GAVIN PUBLISHERS

Advances in Biochemistry and Biotechnology

Gupta A and Sahu PK. Adv Biochem Biotechnol 2: 135.
DOI: 10.29011/ISSN 2574-7258.000035

Research Article

Standardization of the Conditions for Production and Purification of L-Glutaminase from Soil Bacterium *Pseudomonas*sp

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Citation: Gupta A, Sahu PK (2017) Standardization of the Conditions for Production and Purification of L-Glutaminase from Soil Bacterium *Pseudomonas* sp. Adv Biochem Biotechnol 2: 135. DOI: 10.29011/2574-7258.000035

Received Date: 24 July, 2017; **Accepted Date:** 16 August, 2017; **Published Date:** 23 August, 2017

Abstract

L-Glutaminase (L-Glutamine Amidohydrolase, E.C. 3.5.1.2) is the enzyme responsible for hydrolysis of L-Glutamine into L-Glutamic acid and produce ammonia as by-product.Microbial Glutaminases have found applications in several fields. L-Glutaminase has received significant attention recently owing to its potential applications in medicine as an anticancer agent and flavor enhancing agent in food industries. The present study was aimed at isolation, production and purification of L-Glutaminase degrading strain of *Pseudomonas*sp.from soil for the production of L-Glutaminase. Soil samples enriched with the degraded agricultural wastes were collected from 80ft road, Sanjay Nagar and Domlur, Bengaluru which were screened for L-Glutaminaseactivity with the help of media stained with Phenol red dye. L-Glutaminase activity was confirmed by the observation of inhibition zones around the colonies on the media. Optimization of various chemical parameters such as nitrogen concentration (beef extract), carbon source (fructose) and salt concentration ($MgSO_4$) was carried out and higher production of the enzyme was observed. L-Glutaminase enzyme was partially purified by treating with 40% ammonium sulphate. Purification was done by using column chromatography method, using DEAE-Cellulose.

Keywords: Enzyme Production; L-Glutaminase; Optimization Conditions; Pseudomonassp.

Introduction

Enzyme industry is one of the major industries having greater market value in the world. Enzymes are in great demand for use in several industries, such as food, beverage, starch and confectioneries production as well as in the textile and leather processing, pharmaceuticals and waste treatment. L-Glutaminase is the enzyme deaminating L-glutamine [1,2]. The action of Glutaminase plays a crucial role in the nitrogen metabolism of both prokaryotes and eukaryotes. L-Glutaminase or Glutaminase (L-glutamine amido hydrolase) have got application in several crucial fields. Recently, its life saving role in cancer treatment has become the area of great concern in biomedical sciences as therapeutic agent [3].This has attracted hugeattention in pharmaceuticals as an antileukemic agent and also asflavor enhancing agent in food industry as. L-Glutaminase in combination with or as an alternative to asparaginase could be of significance in enzyme therapy for cancer especially in the situation of acute lymphocytic leukemia. Its commercial importance as anticancer and flavor enhancing agent demands not only the search for better yielding viable strains, but also economically viable bioprocesses for its large scale production. Another important application of L-Glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid. This enzyme catalyze the deamidation of L-Glutamine to L-Glutamic acid and Ammonia. L-Glutaminase plays an important role in plants, animal tissues and microorganism including bacteria, fungi and yeast [4].

L-Glutaminase has found a crucial role in nitrogen metabolism of cell. This enzyme widely used in industrial and pharmaceutical sector as an effective therapeutic agent in the treatment of HIV 5-6 and acute lymphocytic leukaemia. L-Glutaminase causes selective death of glutamine dependent tumor cells by starving these cells of glutamine. The use of L-Glutaminase to blocking neoplasms of essential nutrients helps in the treatment of malignancies and also used as an analytical reagent in the determination

of glutamate and glutamine, as a bio sensing agent in biosensor. L-Glutaminase is also a good substitute for flavor and aroma enhancing agent in the food industries [4,5].

L-Glutaminase has now replaced the use of Monosodium Glutamate (MSG) for imparting taste in Chinese foods and also used forthreonine manufacture by gamma glutamyl transfer reactions. Its commercial demands give much attention to search the viable bio processing technology for its large scale production. Glutaminases have also been detected in various mammalian tissues, where they are the major enzymes responsible for catabolic glutamine breakdown. It has gained importance due to its potential application as anti-cancer and flavorenhancing agent. This is an essential enzyme for the synthesis of various nitrogenous metabolic intermediates. Glutaminase is synthesized by various bacteria, fungi, yeast, moulds and filamentous fungi [5]. L-Glutaminase has attracted much attention with respect to proposed applications in both pharmaceutical and food, industries. The major advantage of using microorganisms for the production of L-Glutaminase is the economical bulk production capacity and also microbes are easy to manipulate to obtain enzymes of desired characteristics.

