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Production and optimization of L-asparaginase by an actinobacterium isolated from Nizampatnam mangrove ecosystem

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Abstract

The aim of the present study was to isolate and screen actinomycetes from the mangrove sediments of Nizampatnam that are potent to produce L-asparaginase, an enzyme that catalyses the hydrolysis of asparagine. A total of 31 actinomycetes strains were isolated, of which 6 strains were positive for L-asparaginase. Several physico-chemical parameters were optimized for maximizing L-asparaginase production by the potent strain identified as *Pseudonocardia endophytica* VUK-10. Production of L-asparaginase by the strain was high in modified Asparagine glucose salts broth (FM-4)(3.96 IU/ml) as compared to other tested media. Maltose(6.99 IU ml^{-1}) and L-asparagine (7.42 IU ml^{-1}) were found to be the most suitable carbon and nitrogen sources for optimum enzyme production. Maximum production of L-asparaginase was found in the culture medium with pH 8 and temperature 30ºC incubated for four days. This is the first report on the production of L-asparaginase by *Pseudonocardia endophytica* VUK-10 from Nizampatnam mangrove sediments.

Key words

L-asparaginase, *Pseudonocardia endophytica*, Optimization, Mangrove sediments

Introduction

The anti-carcinogenic potential of L-asparaginase has received increased awareness in the current years because of its use as an effective therapeutic agent against lymphoblastic leukaemia and other kind of cancers. The cancer cells depend on the circulatory L-asparagine from plasma pool (Swain *et al*., 1993; Jayaramu *et al*., 2010). Lack of exogenous supply of L-asparagine coupled with impaired protein synthesis is the prime factor for causing the death of malignant cells (Duval *et al*., 2002; Thomas *et al*., 2010). The chemotherapeutic activity of L-asparaginase is due to its ability to convert L-asparagine to L-aspartic acid and ammonia (Fisher and Wray, 2002; Sushrut *et al*., 2011; Thenmozhi *et al*., 2011; Dhanam and Kannan, 2013).

L-asparaginase supplementation results in continuous depletion of L-asparagine. In such environmental conditions the cancerous cells do not survive. This biological defect of cancerous cells was trapped by the scientific community to treat neoplasias using L-asparaginase (Story *et al*., 1993). This enzyme is also a choice for acute lymphoblastic leukemia, lymphosarcoma and many other clinical experiments relating to tumour therapy in combination with chemotherapy (Wafaa and Maysa, 2010).

Commercially available enzymes isolated from *Escherichia coli* and *Erwinia carotovora* (Northrup *et al*., 2002; Kamble *et al*., 2006) are the only sources for the front line therapy but long term administration of this enzyme in general produces the corresponding antibody in tissues, resulting in anaphylactic shock or drug neutralization. So there is an urgent need for using new serologically different L-asparaginase with similar therapeutic properties (Saleem *et al*., 2008; Pokrovskaya *et al*., 2012).

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A programme for screening and isolation of novel bacteria and actinomycetes producing new natural compounds and therapeutic enzymes from the unexplored environment is an important task to be undertaken. In this entourage for virtuous utilization of any microbial system at the bioprocess level, it is legit to screen and audit various nutritional and environmental requirements for the microbial growth and biocatalyst production (Sreenivas *et al*., 2004; Prakasham *et al*., 2005), as the culture conditions that assist optimum enzyme production contradict considerably due to the molecular nature of the microorganism (Prakasham *et al.,* 2007). Actinomycetes are an important group of bacteria that are highly explored for the production of antibiotics, bioactive metabolites and anticancer agents (Amena *et al*., 2010; Mohana Priya *et al*., 2011; Anupa *et al*., 2013).

