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## RESEARCH ARTICLE

# PRODUCTION OF L- ASPARAGINASE AS ANTICANCER AGENT BY *HALOMONAS ALKALIANTARCTICA* ISOLATED FROM MARINE SAMPLES

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**ABSTRACT**

L-Asparaginase enzyme that proved clinically as a treatment of Acute Lymphoblastic Leukemia (ALL). This study focused on screening, optimization, and partial purification of L-asparaginase. Six bacteriial strains isolated from six marine samples, was screeened for L-asparaginase production isolates. Screening was conducted on Glucose Asparagin agar medium supplemented with L-asparagine and phenol red as an indicator dye pH7. Pink colour around the colony was a sign of L-asparaginase activity. Resulting from16S rDNA sequencing of highest L-asparaginase production isolate was identified as *Halomonas alkaliantarctica*. Optimizing of L-asparaginase activity was done by studying the effect of nutritional and inoculum size parameters. *H. alkaliantarctica* recorded maximum L-asparaginase production (126.67U/min/ml) in medium supplemented with asparagine 0.1% and 1% tryptone without carbon source inoculated with 20% of (3.020×107CFU/ml) incubated for 48h, in shaking incubator (150rpm). The extracted l-asparaginase of *H.alkaliantarctica* was partially purified using ammonium sulfate fractionation 80%.



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## INTRODUCTION

Current cancer treatments causing side effects on the human in some instances. Extracted natural products from medicinal plants considered as a significance cancer treatment. The marine organisms able to produce novel chemicals against extreme variations which are unique in diversity, structural, and functional features (Kathiresan *et al*., 2008). L-asparaginase was known as L-asparagine amidohydrolase (EC. 3.5.1.1). L-asparaginase is useful anti-leukemia enzyme because of its ability in hydrolyzing L-asparagine into aspartic acid and ammonia (Dias and Sato, 2016). In the proliferation of leukemic cell L-asparagine is an important amino acid (Narta *et al*., 2007). Researchers mentioned that it can be isolated L-asparaginase producer from a wide diversity of microbe especially bacteria which are most studied because of the high stability of isolation as claimed (Sanjeeviroyar *et al*., 2010). *Halomonas daceae*, *Halomonas* is one of the genera most frequently isolated from hypersaline waters and soils by conventional-culture techniques (Ventosa *et al.*, 2008; Kaye and Baross, 2000). The ecological role that *Halomonas* species play in these habitats and their relationships with other halophilicand non-halophilic microorganisms are still unknown. Most species of the genus *Halomonas* have been recognized as having potential applications in biotechnology due to their capacity to produce compatible solutes, enzymes or exopoly saccharides and for their role in the degradation of pollutants, such as several aromatic compounds (Ventosa, 2006; Rafael *et al*., 2011; Oren, 2010).

## MATERIALS AND METHHODS

**Samples:** Save marine samples were collected from the west coast of Saudi Arabia at red sea. At shore (*Nerita* sp.) and (*Atactodea glabrate)*, from depth of 50 cm one species of brown algae (*Dictyota* sp.), one species of green algae (*Ulvalactuca*) and at the depth of 20 m, one species of solitary disccoral *(Ctenactiscrassa),*and two species of soft coral (*Sarcophton*s p, *Xenia umbellate)*.

**Isolation of L-asparaginase producing bacteria:**
Suspensions were prepared by mixing 10g of sediment samples into conical flasks containing 100mL of sterile phosphate buffer, rotated at 50 rpm/min for 30min, the suspending matter and the clear supernatant was decanted and serially diluted. Further, 100UL of the different dilutions spread on Glucose Asparagine solid medium (glucose 20g, asparagine 5g, KCl 0.5g, $K_2HPO_4$ 1.0g, $MgSO_4$. $7H_2O$ 0.5g and pH7.0±0.2 in 1000ml sea water). The medium was supplemented with phenol red (2.5%): 0.04-0.36 mL indicator. The plates were then incubated at 35±2°C for 24-72h, to obtain colonies with pink zones around them (Gulati *et al.*, 1997).The more potent strains were selected for fermentation process.

**Preparation of inoculums:** The bacterial strain was inoculatedin nutrient agar plates and incubated at 35±2°C for 24-48h. One or two colonies of bacterial growth were inoculated into 50 ml of nutrient broth in 250 ml flask and kept in a rotary shaker (150 rpm) at 35±2°C for 24h.