Material and Methods

Sampling and Isolation

Soils samples were collected from areas of 80ft road, Sanjay Nagar and Domlur Bangalore. The samples were collected in sterile polythene bags which were preserved in refrigerator until further investigation. Standard microbiological methods were followed for the purpose of isolation [6]. One ml of the desired dilution was transferred aseptically on Nutrient agar media for bacteria. Plates were incubated for 24-48hrs for bacterial growth. The isolates thus obtained were characterized (Figure 1).

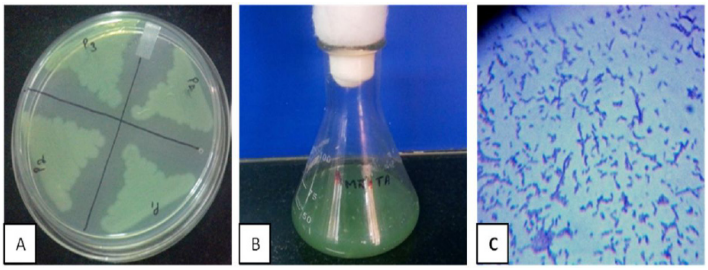


Figure 1(A-C): Pseudomonassp. (A) Mother culture, (B) Culture in Kings' B broth and (C) Microscopic image of Gram negative short rods.

Morphological and Biochemical Characteristics

Biochemical characterization of *Pseudomonas* sp. was carried out by Indole production test,voges proskauer's test, citrate utilization, starch hydrolysis, methyl red test, triple sugar ion test, mannitol motility test and catalase production as described by Dubey and Maheshwari [7](Figure 2, 3).

Detection of L-Glutaminase

Minimal agar medium (KCl 0.5g, $MgSO_4.7H_2O$ 0.5g, FeSO4.$7H_2O$ 0.1g, $ZnSO_4.7H_2O$ 1.0g, KH_2PO_4 1.0g, L-Glutamine 0.5%, Phenol red 0.01 2g, Distilled water 1000 ml) contains 0.5% L-glutamine as the sole carbon and nitrogen source and phenol red as pH indicator. The color change of the medium from yellow to pink is an indication of the extra cellular L-Glutaminase production by the colony. This color change is due to change in the pH of the medium, as L- Glutaminase causes the breakdown of amide bond in L-glutamine and librates ammonia. Bacterial culture was streaked in minimal agar medium. After two days of incubation at 37°C the plates turned pink and. Inhibition zones were observed around the cultures that showed L-Glutaminase activity and were maintained on Nutrient Agar for further use.

Optimization of Process Parameters

The strategy adopted was to optimize one particularparameter at a time and then include it at its optimum valuein the next optimization step. The parameters optimizedwere: carbon source(Glucose, Sucrose and Fructose),nitrogensource (Beef extract, Yeast extract and Peptone) and salt concentration ($MgSO_4$), was also optimized. Here theconcentration of the salt was varied ranging from 0.5-0.8 g/L, in the fermentation broth. The Fermentation broth contains (g/L)Carbon source: 30 Nitrogen source: 5 Magnesium sulphate ($MgSO_4$): 1Potassium Di hydrogen phosphate (KH_2PO_4) pH – 6.0.

Purification of L-Glutaminase

Solid ammonium sulfate was slowly added to the crude enzymefiltrate with gentle stirring to bring 40% saturation. Themixture was allowed to stand overnight at 4°C. It was centrifuged at10,000 rpm at 4°C for 20 min to remove the precipitate.The enzyme precipitate obtainedfrom saturation was dissolved in a minimal volume of 0.01Mphosphate buffer (pH 8) and dialyzed against 0.01M phosphate buffer (pH 8) for 48-72 h at 4°C and the buffer were changed occasionally.

Ion-exchange chromatography

Anion-exchange DEAE-cellulose (Diethylaminoethyl-cellulose) chromatography was performed for further purification of the L-Glutaminaseenzyme obtained from the previous ammonium sulfate precipitation. The fractions then underwent Bradford's test to determine the presence of protein.

Results and Discussion

Morphological and biochemical characteristics

The culture was gram negative rod shaped as revealed by Gram's reaction. Biochemical characterization of *Pseudomonas* sp. was carried out and found that it is negative for indole production

test, voges proskauer's test, starch hydrolysis and methyl red test. It was posive for the citrate utilization, triple sugar ion test, mannitol motility test, L-Glutaminase production and catalase production (Table 1; Figure 2, 3).Based on this results the culture was taken for further optimization of enzyme production parameters.

