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Isolation and identification of marine *Bacillus altitudinis* KB1 from coastal Kerala: asparaginase producer

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Original Article

Abstract

L-asparaginase is a target for many researchers as its properties against cancer, especially leukaemia, and protective agents reduce acrylamide in fried food. In this study, the water samples from Thumba Arattuvazhi Beach in Kerala were screened for l-asparaginase producing microorganisms. This was followed by colourimetric screening using modified M9 media with 0.009% Phenol red dye and using l-asparagine as a sole nitrogen source. Then, the Nessler assay was performed to quantify the enzyme. Molecular identification was made by 16SrRNA sequencing and aligned the sequence with GeneBank for phylogenetic tree construction using BLAST. Seawater was serially diluted for 10^{-1} to 10^{-6} using nutrient agar plates. A total of 19 bacterial colonies were isolated. The colonies were evaluated to produce l-asparaginase according to the pink zone around the colonies on the modified M9 medium using a red phenol indicator. The KB1 sample was selected for further studies according to plate colour assay. Nessler assay of L- asparaginase quantified as 2.537 IU/ml. Molecular characterisation showed the sequence association with *Bacillus altitudinis* the sequence submitted in Genebank as *B. altitudinis KB1* strain. The l-asparaginase II gene (AnsB) was amplified based on the entire length of the hypothetical protein of annotated genome with accession number CP022319.2. The l-asparaginase activity in this study was 57% higher than the reference organism *B. altitudinis* BITHSP010. The l-asparaginase producing bacterium *B. altitudinis* KB1 from a marine source in Kerala can produce asparaginase, which can be utilised for biotechnology applications.

Keywords*: L-asparaginase, bacillus altitudinis KB1, Kerala, marine bacteria, acrylamide*

Introduction

All living things can produce enzymes and commercial enzymes are mainly derived from animals, plants and microorganisms. Among these sources, microorganisms are preferred over animals and plant sources, for industrialised enzyme production as it is cost-effective, has manageable enzyme content, and raw material availability. Microbial diversity in the natural environment can selectively isolate a specific enzyme and optimise production by controlling fermentation conditions (Kuddus, 2019). Microbial catalysts originate from microbial sources used in industrial applications (Niyonzima *et al.*, 2020). Different biological sources include bacteria, fungi, algae, and plants, sources for asparaginase enzymes (Muneer *et al.*, 2020). Asparaginase (asparagine

aminohydrolase; E.C.3.5.1.1) is amino acid amidohydrolase that catalyses the conversion of l-asparagine to l-aspartate and ammonia. This enzyme is essential for the metabolic reactions in all living organisms (Michalska and Jaskolski, 2006). Experiments conclude that l-asparaginase originated from guinea pig serum and *Escherichia coli* is responsible for the anticancer effects (BROOME, 1963). Broom findings prove the tumour inhibitory activity of asparaginase from *E. coli* source (Mashburn and Wriston, 1963). Asparaginase is a chemotherapy agent that is routinely used to treat acute lymphoblastic leukaemia (ALL); widely used for the cure of juveniles and adults (Pieters *et al.*, 2011). For more than three decades, l-asparaginase is routinely used as anti-leukaemia in cancer treatment and the food industry. L-asparaginase improves food quality and decreases acrylamide formation (the carcinogenic agent) (Cachumba *et al.*, 2016). It inhibits the synthesis of protein in tumour cells by depriving them of asparagine. It targets a G1 phase of the cell cycle (Kwok *et al.*, 2017). As a therapy for acute lymphoblastic leukaemia, asparaginase hydrolyses asparagine from blood serum, causing cancer cells apoptosis and depleting P53-dependent apoptosis (Protein synthetase). Normal cells do not affect as they use l-asparagine synthetase (Cachumba *et al.*, 2016; Egler *et al.*, 2016). Asparaginase minimises the level of the Millard reaction formed between asparagine and the reduced sugar in the cooked food. Asparaginase slashes down the formation of carcinogenic acrylamide and enhances food flavour (Xu *et al.*, 2016). The toxicity side effects include hypersensitivity, pancreatitis, thrombosis, encephalopathy, and liver issues in one-third of patients treated with *E. coli* derived asparaginase. (Hijiya *et al.*, 2016). The toxicity of l-asparaginase has been linked to glutamine depletion caused by glutaminase activity. The novel isolated organisms with free glutaminase activity have been recommended (Beckett and Gervais 2019). In terms of physicochemical qualities, such as thermophilic, barophilic, halophilic, pH tolerance, and marine l-asparaginases have advantage over terrestrial organisms. They are thermotolerant, stable at room temperature and adaptable to harsh environments, making them suitable for medicinal and food applications (Izadpanah *et al.*, 2018). The need for new organisms to produce l-asparaginase from marine sources promise a better yield and cost-effective production process. In the present study, seawater from the southern coastal area of Kerala was screened for l-asparaginase producing bacteria. The KB1 sample was screened and identified as *Bacillus altitudinis* KB1 *strain* for the production of l-asparaginase.

