

Immobilization of *pseudomonas* sp. KLM9 in sodium alginate: a promising technique for L-glutaminase production

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ABSTRACT

Microbes and process development are gaining considerable importance owing to their exploitation and suitability for the production of the enzymes of therapeutic and industrial significance. In the present study attempts were made to immobilize the *Pseudomonas* Sp. KLM9 in different supporting matrix to analyze the possible enhancement in the production of L-Glutaminase. The consequences of the study reveal that, sodium alginate at 3.5% concentration was found to be the better supporting matrix than the others. Further, initial pH 7 of the incubation media, incubation temperature of 37 °C were supported good yield of L-Glutaminase by immobilized *Pseudomonas* Sp KLM9. The fermentation period was reduced to 36 hr with immobilized cells. There is moderate increase in the production L-Glutaminase by the immobilized cells than the free cells of *Pseudomonas* Sp KLM9. Biomass obtained from the 18 hr old culture of *Pseudomonas* Sp KLM9 was found better to immobilize for the production of L-Glutaminase.

Keywords: *Pseudomonas* sp. KLM9; L-glutaminase production; sodium alginate

1. INTRODUCTION

L-Glutaminase [E. C. 3.5.1.2] belongs to the class of hydrolyases and catalysis the hydrolysis of L-Glutamine to L-Glutamic acid liberating ammonia. In the present scenario this enzyme lot of significance owing to its diverse application in several fields such as pharmaceutical and food industry. Studies have revealed that, L-Glutaminase has inhibitory effect on tumor growth (1). L-glutamine is one of the vital amino acid, the tumor cells are the major consumer of L-glutamine, but they are not capable to synthesize this amino acid therefore they compete with host cells for circulating L-glutamine. The L-Glutaminase is used as anti-neoplastic therapeutic agent as it acts on L-Glutamine and causes the depletion of this amino acid for the tumor cells (2). The L-Glutaminase was found with anti-retroviral effect as it lowers the L-Glutamine level in serum and cause reduction in reverse transcriptase activity of Human Immunodeficiency Virus (HIV) (3).

L-Glutaminase has biotechnological application as it is used as biosensor for the detection and monitoring of Glutamine concentration in cell culture. L-glutamic acid is one of the key in gradient in soya sauce. This amino acid is formed by two mechanisms; firstly hydrolytic release from raw materials due to protease and peptidase activity secondly, deamidation of Glutamine by Glutaminase (4). The enzyme L-Glutaminase was reported for its use in food industry for the production of soya sauce as it converts L-Glutamine to glutamic acid and provides toothsome taste to the sauce by increasing desirable acidity. Therefore L-Glutaminase has a considerable application in food industry too.

Whole cell immobilization technique is often used improve the bioprocess to get enhanced yield of aimed product. Several matrices were reported as supporting material for immobilizing different microbes including bacteria actinomycetes, fungi and yeast for the production of various biomolecules like antibiotics, enzymes and solvents like alcohols and organic acids (5). Immobilization of whole cell for fermentation purpose has got significant advantage. The immobilized microbial cell can be used repeatedly in many cycles. Immobilized *Pseudomonas* Sp BTMS-51 was able to produce average yield of L-glutaminase over 20 cycles under repeated batch fermentation (6). *Pseudomonas* sp was immobilized in different supporting matrix and evaluated for the production of alkaline protease (7). Kuo-Ying-Amada Wu, *et al.*, studied the immobilization of *Pseudomonas* in polyvinyl alcohol(PVA) and sodium alginate and its application in phenol degradation (8) and reported that, PVA-alginate beads containing *Pseudomonas* were strong and durable for phenol degradation in fluidized bed reactor. PVA was proven to be the best supporting matrix in immobilization activated sludge (9). Immobilized *Pseudomonas aeruginosa* and *Bacillus subtilis* agar were reported for bioremediation of textile effluents (10). Promod *et al.*, reported the increased production of citric acid cells than free cells of *Aspergillus niger* (11).

