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Production and Partial Purification Of L-Asparaginase from Bacillus Sp.

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Abstract: The present study focuses on the production & partial purification of L-asparaginase enzyme from Bacillus sp. The rhizosphere soil sample was collected and screened for L-asparaginase producing bacteria on M9 media respectively using phenol red indicator growth medium. The potential isolate was selected and tested for morphological and biochemical characteristics, which confirmed Bacillus sp. The enzyme production was carried out by submerged fermentation. The different carbon and nitrogen sources were used for the enzyme production and xylulose was found to be the better carbon source, ammonium sulphate was used as a nitrogen source. The activity of crude enzyme was checked in different physical conditions of varying pH and temperature, The characterized enzyme exhibited maximal enzyme activity at pH 7 and temperature 30°C. The crude L-asparaginase was partially purified with ammonium sulfate precipitation and dialysis. Optimization of growth parameters showed significant effect on production of enzyme L-asparaginase from 2.18 to 2.92 µmol/mL/min. Thus, use of optimized M9 broth increased enzyme activity by 33.94%.

Keywords: L-asparaginase, Bacillus sp. Partial purification, submerged fermentation.

1. INTRODUCTION:

L-Asparaginase (ASNase) is one of the vital components used in the treatment of acute lymphoblastic leukemia (ALL), an aggressive type of blood cancer expected to afflict over 53,000 people worldwide by 2020. The ASNase has the potential for preventing metastasis from solid tumors, however it's characterized by a plethora of potential side effects, ranging from immune reactions to severe toxicity. Consequently, in accordance with Quality-by-Design (QbD) principles, ingenious new products are tailored to minimize adverse reactions while increasing patient survival [1].

L-asparaginase as a chemotherapeutic agent represents a milestone in the field of medicine due to the ratio of acute lymphoblastic leukemia children's patients who achieve complete remission after treatment incorporating ASNase (93%) due to its selectivity against the tumor cells. Its main mechanism of action is the depletion of the amino acid L-asparagine (LAsn) from the bloodstream, which is hydrolyzed into aspartic acid (ASP) and ammonia (NH_3). Since tumor cells lack the enzyme asparagine synthetase they are unable to synthesize enough L-asparagine for their maintenance and accelerated growth, which compromises its cellular functions and leads to cell death [2].

Although L-asparaginase is present in mammals, birds, plants, yeast, and bacteria, microorganisms are considered as the main source for L-asparagine synthesis. The production of this enzyme is mainly preceded by submerged fermentation from bacteria such as Pseudomonas fluorescens [3], Escherichia coli [4], Streptomyces albidoflavus [5] and Streptomyces ginsengisoli [6] and several genera of fungi as Aspergillus [7], Penicillium [8] and Fusarium [9]. In pharmaceutical industry, the best producers of L-asparaginase are members of the Enterobacteriaceae, such as Escherichia coli however in most of the patients receiving bacterial enzymes it results in immunological sensitization and immune inactivation. Most of asparaginase have low stability and are active in a narrow pH range and also the glutaminase activity generated by these enzymes can cause secondary effects such as allergic reaction, nausea, pancreatitis, diabetes, and coagulation abnormalities [10].

The medical use of ASNase is, however, not without risks, being associated with allergic reactions and several types of toxicity [11], hence there is a current need for novelbio better ASNase in the market. The new drugs designed from existing peptide or protein-based biopharmaceuticals by improving their properties such as affinity, selectivity, and stability against degradation are termed biobetter or biosuperior [12]. The present investigation deals with isolation and characterization of L-asparaginase from rhizosphere of paddy field and optimization of different parameters in its production.

