

Original Research

Efficient Production, Purification and Characterization of Therapeutically Significant L-Asparaginase from *Bacillus licheniformis* ASN51

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Abstract

L-asparaginase (L-ASNase) is a versatile anticancer and acrylamide reducing enzyme used in medical and food industries. Exploration of new sources of L-asparaginase is in demand due to immunogenic issues, short half-life and narrow range of pH and temperature stability of available formulations. The present study describes extracellular Type II L-ASNase production from soil *Bacillus licheniformis* ASN51 strain. Optimized asparaginase production with a specific activity of 499 U mg⁻¹ was obtained at pH 8 and 37°C in M9 medium containing 0.2% glucose and 1% L-asparagine. L-ASNase was precipitated using acetone and purified through Sephadex G-100 column yielding 61.2 and 34% recovery, respectively. The purified asparaginase has a molecular mass of 38 kDa and specific activity of 8,333 U mg⁻¹. L-ASNase showed stability at a wide range of pH (4-9) and temperature (10-50°C) while retained 100% of its activity for 24 h at 37°C, pH 7. Enzyme kinetics revealed a *V*_{max} of 7750 U and *K*_m of 0.04 mM. Purified L-ASNase from *B. licheniformis* ASN51 showed antioxidant activity (IC₅₀ value 53.1 µg mL⁻¹) against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical and anticancer activity (53.3%) against HepG2 cell line. To the best of knowledge, among reported strains of *B. licheniformis*, ASN51 is the highest asparaginase producing strain. Purified L-ASNase

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showed long-term stability at physiological pH and temperature indicating its suitability for therapeutic applications.

Keywords: *Bacillus licheniformis*, L-asparaginase, production, optimization, characterization, therapeutic applications

Introduction

L-asparaginase (L-ASNase, E.C.3.5.1.1) is a commercially important enzyme that catalyzes the hydrolysis of L-asparagine (L-ASN) into L-aspartic acid and ammonia [1]. There are two types of L-asparaginase, namely type I and type II [2]. Type I asparaginase is a cytoplasmic (intracellular) enzyme that shows low affinity for L-asparagine. Whereas, only Type II L-asparaginase is extracellular and have higher L-ASN specificity and anticancer activity [3]. It is commonly found in animals, plants, fungi, yeast and bacteria [4-7]. The enzyme is primarily used in the treatment of acute lymphoblastic leukemia (ALL), hematological and non-hematological disorders [8]. It is also used in food industry to neutralize carcinogenic acrylamide and as a biosensor to detect asparagine levels during chemotherapy [9, 10].

L-ASNase targets cancer cells by starving them of asparagine. Cancer cells lack asparagine synthetase, hence cannot carry out de novo synthesis of asparagine. Thus, asparaginase (L-ASNase) activity causes depletion of asparagine in blood, resulting in the inhibition of protein synthesis for cancer cells [11]. Five commercial formulations of asparaginase are available for ALL treatment: three formulations are based on *E. coli* asparaginase, a pegylated form of *E. coli* asparaginase (Oncaspar) and recombinant asparaginase from *Erwinia chrysanthemi* (Erwinase) [12, 13]. L-ASNase is also a promising acrylamide mitigating agent that reduces carcinogenic acrylamide production in food industry. The carcinogenic acrylamide is produced through the Maillard reaction when starch-rich foods (potato fries, cookies) are treated above 100°C [13-15]. Currently, two commercial L-ASNase (Acrylaway and PreventASE) are available for processing starch-rich food at the industrial level [12]. Different challenges are associated with the current applications of this versatile enzyme. One of the major challenge in the therapeutic application is hypersensitivity reactions in ALL patients [16]. The prevalence of clinical asparaginase hypersensitivity is higher in children than in adults. L-ASNase associated hypersensitivity often results in therapy termination because it causes anaphylaxis or symptoms like dyspnea, pruritus, oedema, rash, coughing, and vomiting [17]. Similarly, L-ASNase is a thermolabile enzyme that hinders its application in food sector; PreventASE and Acrylaway can process food ingredients only at 50°C and 37°C respectively [12]. The inevitable use of L-ASNase in pharmaceutical and food industry and constraints associated with the commercially available

formulations require exploration of new sources of asparaginase with improved specific activity that could be produced through commercially viable processes.

Bacterial L-ASNases are preferred because bacteria can be cultured and manipulated in economical ways that can yield large-scale production in a short time [18]. Moreover, bacterial asparaginases are much easier to extract and purify, boosting their application at the industrial scale [19]. The composition of culture medium directly affects the final cost of a bioprocess. Each organism has different nutritional (carbon and nitrogen source) requirements for maximum production of enzyme. Some of the media commonly used at the laboratory scale are not cost-effective for application at a large scale. Hence, the exploration of cost-effective sources of carbon and nitrogen is important for scale-up production of industrial enzymes [20]. Considering this demand for bacterial asparaginase with improved enzymatic properties, this research was carried out to isolate an efficient bacterial strain from environmental samples for L-ASNase production. The enzyme was purified and characterized for its optimal functioning and effective therapeutic applications.

Materials and Methods

Isolation and Characterization of Asparaginase Producing Bacteria

For isolation of asparaginase producing bacteria, ten soil and nine water samples were collected from different cities of Pakistan. All the samples were serially diluted and spread on nutrient agar plates. After incubation at 37°C for 24 h, morphologically different colonies were selected. For qualitative screening of asparaginase producing bacteria, colonies were streaked on modified M9 minimal medium containing (g L⁻¹) Na₂HPO₄·2H₂O (6), KH₂PO₄ (3), NaCl (0.5), MgSO₄·7H₂O (0.01), CaCl₂·2H₂O (0.001), glucose (2), agar (20), L-ASN (10) and phenol red (0.09) as indicator [21]. For initial screening of L-ASNase producing bacteria, pH of L-asparagine supplemented M9 agar medium was kept acidic (6). After 48 h of incubation at 37°C, colonies having pink zone around them were selected [20]. Morphological, biochemical, and molecular characterization of the selected strain was carried out through standard Gram's staining, biochemical tests and 16S rRNA gene sequencing respectively. Gene sequence was submitted to the GenBank database for accession number.

