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Optimization of fermentation conditions for L-glutaminase production by marine halotolerant*Pseudomonas aeruginosa* MM-2

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Abstract: Optimization of fermentation conditions for improved production of L-glutaminase by *Pseudomonas aeruginosa* MM-2 was carried out. Effect of different physicochemical parameters namely incubation period, initial pH, temperature, Nacl concentration, L-glutamine concentration, carbon and nitrogen sources were studied. The isolated marine bacteria *Pseudomonas aeruginosa* MM2 showed the maximum production of enzyme after 48 hours of incubation, at pH 8, temperature 35°C, 3% Nacl concentration, 1.5% L-glutamine concentration. The glucose as carbon source and beef extract as nitrogen source has supported the maximum yield of L-glutaminase. After optimization of different fermentation parameters the yield of L-glutaminase increased from 267±2 to 437±2 IU/ml. One of the important use of L-glutaminase is in the food industry as a flavor enhancing agent.

Keywords: L-glutaminase, halotolerant, inoculum size, *Pseudomonas aeruginosa*.

Introduction:

L-glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) is a hydrolytic enzyme which catalyses the hydrolysis of L- glutamine to L-glutamic acid and ammonia (Kumar et al., 2012).
L-glutaminase activity is found to be widely distributed in plants, animal tissues and microorganisms including bacteria, actinomycetes, yeast and fungi (Yokotsuka et al., 1987 and Parameswaran et al., 2017). Although L-glutaminase can be synthesized from both plant and animal sources, microbial source is mostly preferred for industrial application because of their short generation time, absence of seasonal variations, feasibility of bulk production and ease of extraction (Singh and Banik, 2013).
Microorganisms from marine environment hold significance in food industry by virtue of their ability to produce salt tolerant L-glutaminase because the salt and thermotolerantglutaminases are needed in soy sauce fermentation (Chandrasekaran M, 1997).
L-glutaminase has many important applications. In food industry it is used as a flavour enhancing agent as it increases the glutamic acid content in food through hydrolysis of L-glutamine to L-glutamic acid and ammonia. It's another important application is in biosensors for monitoring L- glutamine levels in mammalian and hybridoma cell cultures without the need

of separate measurement of L- glutamic acid (Sabu et al, 2000). L-glutaminase commercial importance demands the search for new and potential microbial strains and economically viable bioprocesses for its large-scale production (Iyer and Singhal, 2009). Marine microorganisms represent promising candidate sources of L-glutaminase as they are exposed to salinity, extreme conditions of temperature, pressure and nutrient availability.
The objective of this study was the optimization of different physicochemical parameters such as incubation period, pH, temperature, Nacl concentration, L-glutamine concentration,carbon and nitrogen sources for maximum production of L-glutaminase.

Materials and Methods:

Microorganism and culture condition: *Pseudomonas aeruginosa* MM-2(Accession no: MK334344)isolated from marine habitat was used in this study. The strain was maintained on Zobell marine agar slants at 35±2°C and subcultured for every 15 days.

Inoculum preparation:

The inoculum was prepared by transferring a loopful of 24h old culture of *Pseudomonas aeruginosa* MM-2 into 100 mL of minimal glutamine medium. The inoculated medium was incubated for 24 h at 30^{o}C and 120 rpm for the propagation of bacterial growth up to $10^{8\text{-}10}$ cells/ml.

L-glutaminase Production:

Fermentation was carried out using 250 ml conical flasks each containing sterile 100 ml of minimal glutamine media Glucose – 5.0 g/L, L-Glutamine-5.0 g/L, Na_2HPO_4- 6 g/L, KH_2PO_4-3 g/L, MgSO4 $7H_2O$- 0.49 g/L, $CaCl_2$, 0.01 g/L (pH 8.0). After sterilization the flasks were inoculated with appropriate inoculum and incubated at 120 rpm for 30±2^{o}C.

After incubation, contents of individual flasks were then centrifuged at 10,000 rpm for 15 min. at 4^{o} C and clear supernatant was used as the crude enzyme (Hiremath et al., 2011).

Enzyme Assay:

Enzyme assay was carried out as per the method described by (Imada et al., 1973).

