



Study of oncolytic enzyme production from marine *Streptomyces krainskii* VMB-11

Usha Y.^{1*}, and Vishnuvardhan Z.²

¹Department of Microbiology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

²Department of Botany & Microbiology, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

Abstract: L-asparaginase is an important enzyme, plays a significant role in treating ALL (Acute lymphoblastic leukemia), acute myelocytic leukemia, and other carcinoma treatments. It is well known as an anti-cancerous agent because of its stability in varied environmental conditions. *Streptomyces* sp. is occupied the first position for the producing L-asparaginase from marine and terrestrial sources. In the presence of L-asparaginase, tumor cells get deprived and can't endure. It acts as a potent antitumor or anti-leukemic drug. Because of the urgent need, an attempt was made to isolate and optimize marine actinomycetes for L-asparaginase production. The *S. krainskii* VMB-11 was isolated and inoculated in optimized M-9 media at 40°C, 96 h improving L-asparaginase production from 8.2 IU to 13.2 IU/g dry wt. of biomass. This is often the primary report on production, purification, and characterization of L-asparaginase from marine *Streptomyces* isolate VMB-11 strain to the simplest of our data.

Keywords: Marine actinomycetes; *Streptomyces krainskii* VMB-11; L-asparaginase

Introduction

Marine habitat has been proved as an outstanding and fascinating resource for innovating new and potent bioactive metabolite producing microorganisms (Donia and Hamann, 2003). Recent studies say that L-asparaginase (L-asparagine amidohydrolase, 3.5.1.1) is the example of an organic compound depriving enzyme widely present in nature. The enzyme catalyzes L-asparagine hydrolysis into aspartic acid and ammonia, depriving the leukemic cell, which leads to cell death (Bull *et al.*, 2005).

The L-asparaginase, isolated from microorganisms, is used as an anticancer drug to treat several cancer disorders like lymphomas, leukemia, sarcoma, etc. (Kumar *et al.*, 2014). L-asparaginase is an essential amino acid obtained from diet (or) by the synthesis through asparagine synthetase located on the human chromosome number 7 (Keating *et al.*, 1993). The asparagine synthetase was less active in tumor

cells, cannot synthesis L-asparaginase by on and depends on the external sources. Microbes are very convenient producers of L-asparaginase enzyme production. The L-asparaginase from *E. coli* and *Erwinia cartovora* is a potent anti-leukemic drug to treat Acute lymphoblastic leukemia (ALL) which is common cancer under the age of 5 years in children. Nowadays, L-asparaginase is a crucial drug in treating ALL in children worldwide (Patro *et al.*, 2011; Verma *et al.*, 2007).

Bacteria, plants, and animals were good producers of L-asparaginase, but not found in humans. Due to this, in cancer treatment, the asparaginase effectively controlled tumor growth (Theantana *et al.*, 2009). The L-asparaginase is a biodegradable anticancer agent administered locally quite easily and acts as amino acid L-asparagine. Different fungal (*Fusarium*, *Aspergillus* and *pencillium*) and bacterial species (*E. coli*

*Corresponding Author:

Dr. Usha Y.,

E-mail: smiley.shine13@gmail.com

and *Erwinia carotovora*) are found as an L-asparaginase source products used in medical treatment. Prolonged L-asparaginase usage from these two bacteria causes hypersensitivity, allergic reactions, and anaphylaxis (Patro *et al.*, 2011).

Actinomycetes also a good source for the production of L-asparaginase (Pathom-Aree, 2006). However, very few reports are available on the show of L-asparaginase from marine origin. *Streptomyces* sp. is the most bountiful and immensely present in marine and earth-bound territories and displays a remarkable capacity to create novel metabolites. Individual studies have reportable many marine *Streptomyces* sp. such as *S.karnatakensis*, *S.venezualae*, *Streptomyces* sp. PDK2 produces enormous amounts of L-asparaginase (Dhanam *et al.*, 2014). *Streptomyces* sp. is the best L-asparaginase producer, leading to our point of current investigation is isolation and purification of L-asparaginase from VMB-11 strain from marine soils of Machilipatnam, south-east bank of Andhra Pradesh, India.