Production and Quantification of L-asparaginase through Submerged Shake Flask Method

Extracellular L-ASNase production was carried out using modified M9 minimal medium containing (g L⁻¹) Na₂HPO₄·2H₂O (6), KH₂PO₄ (3), NaCl (0.5), MgSO₄·7H₂O (0.01), CaCl₂·2H₂O (0.001), glucose (2), and L-ASN (10) [21]. Nutrient broth inoculated with a loopful of fresh culture was incubated for 24 h at 37°C in orbital shaker at 75 rpm. 1% of inoculum was transferred to 100 mL of M9 medium. After every 24 h of incubation at 37°C, cells were centrifuged at 10,000 rpm, 4°C for 10 min. Supernatant was taken as crude enzyme and enzyme quantification assay was carried out through Nessler's method till sixth day of incubation [22]. In the first step, crude enzyme (0.5 mL) was mixed with 0.4 mM L-ASN (0.5 mL), 0.5 mL of 50 mM Tris-HCl buffer (pH = 7.4) and 0.5 mL of distilled water. All the contents were vortex mixed and incubated at 37°C for 30 min. Reaction was terminated by adding 0.5 mL of 1.5 M trichloroacetic acid. In the second step, 0.1 mL of previous reaction mixture, 0.1 mL of Nessler's reagent and 3.75 mL of distilled water was mixed and incubated at 37°C for 10 min in water bath. The absorbance was measured at 450 nm against the control. Enzyme units were interpreted in international unit. One international unit of enzyme is defined as the amount of enzyme required to produce one micromole of ammonia per min [23]. Standard curve of ammonia from ammonium chloride was prepared for calculating units of enzyme [24].

Optimization of Extracellular L-asparaginase Production from *Bacillus licheniformis* ASN51 under Submerged Fermentation

Submerged fermentation of L-asparaginase was carried out in M9 modified medium supplemented with 1% L-ASN. Different factors including pH (6, 7, 8), temperature (30, 37, 44°C), incubation time (day 1-6), 0.2% carbon sources (glucose, sucrose, starch), and 1% nitrogen sources (L-ASN, L-ASN + yeast extract, L-ASN + peptone) were optimized for L-ASNase production from *B. licheniformis* ASN51. Each optimum parameter determined for enzyme productivity was applied in the next experiments.

Acetone Precipitation

Chilled (-20°C) acetone was added to crude enzyme (500 mL) with increasing concentration (40, 50, 60, 70 and 80%) at 4°C to precipitate the proteins. After overnight incubation at 4°C mixture was centrifuged, pellet was air dried and suspended in minimal amount of 50 mM Tris HCl (pH 7.4). L-ASNase activity was calculated for each concentration. Concentration of acetone showing maximum enzyme activity in precipitated protein was selected for protein precipitation of crude extract [25].

Sephadex G-100 Column Chromatography

Sample prepared in the previous step was loaded on pre-equilibrated Sephadex G-100 column with 50 mM Tris-HCl buffer (pH 7.4) [26]. Protein elution was done with the same buffer. Total 25 fractions were collected (3 mL each) and assayed for enzyme activity. Chromatographic run was monitored for protein at 280 nm. Molecular mass of purified asparaginase was estimated through 7.5% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [27]. Proteins were stained with Coomassie Blue and then destained in distilled water. The molecular weight of purified L-ASNase was calculated from the inference of the standard prestained protein ladder (Fermentas #SM0671, 10-170 kDa) using GelAnalyzer version 19.1.

Effect of pH, Temperature on Activity and Stability of Purified L-ASNase

The effect of pH on activity of purified L-ASNase was measured over a pH range of 4-10 by using 0.02 mL of 50 mM acetate buffer (pH 4-5), 50 mM phosphate buffer (pH 6), 50mM Tris-HCl buffer (7-9), and 50 mM Glycine-NaOH buffer (10). Stability of the enzyme at different pH was determined by pre-incubating the enzyme (0.02 mL) with 50 mM buffers (0.02 mL) of pH 4-10 for 30 min at 37°C. The enzyme assay was performed with 0.02 mL of 0.4 mM L-ASN. Results were expressed in terms of residual activity, which is the percentage of treated enzyme activity to that of untreated one.

The effect of temperature on L-ASNase activity was determined at different temperatures, ranging from 10-80°C. Stability of L-ASNase was determined by pre-incubating the enzyme at 10-80°C, pH 7 for 30 min. Enzyme assay was performed through Nessler's method and residual activity was calculated in comparison with untreated enzyme [22].

Effect of incubation time on the stability of purified L-ASNase was determined by mixing 0.25 mL of purified enzyme with 0.25 mL of Tris-HCl buffer of pH 7 (optimal pH) and incubating at 37°C (optimal temperature) for different intervals. Sample (0.04 mL) was drawn at different time intervals (0, 0.5, 1, 2, 4, 8, 16, 24, 30, 36, 48 h) and L-ASNase activity was estimated through standard assay [22].

Substrate Specificity and Kinetics of L-ASNase from *B. licheniformis* ASN51

The activity of L-asparaginase towards different concentrations of L-glutamine and L-ASN was compared. The assay was performed by replacing 0.4 mM L-ASN with respective substrate concentration (0.2-0.6 mM). Activity was determined as discussed in enzyme assay.

The kinetics (K_m and V_{max}) of purified L-ASNase were determined by using different concentrations of L-ASN (0.1–1.4 mM) at 37°C, pH 7. Enzyme substrate reaction was carried out and assayed under standard conditions. Michaelis-Menten plot was analyzed by fitting the L-ASNase activity data using Graph-Pad Prism 5 software to determine the kinetic parameters [23].

Therapeutic Applications of L-ASNase

Antioxidant activity of L-ASNase (Free Radical Scavenging Activity Using 2, 2-diphenyl-1-picrylhydrazyl Procedure)

Antioxidant activity of purified L-ASNase was determined through free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [23]. Different concentrations (20–120 $\mu\text{g mL}^{-1}$) of purified L-ASNase (5 μL) and 95 μL of DPPH were incubated at 37°C for 1 h in dark environment. After incubation optical density was recorded at 517 nm in microtiter plate. The concentration of the enzyme required to scavenge 50% of DPPH (IC₅₀) was estimated through linear regression of equation obtained after plotting of percentage scavenging activity against enzyme concentration. Optical density of blank with only DPPH was taken as control. Percentage scavenging was measured by applying the following formula:

$$\text{Percentage Scavenging} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Sulphorhodamine B (SRB) Assay for Anticancer Activity

Anticancer activity of purified L-ASNase was evaluated in vitro by using SRB assay [28]. Pre-seeded HepG2 cells with plate density of 15000 cells/well were exposed to 1% of purified L-ASNase from *B. licheniformis* ASN51 for 24 h under standard culture conditions. Untreated cells (NTC) were taken as control. Cells were fixed with 50% TCA for 1 h at 4°C. Washing was done five times with deionized water. Dried cells were stained with 0.05%

SRB dye for 30 min at room temperature. Cells were washed 5 times with 1% acetic acid. Air dried plates were used to take photographs at 200X using Olympus CK2 light microscope with a camera attached (Optika C-B10 digital camera). Photographs were analyzed using Optika Pro View software (Version: x86, 3.7.13977.20190224).

