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Isolation, Identification of Anti-Leukemic Agent L-Asparaginase Producing Bacteria From Krishna River Soils and Enhancement of Enzyme Production

Tharaka Rama Velisetty[1], Sathish Thadikamala[2], Sudhakar P[1] and Krishna Satya Alapati[1*]

[1]Department of Biotechnology, Acharya Nagarjuna University, Guntur 522510, Andhra Pradesh, India
[2]Koringa College of Pharmacy, Korangi, Kakinada, Andhra Pradesh, India.

ABSTRACT:

Recently WHO listed L-asparaginase as one of the essential medicines for the treatment of acute lymphoblastic leukemia in children. Due to the increase in the usage of this enzyme in the treatment of leukemia and other industries such as food and biosensors, it is gaining more attention by many industries. To meet the demand of the industry's requirement it is necessary to increase the yield as well as to discover new strains which can produce higher titers of this enzyme. In the present study high yielding bacterial strain was isolated from the Krishna River soils. The isolated bacteria were identified as *Bacillus subtilis* by both biochemical and molecular methods. The strain was designated *Bacillus subtilis* THARAKA. Various media were screened for a higher amount of L-asparaginase production by isolated *B.subtilis* THARAKA. Along with media, various fermentation parameters were optimized by one factor at a time method. Overall, by optimization of various fermentation parameters, 1.8 folds of L-asparaginase yield was enhanced.

Key Words: L-Asparaginase, L-Asparagine, Acute lymphocytic leukemia, Acrylamide Isolation, *Bacillus subtilis*

1.0 Introduction

It was estimated that in 2016 therapeutic enzymes occupied the $5.0 billion market globally and are expected to increase the market by 4.7% annually. Among various therapeutic enzymes, L-asparaginase has gained more

popularity (1). L-Asparaginase is listed as a 19th essential medicine in the WHO Model List of Essential Medicines for Children as a cytotoxic and adjuvant medicine for acute lymphoblastic leukemia (2). Acute lymphocytic leukemia is the major type of cancer in children aged between 0-14 years. L-Asparaginase is majorly used in the treatment of childhood acute lymphocytic leukemia, myeloblastic leukemia, lymphoid malignancies in the brain, other central nervous system tumors, and neuroblastoma (3-5). L-asparaginase also administering along with other chemotherapeutic drugs such as vincristine to minimize the deleterious effects of these drugs. Based on these applications L-asparaginase formulations occupied 40% of total enzyme demands worldwide and one-third of the global need for anticancer agents, which is far more than the other therapeutic enzymes (6-7).

L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) hydrolyzes the L-asparagine amino acid into L-aspartic acid and ammonia (8). The Cancer cells lack the L-asparagine synthetase due to this they cannot make enough L-asparagine therefore they are dependent on the external source of L-asparagine for their survival and propagation (9-12). Administration of L-asparaginase reduces the L-asparagine levels in the blood which inhibits the growth of cancer cells, making them starve, and die. While healthy cells they are capable of producing, L-asparagine with L-asparagine synthetase survives (13-15).

L-Asparaginase also uses in the food industries to reduce the acrylamide formation in fries and bakery products. The acrylamide forms by Millard reaction between sugars and L-Asparaginase during frying or baking at high temperatures (16-17). According to International Agency for Research on Cancer, Acrylamide is classified as a probable carcinogenic compound. The addition of L-Asparaginase reduces the L-asparagine in the food and stops the formation of acrylamide.

L-asparaginase also uses in biosensors to detect the L-asparagine in physiological fluids at nano-levels (18).

L-Asparaginase is widely distributed in animals such as mammals, fishes, birds, plants, and microorganisms. Among all, microbes are the best source when compare to plants and animals because of their capability to produce large quantities in a short time, in lesser space, and with inexpensive substrates (19). In microorganisms, fungi (20) and bacteria are capable of producing this enzyme. However, the bacterial L-Asparaginase is approved for therapeutic applications. Among various bacteria, L-asparaginase from *Escherichia coli* and *Erwinia carotovora were* approved for therapeutic application. Evans et al (21), reported the adverse effects of L-Asparaginase from *Erwinia* and *E. coli* in children who have undergone treatment. The adverse effects include hypersensitivity,

anaphylactic shock, allergic reactions, blood clot edema, rash, bronchospasm, hepatotoxicity, pancreatitis, hyperglycemia, and their related reactions (22). These reactions were observed even in PEGylated L-asparaginases also.

Based on current demand and due to the adverse effects of current L-asparaginase from *E. coli* and *Erwinia*, there is a demand to find a new microorganism with the capability to produce a higher titer of L-asparaginase that has similar therapeutic properties. Based on this background, in this current study, various bacteria were isolated from the different sources that have L-asparaginase production capacity, and primary screening studies were conducted.

2.0. Materials and Methods

2.1 Sample Collection

The river bank soil samples from Krishna and Godavari rivers in Andhra Pradesh have been collected aseptically and transferred to the lab at 4˚C. Initially, these samples were stored at 4 ˚C till further use. From these soil samples, L-asparaginase enzyme-producing microbial strains were isolated. The main aim was to isolate the bacterial strains only.

2.2 Screening of soil isolates for L-asparaginase production by rapid plate assay

Figure 1 shows the schematic representation of the isolation process of the L-asparaginase-producing microorganisms. Soil samples collected from various places were used for the isolation of L-asparaginase-producing bacterial strains. The potent L-asparaginase-producing microbes were isolated from the soil samples according to the method of Prakasham et al (23) and Gulati et al (24). Initially, 1.0 gm of collected soil sample was suspended in 100 ml of sterile distilled water and mixed well for an hour at room temperature. From this solution, 1 ml was transferred to 9 ml of sterile water and diluted from this another 1ml transferred to another 9 ml of sterile water. Similarly, the serial dilution was continued until to achieve the dilution up to 10^{-8}. From 10^{-6} to 10^{-8} dilution tubes, 100 µL of the solution was collected and spread on M9 medium agar plates. The M9 agar medium composition follows as (g/L) - Glucose 2.0; L-asparagine 5.0; Na_2HPO_4 6.0; KH_2PO_4 3.0; NaCl 0.5; $CaCl_2$ 0.12 and $MgSO_4$ 2.46. The M9 media pH was adjusted to 7.0 using 0.1M HCl or NaOH solutions and it was supplemented with 2.5% v/v phenol red as a pH indicator. The Plates were incubated at 37 °C for 24 to 48 hours. After incubation, it is observed that the number of colonies formed on the plates and the zone of color

formation due to the production of L-asparaginase. The colonies having bigger zones were selected for secondary screening.

2.3 Secondary screening of soil isolates for L-asparaginase production by rapid plate assay

The L-asparaginase positive isolates identified in primary screening were further evaluated for their potentiality on enzyme secretion by plating them again on M9 agar medium supplemented with 2.5% v/v phenol red as a pH indicator. The same culture was further streaked on the two control plates, one plate contains the M9 agar media without dye and the other plate contains M9 agar media without L-asparagine. The plates were incubated at 37˚C for 24 hours.

The potent L-asparaginase producers were identified based on the pink zone formed around the colonies and compared with other control plates. Further confirmation studies were carried out in the liquid media. Transferred the loop full of each organism in 50 ml of M9 liquid media and incubated in a shaker incubator at 37°C temperature and 100 rpm. After 24 hours of incubation, the L-asparaginase activity was determined in cell-free broths.

The selected microbial strain was grown using 100 ml of M9 medium composed of (g/L) Glucose-2; L-asparagine-5.0; Na_2HPO_4 - 6.0; KH_2PO_4 -3.0; NaCl -0.5; $CaCl_2$ - 0.12 and $MgSO_4$ - 2.46, in 250 ml of Erlenmeyer flask. The sterilized medium flasks were inoculated with a loop full of culture under a sterile environment and these culture flasks were incubated for 24 hours, at 37 ˚C in an orbital shaker at 100 rpm for growth and enzyme production. After incubation the

L-asparaginase activity was determined in cell-free broths. All experiments were performed in triplicate and three aliquots. The data presented in this investigation was the average of the results of all the above experimentations. Based on the diameter of the zone formed and obtained L-asparaginase activity one strain was chosen for further studies and it was designated as ‘THARAKA’

2.4 Identification and Characterization of L-asparaginase-producing isolate THARAKA

The isolate THARAKA was characterized and identified based on cultural morphological, physiological, and biochemical reactions according to Bergey’s Manual of Determinative Bacteriology. The morphological studies

were carried out by observing the structure of the isolate under an electron microscope. The isolate was also identified based on molecular methods such as 16 rRNA gene sequencing and homology searching.

2.4.1 Molecular methods of identification of isolate THARAKA

The molecular characterization of bacterial isolate THARAKA was carried out according to Sathish and Prakasham (25).

2.4.2 Extraction of DNA:

5 ml of lysis buffer was prepared which contain the 10 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, and 20 mM EDTA adjusted the pH 8.0. The isolate THARAKA pellet was suspended in the lysis buffer, to this suspension 0.5 mL of 10mg/mL lysozyme was added and incubated at 37 ^{0}C for 30-60min. After incubation, 2 µL of 500µg/mL of concentrated RNase was added to the mixture and incubated for another 15 min, to this mixture 18µL of 10mg/mL proteinase-k and 180µL of 10% SDS were added and incubated for 60 min at 37^{0}C. From this mixture DNA was extracted by 25:24 phenol: chloroform extraction followed by precipitation by ethanol. The acquired DNA pellet was re-suspended in 1mL of DNase and RNase-free water and used for amplification.

2.4.3 Amplification and Sequencing of 16S rRNA Gene

From the isolated DNA, the 16S rRNA gene was amplified by using PCR according to Sathish and Prakasham (2010), Using an Eppendorf AG PCR system (Eppendorf). To amplify the gene following primers were used 16S-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1500r (5'- AAAGGAGGTGATCCAGCC-3'). Chromosomal DNA is used as a template. Agarose gel electrophoresis was used to analyze the amplified DNA fragments. The Qiaquick PCR purification kit was used to recover the amplicon from agarose gel. The purified PCR product was sequenced directly by ABI DNA sequenator model 377a (Applied Biosystems) using the Big-Dye Terminator kit (Applied Biosystems). The obtained sequence fragments were connected by using the DNAsis program and a homology search was carried out using the BLASTN program. The obtained 16S rRNA gene sequence was deposited in the GenBank database.