2. MATERIALS AND METHODS:

2.1 Isolation and screening of bacteria for production of L-Asparaginase by plate assay.

The soil samples were collected from the rhizosphere of Paddy at Siddalaghatta, Chikballapur district to isolate potent L-Asparaginase producing bacteria. A known quantity (1g) of each soil sample was taken for bacterial isolation by serial

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dilution technique using nutrient agar medium. Antifungal agents (Fluconazole 75ug/ml, Ketocanazole-75ug/ml) were added to control the fungal contamination. The inoculated agar plates were incubated at 37°C for 24 hrs.
Selected bacterial strain was inoculated on agar based modified M9 medium containing $Na_2HPO_4.2H_20$, 6; KH_2PO_4, 3; NaCl, 0.5; L-asparagine, 5; 1M $MgSO_4.7H_20$, 2ml; 0.1M $CaCl_2$ $2H_20$, 1ml;20% Glucose stock solution 10ml; Agar, 20; phenol red indicator (2.5%): 0.04-0.36ml; pH 7.0. and incubated at 37°C for 24hrs. The isolate was streaked on nutrient agar slant grown at 37°C and was stored at 4°C. The bacterium was sub-cultured on fresh nutrient agar slants every fortnight. After isolation as a pure culture, the culture was characterized using morphological and biochemical test.
Submerged fermentation studies were also carried using M9 broth, prepared in 75ml conical flask. After sterilization, about 10 % of bacterial inoculum was aseptically transferred into one of the production medium flasks and the flasks were kept at 37ºC for 48 hours. The un-inoculated flask serves as negative control. After incubation, a small quantity of broth was taken in the test tubes, were added with few drops of phenol red solution [0.012 gm. /liter]. A color change of phenol red to pinkish red color observed in test flask indicates positive result for production of L- asparaginase enzyme.

2.2 Enzyme assay for L-asparaginase

The L-asparaginase activity was determined by hydrolysis of L-asparagine to release the ammonia which was measured by using Nessler's reaction. A mixture of 0.1ml of enzyme extract; 0.2ml of 0.05M Tris HCl buffer (pH 8.6), and 1.7ml of 0.01M L-asparagine was incubated for 10min at 37°C. The reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA). After centrifugation at 1000rpm, 0.5ml of the supernatant was diluted to 7ml with distilled water and treated with 1ml of Nessler's reagent. The color reaction was allowed to develop for 10minutes, and the absorbance read at 480nm with a spectrophotometer by nesselerization. Tubes kept at 0-time incubation acts as control. Enzyme activity was determined based on liberation of ammonia calculated with reference to a standard curve of ammonium sulphate 1mM in concentration.

2.3 Optimization Studies for L-Asparaginase Producers:

The enzyme activity of crude enzyme was checked in different carbon and nitrogen sources also with the physical conditions of varying pH ranges (4, 5, 6, 7, 8, 9) and temperatures in ° C (10, 25, 30, 35, 40). The isolate was grown in M9 broth in 50ml flask. Optimization of Carbon source (1%W/V) like Glucose, Sucrose, Maltose, Glycerol and xylulose and Nitrogen sources (0.5%W/V) like Ammonium chloride, Ammonium sulfate, Sodium nitrate, Urea and Casein was studied. The OD was measured at 480nm.The enzyme activity was determined by nesslerization.

2.4 Mass production of Crude enzyme preparation:

The enzyme was mass produced by submerged fermentation method. In a clean 250ml conical flask M9 broth is prepared along with the nitrogen (0.5% W/V) and carbon source (1%W/V), optimum temperature and pH were maintained. After sterilization, about 10 % of bacterial inoculum (Bacillus spp.) was aseptically transferred into one of the production medium flasks and the flasks were kept at 40ºC for 72 hours. M9 broth (minimal) inoculated with the culture acts as reference for before optimization. The un-inoculated flask serves as negative control. After the fermentation for 72hrs broth was separated by centrifugation at 12,000rpm for 12 minutes to separate the biomass. The clear supernatant was collected in a screw cap tube and stored at 4°C until further use. The clear supernatant was used as crude enzyme.

2.5 Partial Purification of Crude Enzyme L-asparaginase:

The L-asparaginase crude enzyme is partial purified by using ammonium sulfate precipitation method. It is a method that involves the precipitation of proteins from the sample extract by the ammonium sulphate salts. When ammonium sulphate salt is added into the protein extracts, the solubility of proteins increases with the concentration of ammonium sulphate salts added. The proteins are surrounded by the hydrated counterions, which prevents the aggregation of the proteins in the solution. This is known as salting in effect. When the concentration of salts added reaches a limit, the salts withdraw the hydrated water molecules from the proteins, and thus leads to aggregation and precipitation of protein molecules due to the hydrophobic effect between each other.