**Estimation of L-asparaginase:** Assayed through Nesslerization method described by (Imada *et al.*, 1973). The measurements was conducted by spectrophotometer at 450 nm. The reaction mixture was assayed in triplicates (Basha *et al.*, 2009).

**Molecular characterization of bacterial isolate:** Genomic DNA isolation using Gene JET Genomic DNA Purification Kit

- Thermo Fisher Scientific. Bacteria isolates identified by16S rDNA universal oligonucleotide primers designed by Stravato and Cappelli, (2001).

- **PCR mixture**: 2µL DNA, 1µL of each primer, 12.5µL Master mix and 9.5µL sterile $dH_2O$.
- **PCR amplification conditions:** Initial: 5 min at 94 °C,Denaturation: 1 min at 94 °C, Annealing: 1 min at 55 °C and Extension: 2 min at 72 °C, Number of cycles: 35. Final extension: 10 min at 72 °C.

**Optimization of L-asparaginase production by *Halomonas alkaliantarctica***

**Effect of shaking incubation onL-asparaginase production:** Two sets of cultures flasks; one was incubated in static incubation another on a rotary shaker 150rpm at 35±2˚C. After 48h of incubation cultures filtrates were collected and centrifuged at 4°C for 30 min at 10000 rpm. The supernatant used as a source of the crude enzyme.

**Carbon Sources:** To investigate the effect of carbon sources on L-asparaginase (ASNase) production by *H. alkaliantarctica*, the carbon source(glucose) had been replaced by different carbon sources in AG broth medium such as lactose, sucrose, and glucose, each added at a concentration of 1% (w/v) (Amena*et al.* 2010; Narayana *et al*., 2008), keeping other components constant.

**Amino acids and other Nitrogen Sources**: Deferent aminoacids, asparagine, arginine, glutamine, glycine and methionine, and nitrogen source rather than amino acids, yeast extract, peptone, tryptone, were added at a concentration of 0.5 % (w/v) to the fermentation medium broth, keeping other ingredients constant (Amena *et al*. 2010). Inorganic nitrogen sources were not tried as it would interfere in enzyme assay. In another study L-asparagine as the best amino acid, was added in different concentrations, 0.25, 0.5, 0.75 and 1.25% (w/v). Trypton was the best nitrogen source other than amino acids, was used in different concentrations varied from 0.25, 0.5, 0.75, 1.0 and 1.25% (w/v), and 0.1% (w/v) of L-asparagine was added.

**The inoculum size:** The effect of inoculum size on the production of L-asparaginase was studied, four different size (10 ml, 20ml, 30ml and 40ml/100ml medium) of inoculum was examined. After inoculation, the flasks were incubated at 35±2˚C on a shaking incubator (150rpm) and the Free-Cell Filtrate (FCF) assayed for ASNase accumulation after 72h.

## RESULTS

**Screening of L-asparaginaseproduction of isolated bacteria by plates:** The primary screening was carried out using phenolred as pH indicator. Colonies with pink zone were selected as asparagine degrading bacteria. After 72hof incubation at 35±2˚C.Six bacterial isolates of sex marine samples had apinkcolour around its colonies, Isolates showed a great ability to change the color of the medium to pink in all incubation periods Table1 and Fig.1.

**Molecular identification of bacterial isolates based on 16S rDNA:** DNA sequences analyzed using Nucleotide BLAS Talignment tools identified the highest L-asparaginase production isolate. It was identified as *H. alkaliantarctica.* The sequence submitted into the Bacterial or Archaeal 16S ribosomal RNA sequences database under accession number MK072693.

**Optimization the production of L-asparagenase by *Halomonas alkaliantarctica:*** The highest production of the enzyme 59.48 U/min/ ml, when the culture of *H. alkaliantarctica* was incubated in shaking incubator after 72h at35±2ºC at 150rpm, while under static conditions it was 13.79 U/min/ ml. The isolate produced different amount of the enzyme in the presence of different carbon sources, but compared with control, the amount of the enzyme was higher in the presence of asparagine as sole organic source, 70.69 U/min/ml, Fig.3.

