

Original Research Article

Screening and characterization of L-asparaginase producing bacterial strains from the soil in Bangladesh

ABSTRACT

Although the enzyme L-asparaginase (L-ASNase) from *Escherichia coli*, *Erwinia* and *Serratia* has been applied to treat certain lymphomas and leukemias, several medical complications such as severe immunological responses leading to hypersensitivity, anaphylaxis, etc. have limited its application. The researchers have documented that such impediments are due to the different biochemical and kinetic properties of L-ASNase, which are directly dependent on genetic variations in microbial strains. Therefore, there is a compelling need to explore novel L-ASNase producing microorganisms that would exhibit different serological properties while retaining similar and/or better therapeutic effects against cancer cells. Heretofore, L-ASNase producing bacterial strains from Bangladesh have never been isolated and characterized. Therefore, the main objective of this research was to isolate and characterize these strains from unexplored and ecologically different habitats that could lead to developing a potential therapeutic drug with fewer immunological responses and side effects over the existing drugs in order to treat cancer patients in the near future. Two L-ASNase producing bacterial strains were successfully isolated from the soil of Hatirjheel lake in Dhaka for the first time. Molecular characterization revealed that both strains belonged to *Pseudomonas aeruginosa* and their DNA sequences were submitted to NCBI GenBank. The accession number OK446669 was obtained for the strain of *P. aeruginosa* EWUKR-1 and OL307081 for *P. aeruginosa* EWUKR-2. The specific activity of L-ASNase from EWUKR-2 (212.1 ± 14.8 U/mg protein) was significantly higher than that of EWUKR-1 (16.3 ± 0.8 U/mg protein) when they were grown in modified M9 media containing 0.5 g/l glucose at 37°C for 24 hours. The experimental results revealed that both of these bacterial strains were extracellular L-ASNase producers. The enzyme from *P. aeruginosa* EWUKR-2 was partially purified using saturated ammonium sulfate followed by dialysis and concentrated using Vivaspin-20 centrifugal concentrator having MWCO of 30 kDa. The optimum temperature and pH of the partially purified enzyme were 37°C and 7.5, respectively. The purification-fold after ammonium sulfate precipitation and yield of the

concentrated enzyme were 2.8 and 101%, respectively. SDS-PAGE analysis revealed that the molecular weight of L-ASNase from *P. aeruginosa* EWUKR-2 was around 43 kDa.

Keywords: Bangladesh, L-asparaginase, soil, bacteria, therapeutic drugs, cancer

1. INTRODUCTION

Enzymes-based medications exhibit two critical features that make them superior over other drugs. Firstly, the enzymes have the property to bind specifically with their targets (ligands) with greater affinity. Secondly, the catalytic properties of enzymes can convert multiple ligands to the targeted end products. Thus, these two important features have made enzymes explicit and powerful medications that could achieve helpful organic chemistry in the body while other chemical drugs cannot do. These qualities have brought about the improvement of numerous enzyme drugs to treat a wide range of human diseases. Therefore, pharmaceutical research has now been focused on the application of enzyme technologies and exploring the enzymes as drugs have been growing in recent years. The concept of using enzymes as therapeutic drugs has been around for the last forty years. For example, the enzymes were suggested as an alternative therapeutic drug for genetic disorders in the 1960s [1]. In 1963, the research works showed that L-asparaginase (L-ASNase) presence in the sera of guinea pigs exhibited antineoplastic activities [2]. Partial purification of L-ASNase from the sera of guinea pig reconfirmed this antineoplastic activity by the scientist Broome [3].

The enzyme L-ASNase is now an acceptable therapeutic agent to treat certain leukemias and lymphomas. This enzyme based drug has also been explored to treat lymphoblastic leukemia and Non-Hodgkin's lymphoma in humans with higher efficiency. Moreover, this enzyme has been used to treat other diseases such as childhood acute lymphoblastic leukemia (ALL), reticulosarcoma, Hodgkin disease, acute myelocytic leukemia (AML), acute myelomonocytic leukemia (AMML), chronic lymphocytic leukemia (CLL), lymphosarcoma and pancreatic carcinoma as well as melanosarcoma in combination with different chemotherapeutic agents [9, 10]. Furthermore, this enzyme has shown growth inhibition in two different types of human cells, such as carcinoma of hepatic cells (Hep-G2) and carcinoma of intestinal cells (Hct-116) with the half maximal inhibitory concentration (IC_{50}) value of about 8.4 $\mu\text{g/ml}$ and about 4.7 $\mu\text{g/ml}$, respectively [11]. The research work has also shown that the treatment with

low frequency laser combined with administration of L-ASNase and polyphenoloxidase was able to decrease the adhesion capability of pathogenic *E. coli* to human red blood cells [12].

L-ASNase enzyme is available commercially as pegaspargase (trade name oncaspar manufactured by Exelead Inc.), crisantaspase, kidrolase (manufactured by Jazz Pharmaceuticals Inc.), erwinase (produced by Porton Biopharma) and elspar (manufactured by Merck and Company, Inc.) [14]. L-ASNase can also be used as a model enzyme for the development of new drug delivery systems [16], and as a biosensor for the early detection of leukemia [17]. L-ASNase production has been reported from the bacteria *E. coli* [9]; the bacteria *Aerobacter* and *Erwinia* [18]; the bacteria *Serratia* and *Xanthomonas* [19]; the bacteria *Pectobacterium* and *Photobacterium* [20]; the strains of *Pseudomonas* [32-34]; the gram-positive bacterium *Streptomyces* and the fungus *Aspergillus* [18, 20]. The purified L-asparaginase enzyme from the bacteria *E. coli* and *Erwinia* as well as *Serratia marcescens* was applied successfully to treat tumor and leukemia [19]. However, these enzyme drugs from the above reported sources pose several limitations, such as inducing severe immunological responses that lead to hypersensitivity whenever they are used for long-term continued treatment, hyperallergic reactions and anaphylaxis [21].

The researchers have pointed out that the different biochemical and kinetic properties of L-ASNase are due to the genetic variations in microbial strains [22], which suggest that there is a compelling need to explore a new source of this enzyme from other microorganisms with different serological properties while retaining similar and/or better therapeutic effects to treat cancer patients. Therefore, the main objective of this research was to search for novel L-ASNase producing bacterial strains from unexplored and ecologically different habitats like the soil samples in and around the Dhaka city, followed by characterization that might be helpful to develop a potential biopharmaceutical drug with fewer immunological responses and side effects over the existing drugs for treating the cancer patients in near future. To the best of our knowledge, this is the first time to isolate and characterize the L-ASNase producing bacterial strains from the soil in Bangladesh.