This phenomenon is known as salting out. The ammonium sulfate salts within the crude extract were then removed by conducting dialysis against 0.1M sodium phosphate buffer at pH 7. During this process, the crude enzyme was centrifuged at 10,000rpm for 10min then brought to 85 % saturation with ammonium sulfate at pH 8.4 and kept overnight in a refrigeration condition at 4°C. The precipitated solution was there after subjected to centrifugation at 10,000 rpm for 12 minutes, ammonium sulfate salts was then removed by using sodium phosphate buffer at pH 7.4. The obtained precipitate was transferred into dialysis membrane and re-suspended in a dialysis buffer solution. Finally, the enzyme activity of dialysate was quantitatively assayed. The amount of protein was estimated by the Lowry's method using bovine serum albumin as the standard.

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3. RESULTS AND DISCUSSION:

3.1 Enrichment, Isolation, and Screening for L-Asparaginase producing bacteria.

Bacterial strains producing L-Asparaginase were screened by plate assay on M9 agar medium. Pink coloration around colonies with phenol red is an indicator for L-Asparaginase producing organisms. Among the isolated bacterial colonies, the bacterial strain, S1 produced maximum pinkish red colored zone and this strain was selected for further studies. The isolated strain was characterized by morphological and biochemical tests. Gram characteristics revealed that the organism is gram positive endospore forming bacilli with short chains, biochemical tests resulted in its identification as Bacillus species. Moorthy et al [13] and Maysa et al [14] have also found Bacillus sp. to be a good source of asparaginase.

Optimization of enzyme production at different physico-chemical parameters.

Optimization of physico-chemical parameters like pH, temperature, carbon, and nitrogen sources plays an important role in production of biocatalysts. Temperature is one of the most critical parameters to be optimized in any bioprocess. pH of the medium strongly affects many enzyme processes and transport the various components across the cell membrane. In present study optimization revealed that maximum production is **2.93±0.06µg/ml/min** at **30°C** after 72 hrs. of incubation (Figure 1).

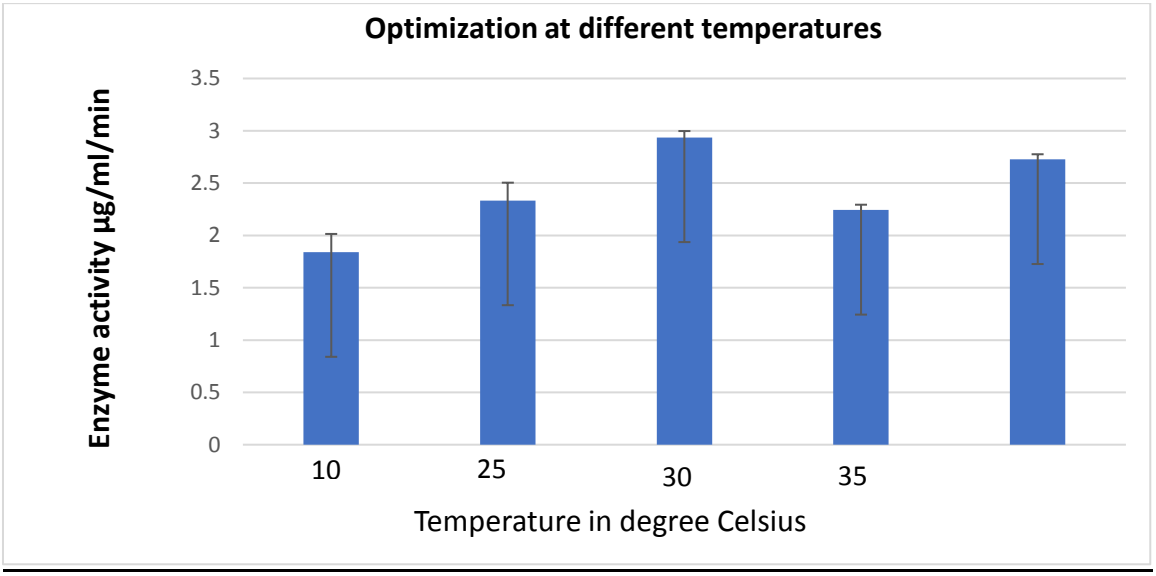

Figure 1: Effect of different incubation temperature on the production of L-asparaginase by Bacillus Sp. Values are the mean of three replicates ± SD.

