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Research Article

ISOLATION AND SCREENING OF L-ASPARAGINASE PRODUCING SPHINGOMONAS SP. FROM SOIL SAMPLE

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ABSTRACT

Objectives: In recent years, L-asparaginase has gained attention due to its anticancer activity. Different types of asparaginase possess different characteristics and different applications. The study is designed to screen potential L-asparaginase producing bacteria from soil samples.

Methods: Soil samples were collected from different locations in Tamil Nadu and Kerala for the isolation of L-Asparaginase-producing bacteria.

Results: About 689 isolates were obtained from 114 samples. Among these, 214 isolates possess the ability to synthesize L-asparaginase. Fifteen isolates were selected for quantitative screening based on the intensity of the pink color formation M9 basal medium.

Conclusion: The isolate VN01 exhibited a higher degree of enzyme activity (156 IU/mg). The isolate was further categorized by phenotypic and genotypic method and the isolate was identified as *Sphingomonas leidyi*. As a future aspect, the study will be continued for optimization of increased production of L-asparaginase and in characterizing the potential of L-asparaginase synthesized by *S. leidyi*.

Keywords: *Sphingomonas leidyi*, L-asparaginase, Anticancer, Acute lymphoblastic leukemia.

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INTRODUCTION

Enzymes are proteins that catalyze the biochemical reactions. There are many enzymes which contribute to the improvement of different functions in this world. The enzyme of interest for our studies was L-asparaginase enzyme foreseeing its importance in the treatment of acute lymphoblastic leukemia (ALL) cancer. It is also combined with highly intensive chemotherapeutic agents for the treatment of the pancreatic cancer, myelomonocytic leukemia, reticulum sarcoma, melanoma sarcoma, non-Hodgkin's lymphoma, and bovine lymphoma sarcoma [1,2]. Its also used in food industry, asparaginase is used as a food processing that aids to reduce the formation of acrylamide [3].

L-asparaginase (EC 3.5.1.1) is an amidohydrolase enzyme that catalyzes the hydrolysis of the amide bond in the L-asparagine amino acid to form L-aspartic acid and ammonia. The threonine residues in the active site play a critical role in the catalytic reaction mediated by the L-asparaginase enzyme. In the characteristic L-asparaginase reaction mechanism, these threonine residues can act as a primary nucleophile by attacking the C atom of the amide substrate, resulting in the formation of the product L-aspartate and liberation of ammonia [4]. These active site threonine residues are found to be highly conserved among the L-asparaginase family [5,6]. The important function of the enzyme is to catalyze the conversion of L-asparagine to L-aspartic acid and ammonia, which deprives the growth of certain types of cancer cell and subsequently reduces the tumors [7].

In April 2002, Swedish researchers stunned the food safety community by revealing preliminary findings of acrylamide in certain fried and baked foods. Acrylamide forms during the Maillard reaction, a process in which the amino acid asparagine, naturally found in starchy foods, transforms when exposed to high temperatures. This reaction is responsible for the characteristic brown color, crust, and toasted flavor of baked or fried foods. However, by adding the enzyme asparaginase before cooking, asparagine is broken down into aspartic acid and ammonium, preventing its participation in the Maillard reaction. As a result, acrylamide formation is significantly reduced [8,9].

For instance, some food goods made with cereal or potatoes and heated through include significant levels of acrylamide. Pretreatment methods such as timing and temperature adjustments, the use of potatoes with low asparagine content, adjusting the pH, blanching, and submerging in water to reduce the amount of reducing sugars and amino acid content are some of the pretreatment recipes and strategies that have been suggested to reduce the formation of acrylamide. It has been demonstrated that prolonged blanching and immersing are beneficial in lowering the production of acrylamide. Nevertheless, these processes might inhibit the production of desired Maillard products in addition to acrylamide, which would be detrimental to the product's flavor and appearance [10]. The use of L-asparaginase is more effective in large scale production.

