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Cloning and Purification of L-Asparaginase from *Enterobacter carcerogenus*

Yakup KOLCUOĞLU[1*] and Ummuhan CAKMAK[1]

ABSTRACT: In this study, the gene coding for *Ec*L-ASP from *Enterobacter carcerogenus* was identified in full sequence and cloned into a mesophilic organism. The gene encoding L-asparaginase was transferred to the pET-28a (+) vector to ensure its expression in *Escherichia coli* BL21 (DE3) pLysS. The recombinant protein containing the N-terminal histidine tail (6xHis tag) was purified by Nickel affinity chromatography. As a result of SDS-PAGE, it was determined that the purified protein consisted of a single type of polypeptide. In the theoretical calculation, the subunit molecular weight of the recombinant protein containing the histidine tail was found to be 37 kDa. It was observed that the cloned enzyme had low L-glutaminase activity. The pH and temperature at which the recombinant enzyme showed the best activity were determined as 7.0 and 37 °C, respectively. From the drawn Lineweaver-Burk graph, it is estimated that the K_m value is 0.06 mM and the V_{max} value is 666.7 U mg^{-1} protein.

Keywords: *Enterobacter carcerogenus*, L-asparaginase, Recombinant DNA, Protein purification

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INTRODUCTION

Enzymes, an important class of biomolecules, have important roles in living organisms and are indispensable for food, agriculture, health, textile, cosmetics and many other fields. To date, many enzymes have been identified and their properties characterized. Researchers have a particular interest on a very small number of these enzymes. According to Hammes (Hammes 2008), these enzymes have specific experimental advantages L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1), which has antineoplastic activity, is among these enzymes (Kumar and Verma, 2012).

Because of its hydrolytic properties, asparaginase is very important for ALL treatment. Normal and cancerous cells (leukemic cells) need L-asparagine. In normal cells, L-aspartate is converted to asparagine by asparagine synthetase. Neoplastic cells cannot synthesize asparagine due to the absence of this enzyme. Therefore, the ability of such cells to grow and multiply depends on asparagine (Savitri and Azmi, 2003). Consequently, combining tumor cells with L-asparaginase results in the conversion of all circulating asparagine before it enters the cancerous cell. This situation causes cancer cells to die slowly due to the lack of resources they need (Dhanam and Kannan, 2013).

To date, L-asparaginase has been reported in a variety of organisms (Patro et al., 2011). L-asparaginases obtained from gram-negative bacteria can be divided into two classes as Type I and Type II. Type II L-asparaginase has higher specific activity on L-asparagine than Type I (Mario et al., 2007). Currently, L-asparaginase from *Escherichia coli* and *Erwinia carotovora* is in clinical use in the treatment of ALL. (Borek and Jaskolski, 2001).

In addition to its therapeutic properties, L-asparaginase is widely used as a food additive. When starchy foods are fried or cooked at 120 °C, acrylamide (neurotoxin) formation is observed as a result of the Millard reaction (Lingnert et al., 2002; Mottram et al., 2002). Since L-asparaginase can convert L-asparagine to L-aspartate, it is used in the processing of starchy foods to prevent the Millard reaction. Therefore, the risk of formation of acrylamide is reduced (Pedreschi et al.,2008).

In this study, L-asparaginase gene from *Enterobacter carcerogenus* strain, which was determined to be a new subtype by various morphological, physiological, biochemical, molecular and DNA hybridization analysis (Kacagan et al., 2013), was cloned into the pET-28a (+) vector and expression in Escherichia coli BL21 (DE3) pLysS strain. The expressed protein was purified and the presence of pure protein was determined electrophoretically. In addition, the presence of enzyme activity was determined by spectroscopic methods and its biochemical and kinetic properties were also investigated.

MATERIALS AND METHODS

Crude enzyme extract

Enterobacter carcerogenus (*E. carcerogenus*), was grown under appropriate conditions (Kacagan et al., 2013). Cells were precipitated by centrifugation at 10.000 rpm for 10 min at 4 °C. The harvested cells were suspended in buffer, lysozyme (0.5 mg mL^{-1}) was added to the mixture and left incubated at 37 °C for 30 minutes. The cells were then sonicated at a rate of 80% for 5 minutes at 1 cycle. The resulting crude mixture is centrifuged at 10,000 rpm for 10 minutes at 4 °C and the supernatant obtained will be used as crude enzyme extract. The presence of *E. carcerogenus* L-asparaginase (*Ec*L-ASP) was determined by performing activity assay from the crude extract.

