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Production of L-glutaminase by halotolerant *Bacillus subtilis* MM1

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ABSTRACT

In the present study the L-glutaminase producing halotolerant *Bacillus subtilis* MM1 isolated from marine environment was screened for the production of various industrially important enzymes like lipase, amylase, protease, chitinase, xylanase and cellulase. The optimization of different physicochemical parameters was carried out for the production of L-glutaminase. The *Bacillus subtilis* MM1 produced amylase, lipase, chitinase and cellulase. The optimum pH, temperature, salt and L-glutamine concentration for production of the L-glutaminase was found to be 8, 35°C, 3% and 1%. The xylose as carbon source and yeast 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 47±0.43 to 104±0.24 IU/ml.

Keywords: *Bacillus subtilis*, L-glutaminase, Lipase, media optimization.

INTRODUCTION

The growing demand for enzymes stable under high salt concentration has focused attention on halotolerant microorganisms. These microorganisms are a potential source of enzymes like L-glutaminase, proteases, amylases, nucleases, lipases, cellulases, xylanases, catalases, and esterases which are capable of functioning under high concentrations of salt, wide range of pH values, and temperatures at which other proteins will usually precipitate or denature.

In recent years, there has been a growing interest and demand for enzymes with novel properties. As compared to the terrestrial environment, the marine environment gives marine microorganisms, unique genetic structures and life habitats (Stach *et al*., 2003). The marine environment ranges from nutrient-rich regions to nutritionally sparse locations. The complexity of the marine environment involving high salinity, high pressure, low temperature and special lighting conditions, may contribute to the significant differences between the

enzymes synthesized by marine microorganisms and homologous enzymes from terrestrial microorganisms. These enzymes are used as pharmaceuticals, food additives, and fine chemicals (Bernan *et al*., 1997). The search for enzymes which are capable of catalyzing reactions under more extreme conditions brought about microorganisms from marine environment as a promising source of novel biocatalysts (Aguirre *et al*., 2018).These enzymes possess unique physiological properties such as salt and pH tolerance, thermostability at high temperature, barophilicity, adaptivity to extreme cold conditions, and novel chemical and stereochemical properties, with potential for biotechnological applications (Zhang and Kim, 2010).

Proteases break the peptide bonds of proteins; they are divided into acid, neutral, and alkaline proteases. These enzymes can be obtained from plants, animals and microorganisms. Alkaline proteases of microorganisms possess immense industrial importance due to their wide applications in tannery and food industries, medicinal formulations, detergents and processes like silver recovery, silk degumming, waste treatment, chemical industry and photographic industry (Furhan and Sharma 2014; Mayuri *et al*., 2019). The alkaline proteases find their largest use in the detergent industry (Nehra *et al*., 2002). L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) is an important enzyme that catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia (Mohammed *et al*., 2019). It is used as flavor enhancer in the food industry, due to its involvement in the synthesis of L-glutamic acid, the main compound which is responsible for the delicious taste or flavor and aroma of many fermented products like soy sauce, miso, sufu etc (Ayodeji *et al*., 2019; Unissa *et al*., 2014). Xylanase is ubiquitous in nature and is present in a wide range of living organisms, such as marine, terrestrial and rumen bacteria (Bhardwaj *et al*., 2019). The importance of xylanase has tremendously increased due to its biotechnological applications for pentose production, fruit-juice clarification, pulp and paper industry, baking and brewing industry, improving rumen digestion and the bioconversion of lignocellulosic agricultural residues to fuels and chemicals (Alokika and Singh, 2019).The selection of potential isolate and the optimized physicochemical parameters for production are always preferred at large scale (Kumar and Takagi, 1999). The production strategy includes evaluation of effect of cost-effective carbon and nitrogen sources on enzyme yield. Beside different chemical parameters, evaluation of effect of various physical parameters like pH, temperature, agitation, inoculum level and incubation period is required to improve the enzyme yield. The optimization of fermentation medium for the production of desired product is necessary to maintain a balance between medium components and to reduce the amount of unutilized components after completion of production (Bhunia *et al*., 2012).