The enzyme is present in many genera, including bacteria, fungi, plants, and animals. Huge diversity and versatility of microorganisms make them primary candidates for the screening of the novel and efficient enzyme [11,12]. Both submerged and solid-state cultures are used to manufacture the enzyme all over the world. Extracellular asparaginases are preferable to intracellular ones because they may be inexpensively purified and generated in large quantities in the culture broth under typical circumstances [13]. The mass production of L-asparaginase, various factors play a very crucial role, such as carbon source, nitrogen source, growth factors, micronutrients, and macronutrients. Range of the carbon sources such as starch, sucrose, maltose, lactose, glucose, galactose, mannose, and fructose affects L-asparaginase production significantly. Similarly, range of nitrogen sources such as urea, peptone, casein, asparagine, glutamine, and proline plays an important role in the production of L-asparaginase. Similarly, process parameters such as pH, temperature, gaseous conditions, fermentation time, and inoculum percentage also play vital roles for efficient enzyme production. Therefore, screening of

significant nutritional components and process parameters is very much important for the enzyme production [14,15].

There is significant potential for isolating highly efficient or novel L-asparaginase enzymes from bacteria and other microorganisms. Since each organism has different needs to produce the most L-asparaginase, it is necessary to develop appropriate medium components and environmental circumstances. Because the culture conditions that encourage the creation of enzymes vary widely depending on the nature of the microbe, it is important to optimize the various environmental conditions and medium ingredients needed for microbial growth and production of enzymes [16,17]. This study aims to isolation and screening of L-asparaginase-producing *Sphingomonas* species from soil sample.

METHODS

Isolation of micro-organism

Soil samples were collected from different locations in and around Nilgiris, Coimbatore, Tamil Nadu and Palakkad, Malappuram, Wayanad, and Kannur, Kerala for the isolation of L-Asparaginase-producing microorganisms. The soil samples were collected in sterile bags by digging 15 cm depth and were processed in serial dilution within 24 h of collection. 1 g of soil sample was taken and dispensed in 99 mL of sterile distilled water, this is mixed vigorously which makes up dilution 10^2. From the master dilution, 1 mL was taken and added to another tube with 9 mL of sterile distilled water to get dilution of 10^3. This is serially diluted to get 10^4, 10^5, 10^6, 10^7, and 10^8 for the isolation of microorganisms, 0.1 mL of each dilution was plated onto nutrient agar medium by spread plate method for the preparation of culture, the pH of the media was adjusted with 1N hydrogen chloride (HCL) or 1N sodium hydroxide solution. Then, the media was autoclaved for sterilization. The sterilized agar was cooled and poured into sterile petriplate and aseptic condition and let it for solidification. After solidify a loopful of culture was inoculated into agar plates by streaking method and incubated at 37°C for 24 h and stored.

Screening of L-Asparaginase enzyme producing bacteria

Using modified M9 agar medium, the bacterial strains were tested for L-Asparaginase activity. The M9 media contains Disodium hydrogen phosphate - 6.0 g, Potassium dihydrogen phosphate - 3.0 g, Sodium chloride - 0.5 g, Glucose solution - 10.0 mL, Magnesium sulfate solution - 1.0 mL, Calcium chloride solution - 1.0 mL, Phenol red - 0.3 mL, and Agar - 15.0g, pH - 7.0. The bacterial strains were inoculated and incubated for 48 h at 37°C. The intensity of pink color developed on the streaked plates after incubation allowed for the identification of L-Asparaginase activity [18].

Screening of L-Asparaginase by submerged fermentation

The bacterial strains that showed pink zone around the colonies on modified M9 media then used for submerged fermentation process that produced L-Asparaginase. Bacterial suspension was added to 100 mL M9 broth and incubated in rotatory shaker for 24 h at 37°C. M9 broth contains monopotassium phosphate - 2 g, L-Asparagine - 6 g, Magnesium sulfate heptahydrate - 1 g, Calcium dichloride dehydrate - 1 g, and Glucose - 1 g, Ph - 7. At the end of fermentation period, the crude enzyme was prepared by centrifugation at 8,000 rpm for 30 min. The cell-free supernatant was taken as the crude enzyme and enzyme assay was performed [19].