2. MATERIALS AND METHODS

2.1 Materials and chemicals

All materials were purchased from Hi-Media (India). The ingredients for culture media were from Sigma (USA). Nessler's reagent was purchased from Fluka (Switzerland). L-asparagine, L-glutamine, urea and bromothymol blue (BTB) were purchased from Spectrochem (India).

Other chemicals and reagents used were of analytical grade from Hi-Media (India) and various commercial sources.

2.2 Sample collection and isolation of bacterial strains

Soil samples were collected in sterilized poly-bags from the depth of ~30 cm from the different areas of Dhaka city, such as Aftabnagar, Banasree, Hatirjheel lake and Hazaribagh, as well as the nearby city of Dhaka such as Savar and Gazipur according to the method described by Prescott *et al.* [23]. The above locations were considered because these sites are basically industrial and waste dumping areas, which were assumed to be very rich in microbial flora where the microbes at these sites might have undergone frequent mutations. Isolation of bacteria was performed by the serial dilution method using nutrient agar (NA) medium (pH 7.0). The sample suspensions were prepared by adding 10 grams of soil into conical flasks (250 ml) containing 90 ml of sterile normal saline followed by mixing using a rotary shaker at 50 rpm for 30 minutes. After sedimentation of the suspended materials, the clear supernatant was decanted and used (50 μ L) to spread on NA plates. The inoculated NA plates were incubated at 37⁰C temperature for 24 to 72 hours depending on bacterial growth status.

2.3 Primary screening of L-asparaginase producing bacterial strains

Morphologically different individual colonies were selected and streaked on the agar plates containing modified M9 medium supplemented with 0.001% BTB [10]. The overlapping colonies were re-streaked to obtain pure culture. The pH of modified M9 agar medium was adjusted to 7.0, which contained 6.0 g/l of Na₂HPO₄·2H₂O, 3.0 g/l of KH₂PO₄, 0.5 g/l of NaCl, 10.0 g/l of L-asparagine, 2.0 ml/l of 1.0 M solution of MgSO₄·7H₂O, 1.0 ml/l of 0.1 M solution of CaCl₂·2H₂O, 0.2 g/l glucose and 15 g/l agar. To screen L-asparaginase (L-ASNase) producing bacterial strains primarily, the isolated colonies with green and greenish-blue halos were selected from the agar plates and stored at 4⁰C for subsequent analysis. As a control, the M9 agar plates were prepared using the same compositions without L-asparagine.

2.4 Measurement of L-ASNase, L-glutaminase (L-GLNase) and urease activities

To measure the enzyme activities, L-ASNase positive strains were cultured in M9 liquid media (pH 7.0) without BTB using 250ml Erlenmeyer flasks where the volume of the media was 100 ml. The un-inoculated medium in the flask with the same volume was also cultured to serve as negative control. After incubation at 37⁰C on a rotary shaker (150 rpm), the culture broth was centrifuged at 10,000 rpm for 10 min and the supernatant was collected,

and stored at 4⁰C. The cell pellets were also kept and preserved at -20⁰C. The activities of L-ASNase, L-GLNase and urease were measured based on the principles of Nesslerization reaction [24, 30] with slight modification. Briefly, the released ammonia from the reaction reacts with Nessler's reagent and produces an orange color. Absorbance of the colored solution was measured at 500 nm using a spectrophotometer (T60 UV-Vis, Leicestershire, UK). The enzyme assay mixture consisted of 1.0 ml of freshly prepared 40 mM substrate (L-Asn, L-Gln and urea) in 50 mM Tris-HCl buffer (pH 7.5) and 100 µL of crude extract of the enzyme. The reaction mixture was incubated at 37⁰C for 15 min and the reaction was stopped by adding 1.0 ml of 110 mM trichloro acetic acid. 1.0 ml of Nessler's reagent was then added to the reaction mixture followed by vortex and the absorbance of the final solution was recorded. A blank reaction was prepared in parallel where 100 µL of 50 mM Tris-HCl (pH 7.5) was added instead of sample supernatant. The enzyme activities were calculated based on a standard curve that was constructed using different concentrations of ammonium sulfate dissolved in 50 mM Tris-HCl (pH 7.5), and were expressed as International Unit (IU). One IU represents the amount of enzyme that liberates 1.0 µmole of ammonia per minute at 37⁰C temperature. The Bradford method [35] was used to measure the concentration of total protein from the samples based on a standard curve, which was constructed using different concentrations of bovine serum albumin (BSA) dissolved in 50 mM Tris-HCl (pH 7.5). All experiments were repeated three times and all data were presented as the mean values with standard deviations.

2.5 Characterization of L-asparaginase (L-ASNase) producing bacterial strains

Genomic DNA of L-ASNase producing bacterial strains was extracted according to the established protocol [25]. PCR was performed to amplify the 16S rRNA gene using bacterial universal primers 27-Forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-Reverse (5'-CGGTTACCTTGTTACGACTT-3') [26]. The amplified PCR products were tested on 1.2% agarose gel and sequencing was accomplished by the National Institute of Biotechnology in Bangladesh. BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to identify the taxonomy of the strains [27]. The sequences of rDNA were submitted to NCBI GenBank to obtain the accession numbers and a phylogenetic tree was constructed using MEGA11 software [28]. Some landmark morphological and biochemical tests such as shape, gram staining, catalase and oxidase tests of the identified bacterial strains were also performed according to Bergey's manual [29].

2.6 Protein purification and SDS-PAGE analysis

L-ASNase enzyme was partially purified using 80% saturated ammonium sulfate ((NH₄)₂SO₄) according to the method of El-Bessoumy et al. with slight modification [8]. Briefly, finely powdered (NH₄)₂SO₄ was added to the sample supernatant with mild stirring, and the mixture was allowed to settle for 9 hours at 4⁰C followed by centrifugation at 10,000 rpm for 30 min at 4⁰C. The supernatant was discarded and precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM phenylmethanesulfonyl fluoride (Sigma, USA) as protease inhibitor and dialyzed overnight against the same buffer at 4⁰C using dialysis tube having MWCO of 10 kDa (Spectrum™, Thermo Fisher, USA). The enzyme solution was then concentrated using Vivaspin-20 centrifugal concentrator with MWCO of 30 kDa (Sigma, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight (M_w) of the partially purified L-ASNase [31]. The gels contained 12% polyacrylamide and Coomassie brilliant blue R-250 was used to stain the protein bands. M_w of the purified enzyme was estimated using a standard protein ladder (Bio-Rad, USA).