Although enzyme has been found in a wide variety of bacteria, fungi, actinomycetes and mammals, very few preparations have been found to have anti-tumour activity. Like bacteria, actinomycetes have also been reported to be a good source of L-asparaginase (Narayana *et al*., 2008; Kavitha and Vijayalakshmi, 2010). Actinomycetes isolated from the mangrove sediments have immoderately acknowledge as a abeyant source of untrodden drug candidates and are a pledging source of antitumor drugs and therapeutic enzymes (Jensen *et al*., 2005). Mangrove actinomycetes have been reported to produce functionally unique metabolites and enzymes that are not found in their terrestrial counterparts because of the extreme living conditions within the mangrove environment (Usha *et al*., 2011).

The activity of L-asparaginase produced by mangrove actinomycetes has not been fully explored. Extracellular asparaginases are more advantageous than intracellular ones since they could not only be produced abundantly in culture broth but also purified economically (Amena *et al*., 2010). The present inquest accords with the production and optimization of extracellular L-asparginase from rare actinomycete *Pseudonocardia endophytica* VUK-10 from Nizampatnam mangrove ecosystem.

Materials and Methods

The sediment samples were collected from 10 different sites of the mangrove ecosystem of Nizampatnam located at south-east coastal of Andhra Pradesh, India. The central portion of the 6-10 cm sediment sample was transferred to sterile bags and subsequently transported to the laboratory for further microbiological analysis. The soil samples were air-dried at room temperature for a week.

Actinomycetes were isolated by suspending pre-treated soil sample at 55°C for 15 min in a suspension fluid containing quarter strength ringer's solution, serially diluted up to 10^{-5} and plated on asparagine–glucose agar medium supplemented with nalidixic acid (25 µg ml^{-1}) and secnidazole (25µg ml^{-1}) in order to retard the growth of bacterial and fungal contaminants, respectively. After incubation of the plates at 28°C for two weeks, typical actinomycetes colonies selected on morphological basis were picked out, purified and preserved on yeast extract, malt extract and dextrose agar medium at 4°C.

Screening and identification of L-asparaginase producer strain: L-asparaginase activity of all the strains was evaluated by using modified M-9 medium incorporated with phenol red (pH indicator dye) and adjusted to pH 7.0 and incubated at 30 ºC for 5 days. Colonies that showed pink zone were considered as L-asparaginase positive strains (Gulati *et al*., 1997). Quantitative estimation of enzyme activities was carried out using M-9 liquid medium. Among the various actinomycetes tested, those showing wider zone and maximum enzyme activity were used for further study.

The potent actinomycete strain was identified as *Pseudonocardia endophytica* VUK-10, based on colony morphology, spore arrangement, physiological and biochemical characteristics. Further, the identity of strain was confirmed based on the molecular analysis of 16S rRNA gene sequence with GenBank accession no: JN087501.

Assay of L-asparaginase: Quantitative assay of L-asparaginase was carried out according to the method of Imada *et al.* (1973). The reaction was started by adding 0.5 ml of culture filtrate and 0.5 ml 0.04 M L-asparagine as substrate and 0.5 ml of 0.05 M tris amino methane (Tris-Hcl) buffer at pH 7.2 and incubated at 30 °C for 20 min in a water bath shaker. The reaction was stopped by adding 1.5 M tricholoroacetic acid. The precipitated protein was removed by centrifugation and the liberated ammonia was determined spectrophotometrically at 450 nm after the addition of 0.2 ml of Nessler's reagent. The enzyme activity was expressed in IU. One IU of L-asparaginase is the amount of enzyme which liberates 1 µmol of ammonia per ml per min (µmole ml^{-1} min^{-1}).

The strain was grown in triplicate for 7 days at 37°C on FM-1, FM-2, FM-3 and FM-4 media. The components of fermentation medium (FM) included: **FM-1**:Sodium caseinate-0.2 %, Soluble starch-0.1 %, L-asparagine-0.5%, K_2HPO_4 - 0.2 %, $MgSO_4$-0.02 %, $FeSO_4$-0.01 %, pH – 6.8; **FM-2**: Yeast extract- 0.4 %, Glucose-1%, Malt extract- 0.4%, L-asparagine-0.5%, pH – 7.2; **FM-3**: K_2HPO_4-0.1%, Glycerol-1%, L-asparagine- 0.5%, $FeSO_4$-0.01%, $MnCl_2$-0.01%, $ZnSO_4$-0.01%, pH-7 and **FM-4**: D-Glucose -1%, L-asparagine- 0.5%, K_2HPO_4 – 0.05%, Trace salts solution-0.1%, pH – 6.8, respectively.