Cloning of *Ec*L-ASP

gDNA isolation from *E. carcerogenus* was performed using Genomic DNA Purification Kit. In order to obtain the gene encoding *Ec*L-ASP from gDNA, the sequences of genes of similar organisms previously studied were overlapped using NCBI database and forward and reverse degenerate primers

were designed from the appropriate regions of the gene (Forward: 5'-CAGTAAATAGTGAAGTTTAGT-3'and Revers: 5'-AAWRBSCWKYMMGWCYWSYRS - 3'). Then, inverse PCR was performed based on the identified gene sequence in order to find the missing gene sequence of L-asparaginase gene. Dream Taq master mix was used for PCR. The PCR process was amplified with 30 cycles: denaturation, annealing, extension and extension, respectively, at 95 °C for 2 minutes, at 60 °C for 1.30 minutes; 72 °C for 2 minutes and 72 °C for five minutes. The PCR products were purified using Promega kit and inserted into the p GEM-T easy vector (Promega). They were sequenced after plasmid isolation. Based on the obtained L-asparaginase gene sequence, invers PCR was performed to complete the deficiencies in the beginning and end of the gene. (Forward: 5'-CGCCATTGTGTCGGTACCATG-3', Revers: 5'- TGCATGTCCGGTAAAGTCAAC-3'). Inverse PCR was performed in 30 cycles following the protocol in the presence of Dream Taq master mix: denaturation, 95 °C for 2 min; annealing, 55 °C for 1.30 min; extension, 72 °C and additional extension for 2 minutes. 72 °C for 5 minutes.

In order to amplify the gene encoding *Ec*L-ASP, a set of forward and reverse primers were designed considering the exact sequence of the gene (Forward: 5'-GGCTAGCATGGAGTTTTTGCAGAAGACG-3', *Nhe* I site underlinded; Reverse: 5'-GAAATGTTTAACCAGTACTAACTGACAAGCTTG-3', *Hind* III site underlined). The primers were designed to be suitable linkers for the His tail at the N-terminus of the gene and to include the stop codon at the end, utilizing the pET-28 a (+) vector. The procedure used to perform this PCR: first denaturation, 95 °C for two min; annealing, 55 °C for one min; and extension, 72 °C for two min and additional extension five min at 72°C. The amplified fraction was purified from agarose gel with the aid of the kit (Promega), It was digested simultaneously with NheI and Hind III and then cloned into pET-28a(+). The amino acid sequence of the resulting enzyme was determined using the BLAST tool on the NCBI website (Figure 1).

```
E.coli           MEFFKKTALAALVMGFSGAALALPNITILATGGTIAGGGDSATKSNYTAGKVGVENLVNA         60
E.carcerogenus   MEFLQKTALKSIYVAYTGGTIGMQRGYIPVS-GRHGGRGNSTTK--FSKSLVSVASTVIS         57
                  ***::**** :: :.::*.::.: .  * .: *  .* *:*:**  :: . *.* . * :

E.coli            VPQLKDIANVKGEQVVNIGSQDMNDNVWLTLAKKINTDCDKTDGFVITHGTDTMEETAYF        120
E.carcerogenus   NPLITPARALP-VAPPMLTLPDMHDQVCVRLTTTIPRSL----ALFLPTVPSSFRNSPFC        112
                   * :.    :       :   **:*:* : *:..*  .     .:.:    .::.:: :

E.coli           LDLTVKCDKPVVMVGAMRPSTSMS--------ADGPFNLYNAVV-TAADKASANRGVLVV        171
E.carcerogenus   GALPTPERRISAFTGWRRKLRTTSAEIPGIVTTPSPLPLTRSQSVAASDEQSVRPPCSAQ        172
                   * .   :  .:.*  *   : *        : .*: * .:   :*:*: *..    .

E.coli           MNDTVLDGRDVTKTNTTDVATFKSVNYGPLGYIHNGKIDYQRTPARKHTSDTPFDVSKLN        231
E.carcerogenus   VG-CPKPRRRIWMP-----ASSSGCRFGEAKASKPSACAFVVLLPR------YSRLLKNS        220
                 :.      * :        *: .. .:*     : .   :     *         : * .

E.coli           ELPKVAIVYNYA--------NASDLPAKALV--------DAGYDGIVSAGVGNGNLYKSV        275
E.carcerogenus   DTPKVGIFVNRVICCHIQRVEQVDLPVRTQLRQRNLAGDDDGLAQILQRHEG--------        272
                 : ***.*. * .        :  ***.:: :        * *   *:.   *

E.coli           FDTLATAAKNGTAVVRSSRVPTGATTQDAEVDDAKYGFVASGTLNPQKARVLLQL-----        330
E.carcerogenus   ------ECRGG-----------------------KR--HCVGTMQDHKAVVLVVMMRNMA        301
                       .:.*                       *    . **:: :** **: :

E.coli             ----------------------ALTQTKDPQQIQQIFNQY         348
E.carcerogenus     LWALISSITSEFAAACRSTIWESSQRVELTPDEEMFNQY         340
                                       :*  :    :::****
```