MATERIALS AND METHODS

Microorganism and culture condition:

The L-glutaminase producing *Bacillus subtilis* MM1 (Accession No: MH447353.1) isolated from marine habitat was used in this study. The strain was maintained on Zobell marine agar slants at 30±2°C slants and subcultured for every 15 days.

Enzyme Profile Study:

The halotolerant *Bacillus subtilis* MM1 was screened for the production of industrially important enzymes like lipase, amylase, protease, chitinase, xylanase and cellulase.

Extracellular Lipase Production:

The lipolytic activity of the strain was detected by screening for zone of hydrolysis around colonies growing on tributyrin agar plates. The plates were incubated at 30°C for 48 h (Shringeri and Naik 2016).

Extracellular Protease Production:

The isolate was screened for proteolytic activity on casein agar plates composed of Casein-10 %, Peptone-5gm and agar-15 g/L. The plates were incubated at 30°C for 3 days. After incubation the protease production was detected by the presence of clear zones around the colonies.

Extracellular amylase, chitinase, xylanase and cellulase Production:

The amylolytic activity of the isolate was determined using starch agar plates. After incubation for 48 h the plates were flooded with Lugol's Iodine solution. The excess Lugol's iodine was drained off and plates were observed for clear halo zone around the colony against blue-black background. A clear halo zone around the colony indicates amylase production (Abd-Elhalem *et al*.,2015). The chitinase production was studied on nutrient agar supplemented with 1% colloidal chitin. After incubation the plates were stained with o.1% congo red solution and destained with1% Nacl. To screen for xylanase activity the isolate was spot

inoculated on xylan agar medium (xylan-1g; yeast extract-0.2g; peptone-0.5g; MgSO4-0.05 g, CaCl2-0.2 g, agar-2g/100ml) and incubated at 30°C for 3 days. The cellulolytic activity of the isolate was determined by replacing the xylan with 1% cellulose. After incubation the xylanase and cellulase activity was detected by flooding the plates with 0.1% aq. congo red solution (Bhosale *et al*., 2013).

Production of L-glutaminase:

Screening of different media for enzyme production:

Seven different media were screened for the production of L-glutaminase.

1. MediumM-1: glutamine-10.0; K_2HPO_4-1.0; KH_2PO_4-0.1; MgSO4-1.0; NaCl-0.5 and yeast extract-0.5 g/L; pH- 7.
2. Medium M-2: peptone-5; yeast extract-3; glutamine-5; $MgSO_4, 7H_2O$-1 g/L; pH-7.
3. Medium M-3: glutamine-5; yeast extract-3; tryptone-5; glucose-10 g/L; pH-7.
4. Medium M-4: glucose-5.0; L-glutamine-5.0; Na_2HPO_4-6; KH_2PO_4-3; $MgSO_4, 7H_2O$-0.49; $CaCl_2$-0.01 g/L, pH-7(Gupta *et al*., 2016).
5. Medium M-5: glutamine-5; beef extract-1; yeast extract-2; peptone-5 g/L; PH-7.
6. Medium M-6: glucose-5.0; L-glutamine-5.0; KH_2PO_4 -1; $MgSO_4,7H_2O$-0.5; $FeSO_4$-0.1; $ZnSO_4$ -0.1; KCl-0.5 g/L, pH-7.
7. Medium M-7: glutamine -5, Malt extract-3, peptone-5, $MgSO_4, 7H_2O$-1 g/L, pH-7.

Fermentation was carried out using 250 ml conical flasks each containing 100 ml of sterile medium.

Enzyme Assay:

L-glutaminase assay was carried out as per the method described by Imada *et al*., 1973.

Optimization of different parameters:

The optimization of different parameters for L-glutaminase production was carried out by one-variable-at-a-time (OVAT) approach. In this approach of media optimization 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 pH, temperature, Nacl concentration, concentration of L-glutamine, carbon and nitrogen sources, agitation speed and inoculum level (Krishnakumar *et al*., 2011).