2.4.4 Phylogenetic Analysis and tree construction.

The obtained bacterial strain THARAKA 16S rRNA gene was used to search the homologous nucleotide sequences in the Gene bank database by running the BLASTN program. From the BLASTN results the sequences, which have high similarity scores were recognized and obtained in a FAST format from the GenBank database. Initially,

the collected sequences and THARAKA sequence were aligned by multiple sequence alignment using the CLUSTAL-W algorithm. From the multiple sequences, an alignment phylogenetic tree was constructed by using the Maximum likelihood method. Multiple sequence alignment and phylogenetic analysis were conducted using MEGA version 11 (46).

2.5 Screening of suitable media for L-asparaginase production

Initially, four different media namely Bennett's broth, Yeast extract malt extract media, Asparagine dextrose salt broth (ADS), and M9 media (26) were evaluated for L-asparaginase production by *B. subtilis* THARAKA. All flasks received 2% of inoculum and incubated at 37 °C for 24 hrs.

2.6 Optimization of L-asparaginase production

For enhancement of L-asparaginase production by isolated bacteria various parameters such as incubation time (6,12, 18, 24, 30, 36, 42 and 48h), inoculum age (12, 18, 24, 30 and 36 h) inoculum concentration (0.5 to 3.0 ml), incubation temperature (22, 27, 32, 37, 42 and 47 °C), pH (5.5 to 9.0 with 0.5 increment), agitation speed (100 to 250 RPM with 25 RPM increment) L-asparagine concentration (0.25, 0.5, 1.0, 1.5 and 2.0 % w/v) and various amino acids (Glycine, Alanine, Serine, Cysteine, Tyrosine, Tryptophan, Methionine, Proline, Glutamine, Arginine, Histidine, Aspartic acid, Glutamic acid and mixture of amino acids) were optimized.

2.7 Estimation of L-asparaginase activity

L-asparaginase activity was estimated by the spectrophotometric method developed by Wriston and Yellin (27). The enzyme activity was determined by measuring the amount of ammonia liberated during the reaction, and its absorbance was measured at 436 nm using a UV-Visible spectrophotometer. The assay mixture contains 0.1ml of obtained enzyme solution, 1.0 ml of 0.5M Tris buffer pH 8.6, and 183 mM asparagine as a substrate in a final volume of 2.2 ml. The enzyme and substrate reaction mixture was incubated at 37°C for 30 min. The reaction was, terminated by adding of 0.1 ml of 15% trichloroacetic acid (TCA). A similar procedure was followed to prepare the control where enzyme solution was added after terminating the reaction by TCA. In another tube, 4.3 ml of distilled water was taken, to this 0.2 ml of the above enzyme reaction mixture and 0.5 ml of Nessler's reagent were added. With the addition of Nessler's reagent yellowish-orange color was developed, and the color was measured at 436 nm against the control. One unit of enzyme is defined as one µmol of ammonia liberated per minute under assay conditions.

3.0 RESULTS AND DISCUSSION

3.1 Enrichment and isolation of microorganisms

Various samples collected from the different rivers and their banks are used for the isolation of potent L-asparaginase-producing bacteria. Among the soil samples tested, only the Krishna river sediments collected from Vijayawada, A.P, India have L-asparaginase-producing microorganisms and are grown on enriched media. Figure 1 shows the schematic diagram of the isolation process. The plates, which received the 10^{-8} serial diluted liquid aliquots, produced 5 colonies that have the capability of L-asparaginase production. From these colonies, the isolates were subcultured on other plates for rapid plate assay. The isolates were designated as THARAKA, THARAKA-1, THARAKA-2, THARAKA-3, and THARAKA-4. Based on the morphological characteristics of colonies it was noted that the isolates belonged to *Bacillus* species.

3.2 Secondary screening by rapid plate assay method.

The five bacterial colonies were further streaked on the M9 agar plates that were supplemented with phenol red indicator (Fig 1). The phenol red indicator is pH sensitive, in neutral and acidic pH it gives a yellow color to the media, similarly it gives pink color in the alkali conditions. The pH indicator color changes when the media turns from acidic to pH or vice versa. The pink zone surroundings of isolated bacterial colonies indicate pH alteration, in this study, the isolated bacteria break down the L-asparagine in the media when producing the L-asparaginase enzyme and release the ammonia. The released ammonia accumulates in the media, makes the media become alkali, and turns the media color from yellow to pink. The amount of ammonia released depends on the amount of L-asparaginase produced which could be visually observed as a pink zone surrounded by the bacterial colony. All isolates produced a noticeable pink zone on the M9 media, however, isolate THARAKA produced a large diameter zone compared with other isolates. For further confirmation, these isolates were grown in the M9 liquid media, and produced L-asparaginase was estimated. The preliminary submerged fermentation studies revealed that among isolates the isolate THARAKA produced the higher titers of L-asparaginase. Hence, further studies were continued with THARAKA. Figure 2 shows the pink zone formed surroundings of isolate THARAKA.

3.3 Morphological and Biochemical Characterization of isolate THARAKA:

To study the morphological characteristics of the isolate THARAKA is grown on the nutrient agar plates. It was observed that the isolated bacterial strain THARAKA produced round colonies with wavy margins, rough surfaces,

and opaque density. On gram staining, it was observed that the isolated microbe is gram-positive rod-shaped and spore-forming in nature. It is an aerobic culture. Further, the bacterial isolate THARAKA was observed under a scanning electron microscope (SEM). Figure 3, shows the SEM picture of the isolated bacterial stain THARAKA, from this it was observed that the isolate belongs to the Bacillus genera. Table 1 & 2 shows the various morphological, and biochemical studies conducted, and their results. From this, it was noticed that the isolated bacterial strain cells could live and grow at pH 5 to 11. The isolate THARAKA could grow at 10% NaCl concentration also. Based on various morphological and biochemical test results the bacterial isolate THARAKA could be *Bacillus subtilis* and it was designated as *B. subtilis* THARAKA. Further, the isolate was identified and confirmed by molecular characterization.

3.4 16S Ribotyping (Molecular characterization) of bacterial isolate THARAKA

The morphological and biochemical characterization studies revealed that the isolate THARAKA belongs to the *B. subtilis* genera. Further, it was confirmed by the amplification of the genomic DNA of this isolate. The genomic DNA of this isolate was used as a template in the PCR amplification and the amplicon was used to conclude the 16S rRNA gene. The obtained gene sequence contains *1539 base pairs consisting of Adenine - 24.7%, Cytosine – 23.6%, Guanine 31.3%, and Thymine – 20.4%.* The partial e 16s rRNA gene sequence was submitted to the GenBank database and obtained Genbank accession number as OL804297. The BLASTN analysis reveals that the sequence of isolate THARAKA is 99% similar to the *B. subtilis* RSP-GLU and other Bacillus species. By collecting similar sequences and other group sequences, multiple sequence alignment was done followed by the phylogenetic tree construction. For Phylogenetic tree construction, *Microbacterium indicum* (AM158907) was taken as an outer group. Figure 4 depicts the constructed phylogenetic tree with an isolated THARAKA strain. From this phylogenetic tree, it was observed that the isolate THARAKA is closely associated with *B.subtilis* family.

Based on morphological, biochemical, and molecular characterization the isolated bacterial strain THARAKA is identified as *Bacillus subtilis* and it is labeled as *B. subtilis* THARAKA.

3.5 Screening of suitable media for L-asparaginase production

For the enhancement of L-asparaginase activity, the primary step is a selection of a suitable media. In the literature, various authors used different media for different types of microorganisms. In this current study, four different

media were selected and tested for L-asparaginase production by isolated *B. subtilis* THARAKA. Figure 5 shows the selected media and their potency in the production of L-asparaginase. It is observed that among all selected media M9 media produced the highest enzyme activity at 140 ± 6.02 U/ml, followed by ADS media with 68 ± 2.78 U/ml. Yeast extract malt extract yielded the lowest L-asparaginase activity 25 ± 0.95 U/ml by *B. subtilis* THARAKA.

Based on obtained results M9 medium is selected for further studies. Deshpande et al (2014) also conducted similar experiments, the authors noticed that ADS media supports the L-asparaginase production by *Streptomyces ginsengisoli*. Hymavathi et al (28) used the M9 media for L-asparaginase production by *B. circulans*.

3.6 Effect of incubation period on L-Asparaginase production

Productivity is one of the important economic factors for the production of any product and governs the final price in the market. Along with various parameters, incubation time also plays a critical role in the final productivity. At the industrial scale, less incubation period reduces the power consumption, labor, and utility maintenance costs largely. Hence, the production pattern of L-asparaginase by this isolate is studied in this study. At time different intervals of fermentation, the samples were withdrawn, and estimated the enzyme activity and biomass. From figure 6 it was observed that as incubation time increases up to 24 the L-asparaginase production was gradually raised along with biomass. At 24 hours, the enzyme activity was observed as 143±6.0 U/ml. After 24hrs to 30hrs, the L-asparaginase activity was reduced to 110 ± 3.85 U/ml. After incubation of 30 hrs, an extreme decrease in enzyme activity was noticed. However, the biomass was remaining the same from 24hrs to 36hrs. From figure 5, it was noticed that the production of L-asparaginase by isolated *B.subtilis* THARAKA was effective at 24 hours of incubation. It was also observed that incubation time exceeding more than 30hrs drastically reduces the enzyme titers. In early times, less L-asparaginase production was associated with less growth. At higher incubation time, the reduced enzyme titer was noticed due to exhaustion of nutrients, cells reach to stationary phase and release proteases which breakdowns the extracellular L-asparaginase. Khamna et al. (29) was observed a similar trend of L-asparaginase production by *Amycolatopsis* sp. CMU-H002. Prakasham et al (27) also observed a similar pattern of L-asparaginase production by *Bacillus circulans*. Indicates that L-asparaginase production is a growth associated along with biomass growth the enzyme production was gradually increased and at the stationary phase the enzyme was decreased due to various growth and nutrient-related limitations.