Estimation of L-Asparaginase activity by Nessler's method

L-asparaginase activity was measured by the following method. The cultures in the modified M9 medium supplied with L-asparagine 1.5% broth were centrifuged at 8000 rpm for 30 min and the resultant supernatant, the crude extract, was used to determine the L-asparaginase activity. Reaction was started by adding 1 mL of crude cell-free extract into 0.5 mL of 0.04 M l-asparagine solution and 0.5 mL of 0.05 M Tris-HCL buffer pH 8 and incubated at 37°C for 30 min in a water bath. The reaction was stopped by the addition of 0.5 mL of 1.5 M trichloroacetic acid. The precipitated proteins were removed by centrifugation at 8000 rpm for 15 min at 4°C, and the ammonia was determined spectrophotometrically at 450 nm by nesslerization (by Nessler's method). Tubes kept at zero-time incubation served as control. Enzyme activity was determined based on the liberation of ammonia calculated with reference to a standard curve of ammonium. One unit of L-asparaginase is the amount of enzyme which catalyzed the formation of 1 μmoL of ammonia per minute at 37°C [20].

Identification of L-Asparaginase synthesizing isolate

The potent isolate was characterized based on Bergey's manual of classification. Primarily, the isolate was categorized based on the colony morphology and characterization. The shape, size, margin, and surface characters of the isolate were noted. Gram staining was performed to differentiate the Gram-positive and negative microbe. Then, the isolate was subjected to biochemical characterization. Further, polymerase chain reaction (PCR) based screening of the 16S Ribosomal RNA gene for genotypic identification of the isolate. Genomic DNA was isolated using alkaline lysis method and quantification of DNA was performed [21,22]. Agarose gel electrophoresis was performed to determine the quality of extracted DNA. 16S ribosomal RNA (rRNA) gene amplified with primers 8 F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 R 5'-GGTTACCTTGTTACGACTT-3' [23,24]. The 35 cycle PCR amplification was performed with 20 μl reaction volume with annealing at 56°C for 45 s. Amplified product of 16S rRNA was visualized using an agarose gel using 1% agarose and 1× Tris-acetate-EDTA buffer at 100 V. The amplified product was purified and subjected to sequencing using an automated DNA sequencer (ABI 3730xl sequencer). The sequenced product was analyzed using Basic Local Alignment Search Tool (BLAST). The BLASTN search was performed to match the obtained sequence with the available database of the National Center for Biotechnology Information (NCBI) and constructed phylogenetic tree using neighbor-joining approach. Molecular Evolutionary Genetics Analysis version 4 program was utilized for sequence alignment program and phylogenetic analysis [25,26].

RESULTS

Isolation of bacteria from soil samples

A total of 114 soil samples were collected from different locations in and around Nilgiris, Coimbatore, Tamil Nadu and Palakkad, Malappuram, Wayanad, and Kannur, Kerala irrespective of seasons for the isolation of potent L-Asparaginase. All the soil samples were serially diluted and plated on the Nutrient agar plate and incubated at 37°C for 24 h. After incubation, individual bacterial colonies from each samples were selected and streaked on to agar slants for further studies. About 689 bacterial isolates obtained were subjected to screening of L-Asparaginase production using M9 basal medium.

Qualitative screening of L-Asparaginase production

All the selected isolates were screened for L-Asparaginase production. The isolates were subcultured onto the M9 basal medium and incubated at 37°C for 24 h. The production of pink color around the colony indicates the ability of the isolate to produce L-Asparaginase. The color variation determines the intensity of L-Asparaginase production. Among 689 isolates, 214 isolates were found to L-Asparaginase producers as they exhibit pink coloration around the colony. Among which 15 isolates were selected based on the intensity of pink coloration. These isolates exhibiting positive results with plate assay method were then subjected to quantitative screening for enzyme production.

