

PURIFICATION AND PARTIAL CHARACTERIZATION OF L-ASPARAGINASE ENZYME PRODUCED BY NEWLY ISOLATED *BACILLUS* sp

DZUN NORAINI JIMAT*, INTAN BAIZURA FIRDA MOHAMED,
AZLIN SUHAIDA AZMI AND PARVEEN JAMAL

Department of Biotechnology Engineering, Faculty of Engineering,
International Islamic University Malaysia,
Jalan Gombak, 53100 Kuala Lumpur, Malaysia.

*Corresponding author: jnoraini@iium.edu.my

(Received: 18th March 2016; Accepted: 13th Nov. 2017; Published on-line: 1st Dec. 2017)

ABSTRACT: A new bacterial producing L-asparaginase was successfully isolated from Sungai Klah Hot Spring, Perak, Malaysia and identified as *Bacillus* sp. It was the best L-asparaginase producer as compared to other isolates. Production of L-asparaginase from the microbial strain was carried out under liquid fermentation. The crude enzyme was then centrifuged and precipitated with ammonium sulfate before being further purified by chromatographic method. HiTrap DEAE-Sepharose Fast Flow ion exchange chromatography followed by separation on Superose 12 gel filtration were used to obtain pure enzyme. The purified enzyme showed 10.11 U/mg of specific activity, 50.07% yield with 2.21 fold purification. The purified enzyme was found to be dimer in form, with a molecular weight of 65 kDa as estimated by SDS-PAGE. The maximum activity of the purified L-asparaginase was observed at pH 9 and temperature of 60 °C.

ABSTRAK: Kajian penyelidikan ini telah berjaya menghasilkan enzim L-asparaginase daripada bakteria-bakteria baru yang diambil dari Kolam Air Panas, Sungai Klah, Perak, Malaysia dan ia dikenal pasti sebagai *Bacillus* sp. Teknik isolasi bakteria ini adalah teknik terbaik dalam menghasilkan enzim L-asparaginase berbanding teknik-teknik lain. Penghasilan enzim ini dibuat menerusi proses fermentasi. Kultur bakteria yang diperolehi diempar dan diikuti presipitasi menggunakan ammonium sulfat sebelum proses penulenan seterusnya dilakukan menggunakan kaedah kromatografi. Kolum penukaran ion jenis HiTrap DEAE-Sepharose Fast Flow diikuti dengan pemisahan oleh gel Superose 12 telah digunakan untuk mendapatkan enzim L-asparaginase yang tulen. Hasil kajian mendapati enzim tulen yang diperolehi mempunyai aktiviti spesifik sebanyak 10.11 U/mg daripada 50.07% enzim yang dihasilkan dan berjaya mencapai 2.21 kali ganda tulen dari enzim tanpa proses penulenan. Enzim tulen yang diperolehi didapati dalam bentuk “dimer“ dengan berat molekul sebanyak 65 kDa yang ditentukan menerusi SDS-PAGE. Enzim ini juga menunjukkan aktiviti yang tinggi pada pH 9 dan suhu 60 °C.

KEYWORDS: *Bacillus* sp.; L-asparaginase; ion exchange chromatography; gel filtration chromatography; partial characterization

1. INTRODUCTION

L-asparaginase is one of the important enzymes in therapeutic treatment as well as the food industry. It is also known as asparagine aminohydrolase. L-asparaginase is a generic

denomination for enzymes that catalyse the transformation of L-asparagine into their respective acid and ammonia [1]. As reported by Dominika et al. [2], biochemical and kinetic properties of enzymes vary with the genetic nature of the microbial strain.

L-asparaginase possesses anti-tumour activity and is accepted as chemotherapeutic agents. This enzyme is used as an effective treatment for acute lymphoblastic leukemia, lymphosarcoma and tumor cells or others that are related to tumor therapy in combination with chemotherapy [3]. It is mainly used in the treatment of leukemia in children. As described by Dominika et al. [2] and Borah et al. [4], L-asparagine acts as an important amino acid for the growth of tumour cells. L-asparaginase, lowering the concentration of L-asparagine, retard the growth of cancer cells. They require an external amino acid because they cannot synthesize asparagine by the enzyme asparagine synthetase. The application of L-asparaginase as a drug in the treatment of leukemia is to reduce and eliminate the amount L-asparagine in the blood and subsequently involves monitoring the level of the corresponding amino acid in blood of treated leukemia patients. Besides therapeutic treatment, L-asparaginase also has a significant role in the food industry. As mentioned in previous studies, the level of acrylamide in baked and fried food products (i.e. fried potato) is reduced by the action of L-asparaginase [1]. Acrylamide is known as a cancer causing agent. It presents in heat-derived (fried and baked) foods and contains some reducing sugar. Other studies reported that the contents of acrylamide should be reduced since it is proven to be neurotoxic, genotoxic, carcinogenic, and toxic to reproductive system [5]. As discussed by [3], acrylamide is formed by the result of a Maillard reaction, which is a heat-induced reaction. Heat-induced reactions between the α -amino group of the free amino group of the free amino acid L-asparagine and carbonyl groups of reducing sugar like glucose can derive acrylamide. L-asparaginase helps in hydrolysing asparagine, which in turn significantly reduces the formation of the substance.