3.7 Effect of inoculum age on L-Asparaginase production

The age of the inoculum plays a vital role in fermentation. Based on the product formation conditions it is important for the inoculum cells' conditions. Young cells take more time to adapt to new media and higher volumes. Similarly, mature cells take more lag phase, and the yields also less in growth-associated products. To understand the effect of inoculum age on L-asparaginase production by *B. subtilis* THARAKA different aged inoculums were inoculated into various flasks and incubated. The biomass and enzyme yields were compared. Figure 7 shows the influence of inoculum age on L-asparaginase production. From this, it is noticed that flasks received 24 hours aged inoculum yielded a higher amount of L-asparaginase 156 ± 6.5 U/ml when compared with other ages of inoculum. Higher or lower the 24 hours of inoculum age the noticeable amount of enzyme production was decreased.

3.8 Effect of inoculum level on L-Asparaginase production

The amount of inoculum plays a vital role in enzyme production, it also changes the lag phase time (Sathish and Prakasham 2010). In this study, the inoculum levels varied from 0.5 to 3.0 % (V/V). It is noticed that the enzyme production was increased from 0.5 to 2 % inoculum, further increment reduced the enzyme activity. At 2% (V/V) inoculum concentration 180 ± 7.56 IU/ml of L-asparaginase activity was observed (Fig 8). Above 2% (V/V) inoculum a rapid decrease in enzyme titer was noticed. At 2.5 % (V/V) inoculum 43% of L-asparaginase activity was deceased, however, the amount of biomass is near equal to 2% (V/V) inoculum (Fig 8). At higher inoculum concentrations the cells attained the stationary phase quickly and stopped the production of the enzyme. Similar results were observed with Prakasham et al (27).

3.9 Effect of incubation temperature on L-asparaginase production

Incubation temperature plays a vital role in the microorganism's growth as well as product formation. Improper incubation temperature leads to less growth and lesser yields. To determine the best suitable incubation temperature the inoculated flasks are incubated at different temperatures in the orbital shaker starting from 22 to 47°C. The study reveals that when increasing the temperature from 22 to 37°C the enzyme production was increased and from 37°C a sharp fall was observed. The maximum L-asparaginase activity of 164±5.9 IU/ml by *B.subtilis* THARAKA was obtained at 37°C. At 42°C only 30 ± 0.75 IU/ml activity and at 42°C no enzyme activity was noticed. The biomass at 42 & 47°C is less compared with 37°C, indicating that the isolated B.subtilis THARAKA is not a thermophile. The decreased biomass and L-asparaginase activity at lower and higher temperatures (Fig 9)

indicates that the isolated *B.subtilis* THARAKA is temperature sensitive. Pandey et al (30) also observed the variation of L-asparaginase production with temperature variation. Prakasham et al (27) also observed the maximum L-asparaginase production was observed at 37°C from *Staphylococcus* sp. Mikucki et al (31) and Narayana et al (32) observed the temperature optimum at 35°C from *Staphylococcus* species & *Streptomyces albidoflavus.* However, Liu and Zajic (33) observed the optimum temperature as 28°C for L-asparaginase production by *Erwinia aroideae*. This literature report and study results indicate that the optimum temperature for enzyme production varies from species to species.

3.10 Effect of pH on L-asparaginase production

pH is one of the vital environmental parameters for all living cells. The variation in the pH could severely affect the microorganism's growth and product formation (25). The ionic strength of media also plays a vital role in the release of extracellular compounds in the media. Figure 10 shows the effect of initial pH on L-asparaginase production by *B.subtilis* THARAKA. From this figure, it is noted that at pH 7.0 is best suitable for the release of L-asparaginase by *B.subtilis* THARAKA. At pH 6.5 & 7.5 decreased production was observed. Based on this study it is observed that *B.subtilis* THARAKA can produce a noticeable amount of L-asparaginase in the pH range of 6.5 to 7.5. Above and below the specified pH range the biomass, as well as enzyme production, is decreased. Prakasham et al (23) also observed a similar trend in L-asparaginase production by *Bacillus circulans*. Koshy et al (34) and Khamna et al (29) also observed a higher amount of L-asparaginase production was noticed at pH 7 by *Streptomyces* sp. and *Amycolatopsis* sp. Whereas Narayana et al (32), Heinemann and Howard (35), and Mishra (36) reported optimum pH for L-asparaginase production was 6.5 from *Streptomyces albidoflavus*, *Serratia marcescens* and *Aspergillus Niger* respectively. De jong (37) and Amena et al (38) observed that the highest enzyme production was observed at alkaline conditions (pH 8.5) by *Streptomyces* sp.

3.11 Effect of Agitation speed

The agitation of the flasks creates the mixing of nutrients and gases in the media. Proper mixing is essential for the nutrient's availability for microorganisms and gas exchange. Figure 11 shows the flasks incubated at different agitation speeds and obtained L-asparaginase yield by *B.subtilis* THARAKA. From this, it is observed that at an agitation speed of 200 RPM, higher titers of enzyme 224±6.46 IU/ml were achieved. Above and below this

agitation speed decreased L-asparaginase production by *B.subtilis* THARAKA is observed. Below 175 RPM and above 225 RPM noticeable amount of enzyme titer is decreased.

The literature reports depict that based on the organism the optimum agitation speed is varied for enzyme production. Khamna et al (29) noticed that 125 RPM is optimum for a higher amount of L-asparaginase production by *Amycolatopsis* (CMU-H002). While Robert and Bernard. (1968) (39) and Khan et al. (1970) (40) suggested that high aerated conditions were needed for optimum L-asparaginase production by *E.coli* and *Serratia marcescens*.

3.12 Effect of L-Asparagine concentration on L-asparaginase production.

To understand the role of L-asparagine on L-asparaginase various concentrations of the amino acid are added to the flasks. L-Asparagine act as a both carbon and nitrogen source. From figure 12 it is observed that L-asparagine from 1 % to 2% released higher amounts of L-asparaginase. Below 0.5 % showed lesser yields. From this study, it was observed that *B.subtilis* THARAKA can withstand higher concentrations of L-aspargine however no significant increase in enzyme titers was observed.

Prakasham et al (23) reported that 0.5% of L-asparagine is optimum for higher amounts of enzyme production from *B.circulans*. However, for *Fusarium oxysporum* 1.0% of inducer is needed for optimal concentration of enzyme production (24). A similar observation was also reported by Lapmak et al (41) in *Bipolaris* sp.

3.13 Effect of amino acids on L-asparaginase production

To understand the role of amino acids on L-asparaginase production by *B.subtilis* THARAKA selected amino acids were supplemented as 50% of L-asparagine concentration. The experimental data are presented in figure 13. From this study, it is noticed that the isolated bacterial growth and enzyme production were governed by the selected amino acids.

None of the selected amino acids improved L-asparaginase production. Higher enzyme production is noticed in the control flasks only. Partial replacement of inducer resulted in a lesser enzyme yield. The addition of methionine and proline produced a comparable amount of enzyme with control. The addition of glutamic acid, aspartic acid, and cysteine completely inhibits enzyme production. This indicates that the ready availability of metabolic important amino acids in the media leads to organism growth and suppression of metabolic enzymes. In

the final flask L-asparagine, arginine, and L-glutamine mixture was supplemented and it was noticed that the production of L-asparaginase is nearly 50% compared with the control. In this mixture, the arginine might induce enzyme production.

The obtained results are contradicting Borkotaky and Bezbaruah, (42) where the authors observed that individual and combination of L-Arginine, DL-alanine, L-asparagine, and L-glutamine enhanced the L-asparaginase production in *Erwinia* sp. These results were correlated with Prakasham et al (23) and Boeck et al (43) who explained that the addition of amino acids other than asparagine repressed the synthesis of L-asparaginase. Cedar and Schwartz (44) noticed that L-asparaginase production was increased with media enriched with different of amino acids. Khan et al (40), and Robert et al (39) reported that L-glutamic acid and methionine are essential nutrients for the optimal production of L-asparaginase. Chandra et al (45) reported that 14.49 % and 25.25 % enhancement of L-asparaginase was observed with the supplementation of L-leucine and L-methionine to Alcaligenes *faecalis*.

4.0 CONCLUSION

The present study depicts the efficient method for the isolation of L-asparaginase-producing microorganisms from soil samples. By following the plate method and broth studies one bacteria was isolated and it was characterized by morphological, biochemical, and molecular methods. Based on identification studies the isolate was recognized as *B.subtilis* and it was designated as *B.subtilis* THARAKA. The enzyme production studies were carried out in submerged fermentation. The fermentation conditions were optimized for enhancement of the L-asparaginase production. By optimizing the various parameters 1.8 fold yield is increased. This study signifies the importance of proper isolation methods for the selective screening of microbes and optimization techniques for the enhancement of enzyme production without altering the genetic material.