Optimization of culture conditions for L-asparaginase production: Influence of different culture conditions like pH, temperature, carbon and nitrogen sources on the production of L-asparaginase was determined by growing the strain in Asparagine glucose salts broth (FM-4) for 96 hr. The asparaginase production was carried out in 250 ml Erlenmeyer flasks containing 50 ml production media inoculated with 0.5%

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inoculum and maintained at 30°C for 7 days on rotatory shaker (120 rpm). To evaluate the activity of L-asparaginase, the culture medium harvested at 24 hr intervals was centrifuged at 7000 rpm for 30 min to obtain the supernatant that served as enzyme source. All the experiments were carried out in triplicate and the average values were reported.

The effect of pH on L-asparaginase production was studied by culturing the strain in Asparagine glucose salts broth with pH varying from 6-9 for 96 hr. The optimal pH achieved at this step was used for the subsequent study.

The effect of temperature on L-asparaginase production was examined by culturing the strain in production medium at different temperatures, ranging from 20°C to 60°C for 96 hr. Once a given parameter was optimized, it was kept constant at that level while varying the other parameters individually.

To investigate the influence of carbon sources on L-asparaginase production by the strain, the production medium was supplemented with different carbon sources such as arabinose, fructose, galactose, lactose, maltose, sucrose, starch, xylose and glycerol at a concentration of 1% (w/v). The effect of varying concentrations (0.5-5) of the best carbon source on enzyme production was also investigated. The carbon source which supports high yield of L-asparaginase production by the strain was chosen for further study.

The influence of different nitrogen sources was determined by supplementing both organic and inorganic nitrogen sources viz., lysine, L-arginine, phenyl alanine, glycine, L-glutamine, L-asparagine, tyrosine, malt extract, peptone, ammonium oxalate and ammonium sulphate at a concentration of 0.5% to the production medium containing an optimum amount of the superior carbon source as determined above. Further, the influence of different levels (0.1-2%) of optimized nitrogen source was studied to standardize maximum enzyme production. The nitrogen source found to be good for optimal L-asparaginase production was selected for the subsequent study.

Statistical analysis: Data obtained on the L-asparaginase production under different culture conditions were statistically analyzed and expressed with one-way analysis of variance (ANOVA).

Results and Discussion

The exploration of important L-Asparaginase producing actinomycetes was under taken in the mangrove ecosystem of the Nizampatnam. The soil sediments collected were enumerated for isolation of actinomycetes. 31 isolates obtained from Nizampatnam were evaluated for the production of L-asparaginase by plate screening assay using M-9 media. Six strains were found positive on the basis of the pink zone around the colony. L-asparaginase produced by the positive strains was quantified and the potent strain was identified as *Pseudonocardia endophytica* VUK-10, based on micro-morphology, biochemical and molecular characteristics.

Influence of media on L-apsaraginase production by the strain is represented in Fig.1. The enzyme production was maximum in Asparagine glucose salts broth (FM-4). Though production of L-asparaginase started after 24 hr of incubation, maximum enzyme activity was found after 96 hr (4th day of incubation). Narayana *et al.* (2008) reported that the production