Production of L-asparaginase varies with incubation period at 30°C indicating optimization of incubating period also exhibit a significant role. The enzyme production showed variation at different pH. L-asparaginase production was found to be maximum **2.89±0.02µg/ml/min** at **pH 7** and observed to be gradually decreased below and beyond this range (Figure 2). Optimization studies on Enterobacter sp., by submerged fermentation has also revealed enhanced production in the enzyme when grown at optimum temperature and pH [15].

The enzyme production varied in presence of different carbon sources. L-asparaginase production was found to be maximum in presence of **Xylulose (2.88±0.14µg/ml/min)** followed by **Glycerol (2.50±0.19µg/ml/min),** enhanced production in presence of xylose and glycerol as a source of carbon has been observed in Arthrobacter kerguelensis [16] and Nocardia levis [17] respectively.

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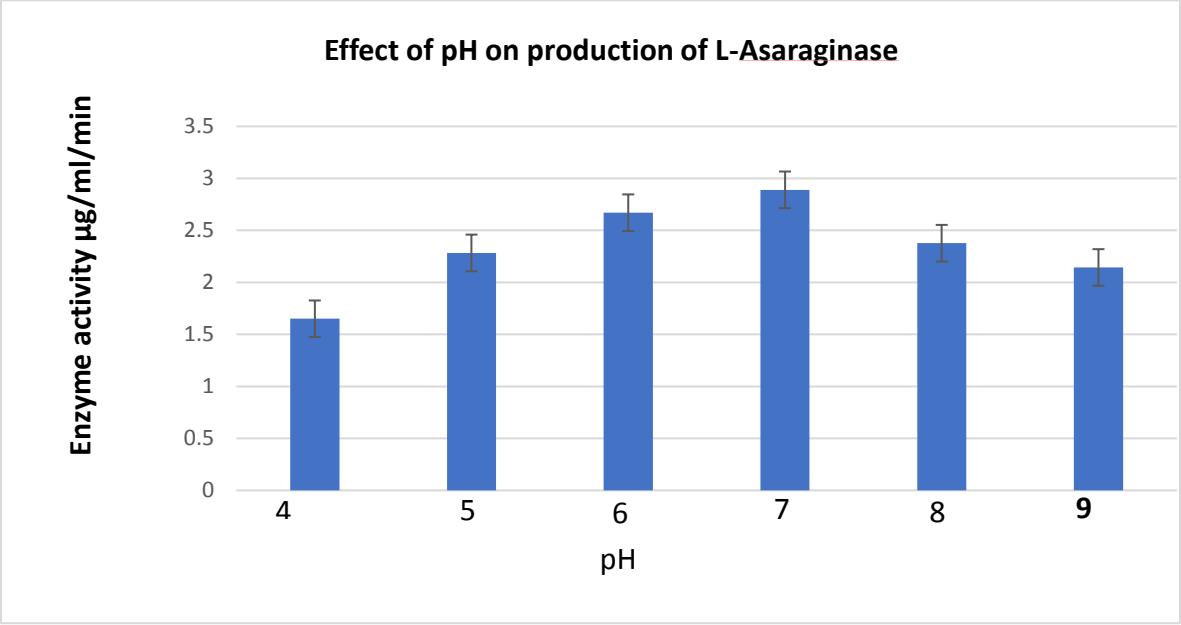

Figure 2: Effect of different pH on the production of L-asparaginase by Bacillus Sp. Values are the mean of three replicates ± SD.

Table 1: L-asparaginase production by Bacillus sp. grown in M9 broth amended with different carbon sources.

Carbon source	Enzyme activity (µg/ml/min)
Maltose	2.18±0.05
Xylulose	2.88±0.14
Glucose	2.15±0.05
Glycerol	2.50±0.19
Sucrose	2.17±0.15

Values are the mean of three replicates ± SD.