2.7 Effect of different pH's and temperatures on the activity of L-ASNase

To determine the effect of different temperatures on the purified enzyme, specific activities of L-ASNase were determined at temperatures ranging between 25⁰C and 49⁰C. For determining the effect of pH, the purified enzyme was mixed in 50 mM Tris-HCl buffer with pH values ranging from 6.0 to 9.0. The specific activities of L-ASNase were then measured according to the protocol mentioned above. All measurements were conducted three times.

3. RESULTS

3.1 Isolation and characterization of L-asparaginase (L-ASNase) producing strains

The soil samples were collected from 51 spots of different areas as mentioned in the section on materials and methods. Based on primary screening, two L-ASNase producing bacterial strains named EWUKR-1 and EWUKR-2 were isolated successfully from 5,000 bacterial colonies. These two strains were found in 2 spots of Hatirjheel lake area, indicating that the other explored spots were not suitable habitat for L-ASNase producing microorganisms. Figure-1 shows the growth of L-ASNase producing strains on modified M9 agar plates containing BTB dye. Since BTB gives a transient green colour at neutral pH and dark blue colour at higher pH [10], green color appeared around the deep blue halo in the case of strain EWUKR-2 because of the enzyme's diffusion throughout the media containing L-asparagine. This phenomenon was not observed for the strain EWUKR-1. The deep blue zone around the

L-ASNase producing bacterial strain EWUKR-2 resulted from the increase in pH due to ammonia release (Fig. 1d), which indicated the potency of L-ASNase synthesis by that strain.

A single band of ~1400bp on the agarose gel was obtained through PCR amplification of genomic DNA from both bacterial strains. The PCR products were sequenced and the obtained DNA sequences were subjected to perform BLAST for identification of the strain's taxonomy and a phylogenetic tree was constructed using MEGA11 software. The phylogenetic analysis revealed that these two bacterial strains belonged to *Pseudomonas aeruginosa* (Fig. 2). The nucleotide sequences of 16 rRNA genes were submitted to the NCBI GenBank and accession numbers for both strains were obtained, which were OK446669 for strain EWUKR-1 and OL307081 for strain EWUKR-2. Subsequently, these two L-ASNase producing bacterial strains were recognized as *P. aeruginosa* EWUKR-1 and *P. aeruginosa* EWUKR-2. Some landmark morphological and biochemical tests such as shape, gram staining, catalase and oxidase of the identified bacterial strains were also performed to make sure of the correctness of their taxonomic identifications (Fig. 3).

3.2 Effect of glucose concentration and culture time on L-ASNase production

L-ASNase producing strains *P. aeruginosa* EWUKR-1 and *P. aeruginosa* EWUKR-2 were cultured in modified M9 liquid media without BTB containing different glucose concentrations under shaking condition (150 rpm) at 37⁰C for 24 hours. The glucose concentrations tested in this study were 0.2, 0.5, 2.0, 5.0 and 10 g/l (Fig. 4). The culture broth was harvested by centrifugation and the supernatant was collected followed by storing at 4⁰C. The cell pellet was also collected and washed three times using 50 mM Tris-HCl (pH 7.5) followed by preserving at -20⁰C. The specific activities of L-ASNase were measured from the culture supernatant and cells where the cell pellet was lysed using lysis buffer. It was found that increasing the glucose concentration from 0.2 g/l to 0.5 g/l resulted in a drastic increase in specific L-ASNase activities for both strains. The higher enzyme activities were observed for *P. aeruginosa* EWUKR-2 compared to that of *P. aeruginosa* EWUKR-1. But, further glucose concentration increasing from 0.5 g/l to 10 g/l significantly decreased specific L-ASNase activities in both strains (Fig. 4). In fact, glucose concentration ranging between 2 g/l and 10 g/l both had no significant effect on L-ASNase activities in both EWUKR-1 and EWUKR-2. The highest enzyme activity of EWUKR-1 was 16.3 ± 0.8 U/mg protein and 212.1 ± 14.8 U/mg protein for EWUKR-2 when cultured at 0.5 g/l glucose concentration. These results indicated that initial glucose concentration in the culture media has a dramatic effect on L-ASNase synthesis in *P. aeruginosa* EWUKR-1 and *P. aeruginosa* EWUKR-2. No

L-ASNase activities were detected in the lysed cells of both bacterial strains, indicating that these strains were the sole extracellular L-ASNase producers.

To explore the effect of cultivation time on L-ASNase production, both strains EWUKR-1 and EWUKR-2 were cultivated at 37⁰C in modified M9 liquid media containing 0.5 g/l glucose under shaking condition. The culture times explored in this study were 18, 24, 42, 48, 66 and 72 hours (Fig. 5). It was found that specific L-ASNase activities significantly increased in both strains when cultivation time was augmented from 18 hours to 24 hours. But, the enzyme activities were found to be decreased dramatically after 24 hours, indicating its degradation in the culture media. The specific enzyme activity of *P. aeruginosa* EWUKR-1 was 11.1 ± 0.9 U/mg protein and 103.0 ± 6.1 U/mg protein for *P. aeruginosa* EWUKR-2 at 42 hours (Fig. 5). The activities of L-glutaminase (L-GLNase) and urease were also measured from the supernatant and cell pellets of both isolated bacterial strains. The results showed no activities of L-GLNase and urease in both *P. aeruginosa* EWUKR-1 and *P. aeruginosa* EWUKR-2. These results indicated that both of these strains were L-ASNase producers.