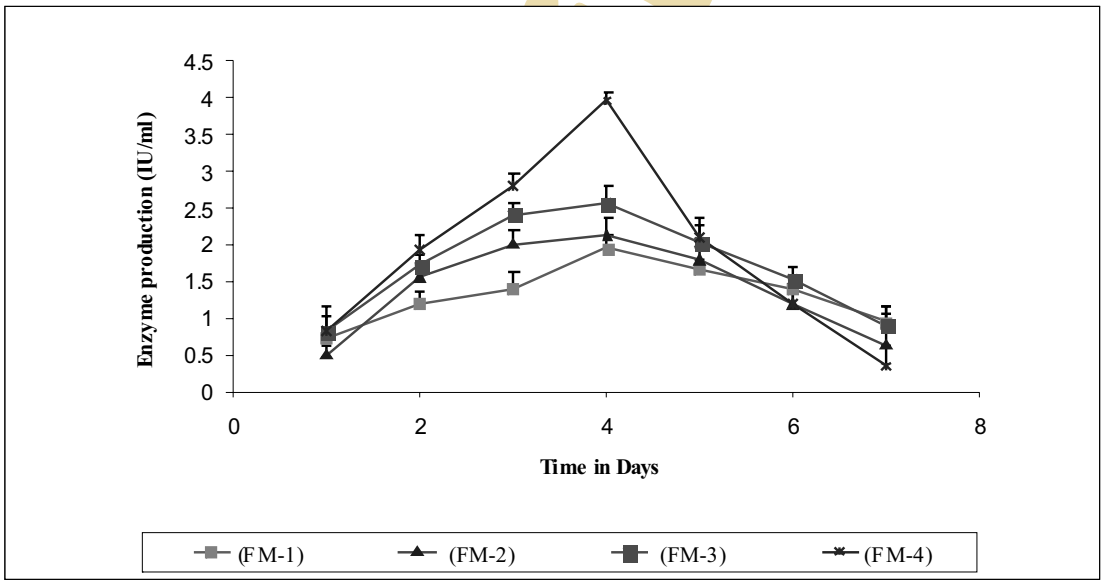

Fig.1: Influence of culture media and incubation period on L-asparginase production by *Pseudonocardia endophytica* VUK-10

of enzyme by *S. albidoflavus* was observed after 24 hrs of incubation but reached maximum after 72 hrs. Sutthinan *et al.* (2009) recorded that maximum production of enzyme was obtained by the strain *Amycolatopsis* CMU-H002 after 172 hrs of incubation in asparagine dextrose salts broth.

The influence of pH on L-asparaginase production was recorded by cultivating the strain at different pH levels between 6 and 9 (Fig.2). The maximum production of enzyme was observed at pH 8 after 96 hrs of incubation. Saleem *et al*. (2008) also recorded the maximum enzyme production at pH 8 for marine actinomycetes. Dhevagi and Poorani (2006) reported that the maximum L-asparaginase activity of *Streptomyces* sp. PDK7 was observed at pH levels between 8.0 and 8.5.

Influence of temperature on L-asparaginase production by the strain is presented in Fig.3. There was a steady increase in the enzyme activity with increase in temperature from 20°C and reached maximum at 30°C. Further, increase in temperature resulted in decline of enzyme production. The results are in conformity with those reported by Jayaramu *et al*. (2010) and Selvam and Vishnupriya (2013). Sutthinan *et al*. (2009) also reported that the optimum temperature for the production of enzyme was between 28°C and 30°C. Amena *et al*. (2010) and Sivakumar *et al.* (2013) recorded optimum pH 8.5 and temperature 40°C for the production of L-asparaginase in *S.gulbargenesis* and *Streptomyces* sp.SS7. The present study revealed that L-asparaginase production by the strain was high when grown in Asparagine glucose salts broth with pH 8 for 96hrs at 30°C.