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Table 1: Morphological & Physiological characteristics of isolated THARAKA

Tests	Results	**Tests**	**Results**
Morphological characteristics		**Physiological characteristics**	
Configuration	Circular	Growth at 4°C	-
Margin	Wavy	Growth at 10°C	-
Elevations	Low Convex	Growth at 15°C	+
Surface	Dull and Rough	Growth at 25°C	+
Density	Opaque	Growth at 30°C	+
Pigments	-	Growth at 37°C	+
Gram's Reaction	+	Growth at 42°C	+
Cell Shape	Fat Rods	Growth at 45°C	+
Size	Long	Growth at 55°C	±
Arrangement	Singles or short chain	Growth at 65°C	-
Spore(s)		**Growth at pH**	
Endospore	+	pH 5.0	+
Position	Central	pH 5.7	+
Shape	Oval	pH 6.8	+
Sporangia Bulging	+	pH 8.0	+
Capsule		pH 9.0	+
Motility	+	pH 11.0	+
Fluorescence (UV)	-	**Growth under Anaerobic Condition**	-

Table 2: Biochemical characteristics of isolated THARAKA

Tests	**Results**	**Tests**	**Results**
Growth on Mac Conkey Agar	-	Adonitol	-
Indole Test	-	Arabinose	+
Methyl Red Test	+	Cellobiose	+
Voges Proskauer Test	+	Dextrose	-
Gas Production from Glucose	-	Dulcitol	-
Citrate Utilization	+	Fructose	+

Casein Hydrolysis	+	Galactose	-
Starch Hydrolysis	+	Inositol	+
Gelatin Hydrolysis	+	Inulin	+
Urea Hydrolysis	-	Lactose	-
Nitrate Reduction	+	Maltose	+
Nitrite Reduction	-	Mannitol	+
H_2S Production	-	Mannose	+
Cytochrome Oxidase	+	Melibiose	+
Catalase Test	+	Raffinose	+
Oxidation/Fermentation/Negative (O/F/-)	_	Rhamnose	-
Arginine dihydrolase	-	Salicin	+
Lysine decarboxylase	-	Sorbitol	+
Ornithine decarboxylase	-	Sucrose	+

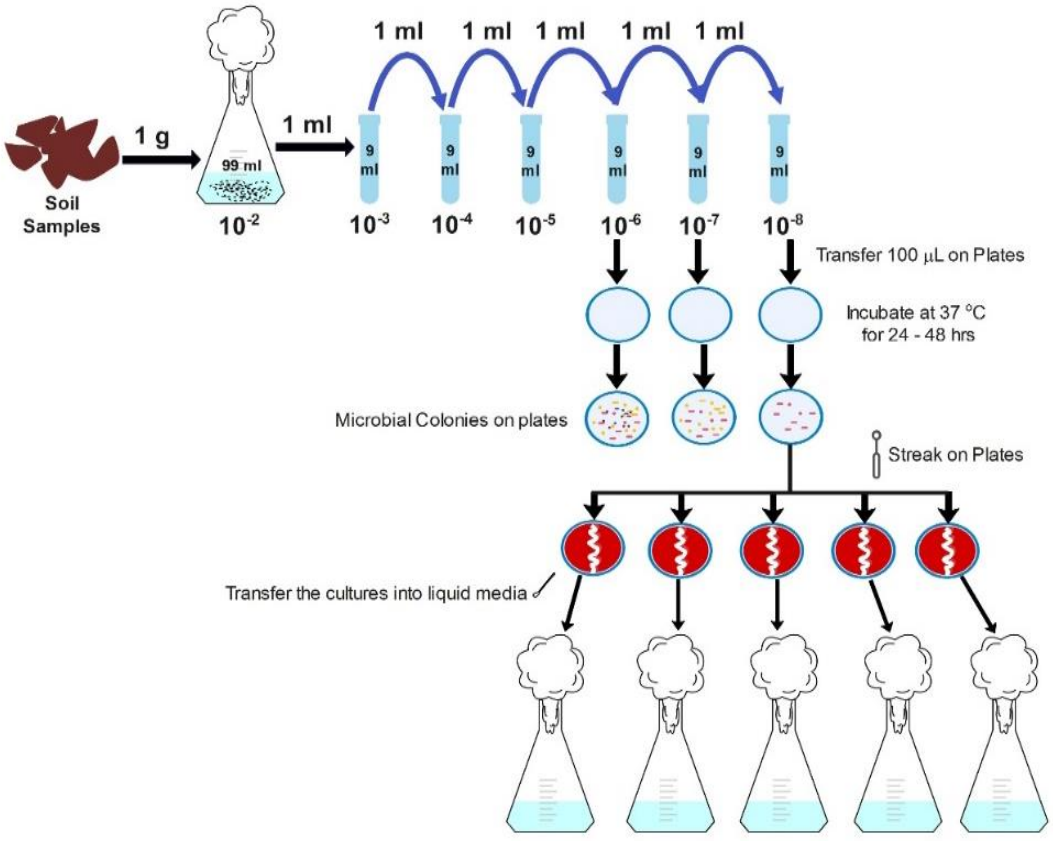

Fig 1: Schematic diagram of isolation procedure of L-asparaginase-producing microbes

Growth medium composition and fermentation conditions

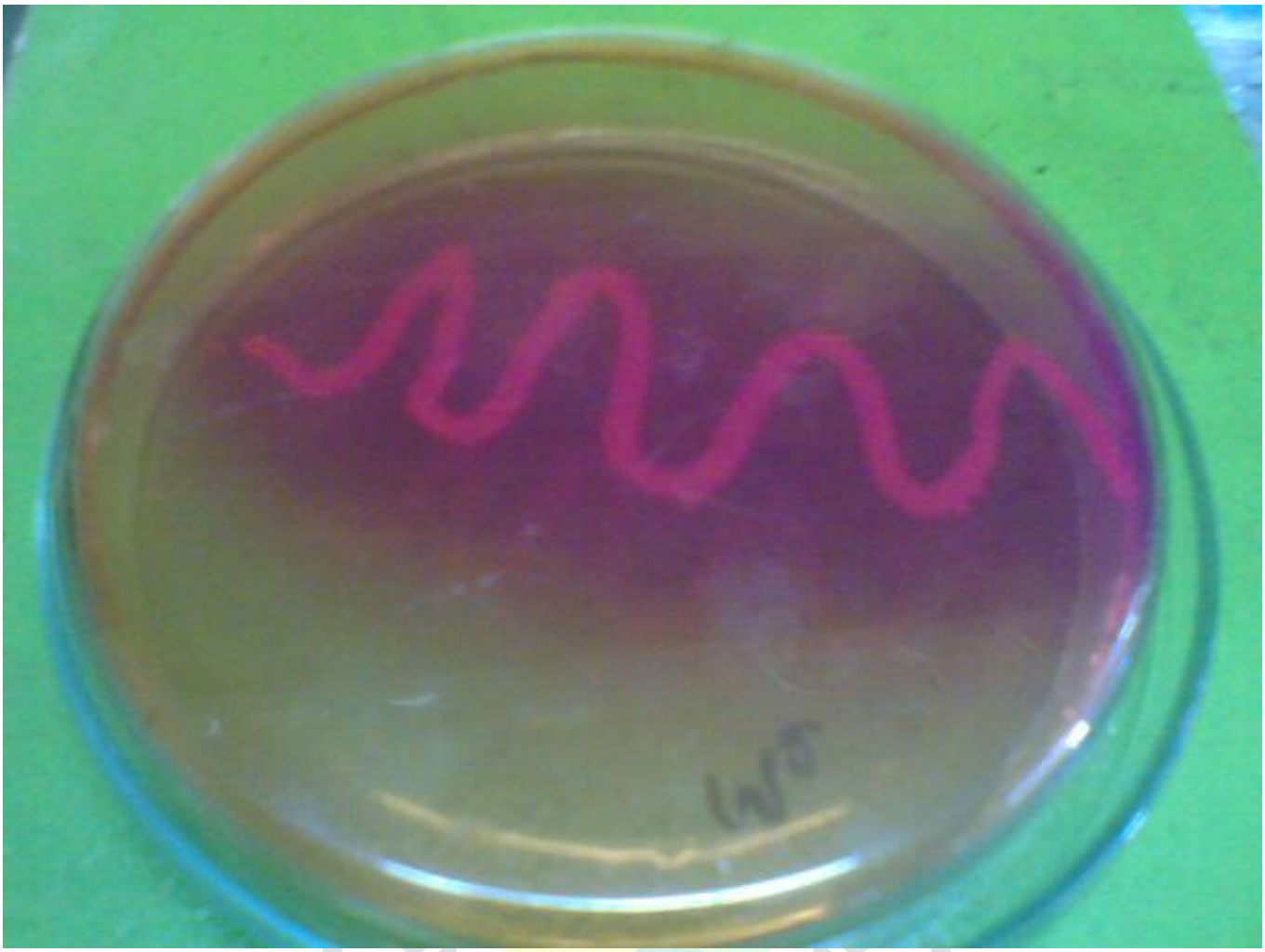

Fig.2: The isolate THARAKA produced a pink zone on the M9 agar plate.