Enzyme production varied in presence of different nitrogen source. L-asparaginase production was found to be maximum **(2.33±0.05µg/ml/min)** when grown in presence of **ammonium sulphate.** Similar promising results was obtained from Bacillus licheniformis isolated from Red sea, Saudi Arabia. Maximal ASNase production was achieved with $(NH_4)_2SO_4$ used as a nitrogen source [18].

Table 2: L-asparaginase production by Bacillus sp. grown in M9 broth amended with different nitrogen sources.

Nitrogen source	Enzyme activity (µg/ml/min)
Ammonium chloride	2.2±0.07
Ammonium sulfate	2.33±0.05
Sodium nitrate	1.86±0.09
Urea	2.23±0.02
Casein	1.1±0.09

Values are the mean of three replicates ± SD.

3.3 Production and Partial purification of enzyme.

The bacterial strain showed maximum growth at 48 h of submerged fermentation in the presence of carbon and nitrogen source, xylulose (1%W/V) and ammonium sulphate (0.5%W/V) respectively. Protein content was calculated from the crude and partial purified culture filtrate before and after dialysis and was found to be 29 and 38 mg/ml; 8 and 12 mg/ml respectively. The maximum enzyme activity was observed in optimized culture medium. There was a **33.94%** increase in enzyme activity due to optimization. Similar kind of enhanced production was observed in Streptomyces ginsengisoli, where there was enhanced enzyme activity by 28% due to optimization of growth parameters [6].

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Table 3: Protein content and enzyme activity in culture filtrate of control and optimized medium.

	Without optimization	With optimization
Protein content (mg/ml) (Crude)	29	38
Protein content (mg/ml) (dialysed)	8	12
Enzyme activity after dialysis (µg/ml/min)	2.18	2.98

CONCLUSION:

L-Asparaginase is an important enzyme that has been industrially produced due to its importance in the treatment of acute lymphoblastic leukemia. The present study aimed at improving the production of L-Asparaginase from Bacillus species. Optimization studies revealed that all the selected parameters examined showed a considerable impact on L-asparaginase production by the bacterium. High activity of the enzyme was obtained at pH 7 and temperature 30°C. Maximum enzyme production was achieved when M9 broth was supplemented with xylose and ammonium sulphate as carbon and nitrogen sources, respectively. Optimization of growth parameters showed significant effect on production of enzyme L-asparaginase from 2.18 to 2.92 µmol/mL/min. Thus, use of optimized M9 broth increased enzyme activity by 33.94%.

REFERENCES:-

[1] Colombo, S., Beck-Broichsitter, M., Bøtker, J. P., Malmsten, M., Rantanen, J., and Bohr, A. (2018).“Transforming nanomedicine manufacturing toward Quality by Design and microfluidics”. Adv. Drug Deliv. Rev. 128 (2018):115–131. doi: 10.1016/j.addr.2018.04.004.

[2] Egler, R. A., Ahuja, S. P., & Matloub, Y. “L-asparaginase in the treatment of patients with acute lymphoblastic leukemia”. Journal of pharmacology & pharmacotherapeutics 7.2 (2016): 62–71. https://doi.org/10.4103/0976-500X.184769.

[3] Fatima, N., Khan, M. M., & Khan, I. A. “L-asparaginase produced from soil isolates of Pseudomonas aeruginosa shows potent anti-cancer activity on HeLa cells”. Saudi journal of biological sciences. 26.6 (2019): 1146–1153. https://doi.org/10.1016/j.sjbs.2019.05.001

[4] Maggi, M., Mittelman, S.D., Parmentier, J.H. et al. “A protease-resistant Escherichia coliasparaginase with outstanding stability and enhanced anti-leukaemic activity in vitro”. Sci Rep 7 (2017):14479. https://doi.org/10.1038/s41598-017-15075-4.

[5] Jayaprakash, K., Kumar, K., Vijayalakshmi, M. “L-asparaginase production by Streptomyces albidoflavus”. Indian journal of microbiology. 48 (2008): 331-6. 10.1007/s12088-008-0018-

[6] Deshpande, N., Choubey, P., Agashe, M. “Studies on Optimization of Growth Parameters for L-Asparaginase Production by Streptomyces ginsengisoli”. The Scientific World Journal. vol 2014: 895167. https://doi.org/10.1155/2014/895167.