Materials and Methods

Isolation of L-asparaginase producers

A particular screening program for the segregation of marine actinomycetes soil samples was gathered from completely different areas in depths in profundities of 6-10 cm *viz.*, Humsaladevi and Machilipatnam situated in Krishna region of Andhra Pradesh. The gathered soil samples were air-dried, sieved, and treated with calcium carbonate (1: 1 w/w) to lessen the rate of microbes and growths utilizing modified M-9 agar plates (El-Nakeeb and Lechevalier, 1963). The structure of M-9 medium ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.6%; KH_2PO_4 0.3%; NaCl 4%; L-asparagine, 0.5%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2%; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1%, Glucose 0.4%, Agar 2% and pH 7.0 distilled water to 100 ml with phenol red (0.009%) conta-

ining antibiotics, for example, 50 µg/ml nalidixic acid and amphotericin - B (25 µg/ml). The soil dilution plate method was utilized for the separation of actinomycete strain according to the modified protocol recommended by Williams and Cross (1971). Aliquots of 10^{-4} dilution was put on the surface of modified M-9 agar plates. The inoculated plates were incubated at 30° C for 10 days. After a period, actinomycetes colonies with pink zones were considered as L-asparaginase producers.

Screening for L-asparaginase activity :

L-asparaginase production by actinomycetes

The strain's enzyme activity was assessed using a modified M-9 medium with phenol red (pH indicator dye) adjusted to pH 7.0 and incubated at 30°C for five days (Gulati *et al.*, 1997). Out of 16 isolates (VMB-1-11), one predominant strain indicates a pink zone around the colony. It is designated as VMB-11 and noted as L-asparaginase positive strain.

Taxonomic investigations of the potent actinomycetes

Cultural, morphological and physiological characterization of the strain and 16s rDNA gene sequencing analysis was studied for its identification. The growth characteristics of strain were analyzed according to the method of Shirling and Gottlieb (1966) on different media, including ISP (International *Streptomyces* project) media like ISP-2 (YMD agar), ISP-3 (oatmeal agar), ISP-5 (glycerol-asparagine agar), ISP-7 (tyrosine agar) and non-ISP media such as glucose asparagine agar, Czapek Dox agar, nutrient agar, and starch casein agar media. Cultural characteristics such as type of growth, the color of aerial and substrate mycelia were recorded. (cross, 1989). The strain was identified up to the generic level by comparing the morphology of spore-bearing vegetative hyphae with the structure of spore chains of actinomycetes (Williams *et al.*, 1983).

Identification of strains by 16 S rRNA analysis

The 16S rDNA was amplified with a forward primer (5'-GTGAGTAACAGCTGGGCACT-3') and reverse primer (5'-TCCTCCTAGATATTGTGCGCAT-3'). The amplified DNA fragment was separated on 1% agarose gel electrophoresis and purified using the Qiaquick gel extraction kit (Qiagen, Germany). The 16S rDNA sequence of the strain VMB-11 generated in this work was aligned with the 16S rDNA sequence of the other closely related *Streptomyces* species retrieved from the NCBI GenBank. A sequence similarity search was done using GenBank BLASTIN (Aitschul *et al.*, 1997). Sequences of closely related taxa were retrieved and aligned using Cluster X program (Thompson *et al.*, 1997). For the neighbor-joining analysis (Saitou and Nei, 1987), the distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985).

Quantitative assay

A quantitative assay of L-asparaginase was carried out with Nesslerization method, according to the procedure followed by Imada *et al.* (1973). Add 0.1 ml of enzyme extract to 0.2 ml of 0.05M Tris - HCl buffer (pH 7.2), and 1.7 ml of 0.01M L-asparagine and incubated for 10 min at 30°C. The reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA), and precipitated protein was removed by centrifugation at 10,000 rpm. The supernatant (0.5ml) was diluted to 7 ml with distilled water and treated with 1 ml of Nessler's reagent and incubated for 10 min. The absorbance was recorded at a wavelength of 450 nm with UV/Visible Spectrophotometer. The enzyme activity was expressed in IU. One L-asparaginase unit is equal to the amount of enzyme that catalyzes 1 μ mole of ammonia per ml per minute (μ mole /ml/min).