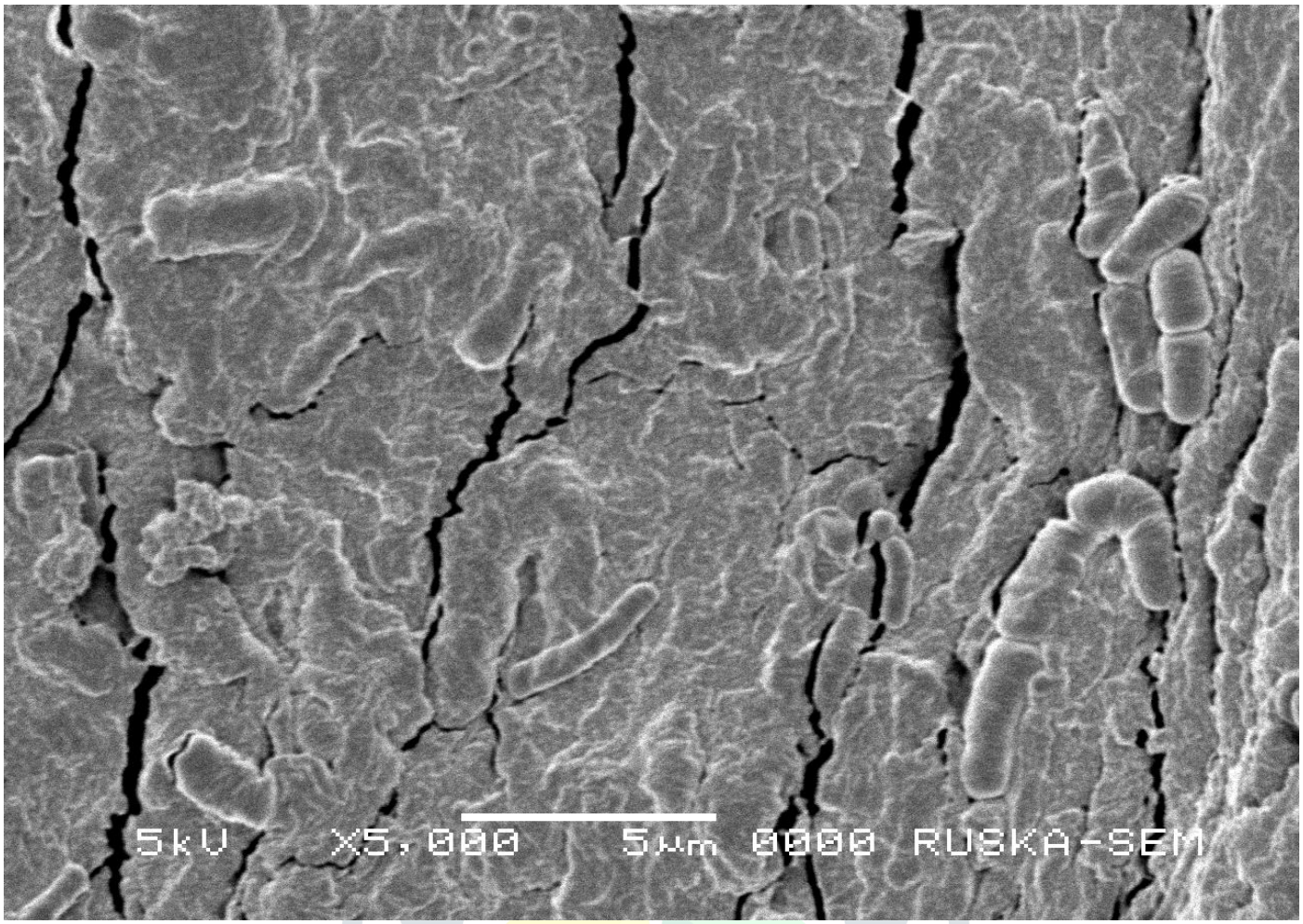

Fig. 3: Scanning electron microscopic (SEM) picture of Isolated bacterial strain THARAKA.

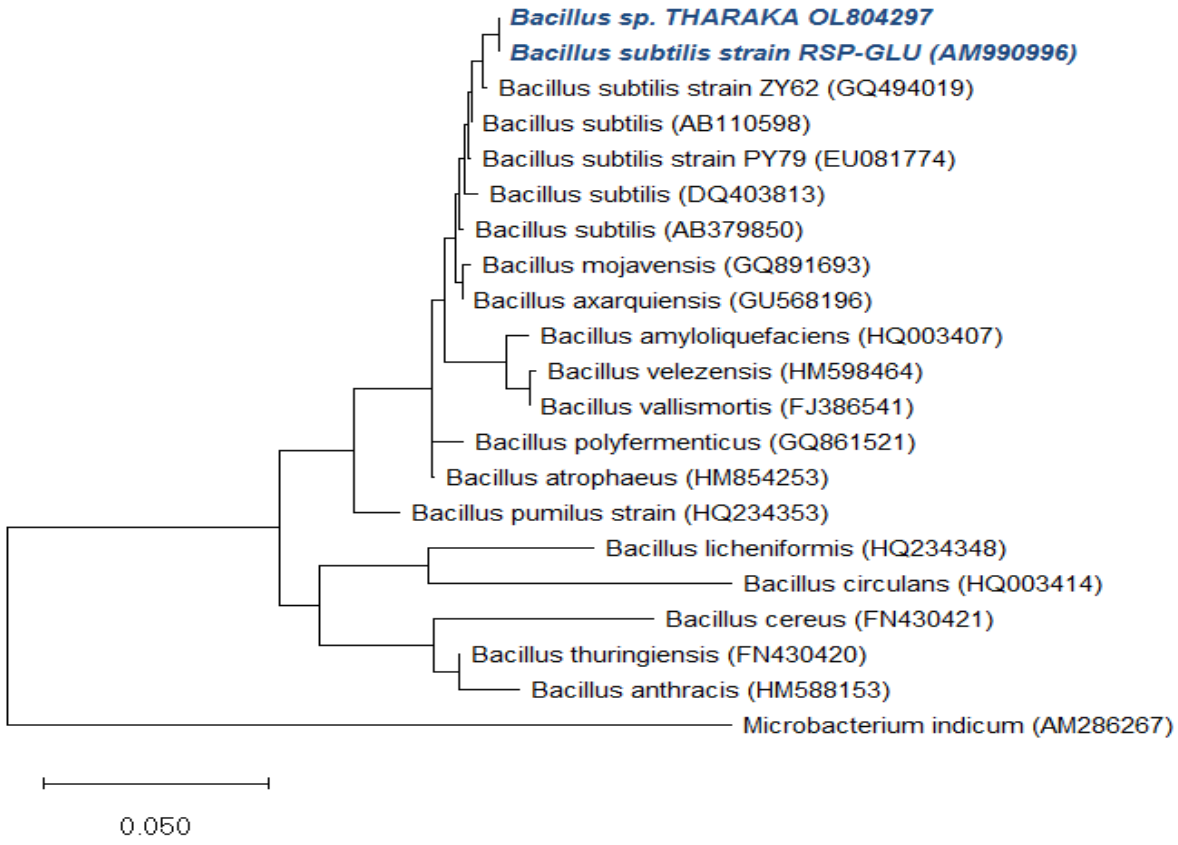

Fig.4: Phylogenetic tree shows the phylogenetic relationship of *Bacillus subtilis* THARAKA with other members of the genus *Bacillus*.