3.3 Effect of pH and temperature on the activity of L-ASNase

As shown in Figure-4 and Figure-5, the strain *P. aeruginosa* EWUKR-2 was found to be very prominent due to its highest enzyme activity over *P. aeruginosa* EWUKR-1. Therefore, L-ASNase enzyme was partially purified from *P. aeruginosa* EWUKR-2 by (NH₄)₂SO₄ precipitation followed by dialysis. A Vivapin-20 centrifugal concentrator with MWCO of 30kDa was used to concentrate the partially purified enzyme after dialysis. The specific activities of L-ASNase were investigated as a function of pH and time (Fig. 6). The pH used in this investigation was 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. It was found that L-ASNase enzyme was very active over the studied pH ranges where the reaction temperature was maintained at 37⁰C. The specific activity of the enzyme increased gradually till pH 7.5 (Fig. 6a). At pH higher than 7.5, the specific enzyme activity was found to be decreased. The maximum specific activity of L-ASNase (599.5 ± 20.3 U/mg protein) was at pH 7.5. These results indicated that the optimum pH of L-ASNase enzyme from *P. aeruginosa* EWUKR-2 was 7.5. The temperatures used in this study were 25, 29, 33, 37, 41, 45 and 49⁰C where the pH was 7.5. The results revealed that L-ASNase was active at a wide range of temperatures. The highest specific enzyme activity was found at 37⁰C (Fig. 6b). The specific L-ASNase activities at 25⁰C and 49⁰C were 254.9 ± 15.3 and 211.3 ± 18.3 U/mg protein, respectively. The specific enzyme activity was found to be decreased when the temperature was increased

above 37°C. These results revealed that the optimum temperature of L-ASNase from *P. aeruginosa* EWUKR-2 was 37°C.

4. DISCUSSION

In 1982, the first genetically engineered drug, insulin, was approved by the Food and Drug Administration (FDA). Then, the second recombinant protein drug Activase® (alteplase, recombinant human tissue plasminogen activator) was approved by the FDA in 1987 to treat heart attacks that were caused due to blockage in coronary arteries by blood clots. The approval of genetically engineered drugs insulin and alteplase opened up a new era for enzymes as therapeutic drugs. Because of the enzyme's characteristic features and enhanced potency, this research work was focused on screening and characterization of novel bacterial strains capable of producing L-ASNase from the soil in Bangladesh. The amino acid L-asparagine (L-Asn) is a very essential nutrient for both normal and tumor cells. The enzyme L-ASNase basically cleaves L-Asn into L-aspartate (L-Asp) and ammonia, and is present in plants, mammals, birds and yeasts as well as in different types of bacteria, but not in humans [4, 5]. Tumor cells have less capability to synthesize L-Asn. The reasons might be either due to the reduced activity of L-asparagine synthetase or the reduced level of its substrates, which are L-Asp acid and L-glutamine. Since tumor cells become dependent on the external source of L-Asn, these cells can be forced to be starved and subsequently eliminated by L-ASNase treatment that will ultimately cause them to deplete the L-asparagine in their proliferation, but not in normal cells. Therefore, the common practice is to provide L-ASNase enzyme through intravenous injection in order to treat cancer patients combined with other chemotherapeutic agents [6-8]. The enzyme L-ASNase is also currently used in the food industry to eliminate acrylamide from starchy fried foods because the compound acrylamide has been known as a very potent carcinogen and neurotoxin [13]. In the food industry, L-ASNase has been marketed as preventase (produced by DSM) and acrylaway (produced by Novozymes) [15]. Preventase (produced from *Aspergillus niger*) is used in bread, biscuits and breakfast cereals. Acrylaway (produced from *Aspergillus oryzae*) is used in baked goods, breakfast cereals, potato-based snacks, coffee and French fries.

Although the enzyme L-ASNase from *Escherichia coli* and *Erwinia* has been marketed to treat certain lymphomas and leukemias, several medical complications such as severe immunological responses leading to hypersensitivity, anaphylaxis, etc. have limited its application. The researchers have documented that such impairments are due to the different

biochemical and kinetic properties of L-ASNase, which are directly dependent on the genetic variations in microbial strains. Thus, there is an urgent need to explore the other L-ASNase producing microorganisms from unexplored and ecologically different habitats. To the best of our knowledge, this is the first study to isolate and characterize the L-ASNase producing bacterial strains from the soil in Bangladesh. Two L-ASNase producing strains were successfully isolated from the soil of Hatirjheel lake located in Dhaka. Molecular characterization revealed that both strains belonged to *Pseudomonas aeruginosa* and their DNA sequences were submitted to the NCBI GenBank. The accession number OK446669 was obtained for the strain of *P. aeruginosa* EWUKR-1 and OL307081 for *P. aeruginosa* EWUKR-2. The maximum specific L-ASNase activity was found in the strain *P. aeruginosa* EWUKR-2 when cultivated in modified M9 media at 37⁰C for 24 hours. The enzyme was partially purified using 80% (NH₄)₂SO₄ saturation followed by dialysis, which was then subjected to concentrate using Vivaspin-20 centrifugal concentrator. The specific L-ASNase activities from crude culture supernatant and concentrated enzyme were 212.1 U/mg protein and 597.8 U/mg protein, respectively. The optimum temperature and pH of partially purified enzyme from *P. aeruginosa* EWUKR-2 were 37⁰C and 7.5, respectively. These results corresponded with the L-ASNase enzyme from other strains of *P. aeruginosa* [32, 34]. The purification-fold after (NH₄)₂SO₄ precipitation was 2.8 and the yield of concentrated L-ASNase was 101%. The molecular weight (M_w) of partially purified enzyme was examined by SDS-PAGE analysis. As shown in Figure-7, the M_w of L-ASNase from *P. aeruginosa* EWUKR-2 was found to be approximately 43 kDa, which was different from the other strains of *P. aeruginosa* [32-34].

5. CONCLUSION

It can be concluded that taxonomic identification of *P. aeruginosa* strains isolated in this study corresponded with basic landmark morphological and biochemical tests such as shape, gram staining, catalase and oxidase. The specific activities of partially purified L-ASNase from *P. aeruginosa* EWUKR-2 at different temperatures and pH revealed its optimum conditions, which were 37⁰C and 7.5, respectively. Since the human body's normal physiological temperature is 37⁰C and blood pH ranges from 7.35 to 7.45, the L-ASNase produced from the above strain would be very suitable to be developed as an efficient drug for the treatment of different cancers. However, further in-depth analysis of this enzyme should be conducted to understand its anticancer properties.