Impact of different carbon sources on the production of L-asparaginase by the strain is presented in Fig. 4. Production medium supplemented with different carbon sources was

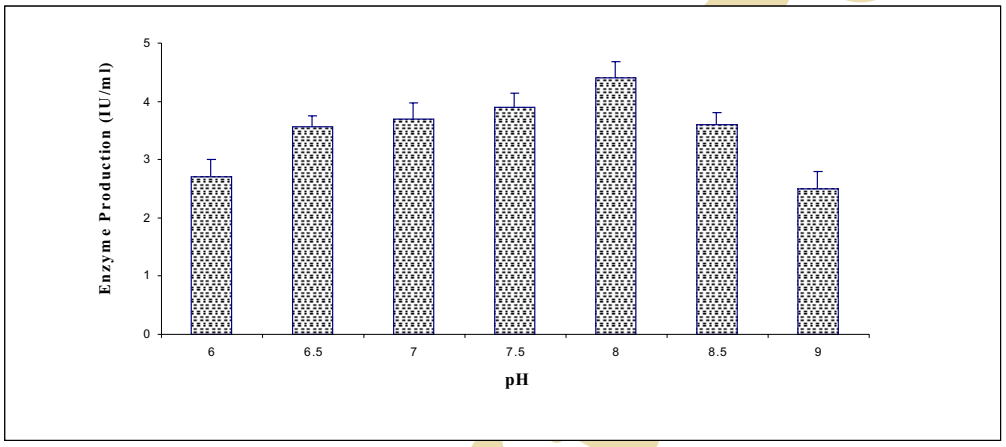

Fig. 2: Effect of pH on L-asparaginase production by *Pseudonocardia endophytica* VUK-10

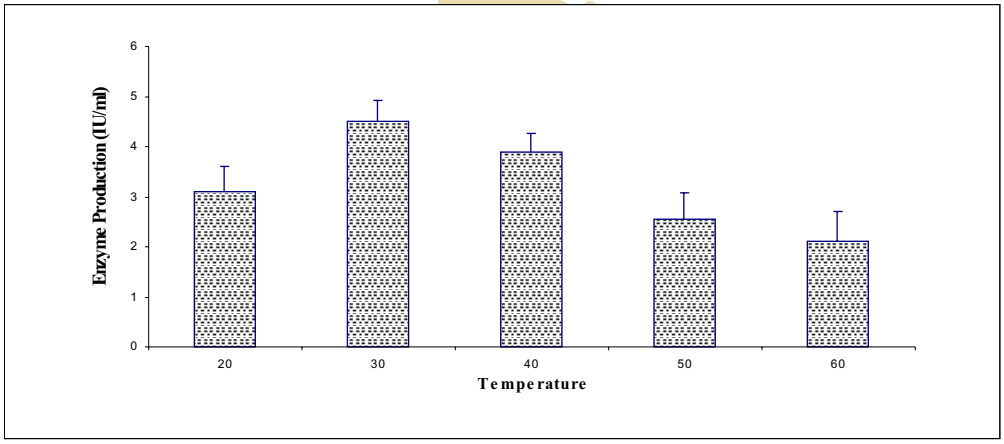

Fig. 3: Effect of temperature on L-asparaginase production by *Pseudonocardia endophytica* VUK-10

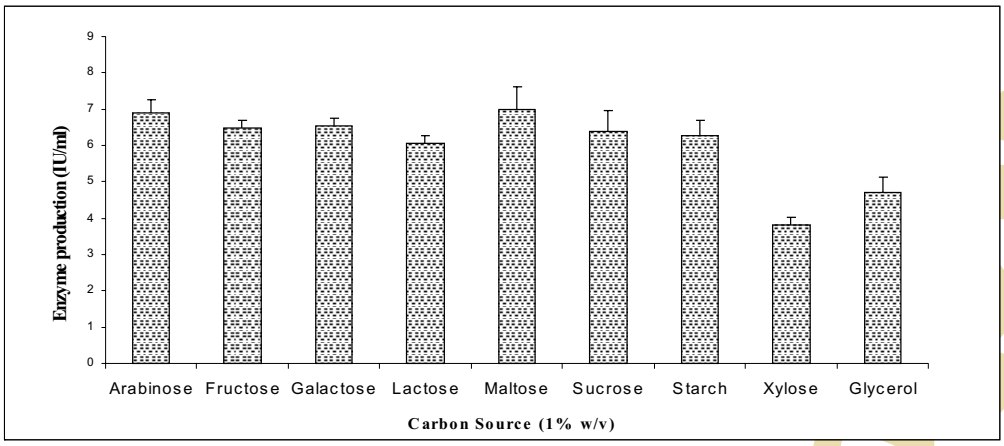

Fig. 4: Effect of carbon sources on L-asparaginase production by *Pseudonocardia endophytica* VUK-10