All of these attempts on immobilization of microbes were mainly targeted to increase the activity of microbe with respect to their application and probability of their reuse. Plenty of studies were conducted and reported on whole cell immobilization and their application to develop possible bioprocess for the production of various biomolecules. In this study *Pseudomonas* sp KLM9 was immobilized in different supporting matrix and evaluated for L-Glutaminase production under submerged fermentation. This study is an attempt to develop bioprocess for the production of L-Glutaminase by *Pseudomonas* sp KLM9.

2. MATERIALS AND METHODS

Chemicals and reagents

Chemicals and reagents which were used in the present study were procured from Himedia Pvt Ltd. India.

Microorganism

The *Pseudomonas* sp KLM9 is a soil bacterium, isolated from Gulbarga University soil sample and maintaining on nutrient agar at the Department of Microbiology, Gulbarga University, Gulbarga, Karnataka, India.

Fermentation media and its Preparation

In this study safflower oil cake (SFOC) extract was used as fermentation media. Media was prepared as per the method prescribed by Amena *et al.* (12). Five grams of powdered SFOC was mixed in 100 ml of distilled water heated up to boil for releasing of nutritional

content. This mixture was cooled to room temperature, filtered with Whatman filter paper No-1 and thus obtained filtrate's volume was made up to 100 ml with additional distilled water. This filtrate was supplemented with 1gm of galactose, glucose and fructose, 0.3gm of ammonium chloride 0.5gm yeast extract (Mahesh *et al.*) (13), sterilized and used as fermentation media.

Preparation of Biomass

The bacterium *Pseudomonas* Sp KLM9 was inoculated in 100 nutrient broth and incubated at 37 °C for 24 hours. Thus obtained culture was aseptically withdrawn in sterile centrifuge tubes, centrifuged at 6000 rpm for 15 minutes cell pellet was collected and washed thrice in phosphate saline buffer (PBS) of pH 7. Thus obtained biomass was mixed in 10ml of sterilized distilled water and used for immobilization.

Immobilization of *Pseudomonas* Sp KLM9 in different matrices

Immobilization in Sodium Alginate

3 gm of sodium alginate was dissolved in 90 ml of distilled water heated for some time for the complete dissolution and this mixture was sterilized by autoclaving. The sterilized slurry was brought to room temperature. Cell suspension of *Pseudomonas* Sp KLM9 was poured into the above mentioned slurry and agitated thoroughly with pre-sterilized glass rod for the uniform distribution of cells. This mixture was taken in sterile syringe and extruded into chilled calcium chloride solution (0.2 M) from the height of 20 cm. Thus formed beads were kept in same solution at 4°C for one hour for curing. After curing the beads were washed thrice in sterile distilled water, stored at 4°C and used for further studies.

Immobilization in Polyvinyl Alcohol (PVA)

Immobilization of *Pseudomonas* Sp KLM9 was done as per the method described by Kuo-Ying-Amada Wu et al, 13 grams of PVA was dissolved in 90 ml of distilled water heated to boil for complete dissolution. This solution was sterilized and brought to room temperature. *Pseudomonas* sp KLM9 cell suspension was poured into the cooled PVA and agitated thoroughly with pre-sterilized glass rod for the uniform distribution of cells. This mixture was taken in sterile syringe and extruded into cold saturated solution of boric acid from the height of 20cm and allowed them for 24hr for hardening. These beads were washed thrice in sterile distilled water used for the production of L-Glutaminase.

Immobilization in Gelatin

The cell suspension was mixed in 1:1 ratio (V/V) with 20% sterile gelatin at 45 °C and mixed properly. Thus obtained gel was poured onto the sterile petridish. This gel was overlaid with 5% glutaraldehyde for hardening. The resulting gel was cut into small cubes (3-4 mm³) aseptically with razor blade. These cubes were washed in sterile distilled water and used for the analysis of L-Glutaminase production.

Immobilization in K-Caraginan

Cell suspension of *Pseudomonas* Sp KLM9 was mixed with 20 ml of 4% K-caragenan solution. This mixture was dropped into the 0.3 M potassium chloride solution with sterile syringe from the tallness of 20 cm. Thus formed beads were washed with sterile distilled water and used for fermentation process.