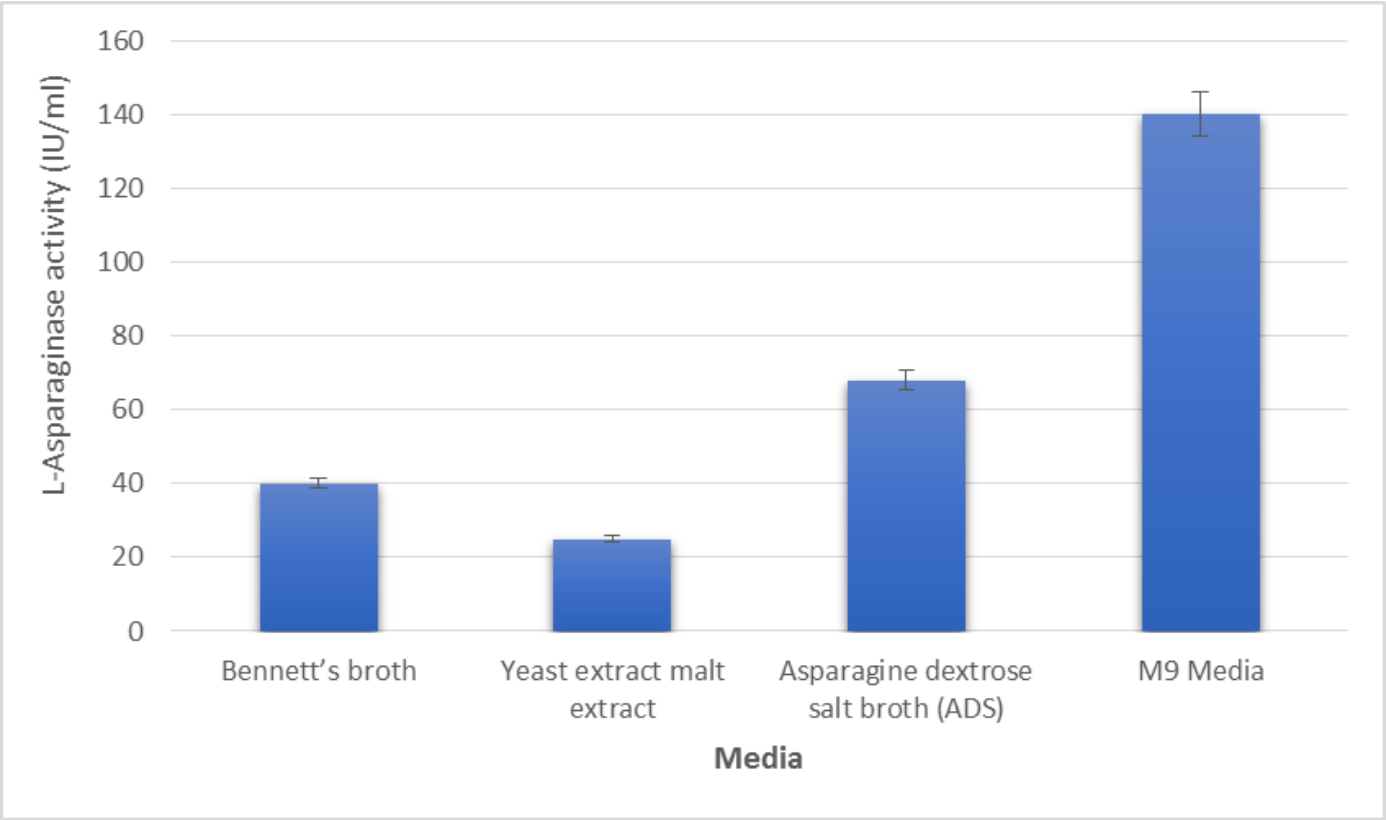

Fig 5: L-Asparaginase production in Different media by *B.subtilis* THARAKA

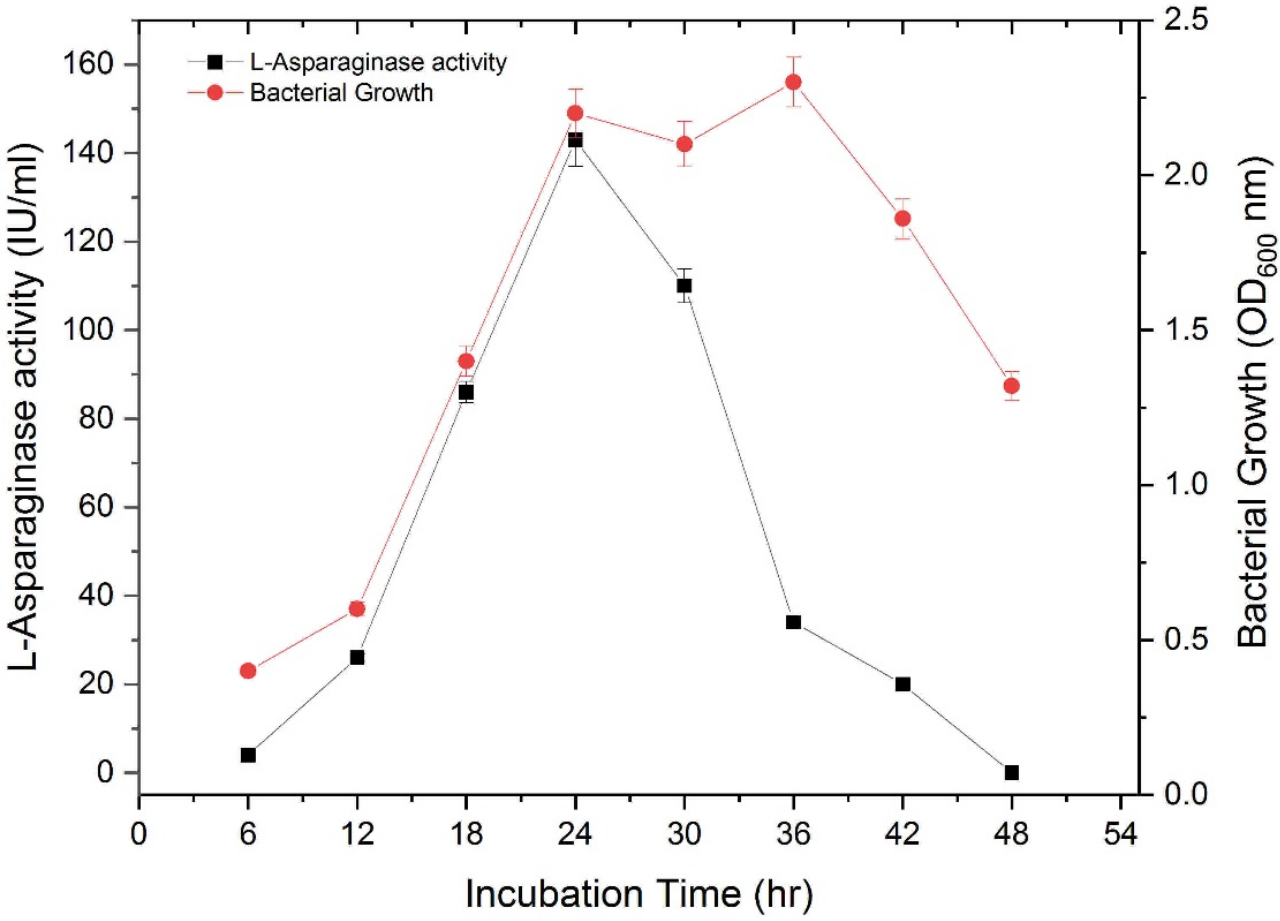

Fig 6: Effect of incubation time of L-Asparaginase production by *B.subtilis* THARAKA

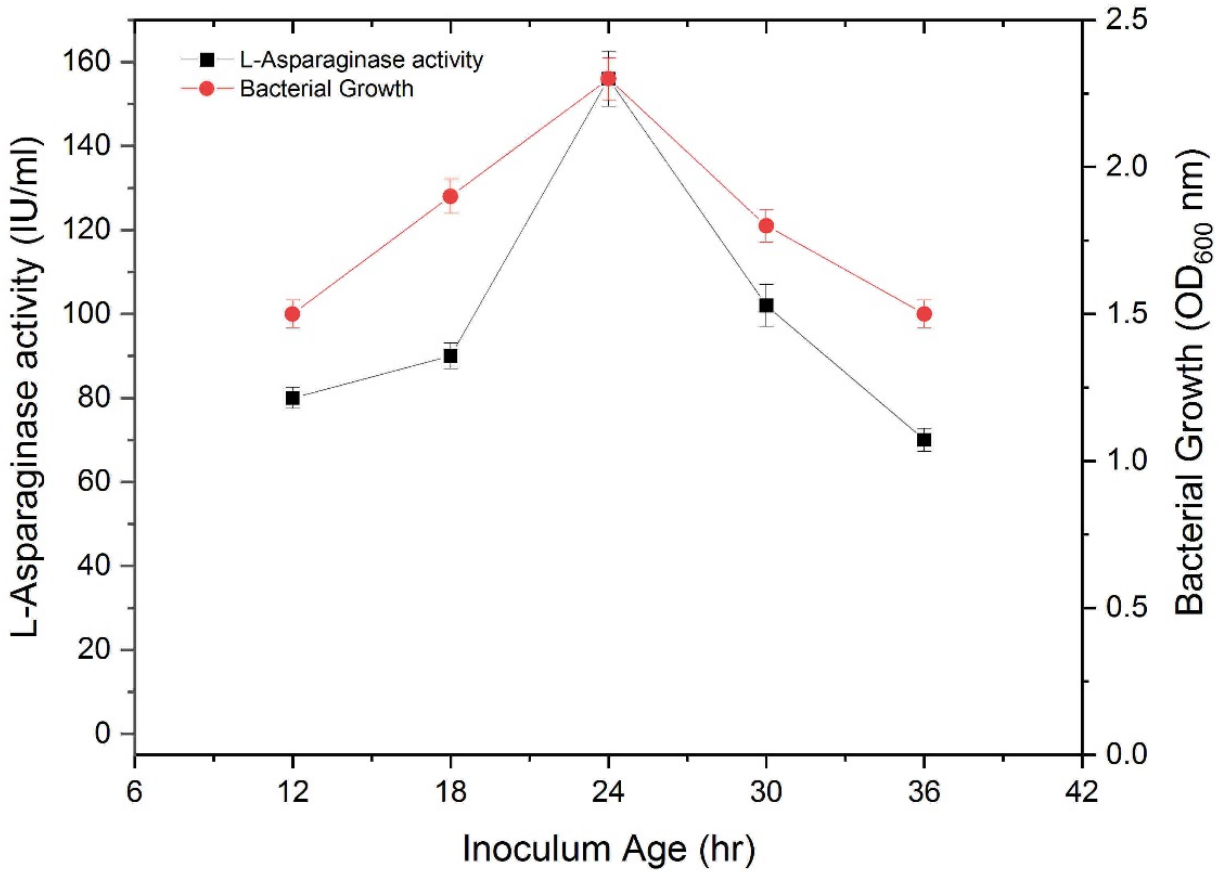

Fig 7: Effect of inoculum age on L-Asparaginase production by *B.subtilis* THARAKA

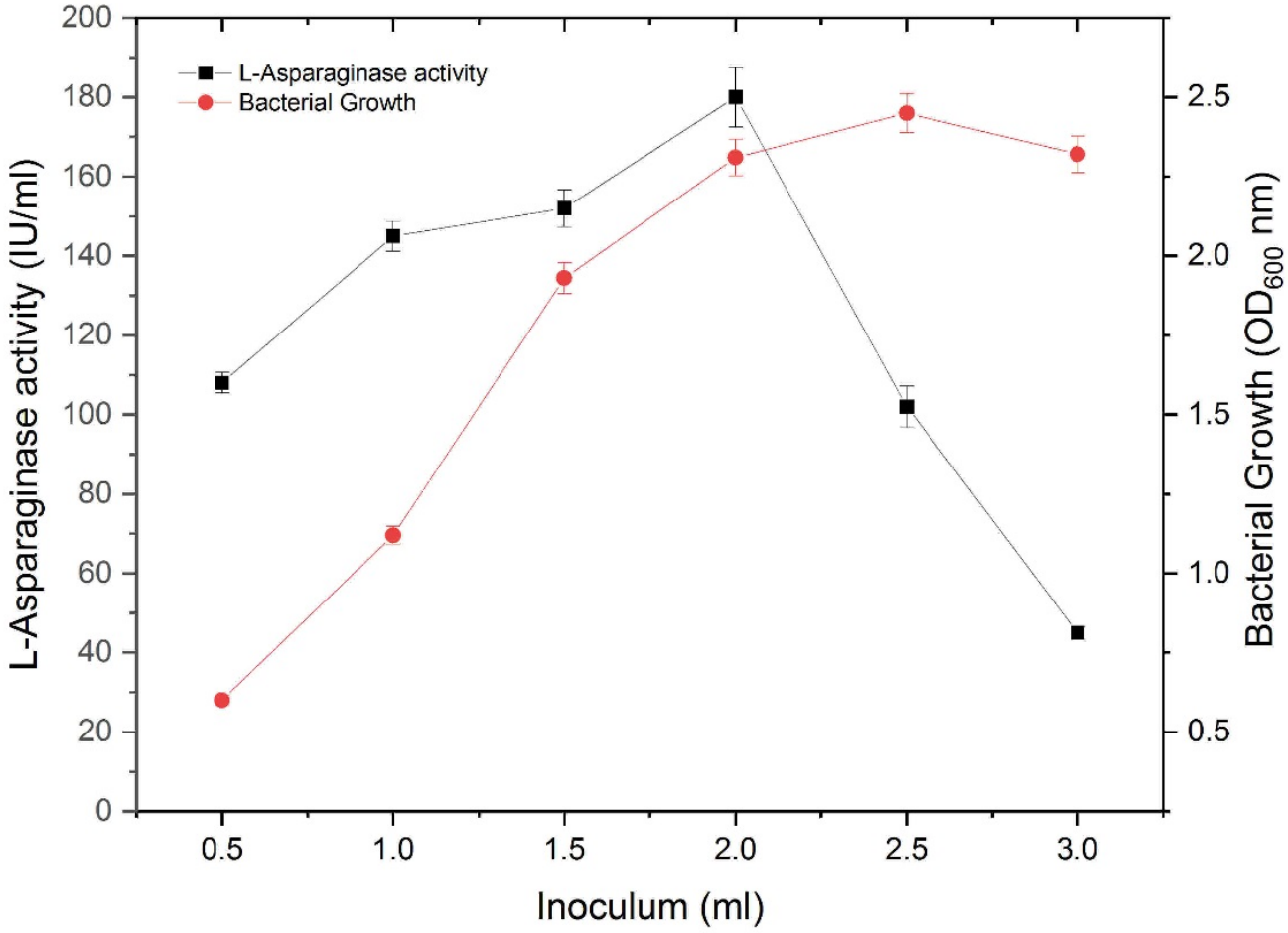

Fig 8: Effect of inoculum level on L-Asparaginase production by *B.subtilis* THARAKA