Nowadays, researchers all around the world are interested in studying the production of thermostable L-asparaginase from various sources. Thermostable enzymes are considered to be of biotechnological and industrial interest as their enhanced stability at elevated temperatures could greatly reduce enzyme replacement costs or permit processes to be carried out in such conditions. The thermostable enzymes are proven to have many advantages over the mesophilic enzymes. It has been reported that L-asparaginase can be produced from several sources such as bacteria, fungi, and animal [6]. A large number of thermophilic microorganisms, especially bacteria, capable of producing the thermostable L-asparaginase have been reported globally. Most of the levels of enzyme activity produced by microbial strains from a natural environment are low and need to be elevated or increased for industrial production. The level of enzyme production by one microorganism differs with other microorganisms. Moreover, levels differ in terms of both composition and properties. L-asparaginase from microbial sources must be purified before it can be characterized in terms of physical and biological characteristics. It is important to remove all contaminants and impurities present in the crude enzyme. Enzyme purification is an important downstream processing step in manufacturing biopharmaceutical products where all the contaminants and impurities need to be removed. Normally, biopharmaceuticals require exceptional purity, making downstream processing a critical step of the overall processes. The selected purification methods must be simple, easy, and adaptable, particularly in large scale. The common purification step that has been applied in protein purification is combination of precipitation (fractionation/salting out) in order to precipitate out the protein. Several chromatographic methods are added to the precipitation as polishing steps.

Therefore, the present work aims to purify and characterize the asparaginase from a local newly isolated strain of *Bacillus* sp. The enzyme was purified using conventional steps of purification. Then, the purified enzyme was partially characterized in terms of pH and temperature stability.

2. MATERIALS AND METHODS

2.1 Microorganism

One liter of modified M9 medium containing: Na₂HPO₄·2H₂O, 6.0 g; KH₂PO₄, 3.0 g; NaCl, 0.5 g; L-asparagine, 5.0 g; 1 M MgSO₄·7H₂O, 2.0 ml; 0.1 M CaCl₂·2H₂O, 1.0 ml; 20% glucose stock, 10.0 ml; agar 20.0 g was prepared and supplemented with 3 ml phenol red as an indicator. The medium was adjusted to pH 7 and autoclaved at a temperature of 121 °C. One hundred microliters of collected water (from the hot spring) was plated onto the agar medium and gently streaked. Then, the plates were incubated at a temperature of 50 °C for 48 hrs. The isolated bacteria, which produced L-asparaginase, were screened based on a wide formation of a pink zone around the colonies on the plates (qualitative assay) as well as a concentration of released ammonia (quantitative assay). The potential bacteria that produced the highest enzyme activity (based on release of ammonia) were selected for further studies. The selected bacterial strain was confirmed as *Bacillus* sp. by 16S rRNA gene sequencing. A single colony of the strain was cultured a few times to obtain a pure strain. The pure culture of *Bacillus* sp. was maintained and preserved in nutrient agar at 4 °C and stock culture (contained glycerol) at -20 °C.

2.2 Production of L-asparaginase from *Bacillus* sp. under Submerged Fermentation

Two percent (1.46×10^8 CFU/ml) of inoculum (*Bacillus* sp.) were inoculated into 50 ml of media: Na₂HPO₄·2H₂O, 6.0 g; KH₂PO₄, 3.0 g; NaCl, 0.5 g; L-asparagine, 5.0 g; 1 M MgSO₄·7H₂O, 2.0 ml; 0.1 M CaCl₂·2H₂O, 1.0 ml; 20% glucose stock, 10.0 ml was placed in 250 ml Erlenmeyer flasks and incubated at 50 °C at 150 rpm for 48 hrs. The medium without inoculum was used as a control. Each experiment was done in triplicates. The culture broth was collected after 48 hrs for determination of L-asparaginase activity and protein concentration.