Results in Fig.4 revealed that, presence ofL-asparagine as the sole nitrogen and carbon source reveeled to the highest amount of the enzyme, 106.09 U/min/ml while glycine was the best second source, 102.16 U/min/ml compared with control. Further, it was equal in the presence of with or without methionine *by H. alkaliantarctic*. Increasing of L-asparagine as nitrogen source in the fermentation medium increased the production ofASNasein the fermentation medium, the highest amount of ASNase was 98.37 U/min/ml when 1.0% of L-asparagine was added after 48h of incubation Fig.5. Fermentation medium supplemented with different nitrogen sources rather than amino acids individually. Results in Fig.6 showed the highest ASNase produced, it was 58.32 U/min/ml in the presence of tryptone as an organic nitrogen source. Results in Fig. 7showed that in the presence of 0.25% (w/v) tryptone mixed with 1.0% (w/v) asparagine maximized ASNase production to 120.66 U/min/ml compared with control. Increasing the inoculum size to 20/100ml medium (v/v) revealed to the highest ASNase production to126.67U/min/ml. Fig. 8.

## DISCUSSION

About six bacterial isolates were obtained from six marine samples from the west coast in Jeddah city, Saudi Arabia, based on the formation of a pink zone around the bacteria as an indication of the ASNase production on the Glucose Asparagin agar (GA agar) plates, supplemented with phenol red (Asselin *et al*., 1993; Siddalingeshwara and Lingappa, 2010).Thepresent study was to screen and evaluate ASNase activity of the selected bacteria that isolated from different marine samples.


**Fig. 1. Screening of L-asparaginase production of 1SFC isolated bacterial by plates**

**Table 1. Screening of L-asparaginase production by isolated bacteria by plaates**

| Marine Samples | Sources of bacterial isolates | Bacterial isolates | Qualitative production of L-asparaginase after | | |
|---|---|---|---|---|---|
| Soft coral | *Sarcophton* sp. | 1SFC | one day | two days | three days |
| | | | +++ | ++++ | ++++ |
| | Xenia umbellaate | 2SFC | – | – | – |
| brown alga | *Dictyota* sp. | 1D | + | +++ | ++++ |
| green alga | *Ulva lactucaa* | 1U | ++ | +++ | ++++ |
| solitary disc corals | *Ctenactis crassa* | 1CC | + | + | + |
| Nerite Snail | *Nerita* sp. | 1N | + | +++ | +++ |
| Bivalvia | *Atactodea glabrrate* | 1A | + | ++++ | ++++ |

**Table 2. Identity percentage of 16S rDNA between the ASNase activity strains isolated from marine samples from different sites in the west coast of Jeddaah in Saudi Arabia and the relative strains in the gene bank.**

| Bacterial | Name and Accession of No. of the most related | | Identity | Coverage | Suggested Name ar |
|---|---|---|---|---|---|
| isolates | strain in NCBI GenBank. | | | | isolatess obta |
| Seq1 | MG456867.1 | *Halomonassalkaliantarctica* | 100% | 100% | MK072693 |