Statistical Analysis

All the experiments were performed in triplicates. Standard error of mean was calculated and represented by bars on graphs.

Results and Discussion

Current study is based on the production, purification, and characterization of an important biopharmaceutical L-asparaginase from *B. licheniformis* ASN51. Bacterial L-asparaginases are preferred on the basis of biotechnological conveniences. Extracellular enzymes can be produced economically on a large scale and are therefore preferred over intracellular enzymes [28]. Immunogenic reactions have been observed in commercially available formulations of *E. coli* and *Erwinia chrysanthemi* asparaginases that are considered best for clinical applications [29]. Therefore, there is a constant need to explore new bacterial sources of L-ASNase with better enzymatic activity and potential therapeutic applications.

Screening and Isolation of L-asparaginase Producing Bacterial Isolates

L-ASNase producing bacteria were isolated from different soil and water samples. Five bacterial isolates (ASN4, ASN12, ASN14, ASN38 and ASN51) produced hydrolytic zone in qualitative assay (Table 1). Among these isolates ASN51 produced largest hydrolytic zone of 36 mm and ASN4 produced smallest zone of 8 mm. Isolates were quantitatively assessed for L-ASNase production in M9 medium at pH 6 and 37°C. Different isolates ASN4, ASN12, ASN14 and ASN38 produced 88, 63, 59, and 85 U mL⁻¹ of L-ASNase respectively

Table 1. Screening of L-ASNase producing bacterial isolates through hydrolytic zone formed on M9 medium containing 1% L-ASN.

| Isolates | Source | Zone of hydrolysis (mm)±SE |
|----------|---|----------------------------|
| ASN4 | Garden soil (Islamabad, Pakistan) | 8±0.7 |
| ASN12 | Garden soil (Okara, Pakistan) | 11±0.1 |
| ASN14 | Agriculture soil from wheat field (Jaranwala, Pakistan) | 11±0.7 |
| ASN38 | Water sample (Islamabad, Pakistan) | 10±0.8 |
| ASN51 | Garden soil (Rawalpindi, Pakistan) | 36±0.3 |

SE presents standard error of mean

(Fig. S1a). In comparison to ASN4, ASN12, ASN14, ASN38, isolate ASN51 produced maximum L-ASNase of 119 U mL⁻¹ on third day when grown in M9 medium supplemented with 1% L-ASN at pH 6 and 37°C under shaking condition. Therefore, only ASN51 was selected for further examinations.

Identification of L-ASNase Producing Bacterial Isolate

Gram's staining identified ASN51 cells as Gram-positive bacilli arranged in chains (Fig. S1b). Morphological characterization showed irregular form and filiform margins (Fig. S1c, Table S1). Biochemical characterization revealed that ASN51 could hydrolyze O-nitrophenyl-b-D-galactopyranoside (ONPG), esculin, arabinose, xylose, cellobiose, sucrose, trehalose and glucose (Table S2). Molecular identification based on 16S rRNA gene sequence revealed the highest similarity of ASN51 to *Bacillus licheniformis* in GenBank database. The 16S rRNA gene sequence has been deposited in GenBank under accession number OL355169.1. Phylogenetic tree showed that ASN51 is closely related to *Bacillus licheniformis* (Fig. S1c).

Optimization of L-ASNase Production from *Bacillus licheniformis* ASN51

L-asparaginase production from *B. licheniformis* ASN51 reported in this study was strongly affected by culture conditions and media composition. Kumar et al. [30] reported a strong influence of culture conditions and composition of fermentation medium on L-ASNase production. Optimization of parameters for L-ASNase production from ASN51 was performed in M9 medium supplemented with 1% L-ASN. The results of improved enzyme production are as follows.

Effect of pH and Temperature on L-ASNase Production

L-asparaginase production was assessed at different pHs (6, 7, 8) and temperatures (30, 37, 44°C). *B. licheniformis* ASN51 showed maximum enzyme activity of 499 U mg⁻¹ at pH 8 and 37°C on third day of incubation which gradually reduced to 95 U mg⁻¹ on the sixth day of incubation. Enzyme production was comparatively low in pH 6 and 7 (Fig. 1a). Similarly, temperatures higher or lower than 37°C resulted in decreased enzyme production (Fig. 1b).