RESULT AND DISCUSSION

The *Bacillus subtilis* MM1 produced lipase, amylase, protease and chitinase on respective media. Fig.1 shows the production of L-glutaminase by the isolate, the change in the colour of media from yellow to pink is due to change in pH. The isolate has produced lipase as shown in Fig. 2 the zone of hydrolysis around colonies indicates lipolytic activity of isolate. Fig.3 shows zone of clearance around the colonies after addition of iodine solution. This is due to the hydrolysis of starch by amylase produced by the isolate (Alka and Neha, 2018). When iodine comes in contact with a medium containing starch, it turns blue. If starch is hydrolyzed, the medium will have a clear zone around the growth.

zone of clearance with iodine solution on starch hydrolysis test.

zone of clearance with iodine solution on starch hydrolysis test.

Fig.4 shows the production of protease by the isolate, which is observed by zone of clearance around the colonies on casein agar plates. Earlier researchers have observed the similar results (Mita and Devyani, 2017).

Among the different media screened M-4 medium has supported maximum production of L-glutaminase as shown in Fig.6.

Fig 1: L-glutaminase Production

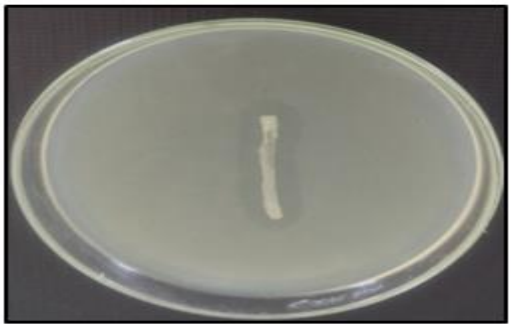

Fig 2: Lipase Production

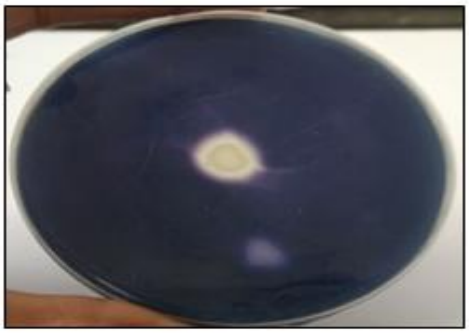

Fig 3: Amylase Production

Fig 4: Protease Production

Fig 5: Chitinase Production

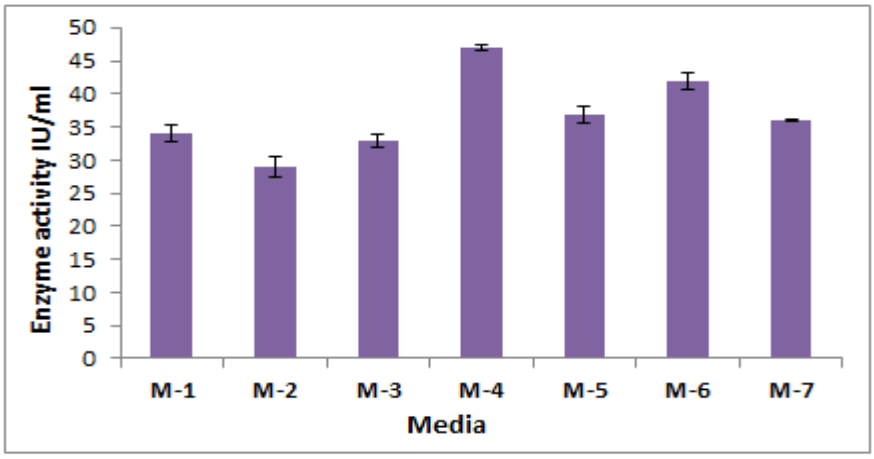

Fig 6: Screening of different media for L-glutaminase production

L-glutaminase production was maximum at pH 8, temperature 35°C, 3% Nacl and 1% L-glutamine concentration as shown in Fig. 7, 8, 9 and 10. The maximum production of L-glutaminase was noticed at 1 % of L-glutamine concentration and the further increase in substrate concentration did not enhance L-glutaminase yield. Temperature is an important parameter which influences the production and it varies for different organisms. The highest production of enzyme was observed at 35°C and least at 20º C. The production of L-glutaminase increased with the increase in the temperature from 20 to 35º C and thereafter it decreased with further increase in temperature. It strongly affects the synthesis either specifically or non-specifically influencing the rates of biochemical reactions (Nathiya *et al*., 2011).