Figure 1. Amino acid sequence alignment of *Ec*L-ASP

Expression of L-asparaginase gene

The gene cloned into the expression vector was transferred to *E. coli* BL21strain. Colonies containing recombinant plasmids were grown in Luria-Bertani medium containing 50 µg mL^{-1} kanamycin and grown overnight at 37 ºC and inoculated to a new medium at a ratio of 1: 100. Induction

was performed by adding 1.0 mM isopropyl-β-D-thiogalactopyranoside when the optical density (600 nm) was in the range of 0.6-0.8. It was then grown for another 3 hours at 37 °C (Kolcuoglu et al.,2010). Induced and uninduced cell proteins were visualized by SDS-PAGE (Figure 2A).

Purification of L-asparaginase

Expressed cells were precipitated by centrifugation at 10,000 rpm for 10 minutes at 4 °C. The collected cells were suspended in 1 mL of 100 mM Tris-HCl (pH 7.4) buffer; lysozyme was added to the mixture containing 0.5 mg mL^{-1} and incubated for 30 minutes at 37 °C. The cells were then sonicated in an ice bath at a rate of 80% for 5 minutes in 1 cycle. The resulting crude mixture was centrifuged for 10 minutes at 10.000 rpm at 4 °C and supernatant was used as crude enzyme extract. The *Ec*L-ASP protein was purified in a single step using the MagneHis Protein Purification System (Promega) containing the nickel affinity column (Figure 2B). Protein purification using the polyhistidine tag relies on the binding affinity of histidine residues for an immobilized metal such as nickel. This affinity interaction is thought to be the result of the coordination of an empty coordination site on the metal and a nitrogen on the imidazole moiety of the polyhistidine. Polyhistidine tags offer distinct advantages for protein purification. The small size of the polyhistidine tag renders it less immunogenic than other larger tags. For this reason, the label does not usually need to be removed in operations following purification.

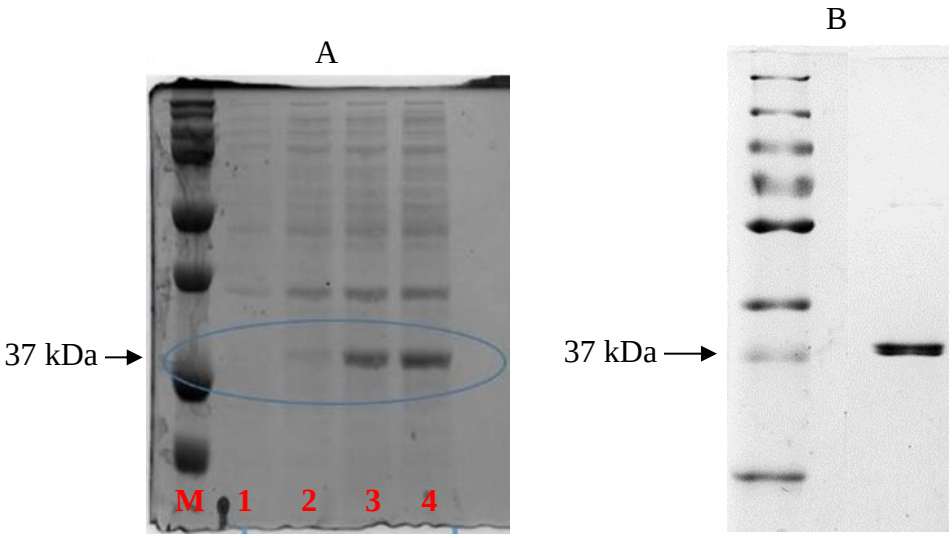

Figure 2. SDS PAGE. **A)** M: Marker, 1: First sample, 2: One-hour induction, 3: Two-hour induction, 4: Three-hour induction. **B)** Purification of recombinant L-asparaginase

Enzyme activity assay

For *Ec*L-ASP activity, 0.9 mL of 0.01 M substrate was prepared in 50 mM Tris-HCl buffer (pH 7.0) and incubated with 0.1 mL of enzyme for 30 min at optimum temperature. At the end of the incubation period, 0.1 mL of TCA (15% w/v) was added to the reaction mixture. The reaction mixture was centrifuged at 10000 x g and the precipitate was removed. The released ammonia was found by measuring the absorbance at 436 nm by adding 0.1 mL of Nessler reagent, 0.1 mL of supernatant and 0.8 mL of distilled water (Singh et al.,2013). In the case of glutaminase activity, glutamine was used as the substrate in the above method (Singh and Banik, 2013).