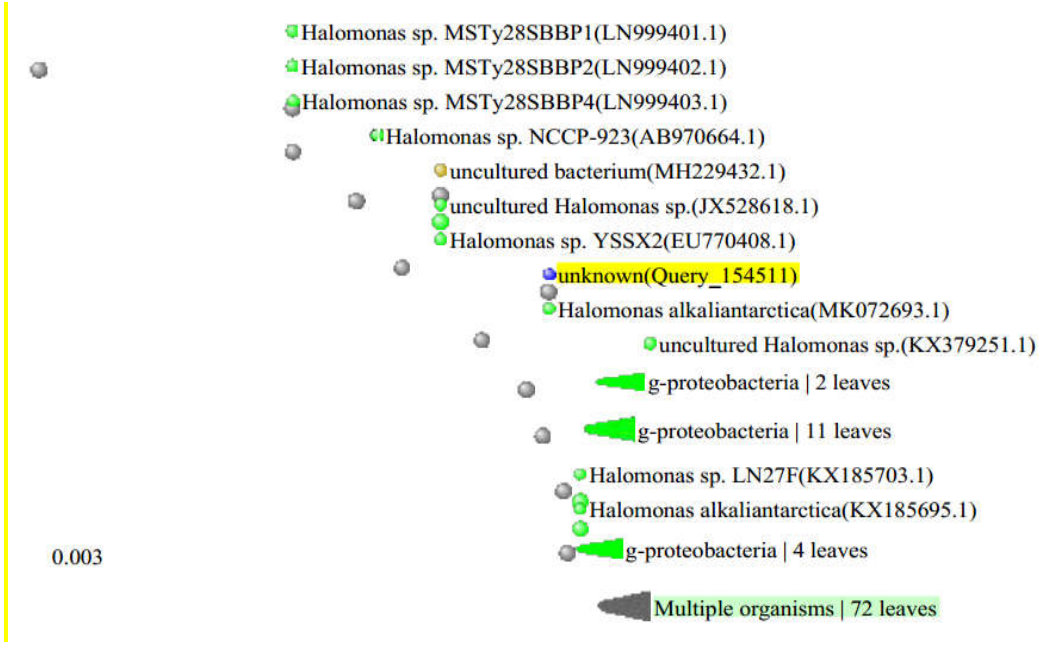


**Fig.2.Phylogenetic tree analysis of ASNase activity isolates based on 16S rDNA**

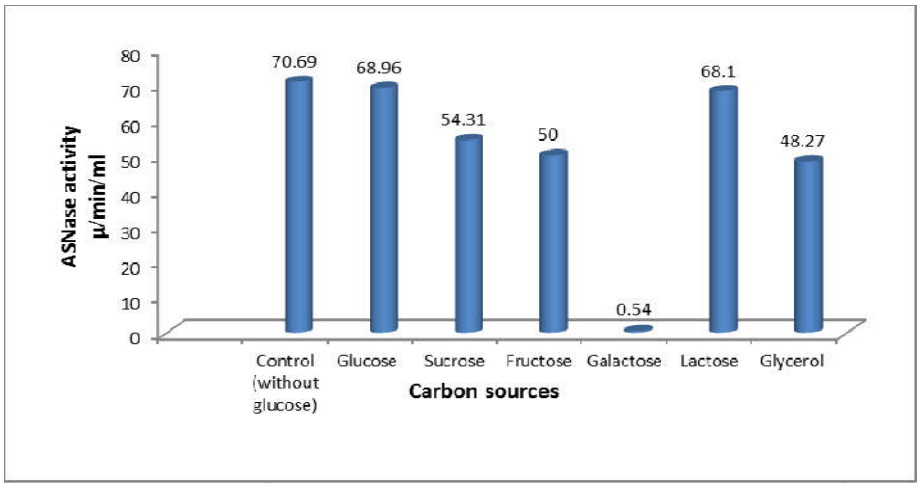


**Fig. 3. Effect of different carbon sources on L-asparaginase by *Halomonas alkaliantarctica.***

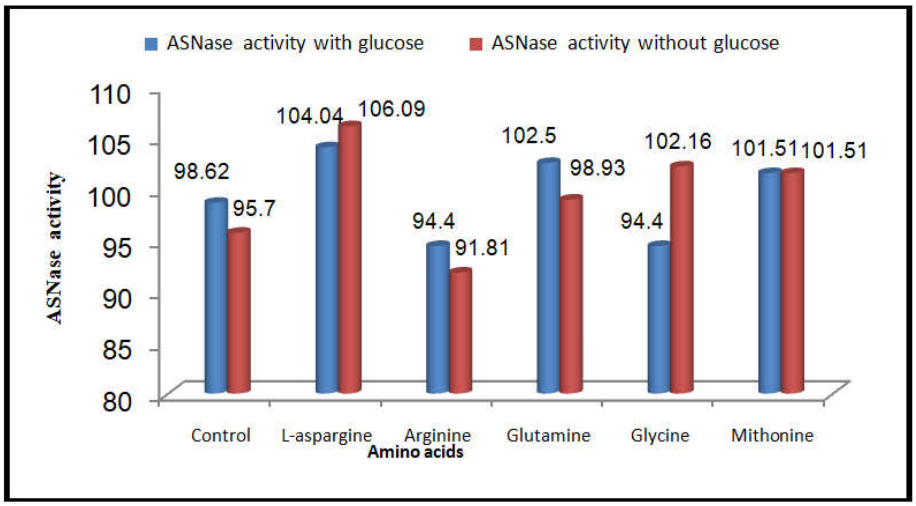