6. REFERENCES

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FIGURES with LEGENDS

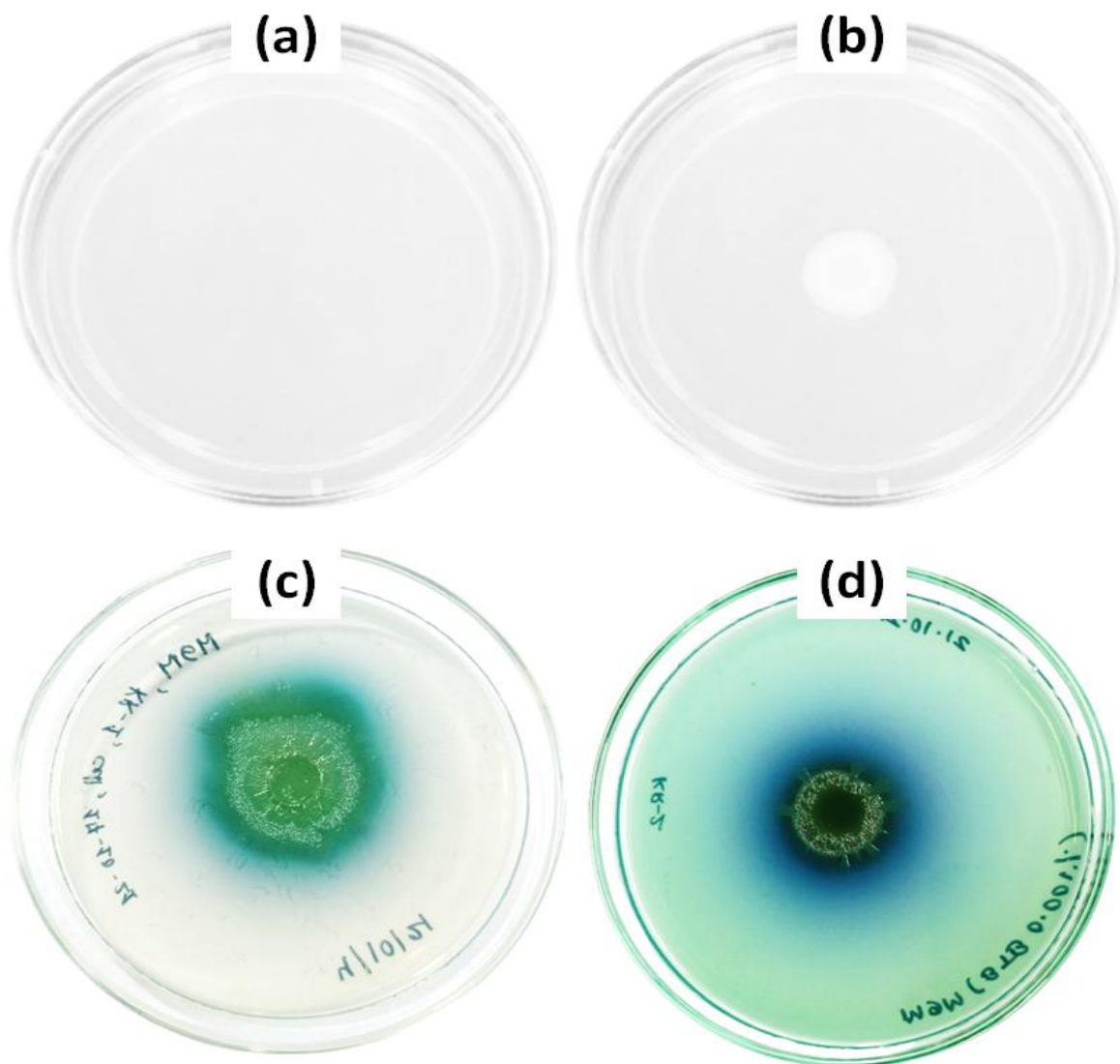


Figure-1. L-ASNase producing bacterial strain on M9 agar plates containing BTB dye: (a) uninoculated plate; (b) plate without L-asparagine inoculated with a L-ASNase producing

strain; (c) plate inoculated with L-ASNase producing strain EWUKR-1; (d) plate inoculated with L-ASNase producing strain EWUKR-2.