In this study xylose as carbon source and yeast extract as nitrogen source has supported maximum production of enzyme as shown in Fig 11 and 12 respectively. The

carbon and nitrogen sources for L-glutaminase production varies with the organisms used for fermentation. Supplementation of media with sucrose and yeast extract as carbon and nitrogen source showed improved L-glutaminase production in *Zygosaccharomyces rouxii* (Iyer and Singhal, 2008). In the present study the optimum agitation speed and inoculum volume was found to be160 rpm and 2% for L-glutaminase production as shown in Fig.13 and 14. Earlier researchers found the maximum yield of enzyme production at 1% inoculum concentration (Jambulingam and Nachimuthu, 2013). All the different physicochemical parameters have influence on the production of L-glutaminase by *Bacillus subtilis* MM1.

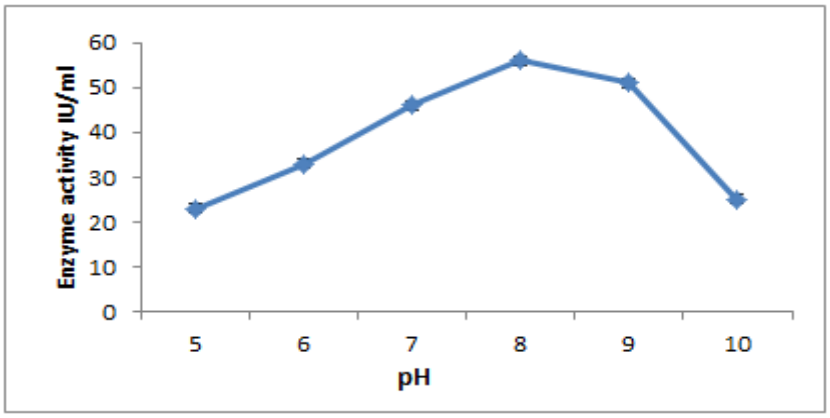

Fig 7: Effect of pH on L-glutaminase production.

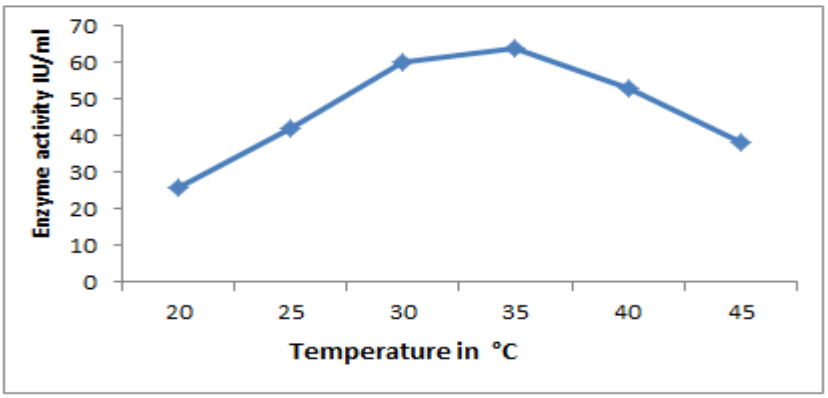

Fig 8: Effect of temperature on L-glutaminase production.