Sn.	Biochemical test	Result
1.	Indole production	-ve
2.	Voges proskauer's test	-ve
3.	Citrate utilization	+ve
4.	Starch hydrolysis	-ve
5.	Methyl red test	-ve
6.	Triple sugar ion test	+ve
7.	Mannitol motility	+ve
8.	Catalase production	+ve
9.	L-Glutaminase production	+ve

Table 1: Different biochemical properties of Pseudomonas culture taken for study.

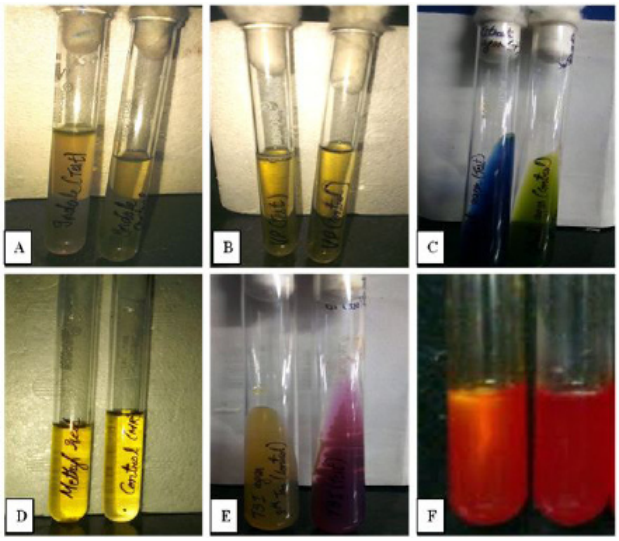


Figure 2(A-F): Biochemical characterization, (A) Indole production test, (B) Voges proskauer's test, (C) Citrate utilization, (D) Methyl red test, (E) Triple sugar ion test, (F) Mannitol motility test.

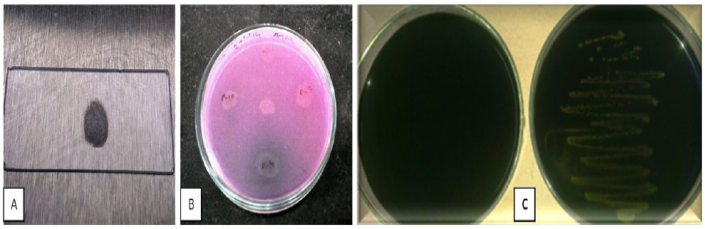


Figure 3(A-C): Different enzyme production tests (A) Catalase production, (B) L-Glutaminase production and (C) starch hydrolysis.

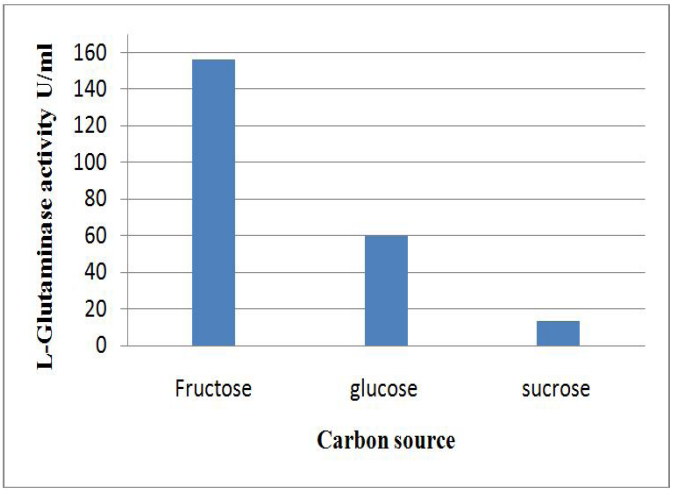
Detection of L-Glutaminase

L-Glutaminase production by the *Pseudomonas* sp. was tested as per protocol described by Imada,et al.[8] and Hartman [9] in brief the L-Glutaminase activity was assayed by measuring the ammonia released by hydrolytic deamination of L-glutamine. The isolates have showed color change on the agar-plate (Figure 3B) and therefore, selected for optimizing enzyme production protocol in liquid broth. The colorchange from yellow to pink indicates the enzymatic reaction in which Glutamic acid is produced from L-glutamine reducing the pH of agar medium. Thestrain is further analyzed for effect of different parameters for efficiency of enzyme production.