0.5 ml of crude enzyme was made to react with 0.5 ml of 0.04 M L-glutamine, 0.5 ml of distilled water and 0.5 ml of phosphate buffer (pH 7.2) for 15 minutes at 37 ºC. The enzyme reaction was stopped by adding 0.5 ml of 1.5 M trichloro acetic acid (TCA), from this reaction mixture 0.1 ml was taken and mixed with 3.7 ml of distilled water, 0.2 ml of Nessler's reagent was added and optical density was measured at 450 nm. One unit of L-glutaminase activity was defined as the amount of enzyme that liberates 1 µmol of ammonia under optimal assay conditions.

Optimization of different parameters:

Production of L-glutaminase was optimized by one-variable-at-a-time (OVAT) approach. In this approach results are recorded with very low experimental errors due to change in a variable at a time (Samuel et al., 2014).

The different parameters optimized in this study were fermentation time, pH, temperature, Nacl concentration, concentration of L-glutamine and effect of carbon and nitrogen sources.

Incubation period: The effect of incubation time on L-glutaminase production during fermentation was studied by incubating the inoculated flasks for a total period of 5 days and estimating the enzyme production at regular intervals of 24 hrs.

pH of the medium: The effect of pH during fermentation was studied by adjusting the pH of the medium in the range of 5-10.

Incubation Temperature: The effect of temperature during fermentation was studied by incubating the inoculated flasks at different temperatures (20-45°C).

Nacl concentration: The effect of Nacl concentration on enzyme production was studied by incorporating different concentrations of Nacl (0-8%) in the media.

L-Glutamine concentration: The influence of substrate concentration on glutaminase yield by bacteria during fermentation was assessed by incorporating L-glutamine at different levels (0.25 - 3.0 % w/v) in the medium.

Carbon and Nitrogen Sources:

The effect of different carbon such as fructose, mannitol, starch, glycerol and nitrogen sources including yeast extract, beef extract, malt extract, casein and peptone was studied on L-glutaminase production.

Results& Discussion:

Optimization of various fermentation parameters and manipulation of media are one of the most important techniques used for the improved production of enzymes in large quantities to meet industrial demands (Tanyildizi et al., 2005).

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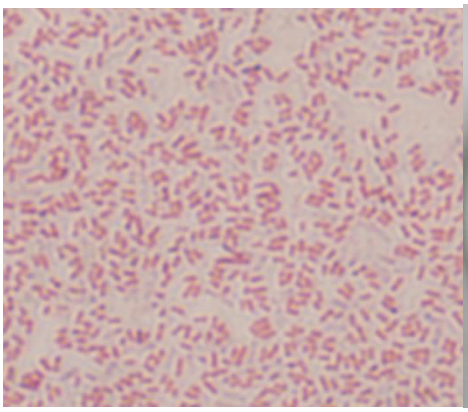

Fig 1: Microscopic observation of *P. aeruginosa* MM-2.

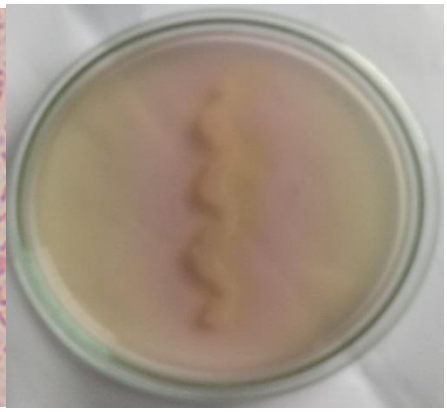

Fig 2: L-glutaminase production on MGA media.

The enzyme production was maximum 267±2 IU/ml after 48 hours of incubation, further increase in incubation period has resulted in decrease production of enzyme. Qadar et al., (2009) have reported 48 hr as ideal incubation period for protease production using *Bacillus* sp. PCSIR EA-3. The incubation period is an important factor which influences the enzyme production by bacteria. The enzyme synthesis is dependent on the stages of growth of the organism and also the period of incubation (Hiremath et al., 2011).