2.3 L-asparaginase Assay

The bacteria cultures were harvested by centrifugation at 6,000 rpm for 15 min. Sample of enzyme extract (0.1 ml) was mixed with 0.2 ml of 0.05 M Tris-HCl (pH 8.6), followed by 1.7 ml of 0.01 M L-asparagine then the mixture sample was incubated for 10 min at 37 °C. Trichloroacetic acid (0.5 ml of 1.5 M solution) was added to the mixture in order to stop the reaction. After that, the mixture was centrifuged at 10,000 rpm, then 0.5 ml of the supernatant was diluted to 7 ml with distilled water together with 1 ml of Nessler reagent. After 10 min, the absorbance was measured at 480 nm. The optical density (OD) readings were then compared to standard curve of ammonia. One unit (U) of L-asparaginase was defined as that amount of enzyme which liberating 1 μmol of ammonia per minute at 37 °C under the assay conditions.

2.4 Protein Concentration

Concentration of protein was determined by mixing the 1.50 ml protein reagent (Pierce™ 660 nm Protein Assay Reagent) with 0.10 ml of sample. The sample was the supernatant that was taken after centrifuging the culture at 6,000 rpm for 15 minutes. After adding the protein reagent into the sample, the OD was measured at 660 nm. Then, the ODs were compared to a standard curve of bovine serum albumin (BSA) concentration.

2.5 Purification of L-asparaginase

2.5.1 Ammonium Sulfate Precipitation

The crude enzyme (supernatant) was prepared by centrifuging the cell cultures at 10,000 rpm for 15 min at 4 °C. Ammonium sulfate powder was added into the supernatant at 4 °C (slowly stirred) to bring it to 90% ammonium sulfate saturation. Then, the precipitated protein was centrifuged at 10,000 rpm for 15 min at 4 °C. The precipitated protein was dissolved in a minimal volume (2-3 ml) of 0.05 M Tris-HCl, pH 8.5. Then, the dialysis was conducted to remove the excess ammonium salt present in the protein solution by putting this protein in a dialysis bag. Principally, the protein was left retained in the dialysis bag while allowing water and salt to permeate out of the bag. After that, this dialysis bag was immersed in a bulky volume of buffer (0.05 M Tris-HCl, pH 8.5) and agitated for 24 hours at 4 °C.

2.5.2 Ion Exchange Chromatography using HiTrap Diethylaminoethyl (DEAE) Sepharose Fast Flow

One milliliter of the dialysate from the previous step was filtered through 0.45 µm filter prior to loading into HiTrap™ DEAE Sepharose Fast Flow chromatography column (dimension: 0.7 x 2.5 cm; volume: 1 ml; bead size: 45-165 µm). Two buffer solutions were used in ion exchange chromatography; 0.05 M Tris-HCl, pH 8.5 (start buffer) and 0.05 M Tris-HCl, pH 8.5 with 1 M NaCl, pH 8.5 (elution buffer). The column was equilibrated with 0.05 M Tris-HCl, pH 8.5 at 1 ml/min prior to use. Then, the enzyme was eluted by the elution buffer, 0.05 M Tris-HCl, pH 8.5 with 1 M NaCl, pH 8.5) at a flow rate of 1 ml/min and 2 ml fractions were collected. The fractions with the highest peak were pooled together. Then, the fractions were analysed for determination of L-asparaginase activity and protein concentration.

2.5.3 Gel Filtration using Superose® 12

The pooled fractions with high L-asparaginase activity from the previous step (Section 2.5.2) were further purified using Superose® 12 (dimension: 1 x 30–31 cm; volume: approximately 24 ml); optimal separation range (globular proteins): 1-300 kDa; average particle size: 11 µm). The column was equilibrated with 0.05 M Tris-HCl (pH 8.5) at 0.8 ml/min. The protein elution was done with same buffer at a flow rate of 0.8 ml/min and 1 ml fractions were collected. The fractions with the highest peak were pooled together. Then, the fractions were analysed for determination of L-asparaginase activity and protein concentration.