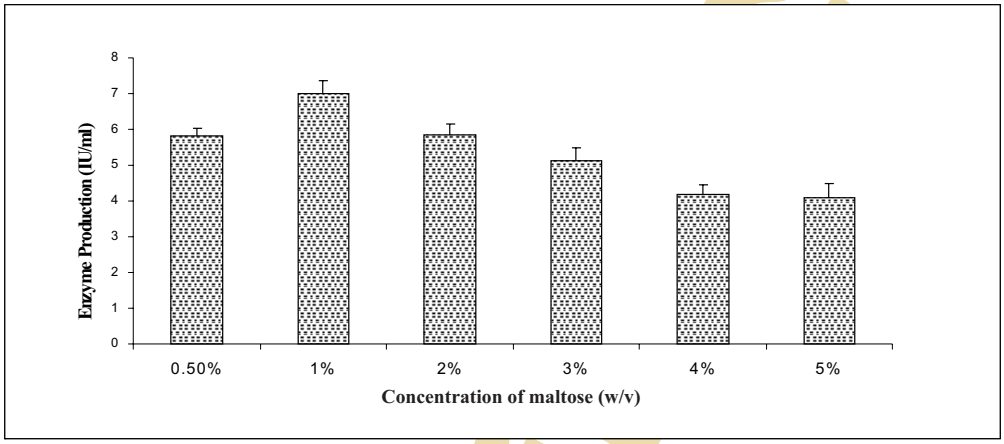

Fig. 5: Effect of different concentrations of maltose on L-asparaginase production by *Pseudonocardia endophytica* VUK-10

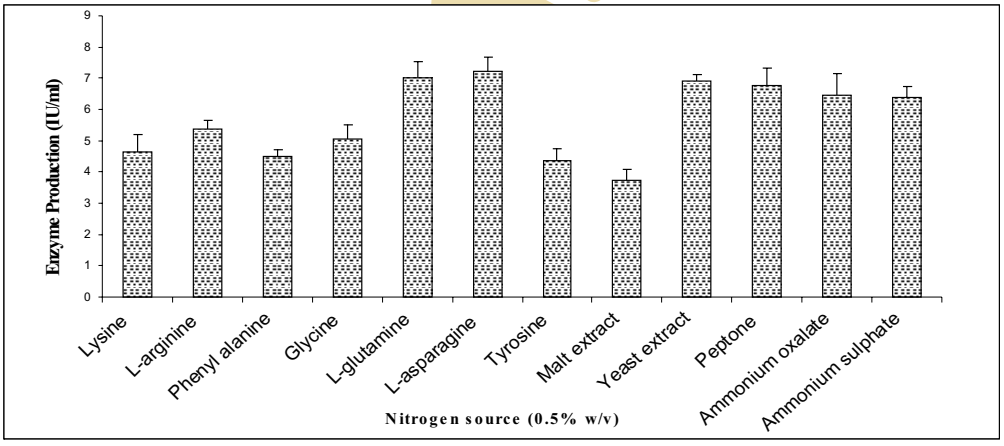

Fig. 6: Effect of nitrogen sources (Organic and Inorganic) on L-asparginase production by *Pseudonocardia endophytica* VUK-10