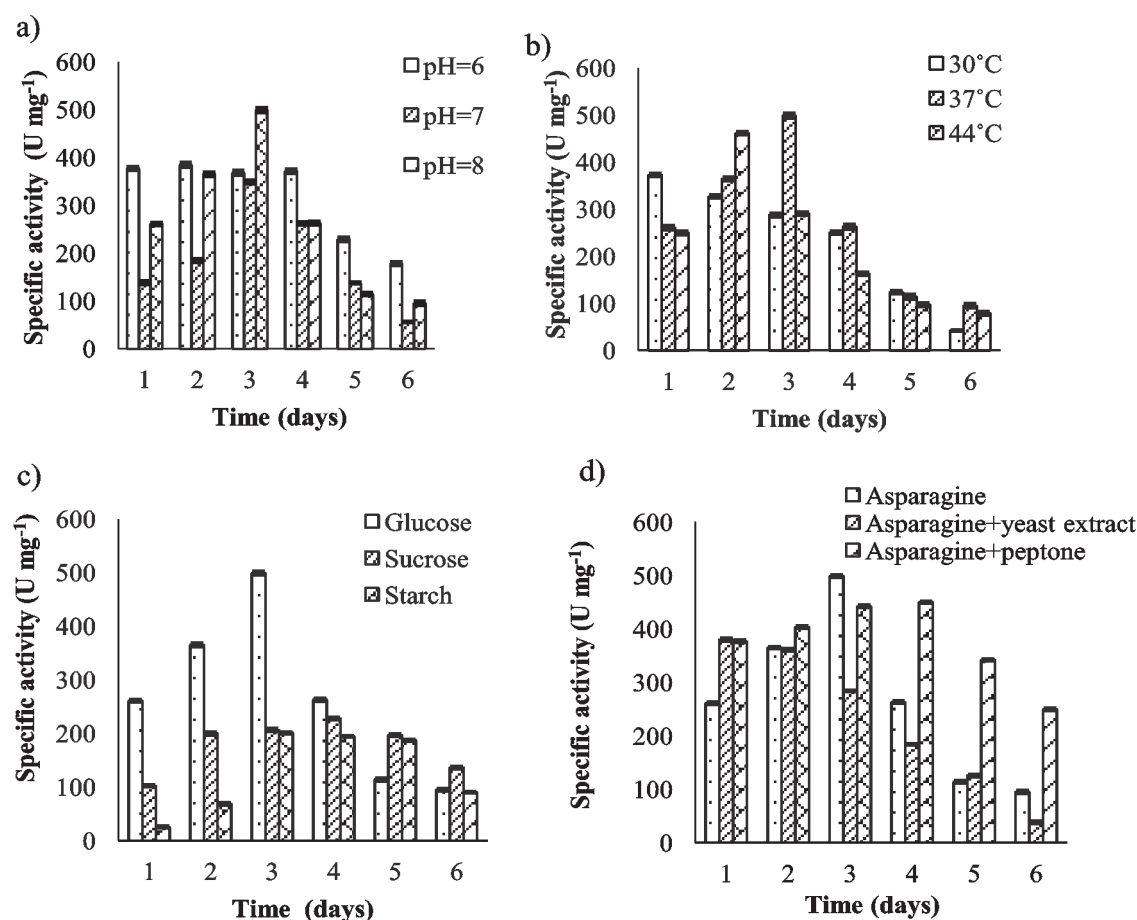


Fig. 1. Effect of a) pH at 37°C b) temperature at pH 8 c) carbon source in pH 8 and 37°C d) nitrogen source in pH 8, 37°C and 0.2 % glucose on the production of L-ASNase from *B. licheniformis* ASN51 grown in M9 medium.

At optimum pH (8), temperature (37°C), carbon (0.2% glucose) and nitrogen (1% L-ASN) source maximum L-ASNase of 1292 U mg⁻¹ was produced on the third day of incubation. Reported time of incubation for optimal L-ASNase production from *B. methylotrophicus*, *Serratia marcescens*, *Lactobacillus salivarius*, *Bacillus subtilis* and *B. licheniformis* (KKU-KH14) was 96, 96, 120, 36 h respectively [31-34]. The optimal pH for L-ASNase production from different bacteria is determined by strain type, genetic features, and fermentation conditions [35]. Contrary to the optimum pH (8) of ASN51; the pH range of 5.5-7.5 has been reported for L-ASNase production from different strains of *Bacillus licheniformis* [34,36]. Morrthy et al. [37] also reported 37°C optimal temperature for L-ASNase production from *Bacillus* sp. In contrast to the current investigation, the optimum temperature for L-ASNase from *E. coli* and *Erwinia carotovora* was 30°C [38]. Strain ASN51 has simpler requirements for production of large amount of extracellular L-ASNase, which establishes its industrial significance.

Effect of Carbon and Nitrogen Source on L-ASNase Production

Three carbon sources (glucose, sucrose and starch) were examined for the optimum asparaginase production. The bacterium showed maximum asparaginase specific activity (499 U mg⁻¹) on third day when grown in M9 medium with 0.2% glucose and 1% L-ASN. Slightly less asparaginase was produced by ASN51 when either sucrose (228 U mg⁻¹) or starch (194 U mg⁻¹) was used as carbon source (Fig. 1c). Optimization of nitrogen sources showed that the combination of L-ASN with yeast extract and peptone decreased the enzyme activity (Fig. 1d).

Purification and Characterization of L-ASNase

L-ASNase must be purified to ascertain its anti-carcinogenic effectiveness [39]. Different techniques including acetone and ammonium sulfate precipitation, ultrafiltration and gel filtration chromatography using Sephadex G-100 have been reported for purification of L-ASNase [23, 40, 41]. Cell free culture supernatant processed with 60% chilled acetone to precipitate the protein had total protein quantity of 16 mg, and maximum specific activity of 2387.5 U mg⁻¹

B. licheniformis ASN51 L-ASNase. L-asparaginase was purified through Sephadex G-100 column. The 16th fraction produced the highest enzyme activity which was 7072 U mL⁻¹ (Fig. S2a), with 16.7-fold purification and yield of 34% (Table 2). The molecular weight of the purified asparaginase was identified as 38 kDa (Fig. S2b). The specific activity of L-ASNase from *B. licheniformis* ASN51 in acetone precipitated protein was 2387.5 U mg⁻¹ with fold purification of 4.8. Ammonium sulfate precipitation had 1.21 fold purification with low L-ASNase activity (9.46 U mg⁻¹) from *B. licheniformis* KKU-KH14 [34]. Sephadex G-100 column chromatography further purified L-ASNase with specific activity of 8333 U mg⁻¹ and fold purification of 16.7. Specific activity determines the purity of enzyme, the value of which increases as the amount of contaminating proteins are removed [34]. Specific activity of L-ASNase increased after acetone precipitation and chromatographic purification, indicating the purity of enzyme [34]. The molecular weight of purified L-ASNase from *B. licheniformis* ASN51 was 38 kDa. Whereas, Mahajan et al. [40] purified L-ASNase (697 U mg⁻¹) of 33.7 kDa from *B. licheniformis* RAM-8 through ultra-filtration, acetone precipitation (1.85 fold purification) and Sephadex G-100 gel (30.17 fold purification). Purified L-ASNase from *B. licheniformis* ASN51 has higher specific activity than those previously reported, making it a more efficient producer (Table 3) [34, 36, 39].

Effect of Different pH and Temperatures on Activity and Stability of L-ASNase

Purified L-ASNase retained its activity over a wide range of pH from 4-9. Maximum specific activity (8333 U mg⁻¹) was recorded at pH 7. Further increase in pH decreased the specific activity of purified enzyme. At pH 9 specific activity of 2800 U mg⁻¹ was observed (Fig. 2a). Stability experiments showed maximum residual activity (100%) at pH 7. L-ASNase showed stability at pH 6 and 8 with 89% and 95% residual activity respectively (Fig. 2b).