Material and methods

Primary screening

The isolation of the sample was done from seawater (20 cm deep) collected from Arattuvazhy Golden Beach Thumba 8°33'16.0"N 76°50'57.9"E Pallithura, Thiruvananthapuram, Kerala, India, in the monsoon season after two weeks of rain in August 2018. Water samples were taken in a 50 ml centrifuge tube and stored in refrigerator at 4°C till use. Water samples were serially diluted to different concentrations from 10^{-1} to10^{-6} and 100 ul from each concentration inoculated to the autoclaved nutrient agar plates. The plates were incubated for 96 hours at 28±2°C.

Secondary screening

The grown colonies from the primary screening were again screened using M9 medium (pH 7·0) (gram/litre): $Na_2HPO_4.2H_2O$, 6·0 g; KH_2PO_4, 3·0 g; NaCl, 0·5 g; l-asparagine, 5·0 g; 1M $MgSO_4.7H_2O$, 2·0 ml; 0·1M $CaCl_2.2H_2O$, 1·0 ml; 20% glucose stock, 10.0 ml; agar 20·0 g. The media was supplemented with 0.009% Phenol red; the dyes were prepared as 2.5% stock in ethanol (Gulati *et al.*, 1997). Control plates of M9 modified media were: (a) without dye and (b) without asparagine ($NaNO_3$ instead of l-asparagine). Plates were incubated for 72 hours at 28±2°C. The colour change of the indicator to red indicated presence of l-asparaginase.

Nessler assay

The Nessler assay uses 50 ml of modified M9 broth incubated at 28°C for 72 hours with agitation at 120 rpm for enzyme quantification. L-asparaginase was estimated by the nesslerisation method as described (Shifrin *et al.*, 1974). After the incubation period, the bacteria-free broth was used as a crude enzyme and stored at 4°C for the enzyme assay. The reagents used for this assay were 50mM Tris pH 8.5 buffer, 189 mM L-asparagine (molecular weight: 132.12), six mM Ammonium sulfate for standard curve, 1.5M Trichloroacetic acid (TCA), Nessler's reagent (sigma) and deionised water. In a 2 ml tube, the mixture of the 0.8 ml tris buffer, 0.1 ml asparagine, and 0.9 ml deionised water for test and blank were equilibrated at 37°C for 10 minutes. Then, 0.1 ml of the enzyme was added to the test, mixed by inversion, and immediately incubated at 37°C for 30 minutes. After incubation, 0.1 ml of TCA was added to stop the reaction. Next, it was mixed by inversion and centrifuged for 2 minutes for clarity. Finally, 4.3 ml of deionised water, 0.2 ml of the supernatant and 0.5 ml of Nessler's reagent were incubated for two to three minutes. Then, the optical density spectrophotometrically recorded at wavelength 436 nm. The optical density was measured in triplicate using a Thermofisher Varioskan lux multimode microplate reader. The standard curve was developed using the same protocol-one international unit (IU) in the assay equivalent to two ammonia units. The calculation equation is: Units/ml enzyme= [(μmol of NH3 liberated) *(2)] / [(0.2) *(30)*(0.1)] : 2 = the volume for the initial step; 0.1 = volume of enzyme solution used in the assay in the first step ; 30 = incubation time in minutes; 0.2= the amount of the supernatant used in the second step with Nessler's reagent.