UNDER PEER REVIEW

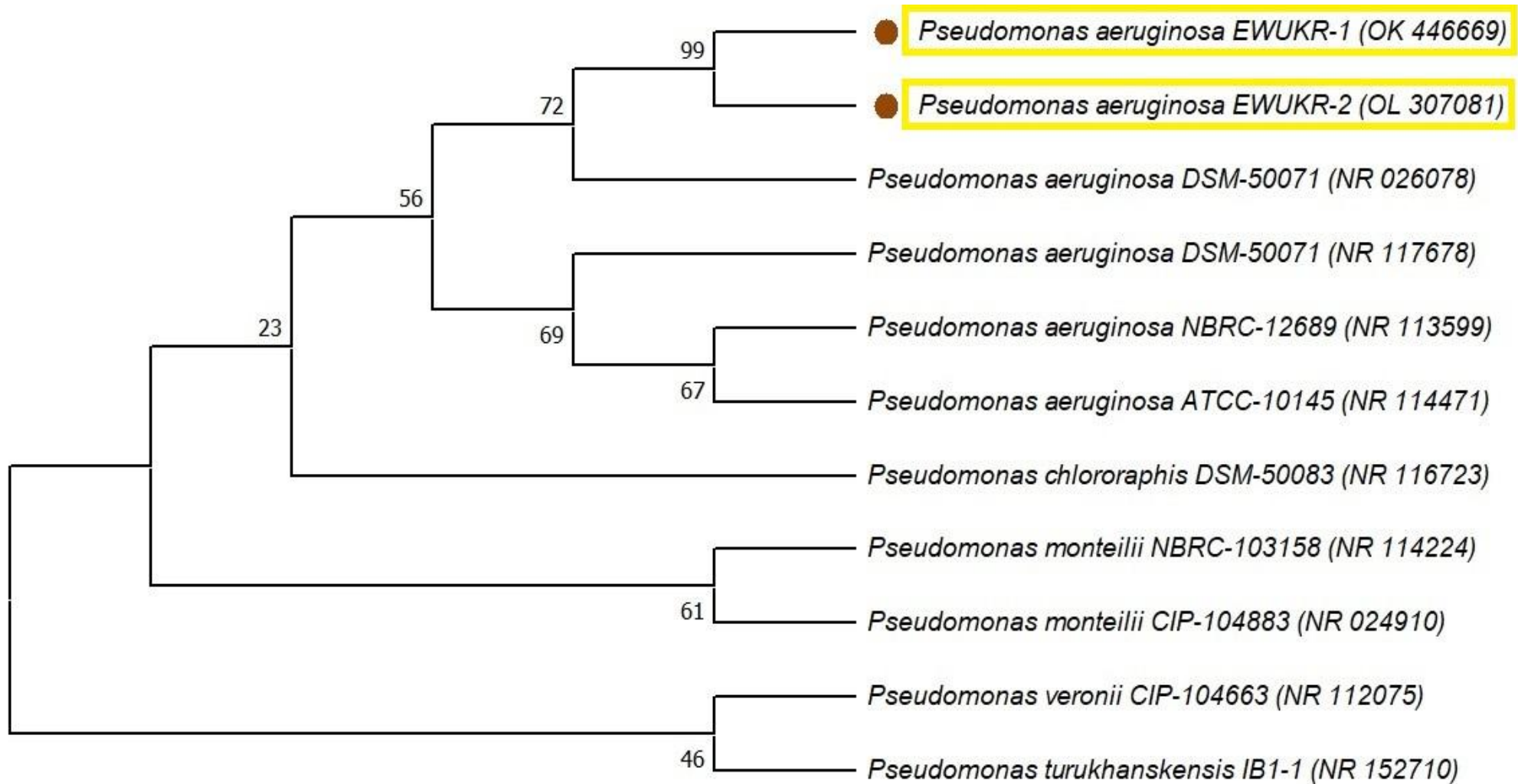


Figure-2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between the isolated L-ASNase producing strains (EWUKR-1 & EWUKR-2) and related species of the genus *Pseudomonas*. GenBank accession numbers are indicated in parentheses after the strain's name. Phylogenetic analysis was conducted using MEGA11 software.

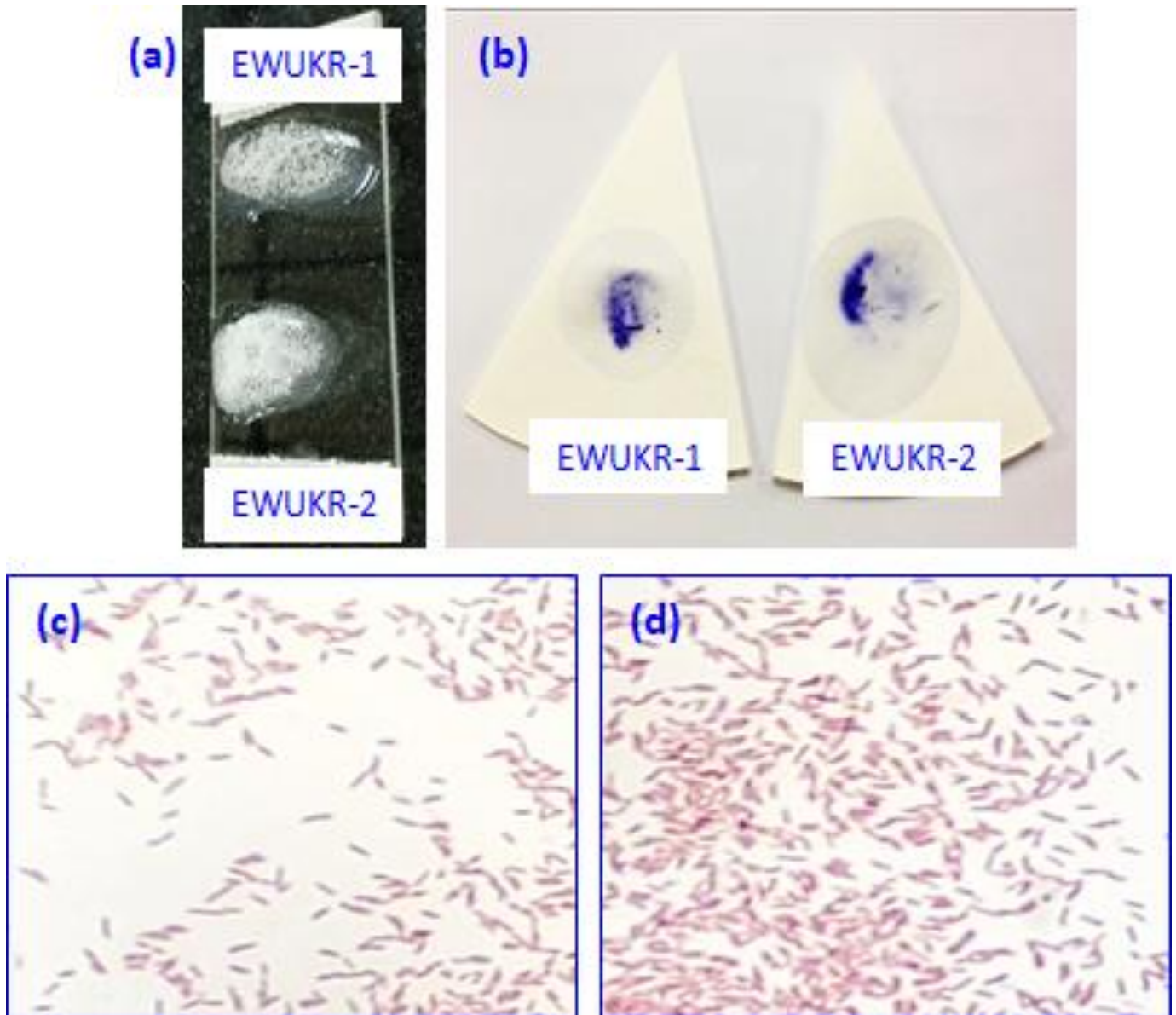


Figure-3. Morphological and biochemical tests of L-ASNase producing bacterial strains: (a) bubble formation indicated positive result of catalase test; (b) blue color indicated positive result of oxidase test; (c) Gram staining of EWUKR-1; (d) Gram staining of EWUKR-2.