Fermentation

Production of L-Glutaminase by immobilized and free cells of *Pseudomonas* sp KLM9

Fermentation was carried out in 250ml conical flasks each containing 100 ml of fermentation media. One ml of 24 hr old culture *Pseudomonas* sp KLM9 was inoculated with a conical flask and other conical flasks were inoculated with immobilized cells of *Pseudomonas* sp KLM9 in different support matrix and kept for incubation at 37 °C. At every 12 hr L-Glutaminase assay was analyzed.

Optimization of L-Glutaminase Production

Fermentation was performed with *Pseudomonas* sp KLM9 immobilized in calcium alginate to analyze the optimum conditions for the production of L-Glutaminase. Fermentation was carried with fermentation media of initial pH ranging from 6-8 with variation of 0.5, incubating temperature ranging from 25-45°C with variation of 5°C and one flask at 37°C, this particular temperature was maintained because it was optimized with free cell in previous studies. The enzyme titer was assayed at every 12 hrs of time interval.

Assessment of suitable matrix concentration and biomass old

To determine the suitable concentration of support matrix, the biomass of *Pseudomonas* sp. KLM9 was immobilized in different concentration of sodium alginate at 3.0, 3.5, 4.0 and 4.5% and used for L-Glutaminase production. Further, biomass obtained at different incubation period such as 12, 18, 24 and 30 hrs were immobilized in 3.5% sodium alginate and performed fermentation to determine the proper old of biomass for better production of L-Glutaminase after immobilization.

Analysis of L-Glutaminase Activity.

To determine the enzyme activity, 5ml of the fermenting broth was withdrawn aseptically from the flasks, was filtered using Whatman filter paper No.1 and then centrifuged (.....) at 10,000 g for 10 min (5). The supernatant thus obtained was used as crude extract for L-glutaminase assay. Assay of enzyme was carried out as per Imada *et al.* (...). The enzyme activity was expressed in IU. One IU of L- glutaminase is the amount of enzyme which liberates 1 μ mole of ammonia per ml per min (μ mole/ml/min).

Repeated Batch Fermentation

Repeated batch fermentation performed to examine the reusability of immobilized cells of *Pseudomonas* sp KLM9 for L-glutaminase production. In repeated batch fermentation, the fermentation medium was aseptically withdrawn from the flasks and fresh medium was added at every 36 hours and further it was kept for fermentation. The same process was continued to run subsequent batches at every 36 hours. The enzyme titers were determined.

3. RESULTS AND DISCUSSION

Table 1 depicts the L-Glutaminase production pattern by free and immobilized cells of *Pseudomonas* KLM9 in different matrices. The analysis of enzyme activity was carried out at every 12 hr of intervals. Among the used matrices sodium alginate supported better enzyme yield with 136 IU than the other tested matrices. There are several report suggest that sodium

alginate is the suitable matrices for immobilizing microbes for the production of biomolecules including enzymes. Study conducted by Adinarayana *et al.*, reported that sodium alginate is the efficient support matrix to immobilize *Bacillus subtilis* for the alkaline protease production. (14). The sodium alginate is the best matrix than the other support matrix to immobilize *Pseudomonas* for the production of protease (7). Productivity of the enzyme was moderately increased by the immobilized cells of *Pseudomonas* Sp. KLM9 from 126 IU to 136 IU. But even though there might not be considerable increase in enzyme productivity but there is significant reduction in fermentation time to 36 hr from 48hr which was observed with free cells.

Table 1. Production of L-glutaminase by free cells and immobilized cells of *Pseudomonas* Sp. KLM9.

Support Matrix	L-Glutaminase Activity (IU)				
	12hr	24hr	36hr	48hr	60hr
Free Cells	09	27	90	126	60
Sodium alginate	20	89	136	48	34
Gelatin	19	65	118	42	32
Polyvinyl Alcohol	02	20	23	18	09
K-Caragenan	10	30	82	48	26

Table 2 shows the effect of the different variables on the productivity of L-glutaminase by *Pseudomonas* sp. KLM9. Immobilized beads with 3.5% of sodium alginate and 18hr old biomass of *Pseudomonas* sp. KLM9 were found to be best for enzyme production. In the study conducted by Sankaralingam *et al.*, reports that, there will be variations in support matrix concentration with respect to matrix used for immobilizing *Pseudomonas*. Further the investigation revealed that, 5% sodium alginate is the best concentration to immobilize *Pseudomonas* for protease production (7). However, *Pseudomonas* sp.KLM9 entrapped in sodium alginate were significantly increased the enzyme yield with the activity from 125 IU to 162 IU. Figure 1 shows the L-Glutaminase productivity by free and immobilized cells of *Pseudomonas* sp KLM9. Lingappa *et al.*, reported the considerable reduction in fermentation time along with considerable increase in the L-Asperginase production with immobilized cells of *Streptomyces gulbergensis* (15).