Genomic DNA isolation

Genomic DNA was isolated using these modifications to the CTAB 2% protocol adapted from (Kalendar *et al.*, 2021). CTAB solution for 100 ml: (2 g CTAB, 2.4 g MOPS, 2 ml 0.5 M Na3EDTA, 30 ml 5 M NaCl). 50 ml nutrient broth in a 250 ml flask was incubated overnight at 28 ±2°C. Following the procedure (1) 2 ml of bacteria culture broth directly was taken to 2 ml Eppendorf microcentrifuge tube. Then, added 600 microliters of CTAB 2% solution were to the tube, followed by aggressive vortexing at 2500 rpm for 3 minutes. (2) Incubated the samples at 80°C for half an hour. (3) Spun at 11000 rpm in a microcentrifuge for 2 minutes, transferred the supernatant to a new 2 ml tube containing an equal volume of chloroform- Isoamyl alcohol (24:1). (4) Mixed by vortexing for 1 minute at 2500-3000 rpm speed. (5) Spun at 11000 rpm in the centrifuge for 2 minutes, transferred the upper layer to a new 2 ml tube, which contained 600 µl propanol (Cold/-20°C), vortex well and incubated tube for a minimum of 15 minutes in -20°C or overnight for better yield. (6) Centrifuged the tubes at 11000 rpm in a microcentrifuge for 10 minutes at 4°C. (7) The supernatant was discarded and the pellet was washed by adding one volume of 70% ethanol (Cold/-20°C), vortexed very well. Then, centrifuged at 11000 rpm for 2 minutes and discarded ethanol. (8) Incubated DNA at 45°C for 5 minutes to dry and dissolved immediately in 100 µl 1xTE, pH 8.0 or (filtered autoclaved distilled water) at 55-60°C for 5-10 minutes in the dry bath. The µDrop plate was used to quantify the genomic DNA using Varioskan LUX Multimode Microplate Reader (ThermoScientific) and a SkanIt RE 6.1 software for curve standard analysis.

16srRNA gene-based identification

The 16srRNA gene-based identification was carried out using universal PCR primers 27F 5′ (AGAGTTTGATCMTGGCTCAG) 3′ and 1492R 5′ (TACGGYTACCTTGTTACGACTT) 3′. The polymerase chain reaction (PCR) done using 25 ml contain 1 ul forward primer, 1ul reverse primer, 21ul PCR Supermix Invitrogen and 2 ul DNA template (50 ng). The thermal cycler setup for 35 cycles; initial denaturation 94°C for 2 minutes, denaturation 94°C for 15 seconds, annealing 55°C for 30 seconds, extension 72°C for 1 minute. The electrophoresis gel runs for verification at 1% agarose. The sequencing performed by Macrogen Inc. company, Seoul, South Korea. Phylogenetic trees were made using ClustalW2 Phylogeny against the assembled full 16SrRNA sequence length, based on BLASTN 2.6.0+, with default parameters, distance model, and the Neighbor-Joining algorithm (Zhang *et al.*, 2000).

PCR amplification of l-asparaginase II gene (AnsB)

The primer designed based on the available NCBI annotated *B. altitudinis* strain SGAir0031 chromosome, complete genome, accession number (CP022319.2) using the sequences (570223-571371) as 1149 bp product (Vettath *et al.*, 2017). Primer3 online web application is used for primers design (Kõressaar *et al.*, 2018). (Forward primer; ASNF ('5 ATGAACGTAACAAAATGGTTTGG 3') and reverse prime; ASNR ('5 TTAATACTGCTCGTAATAGGATTG 3')). Taq 2X Master Mix (M0270) (New England Biolabs, Inc.) was used for PCR reaction with melting temperature 55°C. The PCR product was visualised through 1% agarose gel and a 1KB ladder. The PCR procedure followed the master mix protocol.

Results

Screening and Nessler assay

Seawater was serially diluted for 10^{-1} to 10^{-6} using nutrient agar plates. A total of 19 bacterial colonies were isolated. The colonies evaluated to produce l-asparaginase according to the pink zone around the colonies on the modified M9 medium using phenol red indicator incubated at 28±2 °C. The pink colour change of media refers to the production of ammonia due to the hydrolysis of asparagine. The indicator concentration 0.009% according to Gulati (1997), is used (0.36 ml of 2.5% of stock solution).The KB1 sample was selected for further studies according to plate colour assay (Fig. 1).

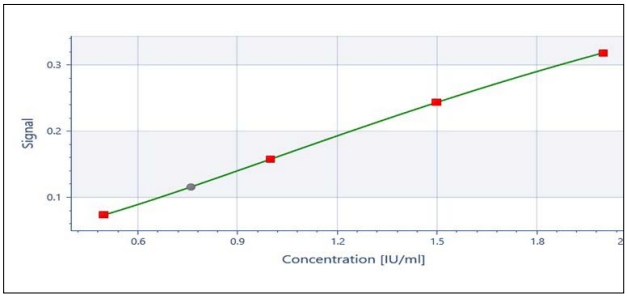

Fig. 1. Nessler assay represents the standard curve with the reading sample at the wavelength 436 nm. The grey colour represents the average of the four replicates