Optimization of Process Parameters

Impact of C-source

The present study indicated that there is variable shift of enzyme production capability from strain to strain [5,10, 11,12]. Impact of carbon (C) source as indicated by the study shows that fructose is better suited source yielding 156.025 U ml^{-1},whereas 59.97 U $ml^{-1}$and 13.375 U ml^{-1}L-Glutaminase was produced using glucose and sucrose, respectively (Graph 1). This shows suitability of fructose as preferred C-source by the strain of *Pseudomonas* used for the study. Sucrose was least preferred C-source. These results are in line with finding of Kiruthika and Saraswathy [13]. Jyothi,et al.[12] also indicated that higher concentration of glucose reduces the yield of L-Glutaminase (Graph 1).

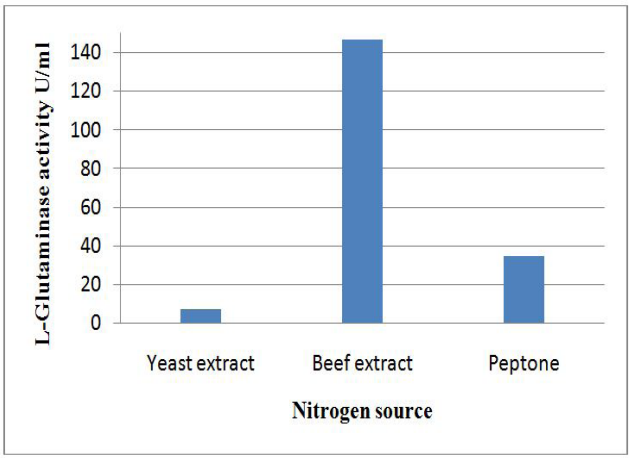


Graph 1: Optimization of Carbon Source.

Impact of nitrogen source

Impact of nitrogen source is also an important element to be considered during the production of L-Glutaminase. In this study, best suited N-source was beef extract giving146.675 U ml-1L-Glutaminase, as compare to 34.47 and 7.33 U ml-1L-Glutaminase

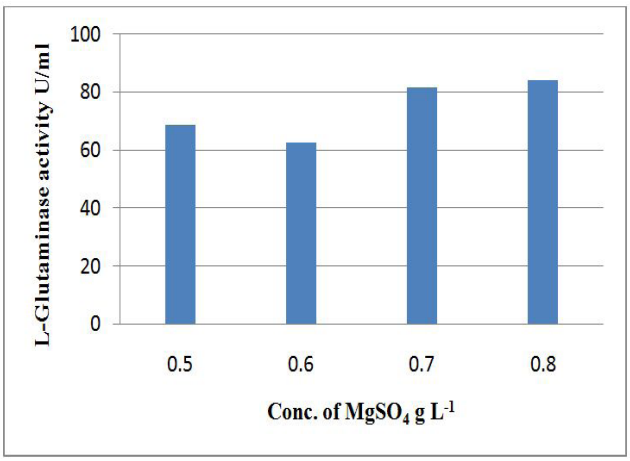
by peptone and yeast extract, respectively. This indicates the suitability of beef extract as N-source for higher L-Glutaminase yield (Graph 2). Kiruthika and Saraswathy [13] also describes higher yield of L-Glutaminase in beef extract but the effect of yeast extract and peptone was different in this study.



Graph 2: Optimization of Nitrogen Source.

Impact of salt concentration

Salt concentration was also one of the crucial parameter affecting the yield of L-Glutaminase. Four different concentrations were tested during the study ranging from 0.5 to 0.8g $MgSO_4$ L^{-1}. Results indicated that $MgSO_4$ is most effective if used in 0.7 or 0.8 g L^{-1}. It has yielded 81.67 and 84.17 U ml^{-1} L-Glutaminasefrom 0.7 and 0.8 g MgSO4 L^{-1}, respectively. The lower concentrations of 0.5 and 0.6 g $MgSO_4$ L^{-1} has given lower yield of 68.75 and 62.5 U ml^{-1}L-Glutaminase, respectively (Graph 3).



Graph 3: Optimization of Salt Concentration.

Conclusion

This study was carried out to isolate, mass produce and purify L-Glutaminase from *Pseudomonas* sp. obtained from soil samples. In the course of this study, various chemical parameters such as carbon source, nitrogen source and salt concentration were optimized that resulted in the mass production of the enzyme. The enzyme was then partially purified by ammonium sulphate precipitation method, followed by dialysis and purified by column chromatography method using Sephadex G100. Conclusively, fructose was found as better C-source, beef extract as N-source and 0.7 and 0.8 g $L^{-1}MgSO_4$ as best salt concentration for the optimum production of L-Glutaminase by the given *Pseudomonas* sp. culture.

Acknowledgements

This study was a part of BCIL training program of Amrita Gupta at Sangenomics Research Labs Pvt Ltd, Bengaluru. Support rendered by DBT, BCIL, Sangenomics Research Labs and Dr. MP Prasad is gratefully acknowledged.

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