[7] Dias, F., Santos Aguilar, J., & Sato, H. H. (2019). “L-Asparaginase from Aspergillus spp.: production based on kinetics, thermal stability and biochemical characterization”. 3 Biotech. 9.7 (2019): 289. https://doi.org/10.1007/s13205-019-1814-5.

[8] Patro, K, R., & Gupta, N. “Extraction, purification and charactri\ation of L asparaginase form Penicillium sp. By submerged ferementation”. In J. of Biotechnology and molecular biology Res. 3.3 (2012).: 30-40.

[9] Cachumba, J. J., Antunes, F. A., Peres, G, F., Brumano, L. P., Santos, J.C, Da Silva, S. S. “Current applications and different approaches for microbial l-asparaginase production”. Braz J Microbiol. (2016):77-85. doi: 10.1016/j.bjm.2016.10.004. Epub 2016 Oct 27. PMID: 27866936; PMCID: PMC5156506.

[10] Brumano, L. P., da Silva, F.V.S., Costa-Silva, T.A., Apolinário, A.C., Santos, J.H.P.M., Kleingesinds, E.K., Monteiro, G., Rangel-Yagui, C.O., Benyahia, B., Junior, A.P. “Development of L-Asparaginase Biobetters: Current Research Status and Review of the Desirable Quality Profiles”. Front Bioeng Biotechnol. 10.6 (2019):212. doi: 10.3389/fbioe.2018.00212. PMID: 30687702; PMCID: PMC6335324.

[11] Ko, R. H., Jones, T. L., Radvinsky, D., Robison, N., Gaynon, P. S., Panosyan, E. H., Avramis, I. A., Avramis, V. I., Rubin, J., Ettinger, L. J., Seibel, N. L., & Dhall, G. (2015). “Allergic reactions and antiasparaginase antibodies in children with high-risk acute lymphoblastic leukemia: A children's oncology group report”. Cancer, 121.23 (2015): 4205–4211. https://doi.org/10.1002/cncr.29641

[12] Lagassé, H.A., Alexaki, A., Simhadri, V.L., Katagiri, N.H., Jankowski, W., Sauna, Z.E., Kimchi-Sarfaty, C. “Recent advances in (therapeutic protein) drug development.” F1000Res. 7.6 (2017) :113. doi: 10.12688/f1000research.9970.1. PMID: 28232867; PMCID: PMC5302153.

[13] Moorthy, V., Ramalingam, A., Sumantha, A., Shankaranaya, R. T. “Production, Purification and Characterization of extracellular L-asparaginase from a soil isolate of Bacillus sp”. African Journal of Microbiology Research.,4.18 (2010):1862-1867.

DOI: 10.17148/IARJSET.2022.9267

[14] Maysa, E- Moharam., Amira M. Gamal-Eldeen and Sanaa T. El-sayed. "Production, Immobilization and Anti-tumor Activity of L-Asparaginase of Bacillus sp R36". Journal of American Science. 6.8 (2010).

[15] Erva, R. R., Goswami, A. N., Suman, P., Ravali Vedanabhatla, P., Rajulapati, S. B. (2017) "Optimization of L-asparaginase production from novel Enterobacter sp., by submerged fermentation using response surface methodology". Preparative Biochemistry & Biotechnology. 47.3 (2017): 219-228, DOI: 10.1080/10826068.2016.1201683

[16] Munaganti, R. K., Muvva, V., Indupalli, M.D. "Studies on optimization of L-Asparaginase production by Arthrobacter kerguelensis VL-RK_09 isolated from Mango orchards" Int J Pharm Pharm Sci. 7.9 (2015): 112-115.

[17] Kavitha, A., & Vijayalakshmi, M. "A Study on L-Asparaginase of Nocardia levis MK VL_113" .The Scientific World Journal. Article ID 160434 (2012): 5 pages.

[18] Alrumman, S.A., Mostafa, Y.S., Al-izran, K.A. et al. "Production and Anticancer Activity of an L-Asparaginase from Bacillus licheniformis Isolated from the Red Sea, Saudi Arabia". Sci Rep **9,** 3756 (2019). https://doi.org/10.1038/s41598-019-40512-x.