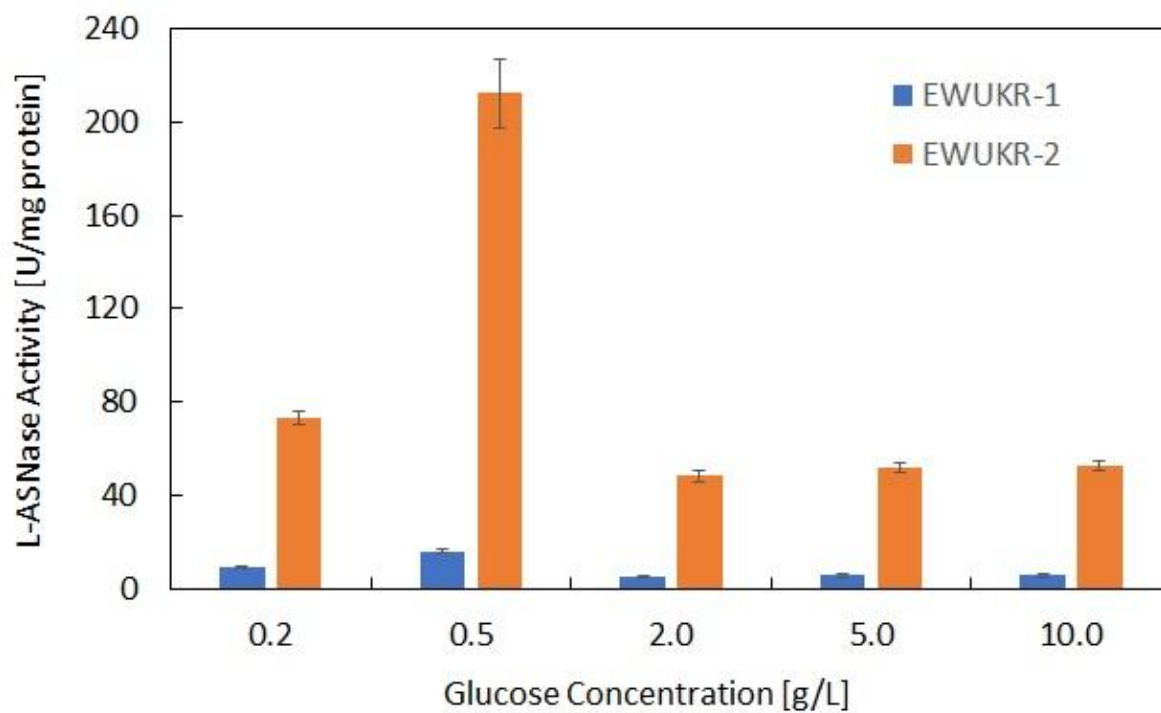


Figure-4. Effect of glucose concentrations on specific L-ASNase production in *P. aeruginosa* EWUKR-1 and *P. aeruginosa* EWUKR-2. The values and error bars represented here were the means and standard deviations from three independent experiments, respectively.

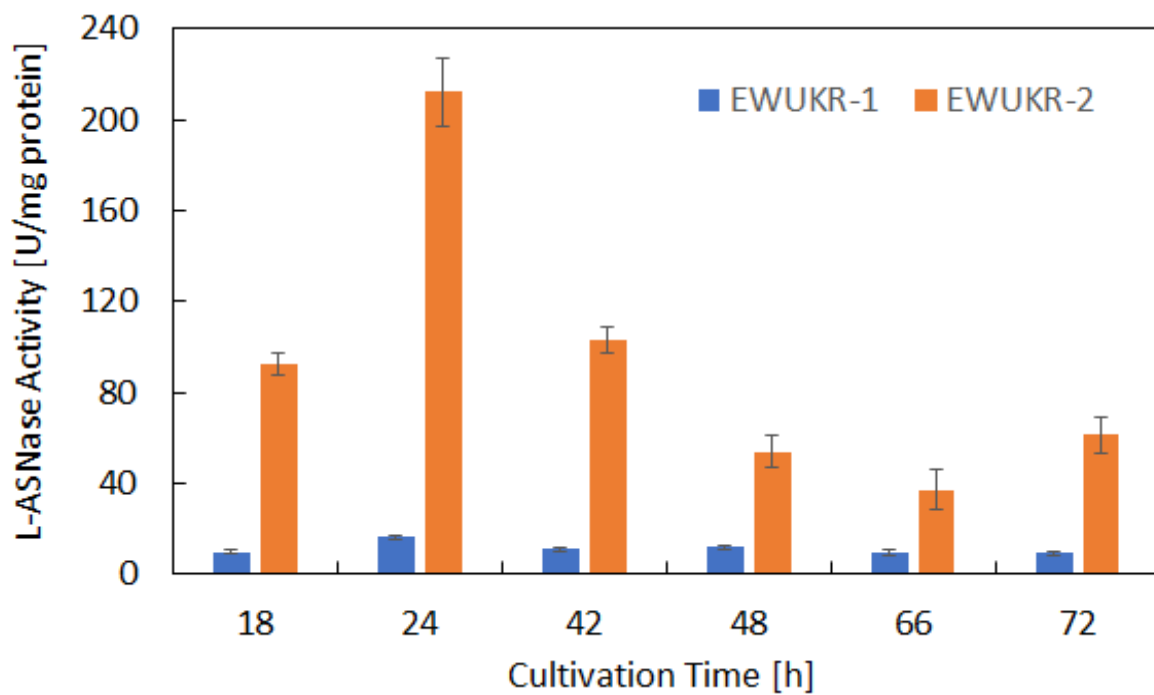


Figure-5. Effect of culture time on specific L-ASNase activity in *P. aeruginosa* EWUKR-1 and *P. aeruginosa* EWUKR-2. The values and error bars represented here were the means and standard deviations from three independent experiments, respectively.