Optimization of L-asparaginase production

Effect of different parameters like pH, temperature, carbon, and nitrogen sources on L-asparaginase production was dictated by growing strain VMB-11 in modified M-9 broth for four days.

Effect of incubation period on enzyme production

The incubation period on growth and L-asparaginase production was evaluated by strain VMB-11 was inoculated in a modified M-9 medium and incubated for different periods like 24h-168h. The strain was assayed at every regular interval of 24-168 hr to determine the optimum incubation period as followed by Abdel-Razik *et al.*, (2019).

Impact of pH on enzyme production

The optimum pH is used to determine the growth and enzyme production. The strain was inoculated in a medium with different pH levels (5.5 -10) and incubated for 96h. After 96h, growth and enzyme production of strain VMB-11 were determined as per the experimental design followed by Dhanam and Kannan, (2014). The optimal pH determined in this step was used for further study.

Impact of temperature

Temperature ranging from 15^o-60^oC with optimum pH was used to study the impact on L-asparaginase enzyme production of strain. M-9 broth was used for the strain growth and production of L-asparaginase (Dhanam and Kannan, 2014).

Effect of carbon sources

M-9 broth was amended with various carbon (sucrose, glucose, mannose, lactose, maltose, xylose) sources, each at a concentration of 1% (w/v) were used to determine the L-asparaginase production (Basha *et al.*, 2009).. The effect of different concentrations of best carbon (0.5-5%) source, which supports high yields of

enzyme production by the potent strain, was chosen for further study.

Influence of nitrogen sources

To determine the impact of nitrogen sources of L-asparaginase production by VMB-11 strain, modified M-9 broth was amended with different types of nitrogen sources (ammonium nitrate, urea, peptone, casein, sodium nitrate, and L-asparagine) at a concentration of 1% (w/v). (as followed by Indira *et al.*, 2015). The effect of different concentrations of the best nitrogen source (0.5-5%) supporting significant yields of enzyme production was studied.

Results and Discussion

Isolation and screening of actinomycete strain

For marine actinomycetes' isolation, the soil samples were amended with CaCO₃ was dried at 45^o C for 1 hr. Serially diluted soil samples were plated on a modified M-9 medium, incubated at 28±2^o C for 10 days. After incubation, leathery and tough actinomycetes colonies are observed. A total of 16 actinomycete strains were isolated from two marine samples. Out of these 16 strains, one of the strain VMB-11 was found to be L-asparaginase producer.

Modified M-9 medium enhanced L- asparagine (0.5%) as sole nitrogen source with phenol red (0.009%) was utilized to screen the strain for L-asparaginase production. The pink zone around the colony indicates the positive result for the production of L-asparaginase. Because of the urgent need for L-asparaginase from different sources in high yields, *Streptomyces kرائسکii* VMB-11 was chosen to produce L-asparaginase. (Picture-1).

Taxonomic studies of potent actinomycetes strain VMB-11

Culture characteristics of the predominant strain designated as VMB-11 were recorded on different ISP media viz; ISP-2, ISP-3, ISP-5, ISP-7, and non-ISP media like glucose asparagine, nutrient, and Czapek-Dox agar media.