**Fig. 4. Effect of different amino acids on L-asparagenase production by *Halomonas alkaliantarctica.***

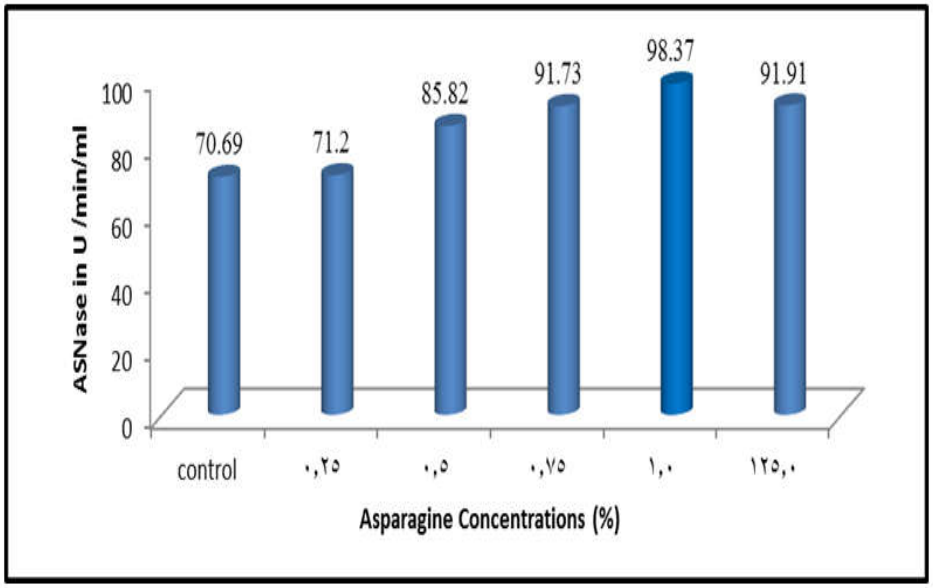


**Fig. 5. Effect of different concentrations of L-asparagine on the production of L-asparagenase by *Halomonas alkaliantarcti***

