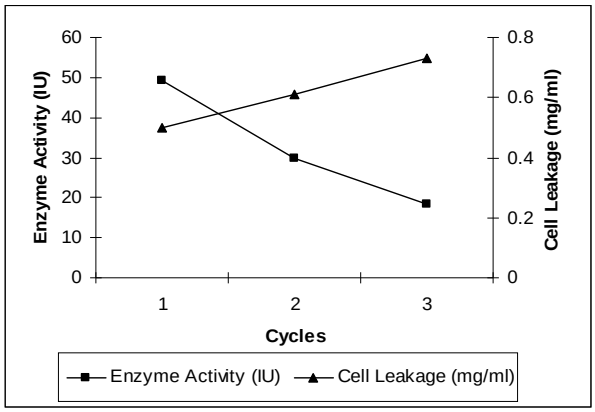

Fig.5: L-asparaginase production by repeated batch fermentation using Gelatin-immobilized cells of *S. gulbargensis* mu24

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 productivity. The separation of products from immobilized cells is easier compared to suspended cell systems (12). Figure 2 shows the L-asparaginase production pattern by free and immobilized cells of *S. gulbargensis*. The analysis of enzyme production was carried out at every 24 h interval. In case of free cells, the enzyme production reached a maximum activity of 30 IU 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. While the gelatin-immobilized cells showed a significant increase in the production of L-asparaginase from the beginning of the cultivation process. Maximum enzyme titer of 33.15 IU was observed at 72 h of incubation. Similar results were obtained with the mutant strain. Figure 3 shows the L-asparaginase production pattern by free and immobilized cells of *S. gulbargensis* mu24. In case of free cells, the enzyme production reached a maximum activity of 44.7 IU at 120 h of incubation. The immobilized cells of *S.gulbargensis* mu24 displayed a maximum L-asparaginase production at 72 h of fermentation yielding 49.4 IU of enzyme. 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. Immobilization studies using gelatin as a support material revealed that L-asparaginase production was enhanced by 1.10 fold as compared to free cells.

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 and the time for each batch/cycle was 72 h. Figure 4 and Fig. 5 show the possibility for reuse of the immobilized *S. gulbargensis* and its mutant to produce L-asparaginase in semi-continuous mode. A gradual decrease in L-asparaginase titer was observed from the first batch onwards. The results revealed that the immobilized cells continued to produce significant enzyme titers for three successive batches. At the same time, the gradual cell leakage from the matrix was determined from first to third batch. With each successive cycle, the cell leakage was found to increase. The cubes were disintegrated during the fourth batch operation. Thus, the repeated batch fermentation was run for three batches. The enzyme activity remained between 49.4 IU-18.3 IU (mutant strain) and 33.15 IU-12.9 IU (prototype) throughout the three cycles.

The present study revealed that strain improvement of *S.gulbargensis* considerably enhanced L-asparaginase production. In addition, production of enzyme by gelatin-immobilized cells of *S.gulbargensis* and *S.gulbargensis* mu24 is potential compared to free cells, because it leads to higher enzyme yield within the same time of fermentation. Further, the long term stability and reusability of the matrix also add to the specific advantages. However, gelatin was the least successful matrix in comparison to the other two matrices employed in our studies (data not shown), the most potent being polyurethane foam (22) followed by calcium alginate (23). The present study yet concludes that gelatin-immobilized cells can serve as promising biocatalysts for the production of L-asparaginase, using groundnut cake extract as substrate.