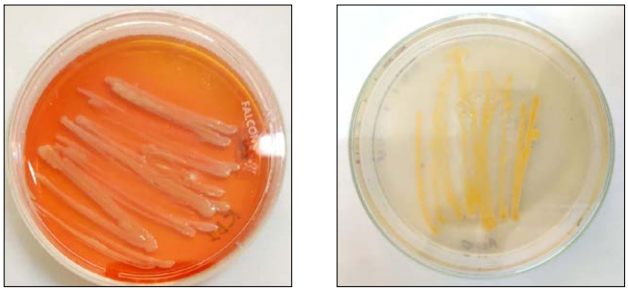

Fig. 2. Primary screening indicates the capability of *B. altitudinis* KB1 to produce l-asparaginase enzyme using 0.009% phenol red dye. The red colour plate indicates the production of l-asparaginase. The colourless plate refers to the control plate

The Nessler assay of l-asparaginase is 2.537 IU/ml after an incubation period of 72 hours using the Shirfin (1974) method at a wavelength of 436 nm; ammonium sulfate was used as the standard for ammonia estimation. The wavelength reading against the concentration of liberated ammonia depicted in Fig. 1.

16srRNA gene-based identification

The molecular identification was performed using the Universal PCR primers 27F and 1492R for 16srRNA bacterial gene restriction. The purified DNA that is used as a template for PCR products is confirmed using gel electrophoresis. The 16SrRNA gene is amplified by PCR and visualised using gel electrophoresis (Fig. 3). The PCR product band aligned with the DNA marker has an estimated 1.5 Kilobase pair of 16SrRNA sequence. The Genbank tools identify the nearest related organisms. The result shows the nearest organisms related to bacillus species with 100 percentage, and a high score shows the affiliation to *B. altitudinis* strain *41KF2bT* (MTCC 7306T &JCM 13350T). According to the phylogenetic tree, the sequence aligned with ClustalW2 Phylogeny, based on BLASTN 2.6.0+, identifies the bacterium as *B. altitudinis* according to the phylogenetic tree (Fig. 4). The 16SrRNA gene is deposited in Genbank with accession number MN897890 as *B. altitudinis* and strained KB1. Complete sequence data can be retrieved online by https://www.ebi.ac.uk/ena/browser/view/MN897890.

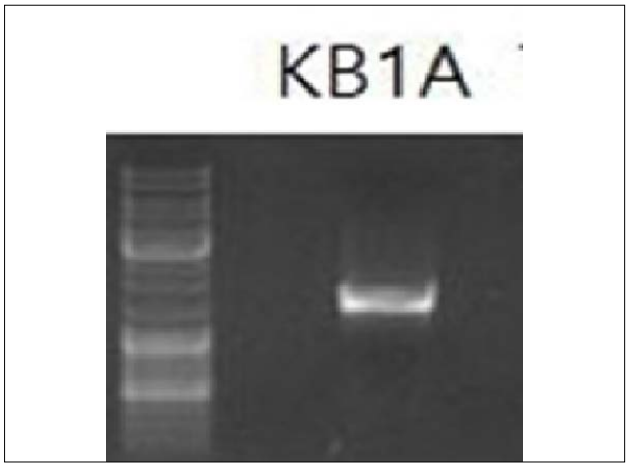

Fig. 3. The 16SrRNA band of KB1 (1.5KB) in 1% gel electrophoresis and 1KB plus ladder

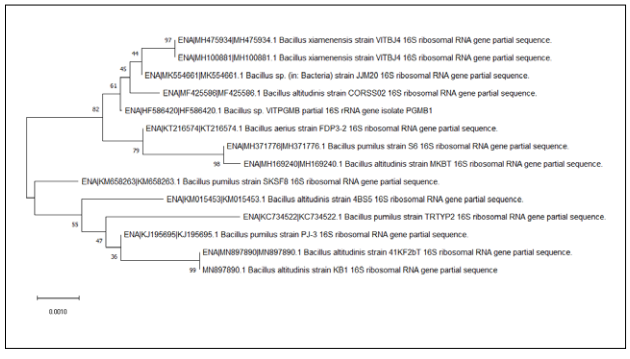

Fig. 4. The alignment of the phylogenetic tree based on the 16S rRNA gene indicates the association with the *B. altitudinis* strain 41KF2bT (=MTCC 7306T =JCM 13350T) close organisms in Genebank. Phylogenetic trees made using ClustalW2 Phylogeny, based on BLASTN 2.6.0+, with default parameters, distance model and the Neighbor-Joining algorithm

PCR amplification of l-asparaginase II gene (AnsB)

The l-asparaginase II gene (AnsB) was amplified using *B. altitudinis* KB1 and the designed primers. The amplified band is visualised using the 1% agarose gel (Fig. 5).