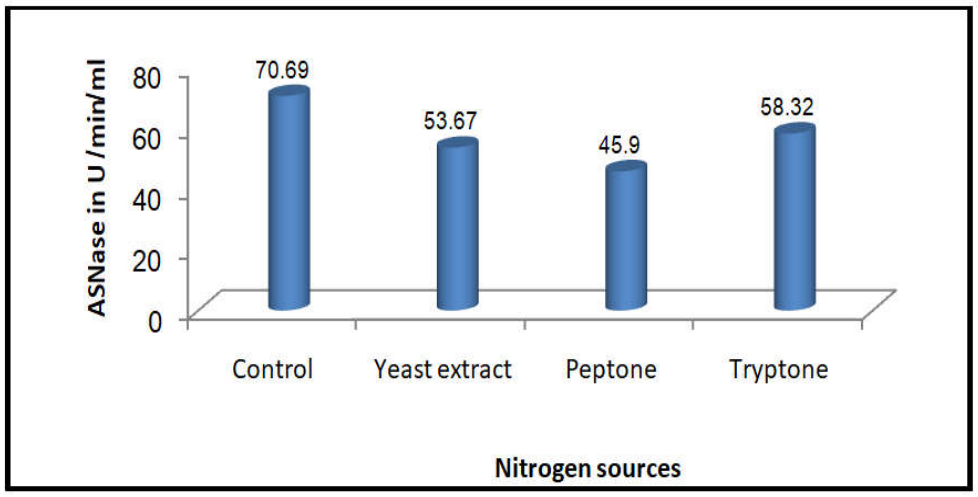


**Fig.6: Effect of different nitrogen sources on L-asparagenase production by *Halomonas alkaliantarctica.***

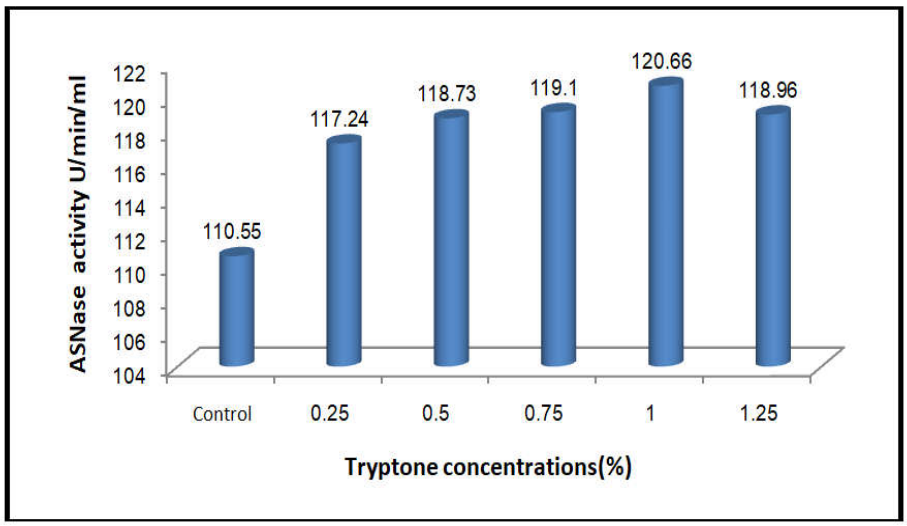


**Fig. 7. Effect of different tryptone concentrations on L- asparaginase production in presence of asparagine by *Halomonas alkaliantarctica.***

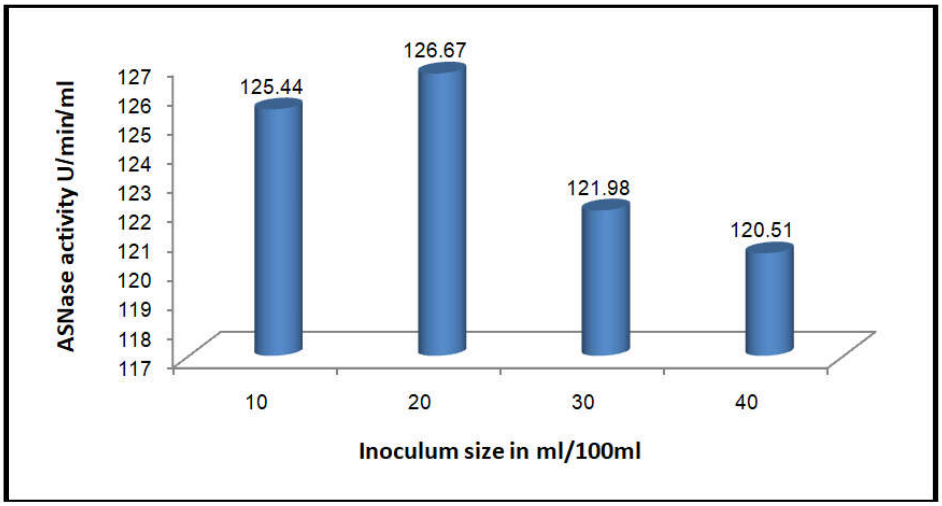


**Fig. 8. Effect of inoculum size on L-asparaginase production by *Halomonas alkaliantarctica***

*Halomonas alkaliantarctica*. (Kalyanasundarram *et al*., 2015) reported, the presence of nitrogen sources along with nitrogenous compounds present in the substrate promotes enhanced growth and consequent enzyme production. Others reported yeast extract supported high yield of ASNase, while tryptone and yeast extract stimulated ASNase synthesis in *Erwiniacarotovora* (Maladkar *et al.,* 1993) and *Escherichia coli* (Kenari*et al*., 2011). This results showed a high level ofASNase production when tryptone at concentration (1%)followed by asparagine (1%). Similar, Sharma and Husain (2015) found culture medium emended withh yeast extract has affirmative effect on enzyme production followed by tryptone by *Enterobacter cloacae*. The inoculum concentration results showed maximum production 126.67U/min//ml at20 ml/100ml inoculums *Halomonasalk aliantarctica*. Palllem *et al.* (2011) reported the maximum production of L-asparaginase with inoculum volume of 1.5 ml of 7 days old *Fuusarium oxysporum* through SSF.

## Conclusion

In conclusion, the marine isolate *Halomonasalk aliantarctica* was identified as a promising candidate for L-asparaginase production and it showed the enzyme activity maximized in nutrition medium supplemented with 1.0% L-asparagine and 1.0% tryptone without carbon source. It is highly recommended that prospects of this enzyme should be explored for its utility in pharmaceutical as anticancer agent and food industry.

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