The strain showed good growth on ISP-2, ISP-3, ISP-5, and ISP-7 followed by moderate growth on glucose asparagine, Czapek-Dox, and nutrient agar media. The aerial mycelium color is white, while that of the substrate mycelium varied from light brown to brownish crimson to light yellow. (Table-1)

Table 1. Cultural characteristics of the actinomycete strain VMB-11 on different culture media

| S.No. | Culture media | Growth rate | Color of the aerial mycelium | Color of the substrate mycelium | Pigmentation production |
|-------|-------------------------|-------------|------------------------------|---------------------------------|-------------------------|
| 1 | ISP 1 | Good | White | Light brown | - |
| 2 | ISP 2 | Good | White | Light yellow | Dark brown - |
| 3 | ISP 3 | Good | White | Light yellow | - |
| 4 | ISP 4 | Good | White | Light yellow | - |
| 5 | ISP 5 | Good | White | Light yellow | - |
| 5 | Nutrient agar | Moderate | White | Light yellow | - |
| 5 | Czapek-Dox agar | Moderate | White | Light brown | - |
| 7 | Glucose asparagine agar | Moderate | White | Light yellow | - |
| 8 | Starch casein agar | Good | White | Brownish crimson | - |



Picture 1: L-Asparaginase activity of *S. Krainskii* VMB-11.

Micromorphological characteristics of the actinomycete strains (Picture 2)

Micro-morphology of the strain was examined by slide culture technique and scanning electron microscope (SEM). Strain VMB-11 showed fragmented aerial mycelium with straight spore chains under Scanning Electron Microscope ($\times 5500$) magnification as the sporogenous hyphae were straight in nature by which the strain was treated under Rectus-flexibilis (RF) group. L-asparaginase produced by *S. noursei* MTCC 10469 showed the spore morphology having Rectus flexibilis hyphae (Selvakumar, 2011).

Picture 2: Micromorphology of *S. krainskii* VMB-11.

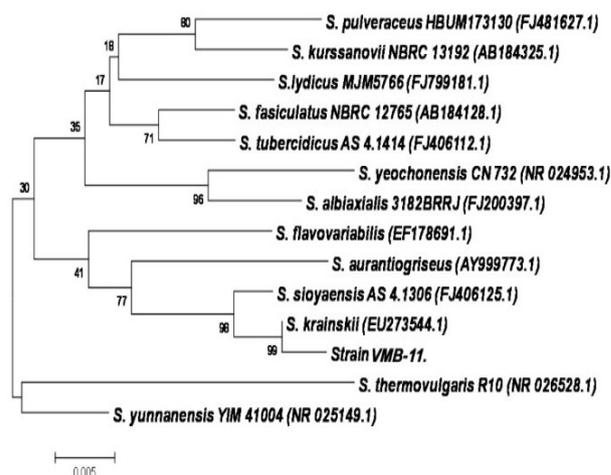


Scanning electron micrograph of Actinomycete strain VMB-11. [X5,000]

Phylogenetic analysis of the actinomycete strains (Picture 3)

The standard protocols extracted genomic DNA of the strain and the 16s rRNA sequences are amplified with the suitable primers in PCR. The phylogenetic portion of the strain was determined by blasting the 16S rRNA gene sequences with the related genera sequences in NCBI GenBank. The total nucleotide sequence (609 bp) is mostly related to the *Streptomyces krainskii* (EU 273544.1) 99% sequence similarity. Therefore, the strain was identified as *Streptomyces krainskii* VMB-11. The sequencing product of strain was deposited in GenBank database under an accession number HQ329080. (Picture 3).

Picture 3. Phylogenetic tree of *Streptomyces krainskii* VMB-11



A phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between strain VMB-11 and species belonging to the genus *Streptomyces* was constructed using the neighbor-joining method. Bootstrap values are expressed as percentages of 1000 replications. Bar, 0.005 substitutions per nucleotide position.

Optimization of L-asparaginase production

Impact of incubation period on enzyme production

The impact of the incubation period on L-asparaginase production by *S. krainskii* VMB-11

is represented in Figure 1. The strain was cultivated in modified M-9 broth and assay at every regular interval of 24 h-168h to determine the ideal incubation period. The enzyme production started after 24 h of incubation; maximum enzyme production was seen after 96 h (10.3 IU/ml) of incubation.

