

Isolation and Screening of Marine Bacteria Producing Anti-Cancer Enzyme L-Asparaginase

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Abstract PURPOSE: The objective of this investigation was to isolation and screening of marine bacteria for L-asparaginase activity. **METHODS:** Marine bacteria were isolated from water samples obtained from Kerala sea coast in India. The isolates were identified as marine bacteria by microscopical and bio-chemical tests. Production of L-asparaginase was carried out in submerged fermentation media (glycerol asparagine media). **RESULTS:** Among three marine isolates subjected to screening only one isolate BKJM-VB showed potential L-asparaginase activity. **CONCLUSION:** The study revealed that marine bacteria may be a potential source of high yield, high substrate specificity L-asparaginase which is an anti-leukemic agent.

Keywords: marine bacteria, asparaginase, sub merged fermentation

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1. Introduction

Enzyme production by microorganisms has been used in various industries in the world. Microbial L-asparaginase has been widely used as a therapeutic agent in the treatment of certain human cancers, mainly in acute lymphoblastic leukaemia. The reason is preferred for the purpose it is bio-degradable, non-toxic can be easy administration at the local site.

Marine microbes represent a potential source for commercially important bio-active compounds. Among marine microorganisms bacteria have gained a special importance as the most potent source of anti-biotics and other bio-active secondary metabolites. While most of the studies on bacteria have focussed on antibiotic production [10]. Only few reports have dwelt on their enzymatic potential. L-Asparaginase production, purification, crystallization of enzyme and has also given the enzymatic properties from *Proteus vulgaris* [3].

Other bacteria includes *Pseudomonas aeruginosa*, [4] *E-coli*, *Citrobacter* spp., [12] and *Bacillus* spp [6] screened the bacteria for enzyme activity.

L-Asparaginase activity was widely reported in plants, animals and microorganisms (bacteria, fungi and actinomycetes) and also in serum of certain rodents but asparaginase was not isolated from human source [1].

In spite of above studies on asparaginase production by bacteria from marine sediments, no literature is available on enzyme production bacteria, isolated from marine water. Hence the present study focuses on isolation and screening of marine bacteria for anti-cancer L-asparaginase production from marine water.

2. Materials & Methods:

1. Sample collection:

Two marine water samples were collected from coastal area of kerala (Shangumugham Beach, veli Back water) at the depth of 10 cm. The samples were collected in sterile air tight bottles and labelled the date of collection and transported to the laboratory for further study.

2. Isolation of bacteria:

For isolation of L-Asparaginase producing bacterial species the samples were serially diluted and plated on starch casein agar, Glucose- asparagine agar and glycine glycerol agar medium and then incubated at 37°C for 7 days.

3. Morphological characterization:

The isolates were characterized morphologically by gram staining and Spore staining methods.

4. Bio-chemical identification of bacteria:

Three isolates were used for bio chemical studies. The various bio-chemical tests (IMVIC tests, starch hydrolysis, urease, citrate utilization test, sugar fermentation, H₂S production, oxidative fermentation & nitrate reduction) were performed for the identification of potent isolates. All the cultures were incubated at 28°C for 24-48hrs.

5. Screening for L-asparaginase positive cultures:

All the bacterial strains were screened for L-Asparaginase production. The isolated strains were inoculated in glycerol asparagine agar (glycerol-1.0%, L-Asparagine-0.1%, K₂HPO₄-0.1%, agar-1.5%) supplemented with 0.3ml of 2.5% phenol red dye at P^H-6.5 [7]. The formation of pink coloured zone around the colony shows the positive results for L-Asparaginase production.

6. Submerged fermentation:

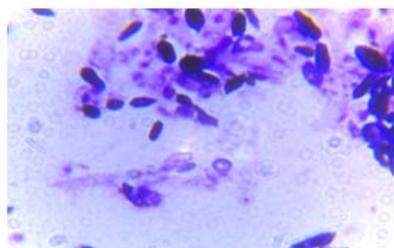
Submerged fermentation carried out for the active bacterial strains using 250ml capacity Erlenmeyer flasks, containing 100ml of glycerol asparagine medium. Each flask was inoculated with 1ml culture suspension (three days old). Inoculated flasks were incubated at 30°C for five days on a rotary shaking incubator at 250 rpm. Samples were taken periodically every day for determination of L-Asparaginase activity [9]. After incubation the fermented media was centrifuged at 10,000rpm or 20 min for crude enzyme preparation.

7. Determination of L-Asparaginase activity:

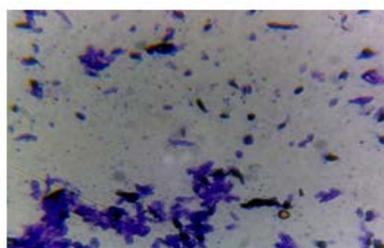
The L-Asparaginase activity was determined according to the method of [5]. A mixture of 0.1ml of enzyme extract, 0.2ml of 0.05M Tris-HCl buffer (pH 8.6), and 1.7ml of 0.01 M L-Asparaginase was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1.5M trichloro acetic acid. The ammonia released in the supernatant was determined spectrophotometrically at 480nm.

8. Molecular characterization of the potent asparaginase producing strain:

Out of three isolates the maximum asparaginase producing isolate were selected and processed for isolation of genomic DNA, 16S rDNA sequencing and phylogenetic analysis. Amplification of 16S rDNA by PCR was done using Universal bacterial primer. Sequencing of 16S rDNA of the isolate was done in Gene's n life health care Pvt.Ltd, Hyderabad.