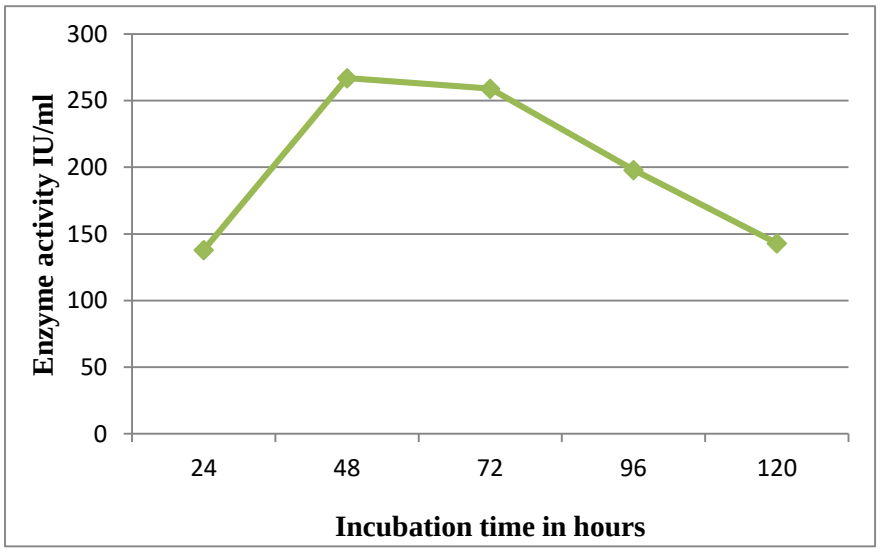

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Fig 3: Effect of incubation time on L-glutaminase production.

pH is one of the important factors to be considered for the enzyme production. Effective pH for enzyme production is different in each microbial source. The enzyme production was maximum 266±2 IU/ml at pH 8 and least106 IU/ml at pH 6.

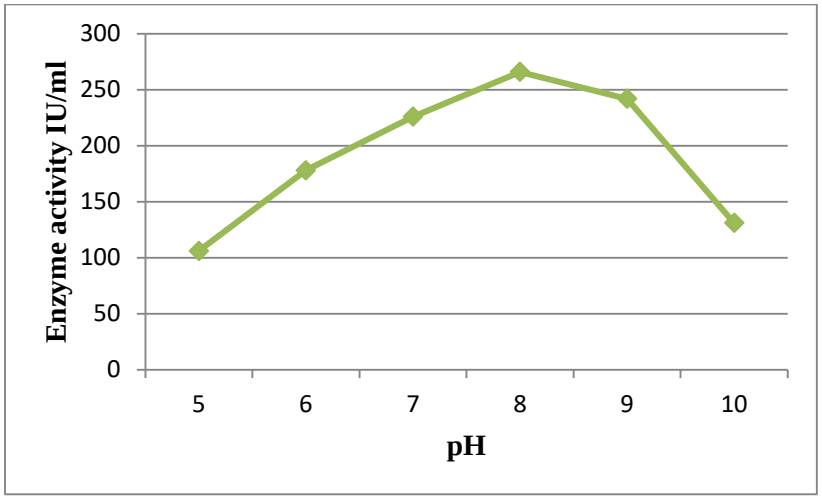

Fig 4: Effect of pH on L-glutaminase production.

Microbial L-glutaminase production is generally observed at mild incubation temperature conditions ranging from 25 to 37°C. In this study the maximum L-glutaminase production 293±1 IU/ml was observed at 35°C. Further increase in incubation temperature above 35°C resulted in the decrease production of enzyme.

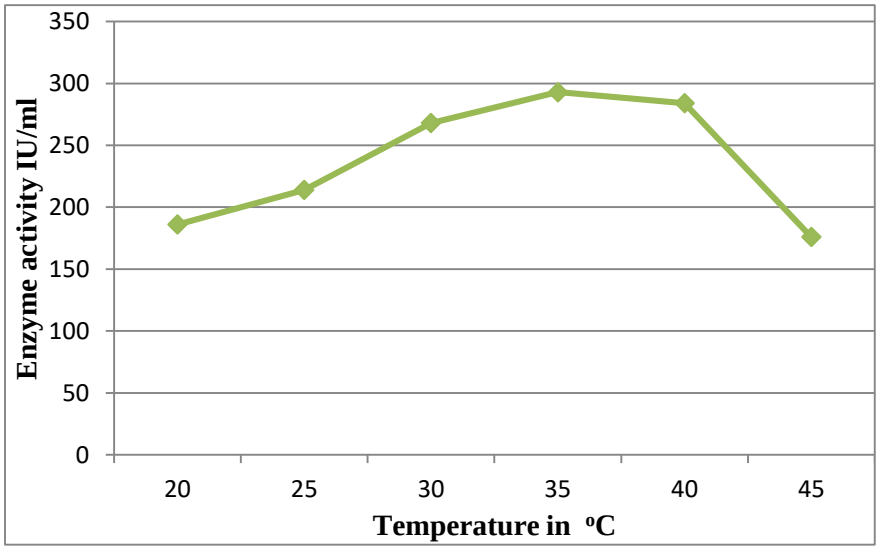

Fig 5: Effect of temperature on L-glutaminase production.