L-asparaginase had specific activity of 8333 U mg⁻¹ at 35 and 37°C. Overall, the enzyme retained residual activity from 10°C (96%) to 40°C (52%). L-ASNase was most stable at 35 and 37°C. At higher temperature of 50°C residual activity was 55% (Fig. 2c). L-ASNase was stable till 24 h at 37°C and pH 7. Incubation (0.5-24 h) at 37°C, pH 7 increased the residual activity of enzyme.

Table 2. Purification scheme for L-ASNase of *B. licheniformis* ASN51.

| <i>B. licheniformis</i> ASN51 | Total Enzyme activity (U) | Enzyme activity (U mL ⁻¹) | Total protein (mg) | Specific activity (U mg ⁻¹) | Fold purification | % Yield |
|-------------------------------|---------------------------|---------------------------------------|--------------------|---|-------------------|---------|
| Crude | 62400 | 124.8 | 125 | 499 | 1 | 100 |
| Acetone precipitation | 38200 | 955 | 16 | 2387.5 | 4.8 | 61.2 |
| Sephadex column fraction (16) | 21216 | 7072 | 1.45 | 8333 | 16.7 | 34 |

Table 3. Summary of biochemical/kinetics of L-ASNase from different strains of *Bacillus licheniformis*.

| Source | Origin | Activity (U mL ⁻¹) | Size (kDa) | Optimum temperature (°C) | Optimum pH | Specific activity U/mg | Glutaminase activity | Km (mM) | References |
|--------------|---------|--------------------------------|------------|--------------------------|------------|------------------------|----------------------|---------|---------------|
| RAM-8 | Soil | 32.26 | ND | ND | ND | ND | ND | ND | 40 |
| RAM-8 | Soil | 32.26 | 33.7 | 40 | 9 | 697 | < 1% | 0.014 | 36 |
| Mutant D103V | - | 2229.17 | ~37 | 37 | ND | 597 | 0 | 0.42 | 56 |
| KKU-KH14 | Red sea | 8.1 | 37 | 37 | 7.5 | 36 | 0 | 49 | 34 |
| PPD37 | Saltern | 17.08 | ND | ND | ND | ND | ND | ND | 57 |
| ASN51 | Soil | 7072 | 38 | 35/37 | 7 | 8333 | < 4% | 0.04 | Current study |

ND (Not determined)

Stability (100%) retained after 24 h of incubation of L-ASNase (Fig. 2d).

Purified L-ASNase from *B. licheniformis* ASN51 showed optimal activity at physiological pH (7) and temperature 37°C. Enzymes that are stable at physiological temperature and pH are suitable for therapeutic applications [34]. Our results are similar to the results of Ghasemi et al. [42] and Alrumman et al. [34] who reported that 37°C is the optimum temperature for L-ASNase activity from *Halomonas elongate* and *Bacillus licheniformis* respectively. Purified L-ASNase showed long-term stability at pH 7, 37°C for 24 h.

Compared with L-ASNase from *Actinomycetales* bacterium (4-10°C) and *Corynebacterium glutamicum* (4-30°C), *B. licheniformis* ASN51 L-ASNase exhibited thermal stability (10-50°C) [43, 44]. Rapid decrease in specific activity of *B. licheniformis* ASN51 L-ASNase at basic pH (9-10) and temperatures higher than 55°C might be due to change in charges of amino acids that can destabilize protein [43]. These results are consistent with previous studies by El-Fakharany et al., [23] Lee et al., [45] and Prakash et al., [46] who found the highest LA activity at pH 8.2, 9.0, and 7.0, respectively.

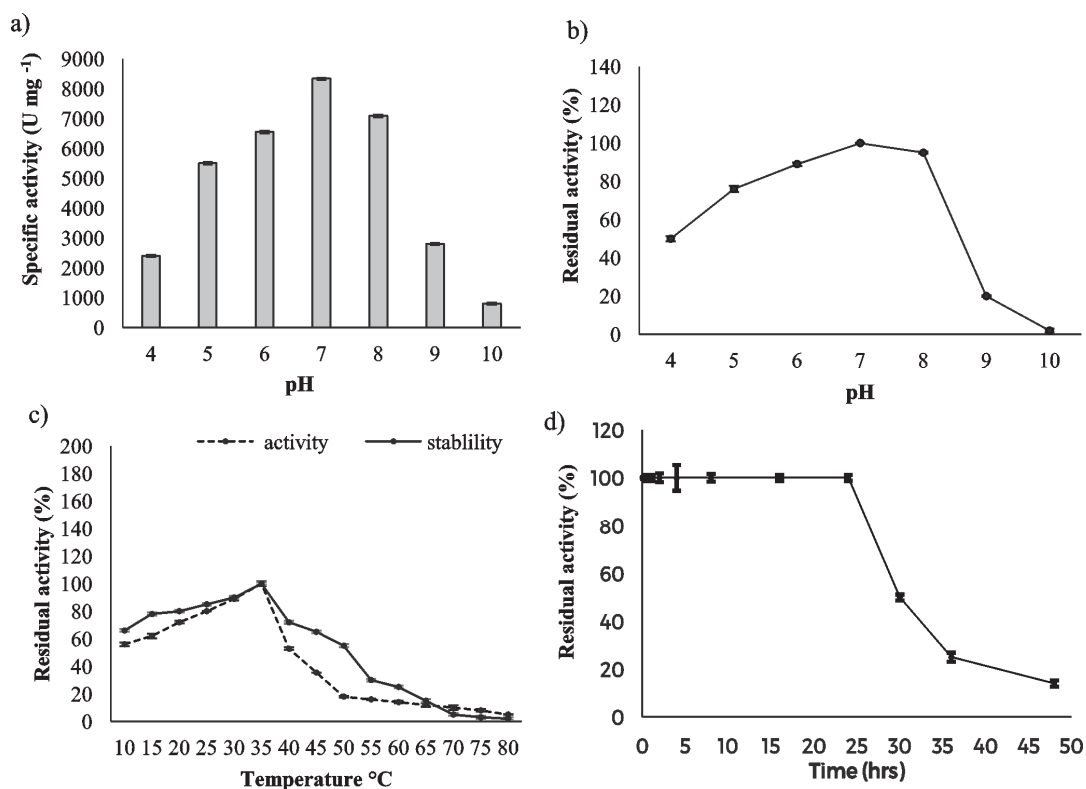


Fig. 2. Effect of a) pH on activity of purified L-ASNase at 37°C b) pH on enzyme stability at 37°C c) temperature on activity and stability of enzyme at pH 7 d) time of incubation on stability of L-ASNase at pH 7 and 37°C (bars represent the standard error of the mean).