Estimation of L-Asparaginase activity

The chosen isolates were tested for L-Asparaginase activity in submerged fermentation using M9 broth and incubated in orbital shaker for 24 h at 37°C. The bacterial cell mass was centrifuged, and the supernatant was used as the crude enzyme source. Then asparaginase activities were measured by nesslerization, where the asparaginase hydrolysis is determined by measuring released ammonia. The yellow color with brown precipitate represents the ammonia. According to Mashburn and Wriston's description, asparaginase activities were regularly measured

by direct nesslerization, where the rate of asparagine hydrolysis is determined by measuring released ammonia, which indicates yellow with brown precipitate. Among the selected 15 isolates VNO1 exhibits higher enzyme activity (156 IU/mg) of L Asparaginase enzyme and was selected for further studies (Table 1).

Identification of potent isolate

The potent isolate was selected and subjected to phenotypic and genotypic identification. Based on cultural characteristics, the colonies were creamy, non-pigmented circular, convex, smooth, and translucent. In Gram staining, the isolate was found to be Gram-negative rods. Biochemical characteristics determine that the isolate was Indole, Methyl Red, and starch hydrolysis positive and negative for Voges-Proskauer and citrate utilization. The isolate was found to positive for catalase and oxidase (Table 2). Further, the isolate was subjected to genotyping using the 16S rRNA. The genomic DNA was extracted and PCR was performed for amplification of 1430 bp amplicon size. The amplified product was further sequenced and analyzed. The neighbor-joining tree was plotted and the sequence of study isolate was highly similar with that of *Sphingomonas leidyi* 16S rRNA sequence available in NCBI. Hence, the isolate was identified as and isolate was identified as *S. leidyi*.

DISCUSSION

The present study was designed to isolate potent L-Asparaginase-producing strain from soil samples collected from different locations around Nilgiris and Coimbatore districts of Tamil Nadu and Palakkad, Malappuram, Wayanad and Kannur districts of Kerala. As Rani *et al*. [27] and Devi and Ramanjaneyulu [28], all the collected samples were serially diluted and plated on the Nutrient agar plate for the isolation of L-Asparaginase-producing bacterial strain. Similarly, the isolation and screening of L-Asparaginase-producing bacteria, fungi, and Actinomycetes from various niches were performed by various researchers [29-33]. About 689 individual bacterial colonies from each sample were selected and screened for L-Asparaginase production in the present study.

Table 1: Enzyme activity of selected isolates

Sample	Units	Sample	Units	Sample	Units
VC05	80.2	VS02	52.7	VC06	90.5
VP03	95.4	VK07	97.4	VA03	65.2
VK02	111.3	VA09	120.5	VNO4	141.4
VS01	71.7	VM04	102.3	VC08	124.6
VM05	130.3	VN01	156.6	VN06	86.1

Table 2: Phenotypic characteristics of the selected isolate

Cultural characteristics of isolate	
Isolate	VN01
Shape	Circular
Elevation	Convex
Surface	Smooth
Pigment	Non pigmented
Optical properties	
Color	Creamy
Opacity	Translucent
Morphological characteristics	
Gram staining	Negative
Shape	Rod
Biochemical characteristics	
Indole production	Positive
Methyl red	Positive
Voges-Proskauer	Negative
Citrate utilization	Negative
Starch hydrolysis	Positive
Catalase	Positive
Oxidase	Positive

In the present study, isolates were streaked onto the M9 basal medium for primary confirmation of L-Asparaginase production. The production of pink color around the colony indicates the ability of the isolate to produce L-Asparaginase. Similarly, Prakasham *et al*. [34] and Darwesh *et al*. [25] screened L-Asparaginase production using M9 basal media. The color variation determines the intensity of L-Asparaginase production [35]. Among 689 isolates, 214 isolates were found to L-Asparaginase producers as they exhibit pink coloration around the colony. In another study, L-asparaginase producers were primarily confirmed by the development of pink coloration around the colony [36,37]. A total of 15 isolates were selected based on the intensity of pink coloration and were then subjected to quantitative screening for enzyme production in the present study.