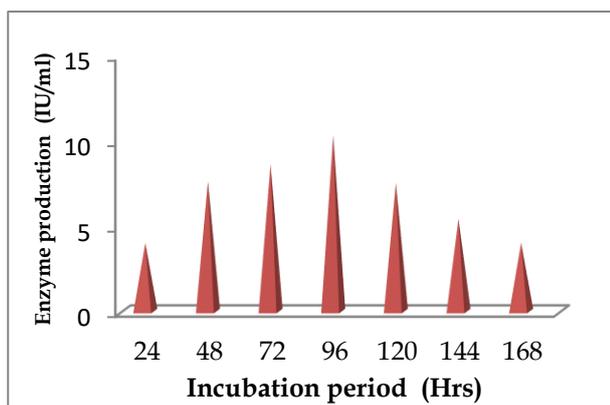


Figure 1: Effect of incubation period on L-asparaginase production by *S. krainskii* VMB-11

Effect of pH on the growth and enzyme production

The impact of pH on L-asparaginase production was studied by developing the medium's strain at different pH levels from 5.5-10.5 (Figure 2). A high yield of enzyme production was recorded at pH 8.5 (10.3 IU/ml) after 96 h incubation. *S. brollosae* NEAE-115 (El-Naggar *et al.*, 2018) produced maximum enzyme production at pH-8.5. At pH- 9 maximum enzyme production from *Streptomyces gulbargensis* has been observed (Amena *et al.*, 2010). Marine *Streptomyces noursei* MTCC 10469 produced high yields of L-asparaginase enzyme at pH-8 (Selvakumar, 2011). The Optimal pH-7 favors maximum enzyme production by marine *Streptomyces griseoluteus* GDJ1 (Dhanam, 2017).

Impact of temperature on growth and enzyme production

The effect of temperature on L-asparaginase production by the strain *S. krainskii* VMB-11 in Figure 3. There was a continuous increment in the enzyme production with increment in

temperature from 20°C and reached a maximum at 40°C (9.8 IU/ml). It was recorded as the ideal temperature for L-asparaginase production. The results are similar to those reported that the optimum temperature for enzyme production at 40°C in *S. radiopugnans* MS and *S. gulbargensis* (Amena *et al.*, 2010).

The current investigation finds that the strain *S. krainskii* VMB-11 produces significant L-asparaginase enzyme amounts when developed in modified M9 broth with pH-8.5, 96h at 40°C. The reports are comparable with *S. fradiae* NEAE-82 produced maximum amounts of L-asparaginase at 40°C, pH - 8.5 (El-Naggar *et al.*, 2016).

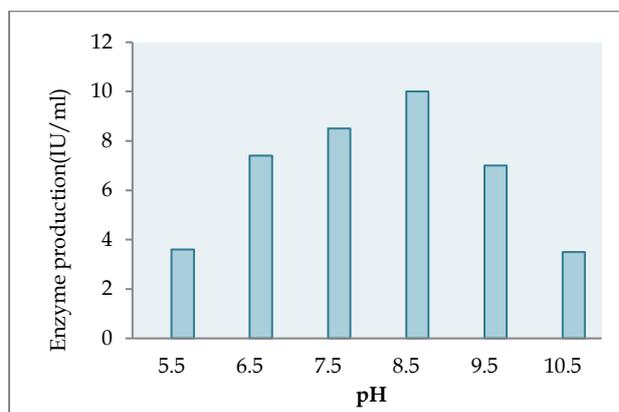


Figure 2: Effect of pH on L-asparaginase production by *S. krainskii* VMB-11

The present study discovered that the strain *S. krainskii* VMB-11 produced high L-asparaginase enzyme yields when grown in modified M9 broth with pH-8.5, 96h at 40°C. The results are similar to *S. fradiae* NEAE-82 produced maximum enzyme production at 40°C, pH -8.5 (El-Naggar *et al.*, 2016).