Maximal L–glutaminase production 338±1 was recorded when the media was supplemented with 3% NaCl. Enhancement in L-glutaminase production at 2% NaCl concentration by Streptomyces sp. strain LG-10 isolated from estuarine fish *Chanoschanos*was reported by Sivakumar et al., (2006). Even though the *Pseudomonas aeruginosa*MM-2 strain isolated from marine environment, it produced low concentration of enzyme at increased concentration 4, 5, 6, 7 and 8% of the same. This indicates that the strain is halotolerant.

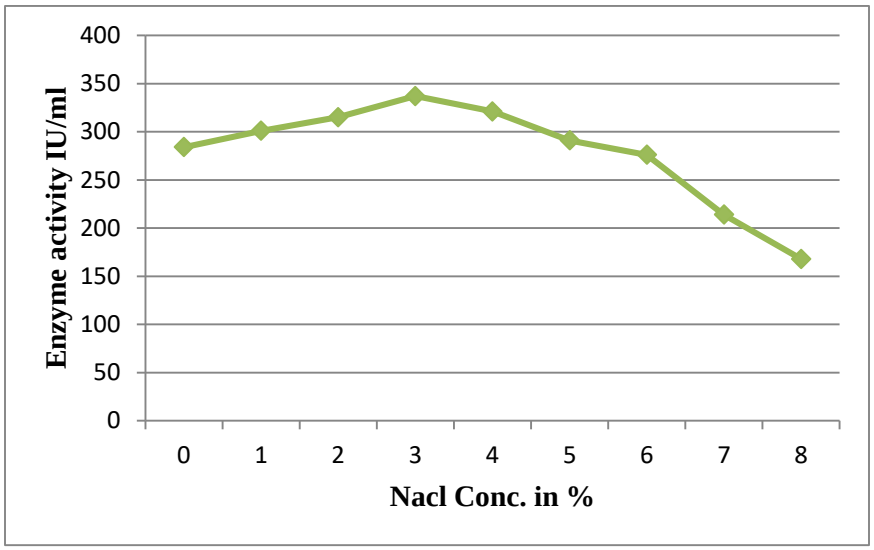

Fig 6: Effect of Nacl Concentration on L-glutaminase production.

The maximum production of L-glutaminase 411±0.6 IU/ml was recorded at 1.5 % of the substrate concentration. L-glutaminase production showed a linear increase along with increase in substrate concentration upto 1.5 %. However, further increase in substrate concentration did not increase L-glutaminase yield.

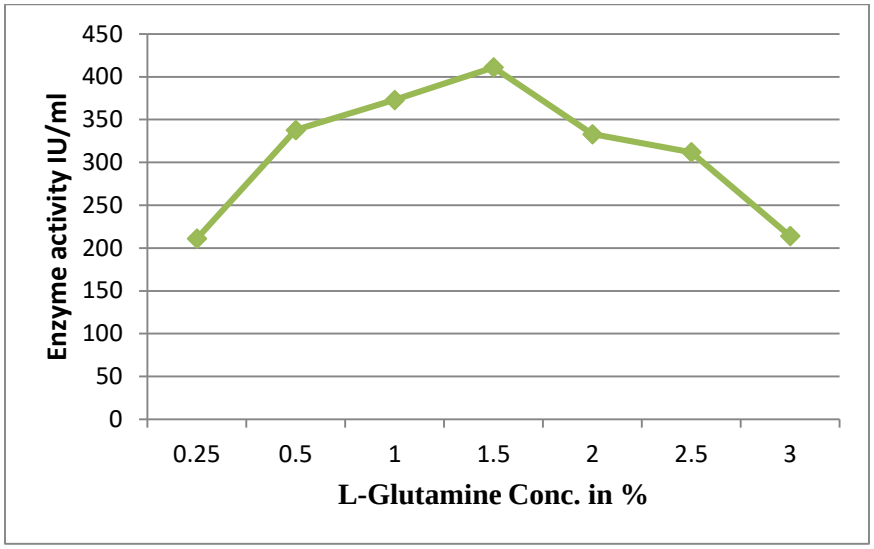

Fig 7: Effect of L-glutamine Concentration on L-glutaminase production.