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REFERENCES

1. Verma N., Kumar K., Kaur G. and Anand S. (2007). L-asparaginase: A promising chemotherapeutic agent. Crit Rev Biotechnol 27: 45
2. Prakasham RS., Subba Rao Ch., Sreenivas Rao R., Suvarna Lakhsmi G. and Sarma PN. (2006). L-asparaginase production by isolated Staphylococcus sp.-6A: design of experiment considering interaction effect for process parameter optimization. J Appl Microbiol (doi:10.1111/j.1365-2672.2006.03173.xpublished online)
3. Pritsa AA. and Kyriakidis DA. (2001). L-asparaginase of *Thermus thermophilus* : Purification, properties and identification of essential amino acids for its catalytic activity. Mol Cell Biochem 216: 93
4. Swain AL., Jasolski MH., Dominique R., Mohana JK. and Wlodawer A. (1993) Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. Proc Nat Acad Sci USA 90: 474
5. Liu FS. and Zajic JE. (1973). Effect of oxygen-transfer rate on production of L-asparaginase by *Erwinia aroideae*. Can J Microbiol 19:1153
6. Manna S., Sinha A., Sadhukan R. and Chakrabarty SL. (1995). Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri* MB-405. Curr Microbiol 30: 291
7. Abdel F., Yasser R. and Olama Zakia A. (2002). L-asparaginase production by *Pseudomonas aeruginosa* in solid-state culture: Evaluation and optimization of

culture conditions using factorial designs. Process Biochem 38**:** 115

8. Mukherjee J., Majumdar S. and Scheper T. (2000). Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*. Appl Microbiol Biotechnol 53: 180
9. Amena S., Vishalakshi N., Prabhakar M., Dayanand A. and Lingappa K. (2010) Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis.* Braz J Microbiol 41: 173
10. Parekh S., Vinci VA. and Strobel RJ. (2000). Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54: 287
11. Stanbury PF., Whitakar A. and Hall SJ. (1995). In: "Principles of Fermentation Technology", 2 nd edition, Pergamon, USA. 1
12. Beshay U. (2003). Production of alkaline protease by *Teredinobacter turnirae* cells immobilized in Ca-alginate beads. Afr J Biotechnol 2: 60
13. Kar S. and Ray RC. (2008). Statistical optimization of α-amylase production by *Streptomyces erumpens* MTCC 7317 cells in calcium alginate beads using response surface methodology. Pol J Microbiol 57: 49
14. Adinarayana K., Jyothi B. and Ellaiah P. (2005). Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique. AAPS Pharmascitech; **6 (3)** Article 48
15. Dastager SG., Li WJ., Dayanand A., Sulochna MB., Tang SK., Tian XP., Zhi XY. (2007). *Streptomyces gulbargensis* sp. nov., isolated from soil in Karnataka, India. Antonie Van Leeuwenhoek 91: 99
16. Gardener JF., James LV. and Rubbo SD. (1956). Production of citric acid by mutants of *Aspergillus niger*. J Gen Microbiol 14: 228
17. Gulati R., Saxena RK. and Gupta R. (1997). A rapid plate assay for screening L-asparaginase producing microorganisms. Lett Appl Microbiol 24: 23
18. Lingappa K. and VivekBabu CS. (2005). Production of lovastatin by solid state fermentation of carob (*Ceratonia siliqua*) pods using *Aspergillus terreus* KLVB28. Ind J Microbiol 45: 283
19. Reyed MR. (2007). Biosynthesis and properties of extracellular amylase by encapsulation of *Bifidobacterium bifidum* in batch culture. Aus J Basic Appl Sci 1: 7
20. Ding CH., Diang ZQ., Li XT., Li LQ. and Kusakabe I. (2003). High activity xylanase production by *Streptomyces olivaceoviridis* E-86. World J Microbiol Biotechnol 20: 7
21. Imada A., Igarasi S., Nakahama K. and Isono M. (1973). Asparaginase and glutaminase activities of microorganisms. J Gen Microbiol 76: 85
22. Lingappa K., Amena S., Vishalakshi N. and Prabhakar M. (2009). Immobilization of *Streptomyces gulbargensis* in polyurethane foam- A promising technique for L-asparaginase production. Iran J Biotechnol 7: 199
23. Amena S., Lingappa K., Prabhakar M. and Dayanand A. (2008). Immobilization of *Streptomyces gulbargensis* mu24 in calcium alginate for L-asparaginase production. The Bioscan 3: 401