2.6 Characterization of L-asparaginase

2.6.1 Determination of Molecular Weight using SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to estimate the molecular weight of the purified L-asparaginase. In the present work, the method was adopted from Gupta et al. [7]. A 12% resolving or separating gel, pH 8.8 (sterilized distilled water: 1.7 ml; 30% degassed acrylamide: 2.0 ml, resolving buffer: 1.25 ml; 10% SDS: 50 µl; 10% ammonium persulfate: 50 µl; TEMED: 5 µl) and 4% stacking gel, pH 6.8 (sterilized distilled water: 3.05 ml; 30% degassed acrylamide: 0.65 ml, stacking buffer: 1.25 ml; 10% SDS: 50 µl, 10% ammonium persulfate: 50 µl; TEMED: 10 µl) were prepared. The casting frames were set (two glass plates were clamped together in the casting frames) on the casting stands. An appropriate amount of separating gel solution was filled into the gap between the glass plates. Water was filled on the top of the separating gel in order to check for any leakage between the glasses and to avoid bubbles.

After separating gel was polymerized, the stacking gel solution was poured until overflow. Then, the well-forming comb was inserted without trapping air under the teeth. The comb was taken out after complete gelation of the stacking gel. After that, the 1X running buffer was poured into the inner chamber until the buffer surface reached the required level in the outer chamber. Ten microliters of samples were mixed with 2 μ l of sample buffer (loading buffer) and the mixture was heated at 95 °C (denaturing the samples) for 10 min. Next, 10 μ l of prepared samples were loaded into each well. The protein marker (Thermo Scientific Pre-stained Protein Ladder 10-170 kDa) was loaded into the first lane. The equipment was operated after everything was set up correctly. The voltage was set at 120 V. When the dye reached to the bottom of the gel, the supplied power was turned off and the gel unit was removed from the electrophoresis unit. The plates were gently separated with the help of a spatula and the gel was carefully transferred into the staining solution for staining overnight. Next, it was destained for 7 hours. The molecular weight of the L-asparaginase was measured with reference to the standard markers used. Protein bands were visualized by staining with Coomassie R-250 blue.

2.6.2 Effect of pH on Stability of Enzyme Activity

The effect of pH on the stability of L-asparaginase activity was determined by incubating the purified enzyme in buffers with various pH values. Purified L-asparaginase (100 μ l) was incubated with 0.2 ml of buffer solutions (different pH) in the absence of substrate (L-asparagine) for 24 hours at 4 °C. The buffer solutions were 0.05 M sodium acetate-acetic acid (pH 4 and pH 5), 0.05 M sodium phosphate (pH 6 and pH 7), 0.05 M Tris-HCl (pH 8 and pH 9) and 0.05 M Glycine-NaOH (pH 10). After that, 1.7 ml of 0.01 M L-asparagine was added into the solutions and the activity of L-asparaginase was determined under assay conditions.

2.6.3 Effect of Temperature on Stability of Enzyme Activity

The temperature stability of L-asparaginase was determined by incubating 100 μ l of purified enzyme at different temperatures (30, 37, 40, 50, 60, 70 and 80 °C) for 60 minutes. Then, the enzyme activity was measured after adding 1.7 ml of substrate under assay conditions.

3. RESULTS AND DISCUSSION

A summary of enzyme activity and yield of purified L-asparaginase from *Bacillus* sp. is shown in Table 1. The specific activity before purification was 4.58 U/mg. Upon precipitation with ammonium sulfate, the specific activity was found to be increased by 1.33 fold with a yield of 98.07% and the total protein content was found to be decreased by 1.36 fold. Enzyme activity was slightly loss (2.13%) at this stage. It indicated some contaminant proteins were removed by the salting out process. The precipitated enzyme was then purified by ion exchange chromatography (IEC) through a HiTrap DEAE-Sephacrose Fast Flow column that was eluted in 50 mM Tris-HCl and 1 M NaCl, pH 8.5 at a flowrate of 1.0 ml/min. In this step, L-asparaginase was purified to 1.58 fold with a yield of 69.62% and specific activity of 7.21 U/mg. A typical elution profile of enzyme from the DEAE-Sephacrose ion exchange column (Fig. 1) shows one distinct major peak at fraction numbers 5 to 6. High enzyme activity was located at the fraction numbers. Both fractions were pooled and tested for L-asparaginase quantitative activity. The fractions containing high enzyme activity were then applied to Superose 12 gel filtration column. The activity was located in peak 2. After ion exchange chromatography, only 1.5% of the total protein from crude extract was eluted (2.26 fold decreased), but the specific activity was found to

be increased by 2.21 fold. The specific activity after the gel filtration column was 10.11 U/mg. The specific activity of purified L-asparaginase of *Bacillus* sp. obtained in the present study was 7.21 U/mg. It was 28% less than previous study which was also performed via two chromatography steps (13.97 U/mg) with 36.204% yield [7]. Another study reported a recovery of 18.46% with a specific activity of 69.43 U/mg of an extracellular L-asparaginase from *Mucor hiemalis* [8].