100X Magnification



40X Magnification

Figure 2. Gram staining of BKJM-VB2

On spore staining the potent isolate shows no spores, so the isolate is non-sporing. (Figure 3).



Figure 3. Spore staining of BKJM-VB2

BIO CHEMICAL CHARACTERIZATION:

Biochemical tests of the isolate shown in the following Table 2.

3. Results

1. Isolation of Bacteria:

From the two marine water samples 10 isolates were isolated. Among 10 isolates one potent isolate selected from 10^{-5} dilution for asparaginase production given in Table 1 (Figure 1)

Table 1. Colony morphology of potent isolate.

Isolate	Colony Morphology
BKJM-VB2	Medium sized pale orange in colour, smooth surface.

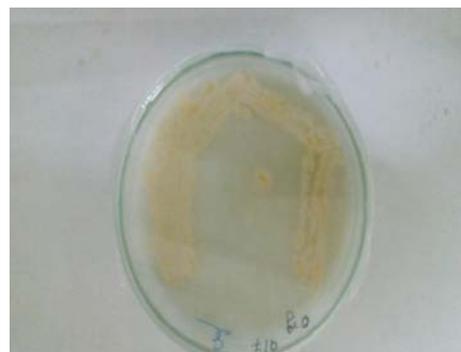


Figure 1. BKJM-VB2 colony morphology

2. MORPHOLOGICAL CHARACTERIZATION:

On gram staining the potent isolate shows gram positive rods with chains and singles (Figure 2)

Table 2. Bio-chemical tests of the L-Asparaginase producing BKJM-VB₂ isolate

Bio-chemical test	BKJM-VB
Indole test	Negative
Methyle red test	Negative
VP test	Positive
Citrate utilization test	Positive
Urease test	Positive
Sugar fermentation test	Positive
Starch hydrolysis	Negative
Nitrate reduction test	Negative
Oxidative fermentation	Negative
H ₂ S production test	Negative

Screening of L-asparaginase producing marine bacteria:

Out of 10 isolates, one strain showed the pink colour around the colony which indicates L-Asparaginase activity on the glycerol asparagine agar medium (Figure 4).

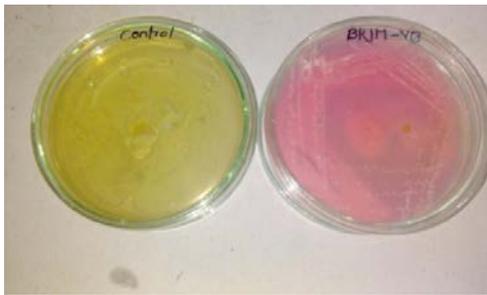


Figure 4. Screening for L-Asparaginase enzyme

Submerged fermentation and L-Asparaginase enzyme activity:

The enzyme activity was tested in positive isolate obtained from submerged fermentation and values taken spectrophotometrically at 480 nm and given in the following Table 3.

$$\text{Units / mg} = \frac{\text{Micromoles ammonia released}}{10 \text{ minutes} \times \text{Enzyme in reaction}}$$

Table 3. OD values and $\mu\text{mol ammonia/ml}$ (enzyme activity) show below.

Days	OD values at 480 nm	$\mu\text{mol ammonia/ml}$
1 st	0.1198	1.198
2 nd	0.2105	2.105
3 rd	0.1812	1.812
4 th	0.7087	7.087
5 th	1.1202	11.202

After five days of incubation the highest L-Asparaginase activity ($11.202\mu\text{mol ammonia/ml}$) was observed.

Molecular characterization of the potent asparaginase producing strain:

The NCBI BLAST Search programme (do not given from gen bank) showed that the sequence of BKJM-VB₂ had homologous similarity with *Lactobacillus salivarius* species on 16srRNA sequencing. (KT970506) is the accession number allotted by Genbank for the submitted nucleotide sequence.

4. Discussion

Marine bacterial strain was isolated and preliminarily characterized morphologically. The strain showed typical morphology of *Lactobacillus* when analyzing shape and spores under light microscope but *Lactobacillus* is non-spore forming bacteria. The nutritional, bio-chemical characterization and 16srRNA sequencing suggest that the strain be classified under *Lactobacillus* genus. The strain was further confirmed and identified as *Lactobacillus salivarius*.

Lactobacillus salivarius screened for L-Asparaginase production by plate method as well as by submerged fermentation. Similarly L-Asparaginase production is reported in different bacterial genus such as *Bacillus circulans* [8] and *Streptomyces* sp. PDK7 [2].

Marine isolates BKJM-VB₁, BKJM-VB₂ and BKJM-VB₃ from Kerala posses L-Asparaginase activity. Out of these three, BKJM-VB₂ shows more activity when compared to other organisms.

Production of L-Asparaginase by submerged fermentation yielded crude enzyme with total activity of $11.202\mu\text{mol ammonia/ml}$. Crude L-Asparaginase production from marine

Streptomyces sp.PDK7 with total activity of 374.6 IU, total protein of 489.5 mg has been reported by Dhevagi and Poorani. [2]. A comparison of submerged and solid-state fermentation shows a significant difference in enzyme activity. This suggests that there may be increased accumulation of intermediate metabolites between substrate and product formation in submerged fermentation. This is also probably due to the difference in the physiological state of the microorganisms in solid-state and submerged fermentation.

5. Conclusion

Based on the results obtained in the present study we concluded that the L-Asparaginase activity is highest ($11.202\mu\text{mol ammonia/ml}$) after 120 h incubation. Finally we concluded that the potent isolate (BKJM-VB₂) belongs to genus *Lactobacillus*, species *salivarius* and the accession number (KT970506) allotted by Genbank.

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