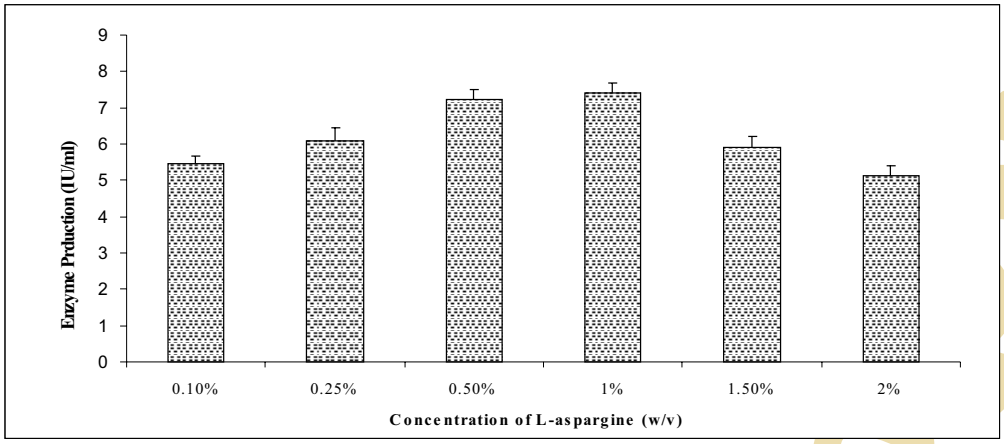

Fig.7: Effect of different concentrations of L-aspargine on L-asparaginase production by *Pseudonocardia endophytica* VUK-10

employed to determine their influence on L-asparaginase production. As compared to other carbon sources tested, culture medium supplemented with maltose stimulated L-asparaginase production at pH 8 and temperature 30°C. These results are in agreement with the results reported by Amena *et al*. (2010) who stated that *S. gulbargensis* showed maximum activity with maltose as the carbon source. Maltose proved to be the best carbon source for the production of L-asparaginase in *Streptomyces* sp. SS7 (Sivasankar *et al*., 2013). As maltose was the most preferred carbon source, for enzyme production by the strain different levels of maltose (0.5-5%) were tested to determine optimal concentration for enzyme production (Fig .5). Maximum enzyme production was obtained in fermentation medium supplemented with 1% maltose. Narayana *et al*. (2008) reported that 1% maltose enhanced optimum enzyme production by *Streptomyces albidoflavus*. On the contrary, Kavitha and Vijayalakshmi (2010) reported 2% sucrose as the best carbon source for L-asparaginase production by *Streptomyces tendae.*

Impact of different nitrogen sources on the production of L-asparaginase by the strain is presented in Fig.6. The effect of nitrogen sources on the production of L-asparaginase by the strain was studied by incorporating different organic and inorganic nitrogen compounds in the production medium. Maximum enzyme production was recorded when L-asparagine was used as nitrogen source. Tryptone and Yeast extract served as good nitrogen sources for L-asparaginase production by *E. carotovora* EC-113 (Maladkar *et al.,* 1993). Effect of different concentrations of L-asparagine on the production of L-asparaginase has been shown in Fig.7. It should be noted that L-asparagine, at 1% concentration, exhibited optimal production of enzyme. Earlier reports also suggested that maximum enzyme production was observed when L-asparagine at 0.5% and 1% concentration, was used as nitrogen source in *Streptomyces gulbargensis* and *Erwinia carotovora,* respectively (Amena *et al*., 2010; Warangkar and Khobragade, 2009). While Narayana *et al* (2008) recorded 2% yeast extract as the optimal concentration for L-asparaginase production.

The present study revealed that actinomycetes isolated from mangrove sediments are rich source of L-asparaginase. The selected parameters examined showed a considerable impact on L-asparaginase production by *Pseudonocardia endophytica* VUK-10. In the present work, Asparagine glucose salts broth at pH 8.0 and temperature 30ºC supplemented with 1% maltose and 1% L-asparagine, greatly supported L-asparaginase production by the strain. The enzyme yield before optimization was 3.96 IU ml^{-1} which enhanced to 7.42 IU ml^{-1} after optimization. This is the first report on L-asparaginase production by *Pseudonocardia endophytica* VUK-10 from mangrove sediments of Nizampatnam. However, further investigation is required to characterize the extracellular L-asparaginase, which may be effectively used as a potential therapeutic agent for pharmaceutical purpose.

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