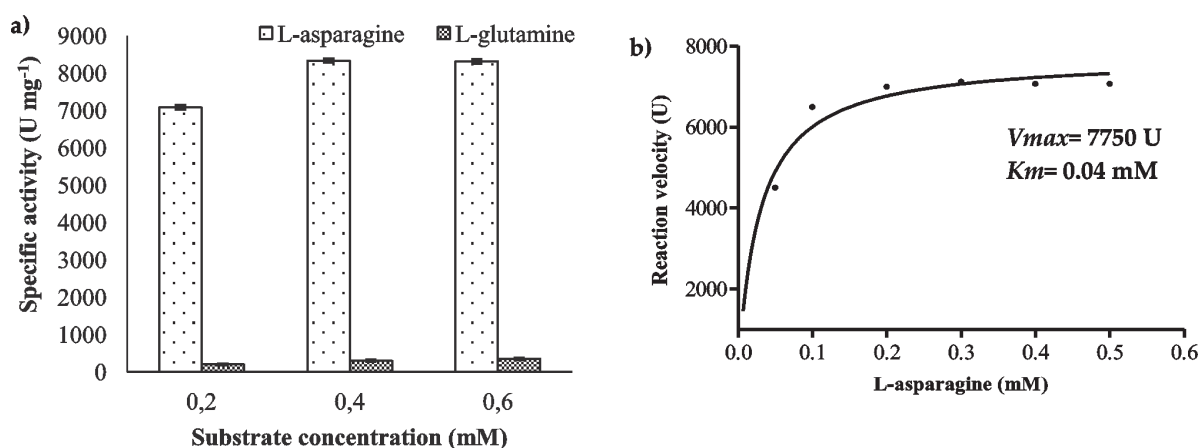


Fig. 3. a) Effect of L-asparagine and L-glutamine concentration (mM) on the activity purified L-ASNase at 37°C and pH 7 b) Kinetics of L-ASNase from *Bacillus licheniformis* ASN51 at pH 7 and temperature 37°C.

Substrate Specificity and Kinetics of Purified L-ASNase from *B. licheniformis* ASN51

Substrate specificity experiments showed that L-ASNase from *B. licheniformis* ASN51 has low glutaminase (200-350 U mg⁻¹) activity against different concentrations (0.2-0.6 mM) of glutamine. L-asparaginase showed a gradual increase in enzyme activity with increase in L-ASN concentration from 0.1 to 0.6 mM (Fig. 3a). L-asparaginase from *B. licheniformis* ASN51 has high L-asparagine specificity and low glutaminase (less than 4%) activity hence demonstrated as a more efficient alternative to *E. coli* and *Erwinia chrysanthemi* L-ASNase in the treatment of ALL [38,47]. Glutaminase activity of L-ASNase is a major determinant of anticancer activity against asparagine synthetase (ASNS) positive cancer cells [48]. Glutaminase activity of L-ASNase is also crucial for pancreatic cancer cells [49]. Chan et al. [51] also reported that L-ASNase glutaminase activity is vital for durable, single-agent anticancer activity in vivo, even against ASNS negative cancer types. Intrinsic glutaminase activity of purified L-ASNase from ASN51 might enhance the anticancer potential of enzyme against ASNS positive and negative cancer cells.

Regarding enzyme kinetics; determination of a lower K_m value is the main criteria for an enzyme to use as a therapeutic drug. The concentration of L-ASN in human serum is in the range of 50–70 μM [48]. Hence, an enzyme with lower K_m and greater affinity for L-ASN is preferred as a protein drug [49]. Kinetics of *B. licheniformis* ASN51 L-ASNase showed K_m value of 0.04 mM with standard error 0.01 and V_{max} of 7750 U from GraphPad Prism with R^2 value of 0.98 (the best fit model) (Fig. 3b). Enzyme kinetics showed K_m for L-ASNase from *B. licheniformis* ASN51 comparable with the commercially available *E. coli* (0.01 mM) and *E. chrysanthemi* (0.06 mM) formulations. For *Bacillus* species K_m values ranged between 0.8-12 mM [1, 3, 50]. The lower K_m value of purified L-ASNase reported in our study signifies its pharmaceutical importance over reported ones (Table 3).

Therapeutic Applications of Purified L-ASNase from *B. licheniformis* ASN51

Assessment of Antioxidant Activity

Antioxidant activity of L-ASNase showed that the scavenging efficiency on DPPH was enhanced by

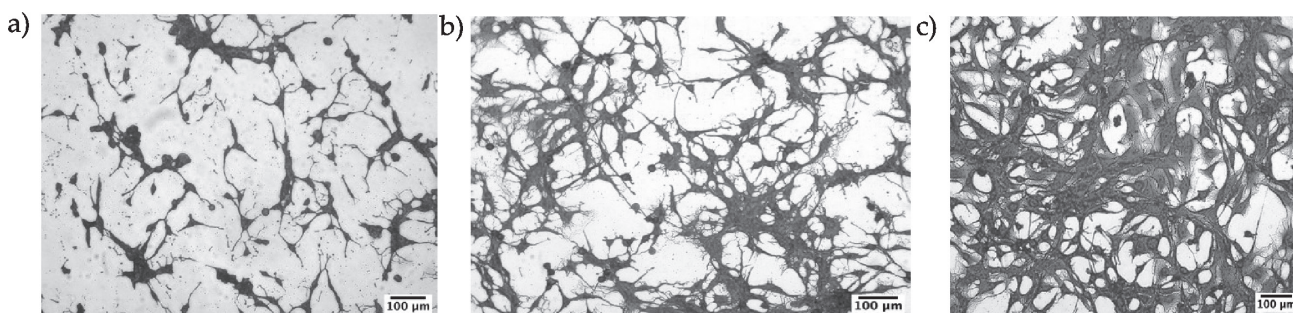


Fig. 4. Microscopic images (scale bar: 100 μm) of HepG2 liver cancer cells treated with a) L-ASNase from *B. licheniformis* ASN51 (0.4 μM) b) DOX (10 μM) c) Non treated cells.

increasing the concentration of L-ASNase from 20 to 120 $\mu\text{g mL}^{-1}$ (Fig. S3). The inhibitory activity of DPPH radicals was calculated in percentage and ranged between 40 to 78% with IC₅₀ of 53.1 $\mu\text{g mL}^{-1}$. This indicates the potential of *B. licheniformis* ASN51 L-ASNase to act as an antioxidant agent. For radical scavenging activity, higher IC₅₀ values (325.4 and 263.63 g mL^{-1}) were reported for *Bacillus* sp R36 and *Aspergillus flavus* (KUFS20) L-ASNase, demonstrating the high antioxidant potential of the ASN51 L-ASNase [51, 52].