Table 2. Effect of Different Variables on L-glutaminase Production by immobilized *Pseudomonas* sp. KLM9.

Variables and Their Range	L-Glutaminase Activity (IU)				
	Fermentation time				
pH	12	24	36	48	60
6.0	16	63	103	28	20

6.5	29	76	114	33	24
7.0	21	91	129	46	26
7.5	20	88	118	44	25
8.0	12	41	76	27	19
Temperature (°C)					
25	06	22	36	18	14
30	11	37	44	22	18
35	21	86	131	52	34
40	20	79	130	54	32
45	17	58	94	38	21
37	29	80	139	54	36
Sodium Alginate concentration (%)					
3.0	28	82	138	55	38
3.5	30	80	145	55	37
4.0	14	56	110	48	30
4.5	06	40	58	42	28
Biomass Old (hr)					
12	10	39	53	39	31
18	35	89	162	59	39
24	29	82	144	48	32
30	20	72	122	46	32

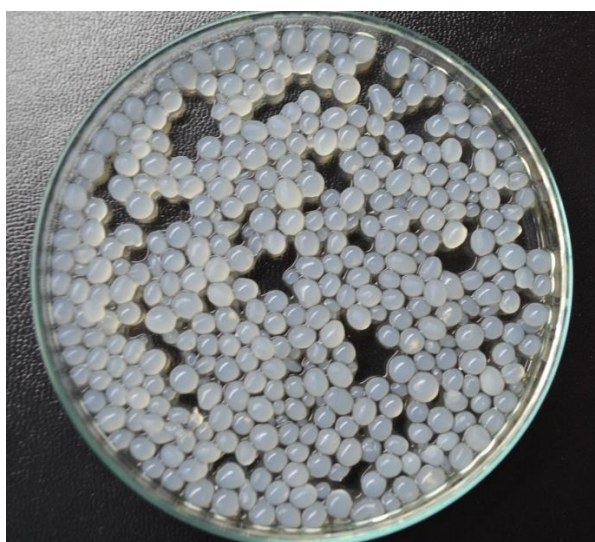


Plate 1. Sodium Alginate beads Containing *Pseudomonas* sp. KLM9.



Plate 2. L-Glutaminase Production by Immobilized *Pseudomonas* Sp KLM9 under Submerged Fermentation.