Table 1: Summary of purification steps of newly isolated *Bacillus* sp. producing L-asparaginase.

Sample	L-asparaginase activity (U/ml)	Protein concentration (mg/ml)	Specific enzyme activity (U/mg)	% yield	Fold Purification
Crude enzyme extract	0.19±0.03	0.041±0.00871	4.57	100	1
Ammonium ppt (90% saturation)	0.18±0.008	0.030±0.0417	6.10	98.07	1.33
HiTrap DEAE-Sepharose Fast Flow column – Ion exchange chromatography	0.13±0.031	0.018±0.00565	7.21	69.62	1.58
Superose 12 column- Gel filtration	0.09±0.0171	0.009±0.0076	10.11	50.07	2.21

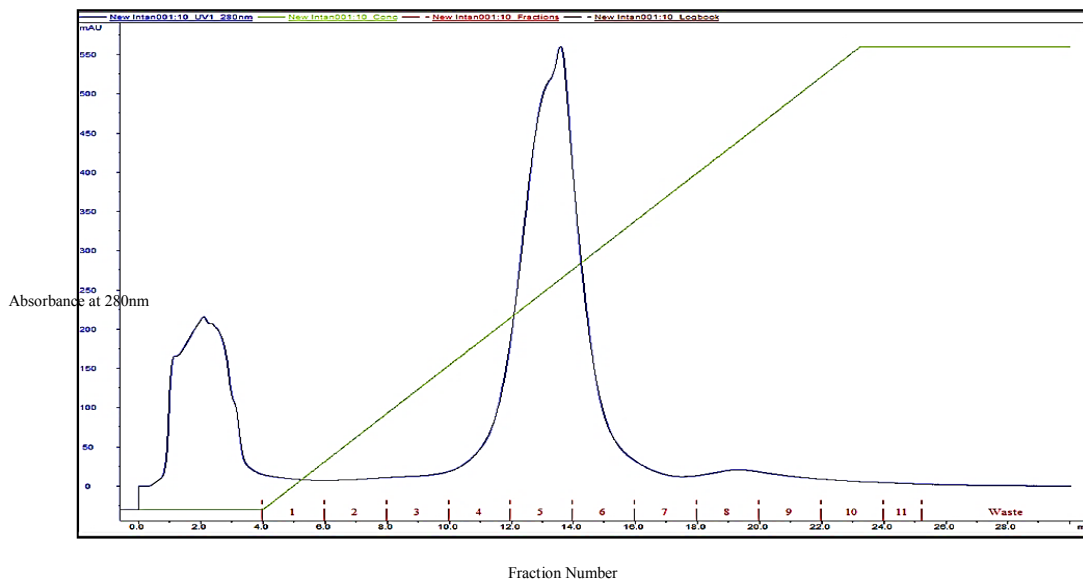


Fig. 1: Elution profile of L-asparaginase from DEAE-Sepharose chromatography column.

Gel filtration chromatography (GFC) was performed as a polishing step in purification. Superose 12 column was used in this chromatography step on a Fast Protein Liquid Chromatography to confirm whether the purified protein was composed of a mixture of other higher molecular weight aggregates or lower monomer forms in addition to the native tetramer state. In the present work, a Superose 12 column (GE Healthcare) was equilibrated with 50 mM Tris-HCl pH 8.5 and 2 ml of the purified asparaginase was loaded onto the column and eluted at a flow rate of 1.0 ml/min. In Fig. 2, the elution diagram of the size exclusion of the purified enzyme also revealed a single peak at a

fraction number of 19 confirming that it was a pure preparation. Previous studies reported the fact that a single sharp peak on the elution diagram refers to the pure target protein [9].