In the present study, selected isolates were tested for L-Asparaginase activity in submerged fermentation using M9 broth and incubated in orbital shaker for 24 h at 37°C similar to the study conducted by Edward *et al*. [38]. The bacterial cell mass was centrifuged, and the supernatant was used as the crude enzyme source [19]. Then, asparaginase activities were measured by nesslerization, where the asparaginase hydrolysis is determined by measuring released ammonia [7,39]. The yellow color with brown precipitate represents the ammonia. According to Mashburn and Wriston's description, L-asparaginase activities were regularly measured by direct nesslerization, where the rate of asparagine hydrolysis is determined by measuring released ammonia, which indicates yellow with brown precipitate as reported by Sindhwad and Desai [40]. Among the selected 15 isolates, VNO1 exhibits higher enzyme activity (156 IU/mg) of L-asparaginase enzyme. Similarly, Noha *et al*. [17] screened L-Asparaginase production by optimizing the enhance medium.

The isolated potent strain was subjected to phenotypic and genotypic identification [41]. Based on cultural and biochemical characteristics, the isolate was identified based on Bergey's manual of classification. Similarly, Darwesh *et al*. [25] and Reddy *et al*. [42] classify *isolates based on their functional and structural attributes.* Further, the isolate was subjected to genotyping using the 16S rRNA [43,44]. The genomic DNA was extracted and PCR was performed and the amplified product was further sequenced and analyzed similar to the analysis conducted by Busse *et al*. [45], Chen *et al*. [24], De Vries [46]. Neighbor-joining tree was plotted and the sequence of study isolate was highly similar with that of *S. leidyi* 16S rRNA sequence available in NCBI. Hence the isolate was identified as *S. leidyi* (Fig. 1).

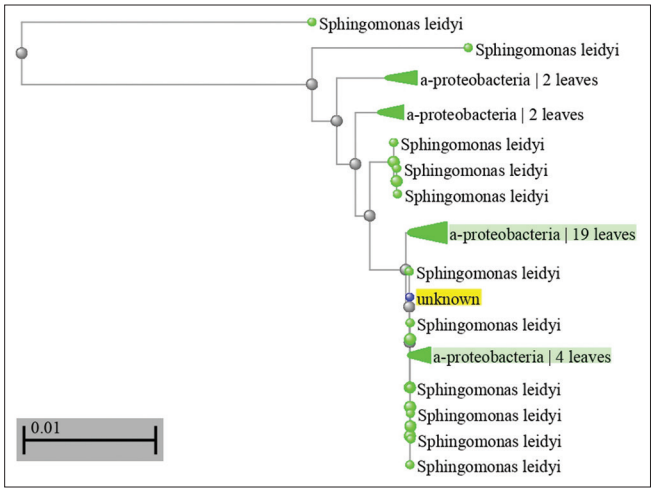

Fig. 1: Neighbor-joining phylogenetic tree of 16S ribosomal RNA

CONCLUSION

The results of the current study reveal that L-asparaginase can be synthesized using numerous microbes. The one third of the isolates obtained in this study possess the ability to synthesis L-asparaginase. Among the isolates, VNO1 exhibited significantly higher degree of enzyme activity. This study is first to report L-asparaginase production by *S. leidyi*. Further, we planned to optimize the production of L-asparaginase by *S. leidyi* and to characterize the enzyme to evaluate the efficiency of enzyme synthesized by each organism.

AUTHOR'S CONTRIBUTIONS

Mr. Deepak V M M – Literature search, writing, sample preparation and analysis. Ms. Vaishnavi L – Literature search and sample preparation. Dr. Radha Palaniswamy – Guidance and correction of manuscript.

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest that may have affected the conduct or presentation of the research.

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