Effect of carbon and nitrogen sources on the L-asparaginase production by *Streptomyces krainskii* VMB-11

The modified M-9 broth was amending with various carbon sources added at a concentration of 1% (w/v) to decide their L-asparaginase production impact. Compared to other carbon

sources, medium supplemented with maltose increased L-asparaginase production (9.3IU/ml) at pH-8.5, 40°C. Results are in accordance with the observations of *S.fradiae* NEAE-82 supplemented with maltose produce high yields of L-asparaginase enzyme (El-Naggar *et al.*, 2016). Maltose proved the best carbon source for the production of L-asparaginase in *Staphylococcus* sp. (Varalakshmi, 2013). Different maltose (0.25% -2%) were tested to decide the ideal concentration for enzyme production; maximum production was observed in a medium with 1% maltose (Figures 4 & 5). Similar reports found in enzyme production by *Streptomyces gulbargensis* (Amena *et al.*,2010).

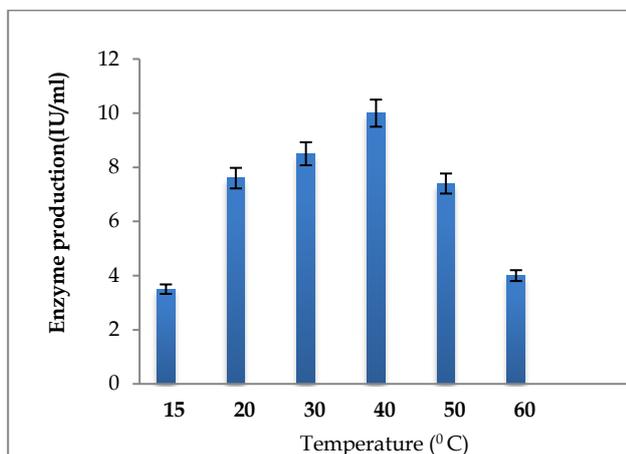


Figure 3: Effect of temperature on L-asparaginase production by *S.krainskii* VMB-11

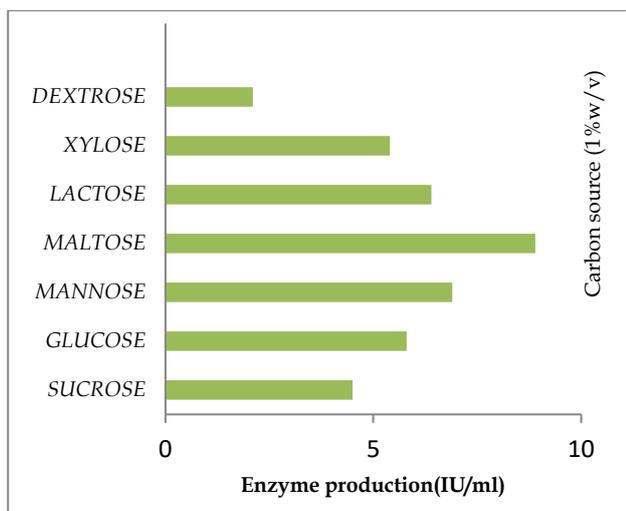


Figure 4: Effect of carbon sources on L-asparaginase production by *S. krainskii* VMB-11

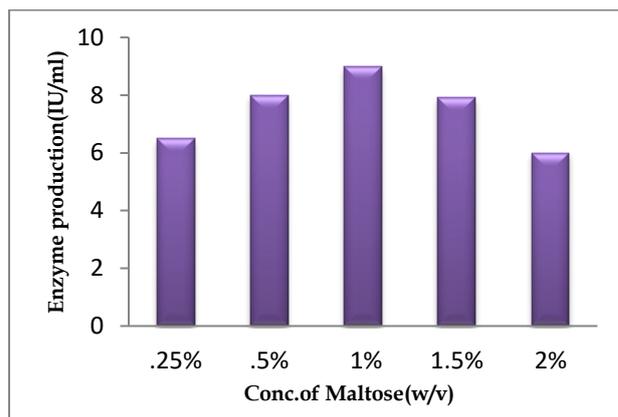


Figure 5: Effect of different concentrations of maltose on L-asparaginase production by *S. krainskii* VMB-11