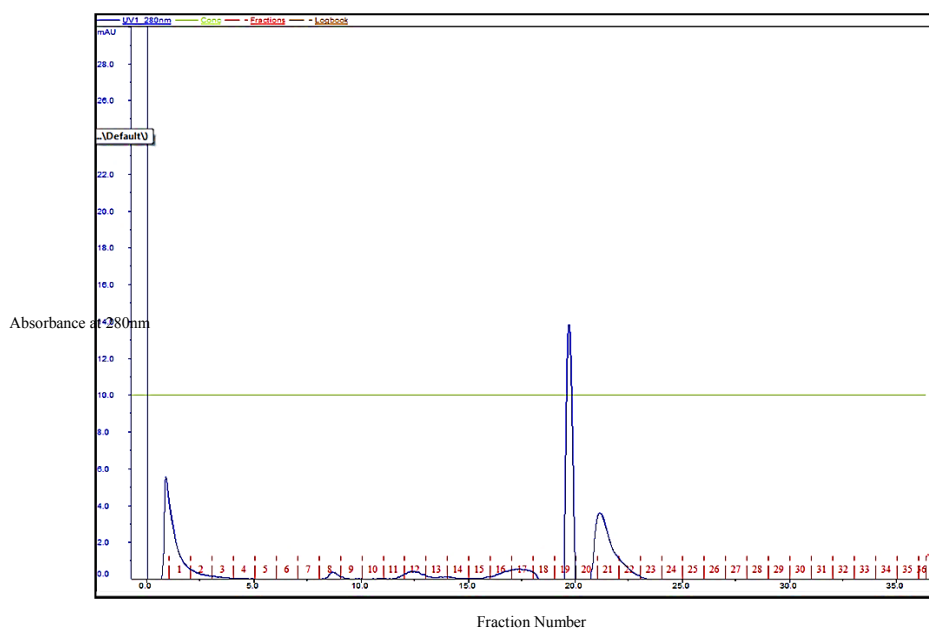


Fig. 2: Elution profile of asparaginase from Superose 12 chromatography column.

The SDS-PAGE analysis of the purified L-asparaginase from *Bacillus* sp. as illustrated in Fig. 3 revealed a protein band migrating at approximately 65 kDa. Three bands were observed in SDS-PAGE after ion exchange chromatography. And after gel filtration chromatography, only one band was observed. From the protein band observed, it can be claimed that the enzyme was purified to near homogeneity (lane 3). The purified enzyme showed a single band in SDS-PAGE gel indicated that it was homogenous [9]. Generally, L-asparaginase is known as a tetramer protein molecule [10]. Tetramer protein is built up of four protein subunits. The similar protein structure of *Escherichia coli* L-asparaginase was discovered by Jha et al.[11]. In the present study, L-asparaginase from *Bacillus* sp. was observed to have dimer form of protein.

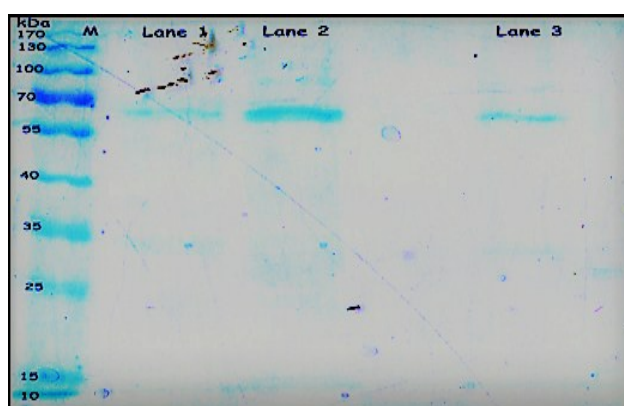


Fig. 3: SDS-PAGE analysis of purified L-asparaginase from *Bacillus* sp. Lane 1: Partial purification by ammonium sulfate precipitation; Lane 2: Eluted fraction contains asparaginase obtained from HiTrap DEAE- Sepharose Fast Flow ion exchange chromatography; Lane 3: Eluted fraction contains asparaginase obtained from gel exclusion by Superose 12 column chromatography.

Figure 4 shows that the L-asparaginase activity secreted by the isolated *Bacillus* sp. was stable at pH 9 after 1 hour incubation at 37 °C. The highest enzyme activity (0.20 ± 0.044 U/ml) was recorded at this pH condition. It seems that the highest enzyme activity was found at the alkaline condition. It was similar to what has been reported by most recent researchers. They found that most of L-asparaginase activities from microbial strains are stable at alkaline environment. The enzyme activity was still comparable and good at pH 8 and pH 10 even though it was slightly lower than activity at optimum pH (pH 9). It is in accordance with the previous studies, the L-asparaginase activity as well as enzyme yield, regardless their sources (L-asparaginase produced by any microbial strains) were found to be low when the enzyme was incubated at pH below and above its optimum pH [9]. Based on the obtained result, it is clear that enzyme activity was very low when the enzyme was incubated in buffers with acidic condition (pH 4-6). This acidic condition probably affected the active site of this enzyme which led to its low activity. As reviewed by other studies, the stability of this enzyme at a wide range of pH is dependent on its sources [11]. Previous studies reported that enzymes' surface exhibit a large number of basic and acidic groups that get ionized depending on surrounding pH condition [8].