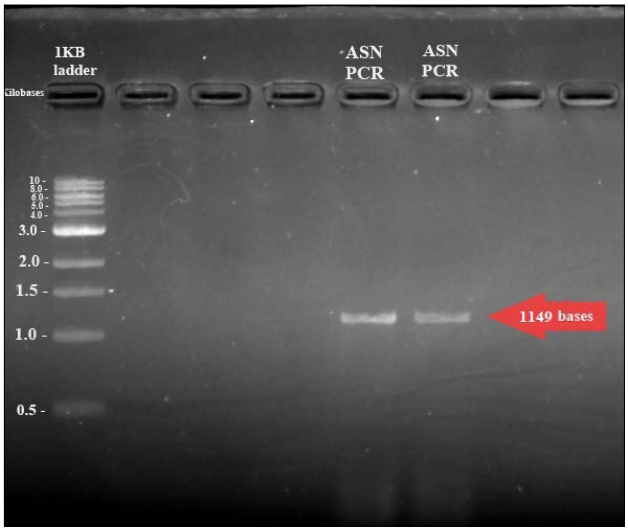

Fig. 5. The amplified l-asparaginase II gene (AnsB) bands (1149 Bases) in 1% gel electrophoresis and 1KB ladder.

Discussion

Asparaginase enzyme has industrial application to protect and treat cancer diseases. It minimises the formation of carcinogenic acrylamide substances and optimises the flavour of food. Also, it is used as a medicine for the treatment of Acute Lymphoblastic Leukemia (ALL). L-asparagine is an essential amino acid for the growth and metabolism of tumour cells. The asparaginase inhibits the synthesis of protein in tumour cells by starving them of asparagine (Xu *et al.*, 2016). The previous literature indicates seawater as a source for this enzyme (Kuddus, 2019). The eastern Arabian Sea of the Malabar region of Kerala holds microbial diversity with industrial application (Sachithanandam *et al.*, 2020).

The marine bacterium was isolated from Kerala's coastal region, and the screening of bacteria based on the modified M9 medium optimised (Gulati *et al.*, 1997). The detection depends on the hydrolysis activity of asparagine to aspartate by l-asparaginase. The shift from acid to base, from yellow to pink colour on rapid plate assay methods, confirms the l-asparaginase enzyme's presence.

According to Shirfrin *et al.* (1974), l-asparaginase estimation is based on Nessler's reaction. It calculates the liberated ammonia produced due to asparagine hydrolysis by the enzyme. In this study, *B. altitudinis* KBI was observed to have a high activity of l-asparaginase compared to the other strains. These findings are strongly correlated to the first report of l-asparaginase obtained from *B. altitudinis* BITHSP010 in 2019 by Prakash *et al.* (2019). While Prakash was using modified M9 media broth for batch fermentation and incubated culture at 37°C, this study did the incubation at 28°C. According to literature, the l-asparaginase activity of this study is 57% higher than the reference organism *B. altitudinis* BITHSP010. The l-asparaginase batch fermentation using M9 media is 2.537 IU/ml compared with 1.45 IU/ml (Prakash *et al.*, 2020) due to strain differences.

The molecular phylogenetic analysis of the bacterial 16S rRNA gene confirms the species as *B. altitudinis*, with deposited strain as *B. altitudinis* KB1. This species belongs to the phylum Firmicutes, family Bacillaceae and genus *Bacillus*. Bacillus is indicated as one of the most dominant species in the marine habitat (Mustafa *et al.*, 2014; Alrumman *et al.*, 2019). The high resistance to the harsh marine environment refers to spores generation (Izadpanah *et al.*, 2018). The most common groups in the eastern Arabian Sea are Firmicutes and Planctomycetes (Sachithanandam *et al.*, 2020). Marine *Bacillus* species reports for the production of both asparaginases I and II (Chakravarty *et al.*, 2021).

The l-asparaginase II gene (AnsB) is amplified using designed primers based on the annotated whole-genome sequence of different *B. altitudinis* in NCBI depository (Ibrahim *et al.*, 2018; Vettath *et al.*, 2017). Previous studies of different strains for the same genus indicate them to be thermophilic Verma *et al.* (2015). Further studies are needed for the improvement of industrial application of l-asparaginase as a thermophilic recombinant protein.

Conclusion

L-asparaginase enzyme has industrial applications in food and medicine. In this study, the l-asparaginase producing bacterium is screened and identified as *B. altitudinis* KB1 from a marine source in coastal areas of Kerala, India. The enzyme assays have shown a significant production level of l-asparaginase with 2.537 IU/ml. The l-asparaginase II (AnsB) amplified PCR product is confirmed using electrophoresis gel. Further studies are required for the AnsB gene to be utilised as a source of l-asparaginase II as a recombinant protein for industrial application.

Conflict of interest

The authors declare no conflict of interest.

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