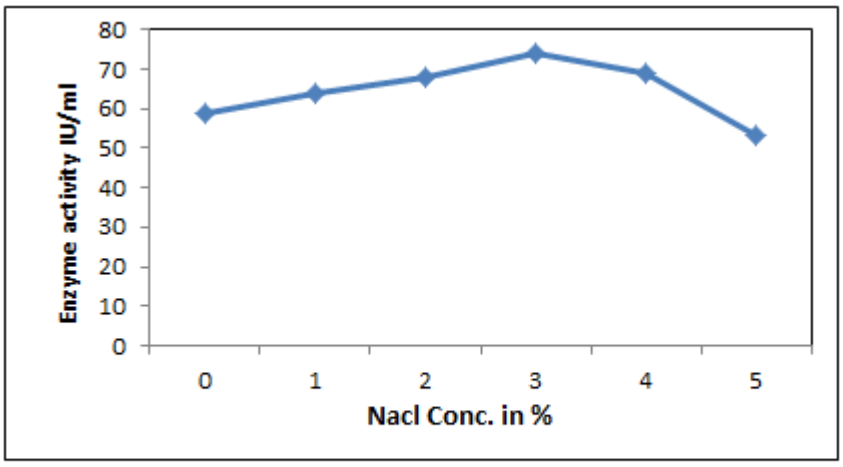

Fig 9: Effect of Nacl Concentration on L-glutaminase production.

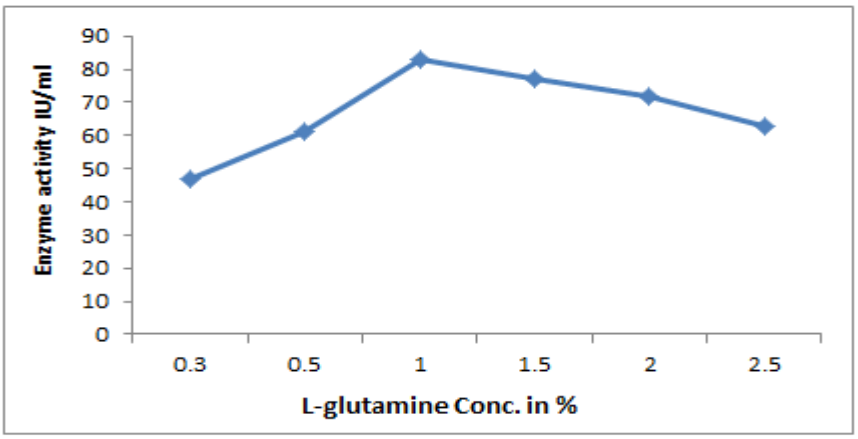

Fig 10: Effect of L-glutamine Conc. on L-glutaminase production.

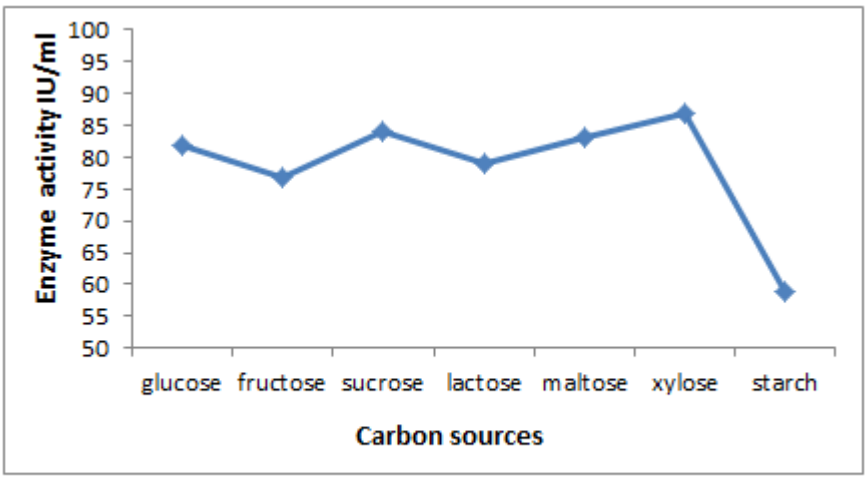

Fig 11: Effect of carbon sources on L-glutaminase production.