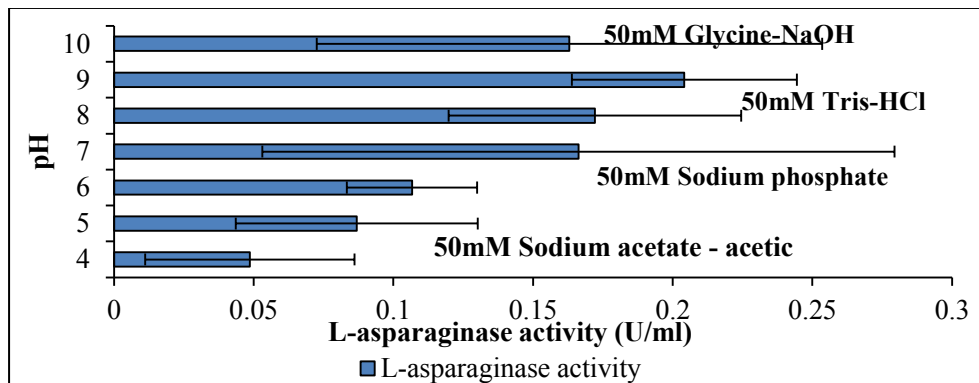


Fig. 4: Effect of pH on L-asparaginase activity.

In general, *Bacillus* sp. is characterized as a bacteria species that grow well in moderate environment; they are known as mesophiles. Mesophiles grow best in moderate temperature, typically between 20 and 45 °C. Figure 5 shows the bacteria that grow in that moderate temperature still can produce the asparaginase which is stable at high temperature (60 °C). The recorded L-asparaginase activity was 0.170 ± 0.01 U/ml. L-asparaginase activity at high temperature (40 °C to 80 °C) is higher than enzyme activity at normal environment (30 °C - 40 °C). Different sources of the L-asparaginase enzymes may have different stabilities of temperature which due to dissimilarity of their amino acid sequences and structures. Denaturation and degradation of proteins or enzymes is likely to occur at temperature above 80 °C. Both may cause loss of enzyme activity.

It was further explained by [12], the extracellular and cell-bound thermophilic enzymes (i.e., saccharidases and proteases) are highly stable and therefore optimally active at temperatures above, or sometimes far above, the host organism's optimum growth temperature. They reported that at a temperature of 117 °C, *Thermococcus litoralis amylopullulanase* was optimally active even though its optimum growth temperature was 88 °C. It is still not understood whether all thermophilic proteins can be expressed in a mesophilic environment or not. Only fewer than 10% of all the thermophilic enzymes obtained by *E. coli* have been found to poses stability, catalytic, or structural properties distinct from the enzyme's purified from the native organism. Other studies reported that thermostable L-asparaginase were produced by *Escherichia coli* and *Pseudomonas*

aeruginosa since the enzyme was found to be maximally active at 55 °C and the L-asparaginase retained 98% activity [9].

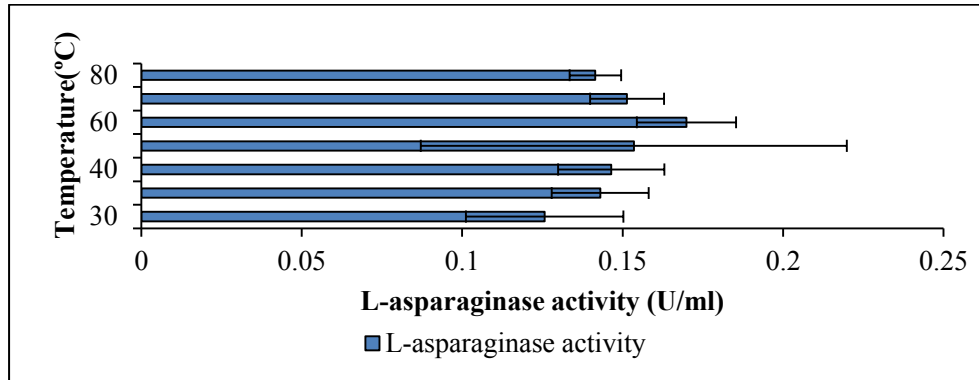


Fig. 5: Effect of temperature on activity of purified L-asparaginase activity.