Anticancer Activity of Purified L-ASNase

Purified L-ASNase (0.4 μM) from *B. licheniformis* ASN51 has 53.3% anticancer activity with percentage viability of 47% for HepG2 liver cancer cells. Percentage viability of cancer cells after DoX (10 μM) treatment was 41.7%. In comparison to non-treated cells, the anticancer activity of purified asparaginase is evident (Fig. 4).

Liver cancer is the fourth most prevalent type of cancer-causing mortality. It is the most aggressive type of cancer that spreads to other organs [53]. Cytotoxicity of 53.3% was recorded for ASN51 L-ASNase at a concentration of 0.4 μM against HepG2 cancer cells. The anticancer activity of ASN51 L-ASNase revealed the effective killing of liver cancer cells because of the deamination of non-essential amino acid L-asparagine [54]. Moharib reported that L-ASNase from *Vigna unguiculata* killed 50% of HELLA, HCT116 and HEPG2 cancer cell lines [5]. Asparaginase from *Capsicum annum* L. had anticancer activity against three different cell lines KB, A549 and HELLA [55]. El-Fakharany reported the cell cycle distribution of NFS60 cells when treated with 10, 15 and 20 mg mL^{-1} of L-ASNase [23].

The present results revealed improved L-ASNase production from *B. licheniformis* ASN51. Antioxidant and anticancer potential of L-ASNase with low glutaminase activity further enhanced its therapeutic applications.

Conclusions

To the best of our knowledge, among reported strains *B. licheniformis* ASN51 is the highest L-ASNase producing strain with specific activity of 8,333 U mg^{-1} . Purified L-ASNase showed activity over a wide range of pH (4-9) and temperature (10-50°C). Antioxidant potential (IC₅₀ value 53.1 $\mu\text{g mL}^{-1}$) and anticancer activity of purified L-ASNase could be helpful in treating oxidative stress related diseases and cancer respectively. Low K_m (0.04 mM) and low intrinsic glutaminase activity (<4%) of purified enzyme suggested its suitability for treating both ASN +/- cancer cells with minimal side effects. These results suggested that L-ASNase from *B. licheniformis* ASN51 could be an efficient substitute for therapeutic applications.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Data

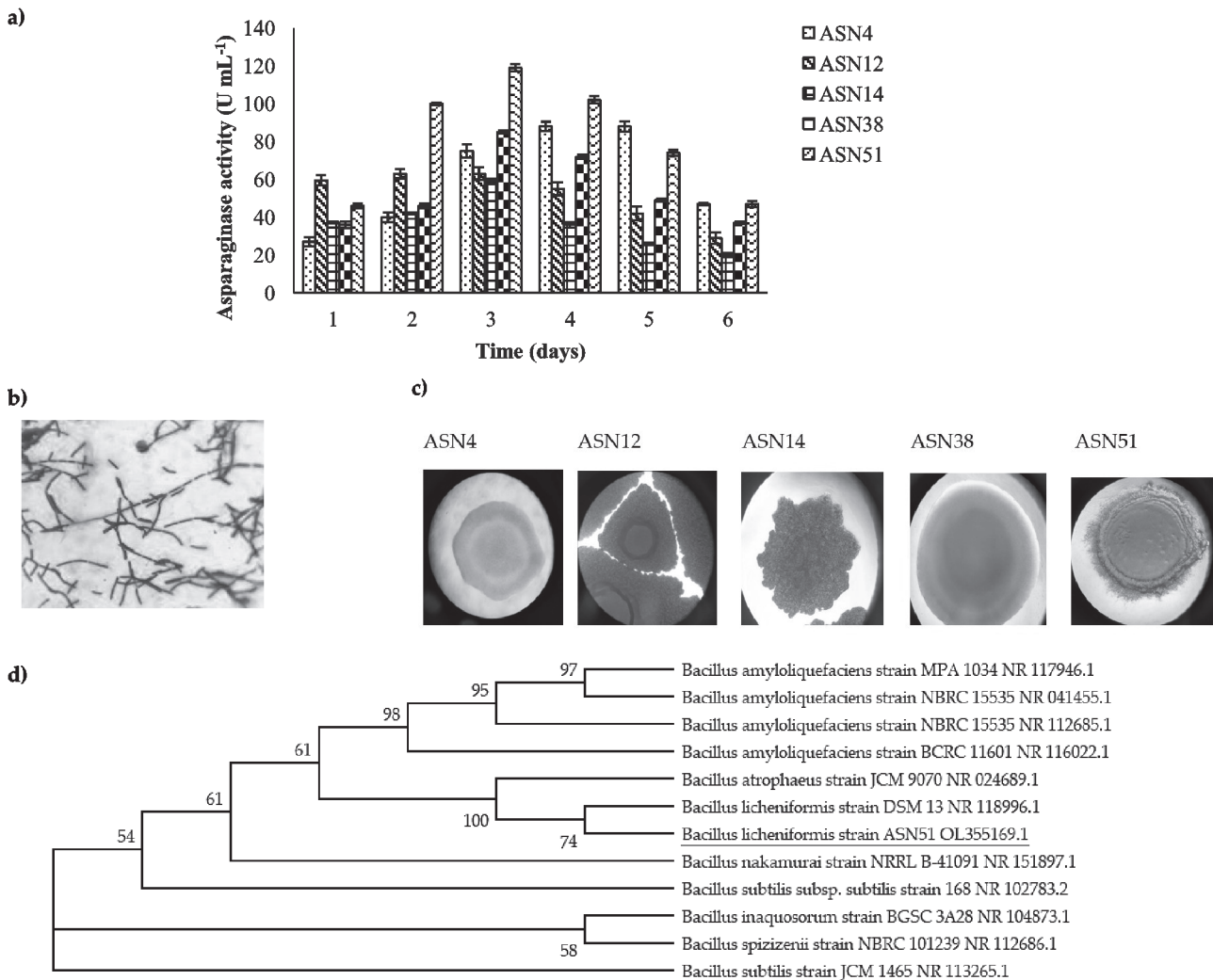


Fig S1 a) L-asparaginase production of five distinct isolates grown in M9 medium at 37°C and pH 6. Results represent a mean of three experimental replicates and error bars represent the standard error of mean.

b) Result of Gram-positive ASN51 isolate under light microscope with magnification 1000x

c) Morphology of ASN4, ASN12, ASN14, ASN38, and ASN51 isolates under light microscope with 400x magnification

d) Phylogenetic analysis of *B. licheniformis* ASN51 16S rRNA gene with closely related *Bacillus* species using neighbor joining method through MEGA11 software. Accession numbers and % similarity is indicated for each entry. Branch points show bootstrap percentages (1000 replicates).