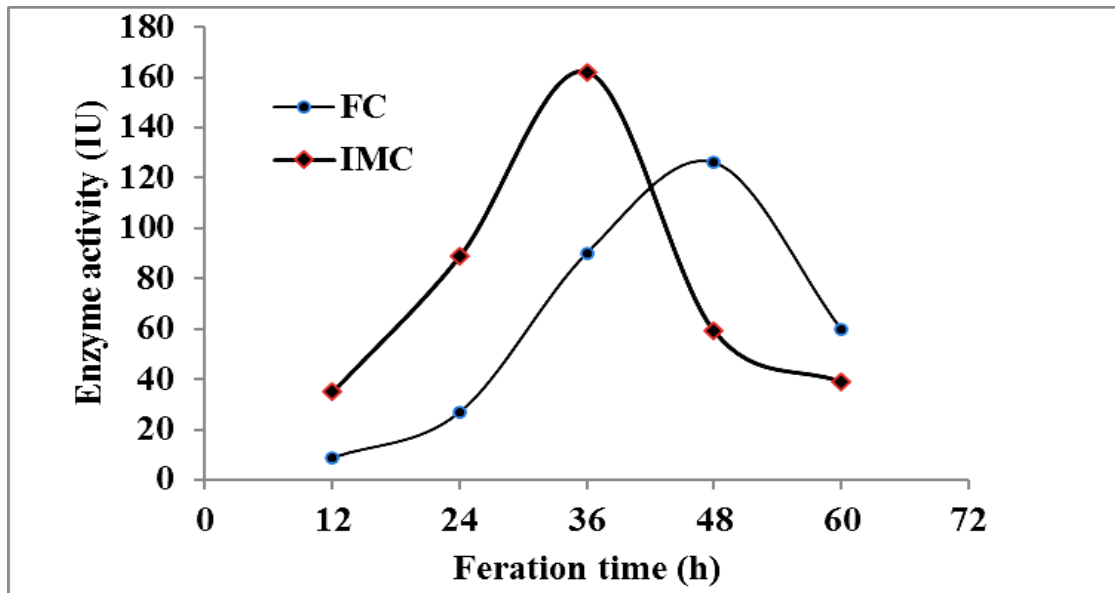


Figure 1. L-Glutaminase Productivity by free and immobilized cells of *Pseudomonas* Sp. KLM9 in Sodium Alginate.

Figure 2 depicts the productivity of L-Glutaminase by immobilized *Pseudomonas* sp KLM9 under repeated batch fermentation. 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 found. A total of seven cycles were found suitable for reusability of immobilized cells of *Pseudomonas* sp. KLM9 for L-Glutaminase production. The average enzyme yield from seven cycles under repeated batch fermentation was 160 IU.

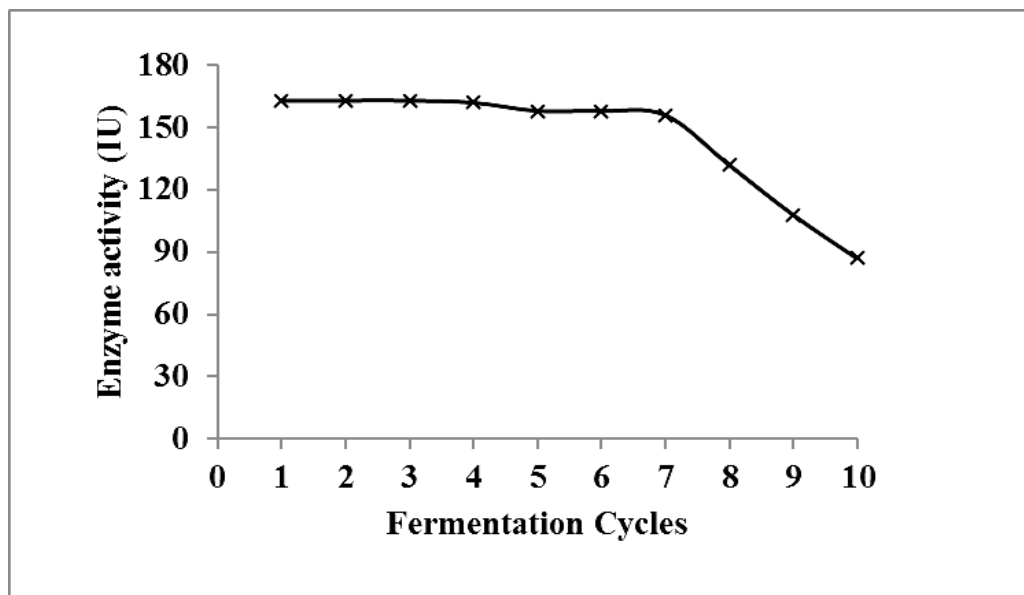


Figure 2. L-Glutaminase Productivity by immobilized cells of *Pseudomonas* Sp. KLM9 in Repeated Batch Fermentation.

4. CONCLUSION

The consequences this study revealed that, sodium alginate is the promising support matrix to immobilize *Pseudomonas* Sp KLM9 for the production of L-Glutaminase submerged fermentation. Further 3.5% of sodium alginate concentration and 18 h old biomass was found better for whole cell immobilization. Under all optimized condition the enzyme production is 162 IU. In repeated batch fermentation, the immobilized *Pseudomonas* Sp. KLM9 produce good enzyme yield up to seven cycles.

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