5. CONCLUSION AND RECOMMENDATIONS

In summary, L-asparaginase from local, newly-isolated *Bacillus* sp. has been purified by a combination of precipitation and chromatography steps. The purified enzyme showed 10.11 U/mg specific activity, 50.07% yield with 2.21 fold purification. The molecular weight of this purified enzyme is 66 kDa in the form of a dimer. This L-asparaginase is stable at a pH of 9 and temperature of 60 °C. Its considerable stability at wide pH range and high temperature make it advantageous for use in biotechnological industries.

The antitumor activity of L-asparaginase from the *Bacillus* sp. should be further examined in order to increase its value and its properties as a potential anticancer enzyme. Later, it could be commercialized in Malaysia as an alternative drug to be used in therapeutic treatment, especially against leukemia, after a few polishing steps are done. It is also recommended to investigate the production of the desired enzyme in a bioreactor or on a large scale.

ACKNOWLEDGEMENT

This project was funded by a Research Acculturation Grant Scheme (RAGS 12-003-0003) by the Ministry of Higher Education, Malaysia.

REFERENCES

- [1] Asthana SN, Azmi W. (2003) Microbial L-Asparaginase : A Potent Antitumour Enzyme. *Indian J Biotechnol.*, 2:184-194.
- [2] Borek D, Jaskólski M. (2001) Sequence analysis of enzymes with asparaginase activity. *Acta Biochim Pol.*, 48(4): 893-902.
- [3] Prakasham RS, Rao CS, Rao RS, Lakshmi GS, Sarma PN. (2007) L -asparaginase production by isolated *Staphylococcus* sp. – 6A : design of experiment considering interaction effect for process parameter optimization., 102:1382-1391. doi:10.1111/j.1365-2672.2006.03173.x.

- [4] Borah D, Sangra A, Shahin L.(2012) Production , purification and process optimization of L-Asparagines (an anticancer enzyme) from *E . coli* , from sewage water. Int J Pharm Pharm Sci.,4(4):560-563.
- [5] Sunitha M, Ellaiah P, Devi RB. (2010) Screening and optimization of nutrients for L- asparaginase production by *Bacillus cereus* MNTG-7 in SmF by plackett-burmann design. African J Microbiol Res., 4(4):297-303.
- [6] Kumar NSM, Shimray CA, Indrani D. (2013) Reduction of Acrylamide Formation in Sweet Bread with L -Asparaginase Treatment. Food Bioprocess Technol., 7(3):741-748. doi:10.1007/s11947-013-1108-6.
- [7] Patro KR, Gupta N. (2012) Extraction, purification and characterization of L-asparaginase from *Penicillium sp.* by submerged fermentation,3(3) :30-34. doi:10.5897/IJBMBR11.066.
- [8] Thakur M, Lincoln L, Niyonzima FN, More SS. (2013) Isolation, Purification and Characterization of Fungal Extracellular L-Asparaginase from *Mucor Hiemalis*. J Biocatal Biotransformation., 2(2):1-9.
- [9] Jois S, Chandru S, Lakshmikantha RY, Satwadi PR. (2013) Study on production, purification and characterisation of L-asparaginase from *Escherichia coli* and *Pseudomonas aeruginosa*. Int J Pharm Chem Biol Sci. ,3(3):565-570.
- [10] Sanches M, Krauchenco S, Polikarpov I. (2007) Structure , Substrate Complexation and Reaction Mechanism of Bacterial Asparaginases. Curr Chem Biol., 1(1):75-86.
- [11] Jha, S. K.;Pasrija, D.; Sinha, R.K.; Singh, H.R.; Nigam, V.K.; Vidyarthi AS. (2012) Microbial L-Asparaginase: A Review on Current Scenario and Future Aspect. Int J Pharm Sci Res., 3(9):3076-3090.
- [12] Vieille C, Zeikus GJ, Vieille C. (2001) Hyperthermophilic Enzymes : Sources , Uses , and Molecular Mechanisms for Thermostability Hyperthermophilic Enzymes : Sources , Uses , and Molecular Mechanisms for Thermostability. Microbiol Mol Biol Rev., 65(1):1-43. doi:10.1128/MMBR.65.1.1.