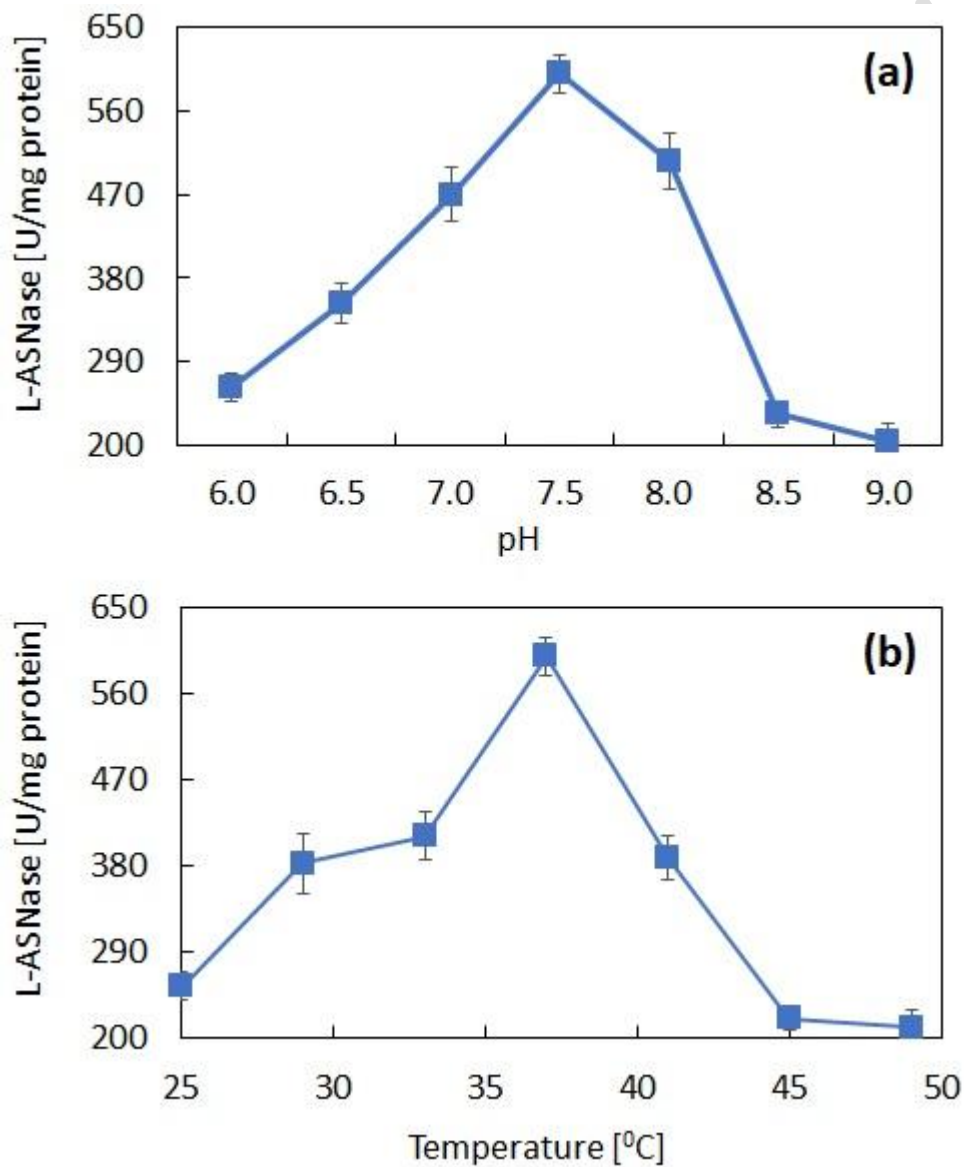


Figure-6. Effect of pH and temperature on L-ASNase activity of *P. aeruginosa* EWUKR-2. The values and error bars represented here were the means and standard deviations from three measurements, respectively.

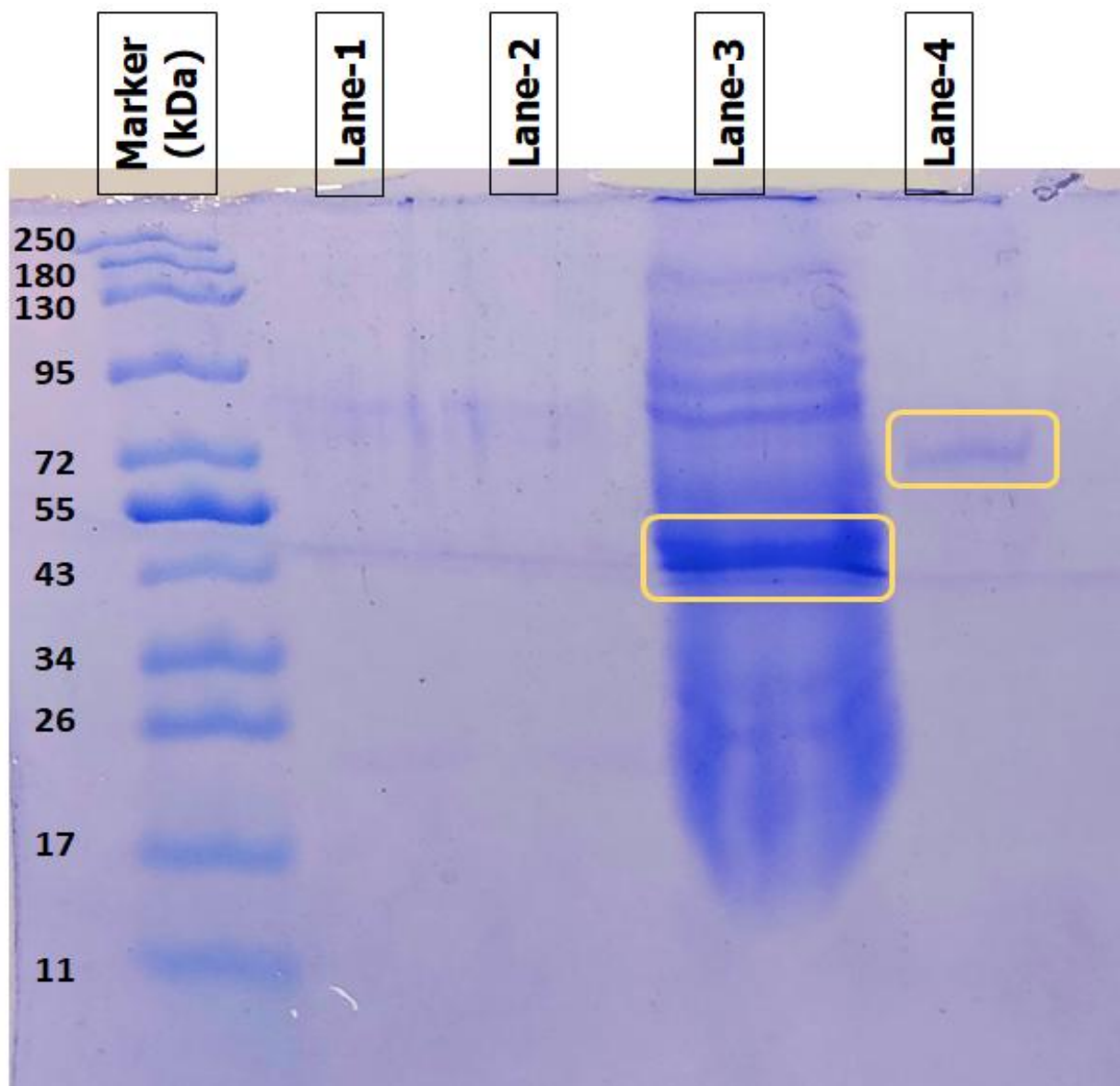


Figure-7. SDS-PAGE analysis of L-ASNase from *P. aeruginosa* EWUKR-2. Electrophoresis was carried out on a 12% polyacrylamide gel. The protein bands were stained using Coomassie blue R-250. 5 μ l of protein marker was loaded. 15 μ l of raw supernatant (Lane-1), filtrate from the Vivaspin-20 (Lane-2) and concentrated protein after dialysis (Lane-3) were loaded. In Lane-4, a total of 0.11 μ g of BSA was loaded. Yellow box in Lane-3 and Lane-4 represents the band of L-ASNase (~43 kDa) and BSA (~67 kDa), respectively.