Table S1. Colony morphology of L-ASNase producing isolates.

| Isolates | Color | | Size (mm) | Elevation | Form | Margin |
|----------|-------------|------------------|-----------|-----------|-----------|----------|
| | Visual | Under microscope | | | | |
| ASN4 | Transparent | Light brown | 1.5-2 | Flat | Circular | Entire |
| ASN12 | Off-white | Dark brown | 5-6 | Flat | Irregular | Undulate |
| ASN14 | White | Dark brown | 3.5-4 | Flat | Irregular | Filiform |
| ASN38 | Transparent | Light brown | 3-4 | Flat | Circular | Entire |
| ASN51 | Off-white | Light brown | 3.5-4 | Flat | Irregular | Filiform |

Table S2. Biochemical characterization of L-asparaginase producing ASN51 strain.

| Test | Substrate | Reactions | ASN 51 |
|---------------|-------------------------------------|-------------------------|--------|
| Catalase Test | Hydrogen peroxide | Catalase | + |
| ONPG | O-nitrophenyl-b-D-galactopyranoside | Beta-D-galactosidase | + |
| LDC | Lysine | Lysine decarboxylase | - |
| ODC | Ornithine | Ornithine decarboxylase | - |
| URE | Urease | Urease | - |
| CIT | Citrate | Utilization | - |
| PPA | Phenylalanine | Phenylalanine deaminase | - |
| MNT | Malonate | Utilization | - |
| ESC | Esculin | Beta-glucosidase | + |
| ARA | Arabinose | Acidification | + |
| XYL | Xylose | Acidification | + |
| ADO | Adonitol | Acidification | - |
| RHA | Rhamnose | Acidification | - |
| CEL | Cellobiose | Acidification | + |
| MEL | Melibiose | Acidification | - |
| SAC | Sucrose | Acidification | + |
| TRE | Trehalose | Acidification | + |
| RAF | Raffinose | Acidification | - |
| GLU | Glucose | Acidification | + |
| IND | Tryptophane | Indole formation | + |
| VP | Pyruvate/creatine | Acetoin production | - |

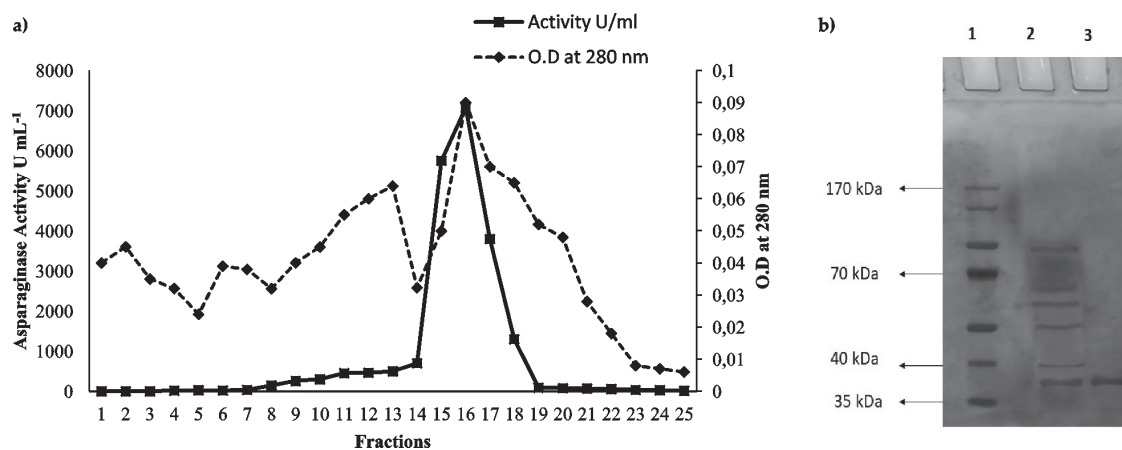


Fig. S2. a) Elution profile for column chromatography of L-asparaginase from *Bacillus licheniformis* ASN51 equilibrated with Tris-HCl buffer of pH 7.4. Elution rate was 3.0 mL/fraction and absorbance recorded at 280 nm; b) SDS-PAGE of L-asparaginase Lane 1. Protein marker Lane 2. Acetone precipitated proteins Lane 3. Purified L-ASNase after Sephadex G-100 column chromatography

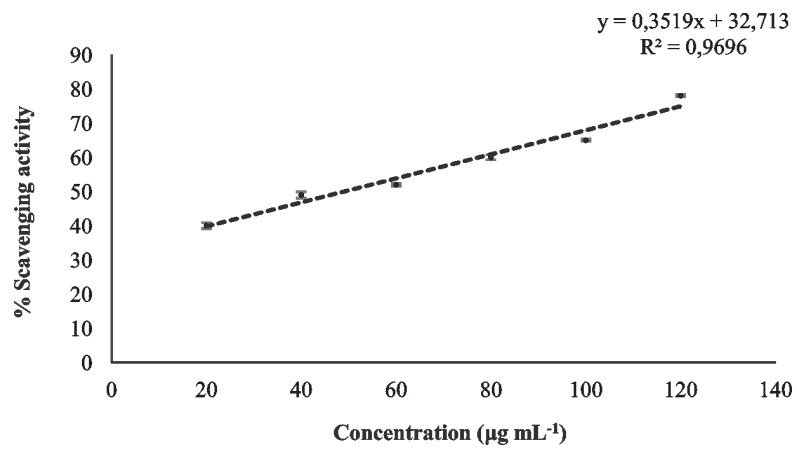


Fig. S3. DPPH scavenging activity of purified L-ASNase from *B. licheniformis* ASN51.