In any fermentation medium, carbon and nitrogen sources influence significantly the rate of growth and product formation. In the present study glucose supported the maximum production of enzyme 408±3 IU/ml followed by mannitol, fructose, glycerol and starch. Similar types of results are reported by S. Krishnakumar et al., (2011).

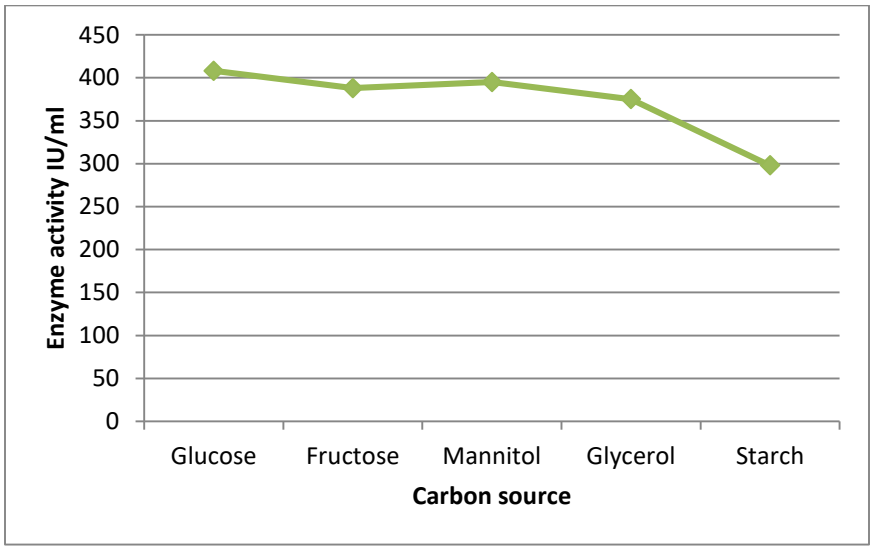

Fig 8: Effect of Carbon sources on L-glutaminase production.

Among the different organic nitrogen sources tested beef extract was observed to be ideal nitrogen source for maximum enzyme production 437±2 IU/ml followed by malt extract, peptone, yeast extract and casein.

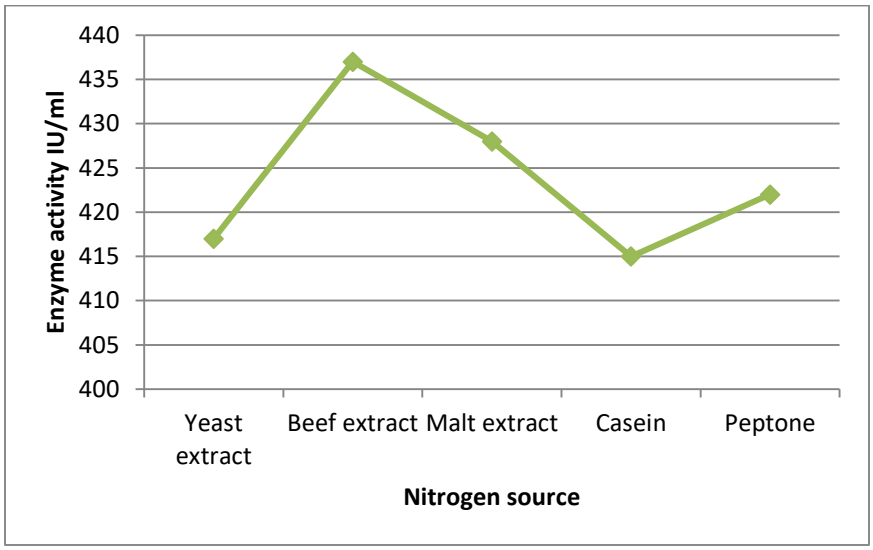

Fig 9: Effect of Nitrogen sources on L-glutaminase production.

All the physicochemical parameters were having influence on the production of L-glutaminase from *Pseudomonas aeruginosa* MM-2. After optimization of allthe parameters the yield of L-glutaminase increased from 267±2 to 437±2 IU/ml.

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