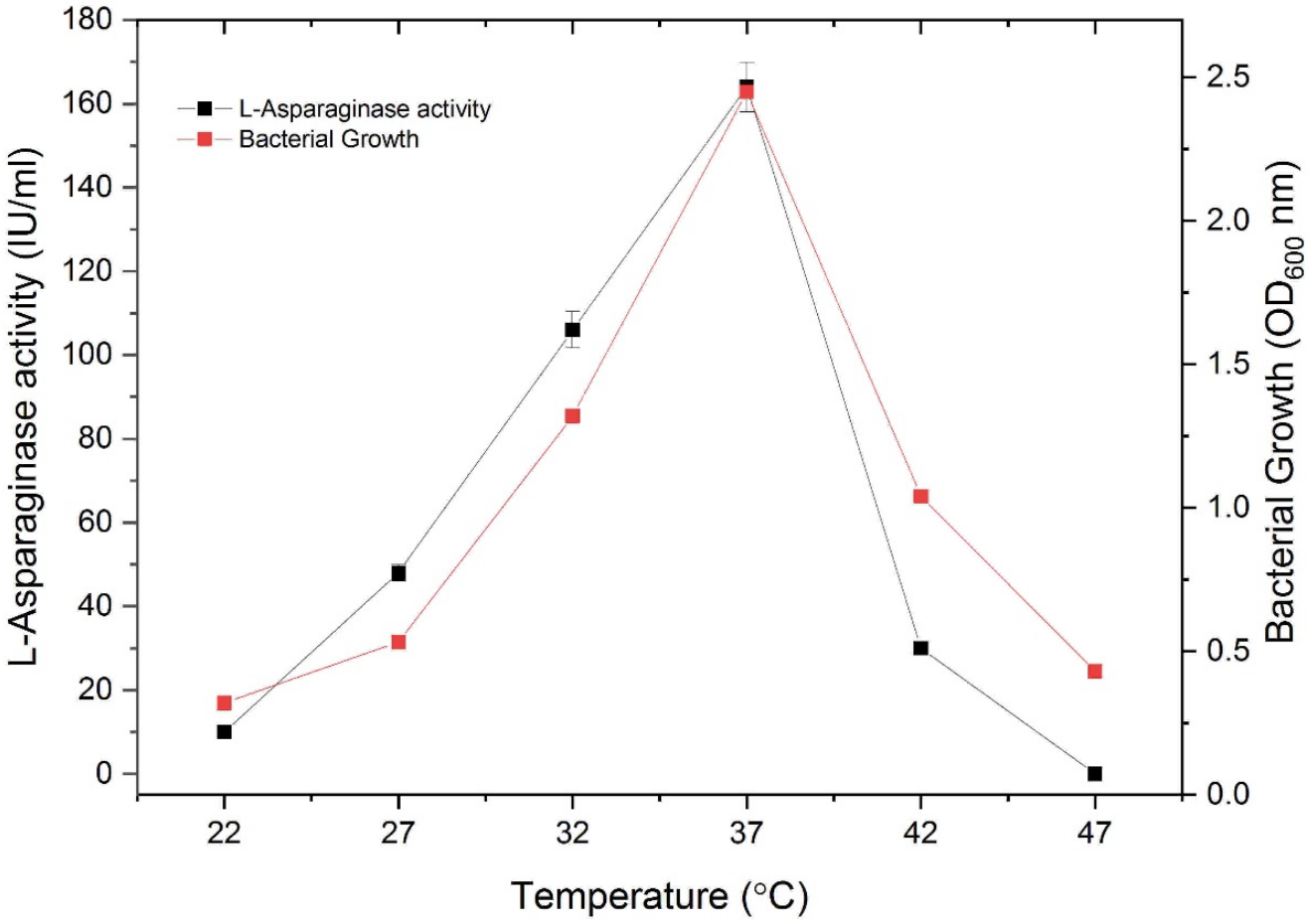

Fig 9: Effect of incubation temperature on L-Asparaginase production by *B.subtilis* THARAKA

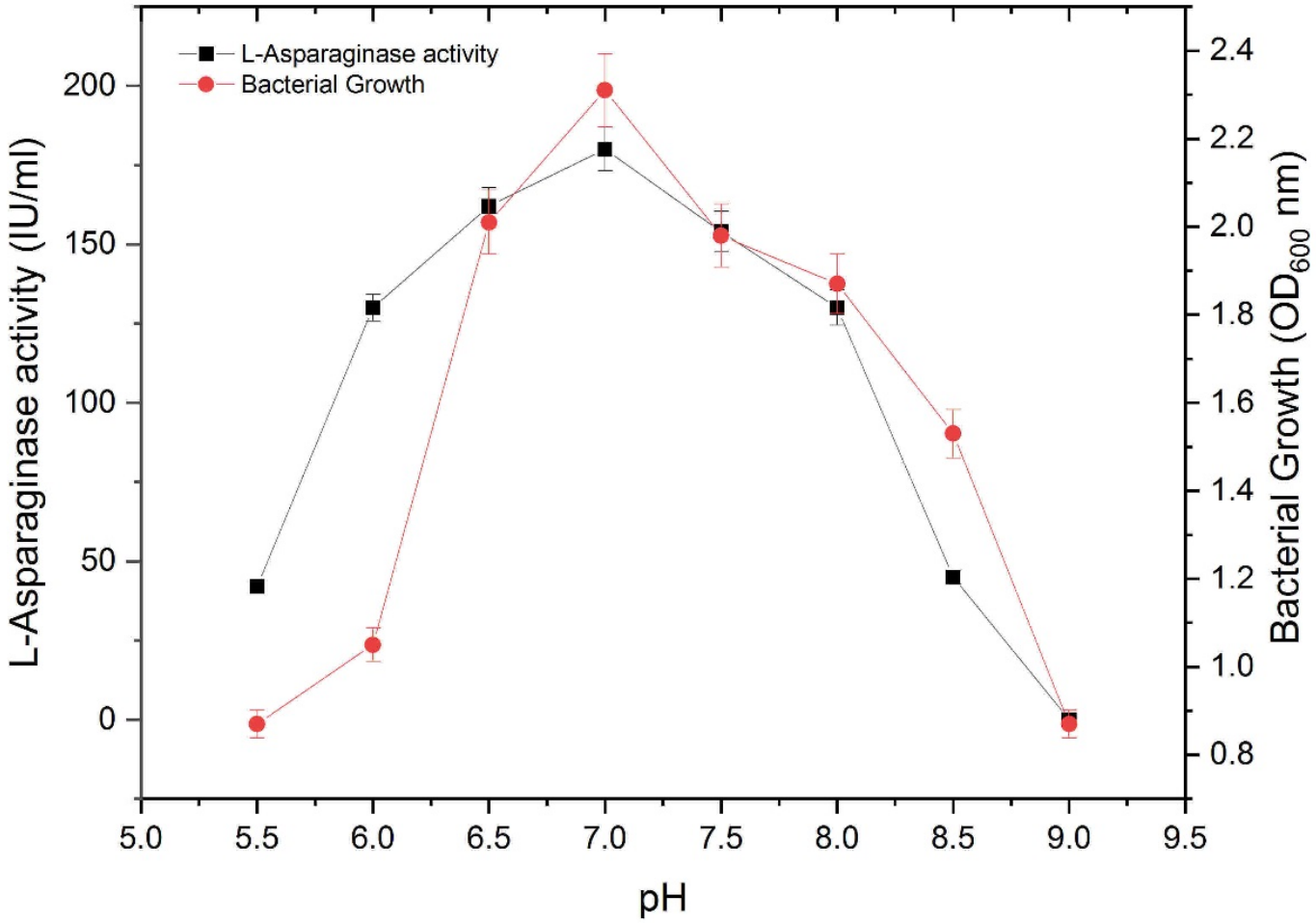

Fig 10: Effect of initial pH on L-Asparaginase production by *B.subtilis* THARAKA