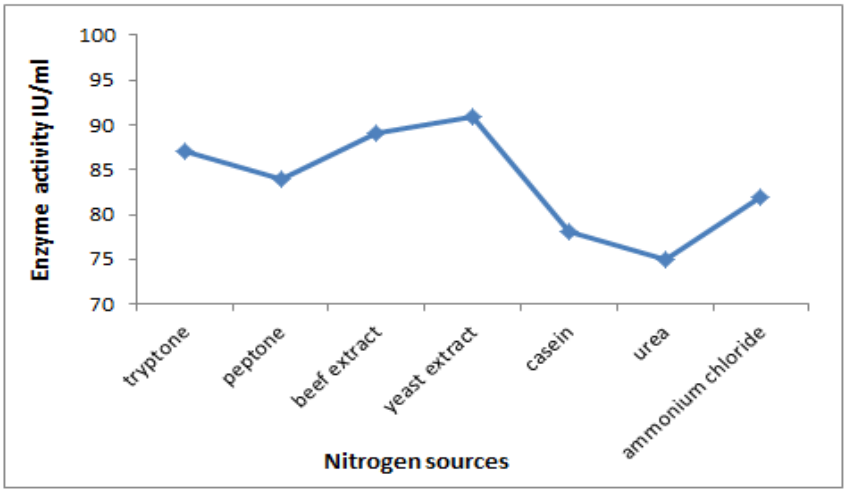

Fig 12: Effect of nitrogen sources on L-glutaminase production

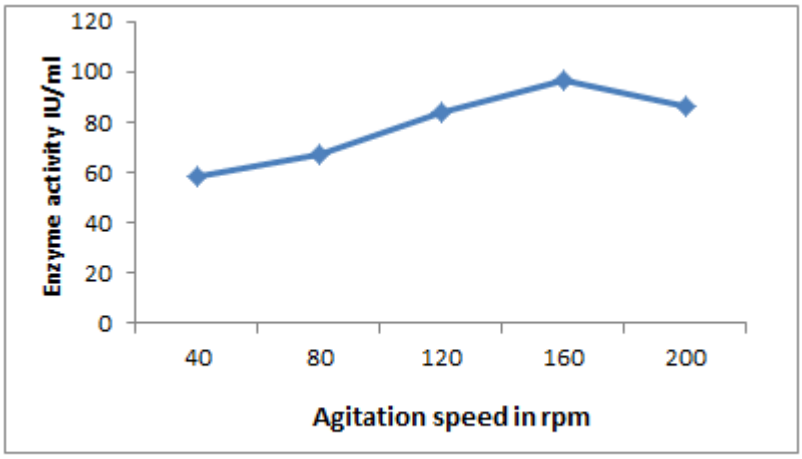

Fig 13: Effect of agitation speed on L-glutaminase production.

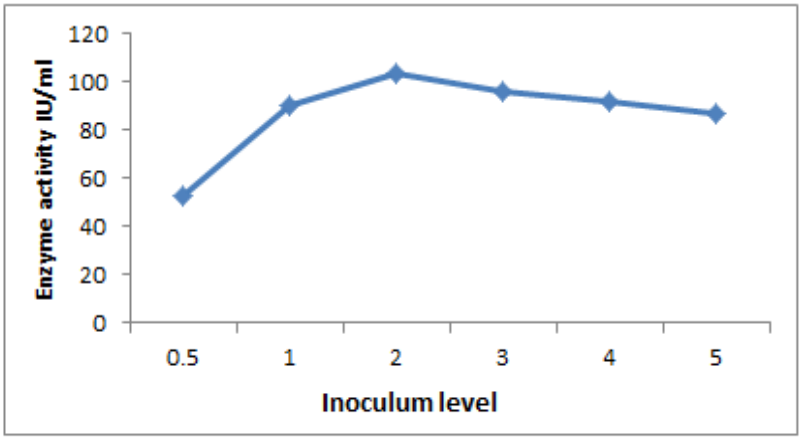

Fig 14: Effect of Inoculum level on L-glutaminase production.

CONCLUSION

The L-glutaminase producing halotolerant *Bacillus subtilis* MM1 also produces other industrially important enzymes including lipase, amylase, protease and chitinase. All the physicochemical parameters were having influence on the production of L-glutaminase under submerged fermentation. After optimization of all the parameters the yield of L-glutaminase increased from 47±0.43 to 104±0.24 IU/ml.

Conflict of interest

The author declares that there is no conflict of interest.

Acknowledgment

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