The highest enzyme production was found when L-asparagine was utilized as the nitrogen source. Further, the influence of the optimal concentration of L-asparagine 1% that supporting high yields of enzyme production (11.3 IU/ml) was observed (Figures 6 & 7). L-asparagine (0.5%) served as an optimal nitrogen source in *Streptomyces gulbargensis* for enzyme production (Amena *et al.*, 2010). *Enterobacter cloacae* produce high rates of L-asparaginase production utilizing L-asparagine as a nitrogen source (Nawaz *et al.*, 1998). *P. endophytica* produced high rates of L-asparaginase with 1%L-asparagine as a nitrogen source (Mangamuri *et al.*,2016).

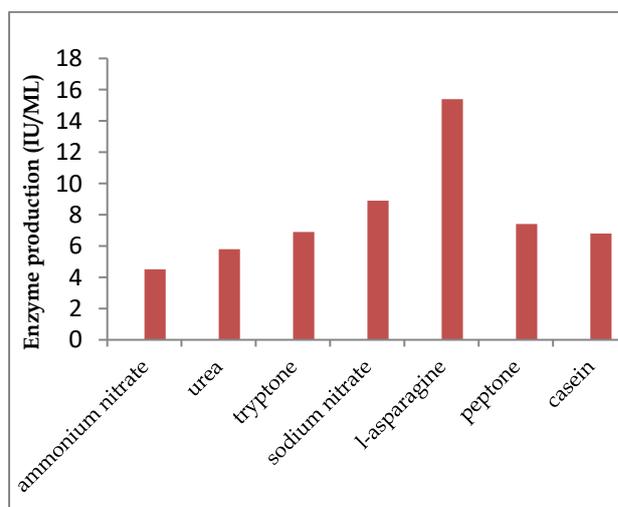


Figure 6: Effect of nitrogen sources on L-asparaginase production by *S.krainskii* VMB-11

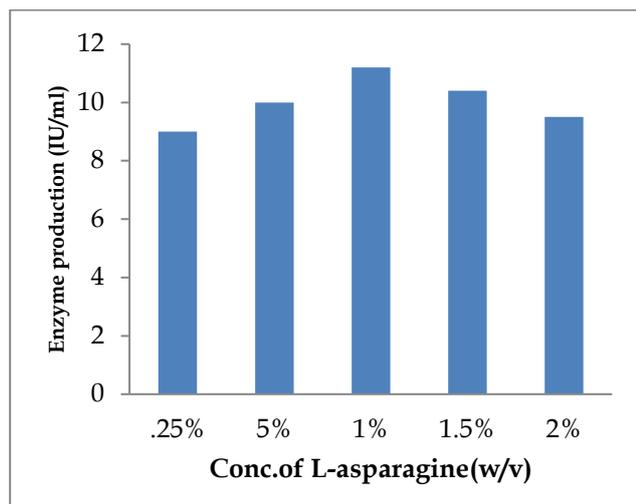


Figure 7. Effect of different concentrations of L-asparagine on L-asparaginase production by *S. krainskii* VMB-11

In the current study, improved enzyme production levels were noted from 8.2 IU to 13.2 IU/g dry wt. of biomass in the modified M-9 broth containing 1% maltose, 1% L-asparagine, NaCl-6%, MgSO₄·7H₂O-0.2%, CaCl₂·2H₂O-0.1%, pH 8.5 incubated at 40°C, 96hr by *Streptomyces krainskii* VMB-11. Previous studies on L-asparaginase enzyme production reported that *S. albioflavus* and *S. griseous* exhibited maximum enzyme production was 11.0 IU/ml and 5.361 IU/ml under optimized conditions (Salimath and Onkarappa, 2016). To the best of our knowledge, this is the first report of L-asparaginase enzyme production by *Streptomyces krainskii* VMB-11 from marine origin.

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