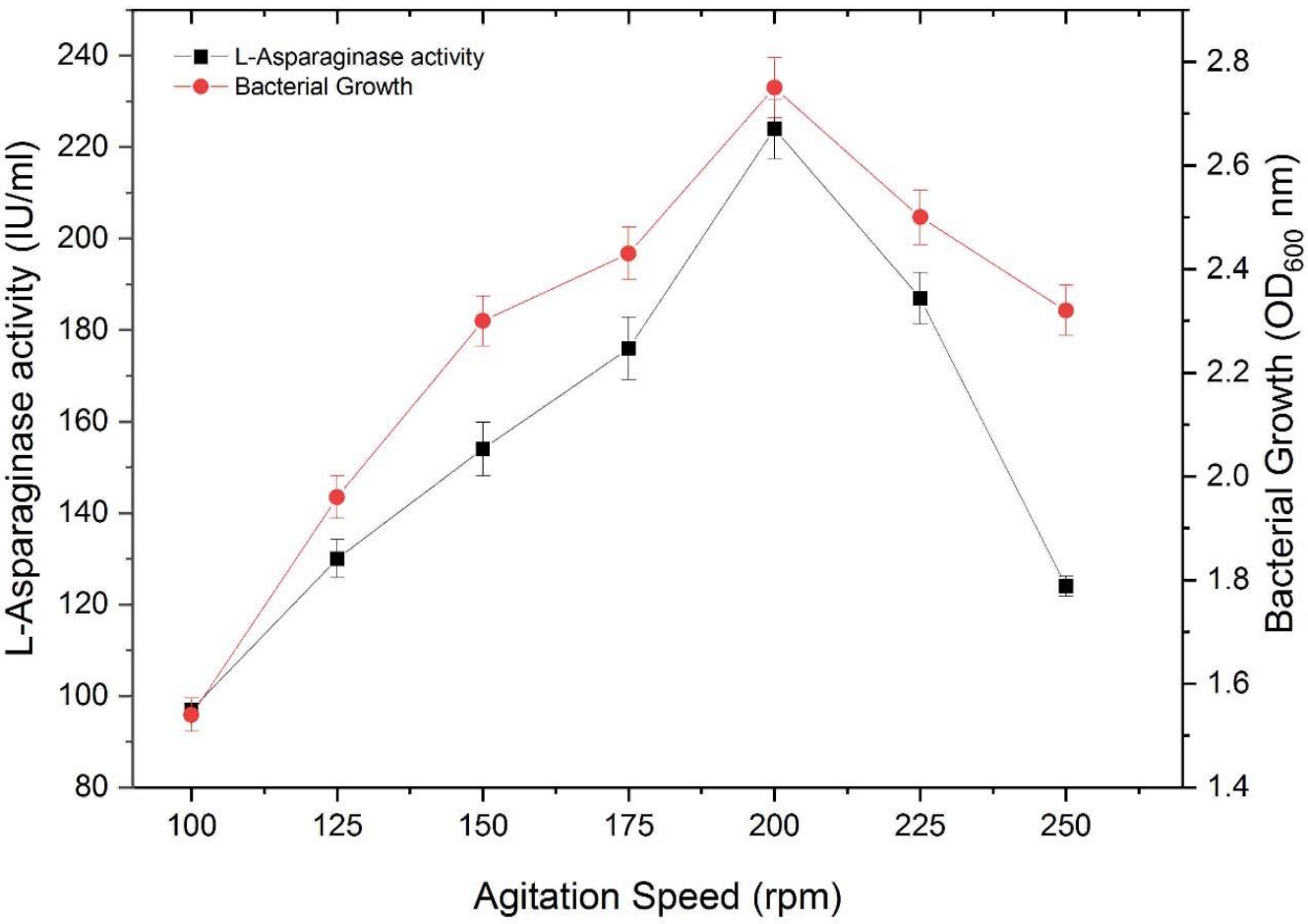

Fig 11: Effect of Agitation speed on L-Asparaginase production by *B.subtilis* THARAKA

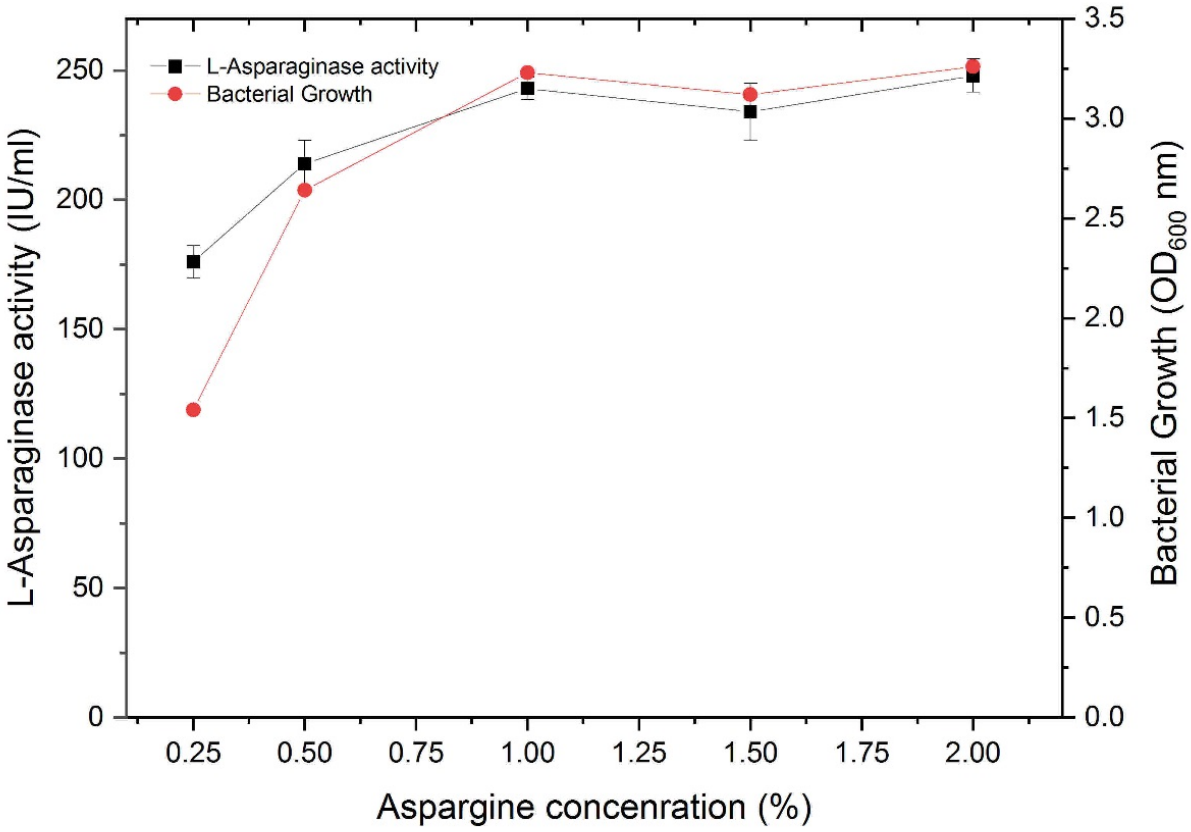

Fig 12: Effect of L-asparagine concentration on L-Asparaginase production by *B.subtilis* THARAKA

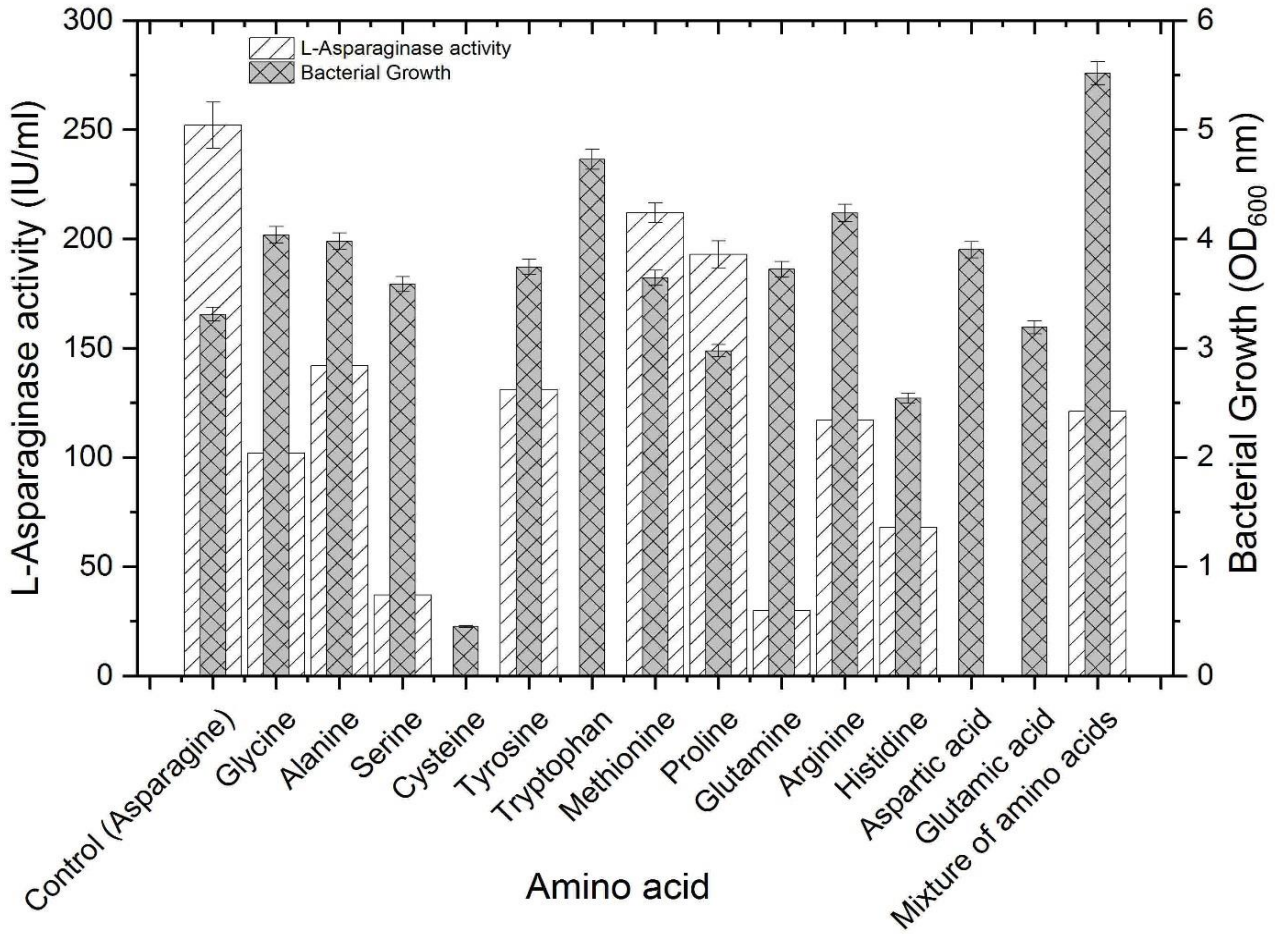

Fig 13: Effect of amino acids supplements on L-asparaginase production by *B.subtilis* THARAKA