Optimum pH and Temperature

The optimum pH of *Ec*L-ASP activity was determined with buffer solutions prepared at different pHs and using L-asparagine as substrate. For this purpose, Mcilvaine buffer (citric acid/disodium hydrogen phosphate buffer, pH 3.0-6.0) and Tris-HCl buffers (pH 7.0-9.0) prepared at 50 mM concentration were used.

To determine the effect of temperature on enzyme activity, activity measurements were made in increments of 10 °C. Buffer and substrate solution mixtures were incubated for 30 min at the optimum pH and specified temperature values. The effect of substrate concentration and protein content on enzyme activity was investigated by using the optimum pH and temperature values.

Effect of enzyme concentration

In order to examine the change in enzyme activity by varying the *Ec*L-ASP concentration and to determine the optimum amount of protein with the highest activity, the activity change was plotted against varying amounts of protein (0.01-1 mg mL^{-1}) at predetermined optimum pH and temperature and constant amount of substrate.

Substrate concentration

In order to determine the optimal substrate concentration at which the enzyme showed activity, enzyme activity was determined against varying amounts of substrate (0.005-30 mM) under the optimum conditions previously described. Using the obtained data, K_m and V_{max} values were calculated with the help of Lineweaver-Burk graph.

RESULTS AND DISCUSSION

Obtaining the Full Sequence of *Ec*L-ASP Gene by PCR

The results obtained from the sequence analysis of the PCR product revealed that the recombinant gene consisted of 1044 nucleotides and encoded 340 amino acid units (www.expasy.org). The molecular weight of the recombinant L-asparaginase expressed in *E. coli* was calculated to be 37.3 kDa (Anonymous, 2021).

Using the BLAST program, it was observed that the sequence of *Ec*L-ASP was similar to those of *Enterobacter* species. As a result of the examination, it was found that it was 81% similar to *E. ludwigii* L-asparaginase, 81% to *E. cloacae* EcWSU1 L-asparaginase and 81% to *E. mori*L-asparaginase.

Expression and purification of *Ec*L-ASP

The recombinant gene cloned into the pET-28a (+) vector was expressed to contain 6xHis-tag in *E. coli* BL21 (DE3)pLysS in the presence of kanamycin and 1 mM IPTG at 37 °C. Expressed protein sample and protein obtained by purification were visualized on SDS-PAGE (Figure 2A). The fact that the pure protein is in a single band on the chromatogram indicates that the enzyme does not comprise or contain subunits of different molecular weight (Figure 2B). It has been reported in the literature that recombinant L-asparaginase has been purified from different sources using Ni-affinity chromatography. L-asparaginase enzyme from *Enterobacteriaceae*, a thermotolerant bacterium, (Vidya and Pandey 2012), from *E. coli* (Zhang et al. 2017) and from *Thermococcus gammatolerans* EJ3 (Zuo et al. 2014) were purified by nickel affinity chromatography. Electrophoretic studies have shown that the enzyme is effectively expressed and purified when compared to non-induced *E. coli* intracellular proteins. Single band was observed in SDS polyacrylamide gel electrophoresis after purification. As a result of the electrophoresis, the monomer molecular weight of *Ec*L-ASP was determined as 37 kDa.

In the literature, the molecular weight of *Bacillus licheniformis* L-asparaginase with glutaminase-free activity was calculated to be approximately 37 kDa from SDS gel electrophoresis (Sudhir et al., 2014).

Moleculer weight of *Erwina chryssanthemi* L- asparaginase 37.2 kDa (Kotzia and Labrou, 2007), *Pseudomanas stutzeri* MB-405 L-asparaginase molecular weight 34 kDa (Manna et al., 1995), *Eschericia coli* L-asparaginase II 37 kDa (Khushoo et al., 2004), *Streptomyces gulbargenis* L-

asparaginase molecular weight 85 kDa (Amena et al., 2010) and Acinetobacter *calcoaceticus* L-asparaginase molecular wight were calculated 25 kDa (Joner, 1976).

Determination of Enzyme Activity

In order to perform characterization studies, L-asparagine and L-glutamine were used as substrates for activity for pure enzyme. According to the findings, the activity of the enzyme against L-asparagine substrate was higher (Table 1).

Table 1. Relative activity values obtained in the presence of L-asparagine and L-glutamine substrates

Substrate	Activity (%)
L-asparagine	100
L-glutamine	40

Effect of pH on *Ec*L-ASP

To determine the optimum pH value of *Ec*L-ASP, enzyme activity was determined by using buffers solutions at different pHs with L-asparagine substrate. Using these obtained activity values, pH-% relative activity curve was plotted (Figure 3). When the pH dependency of recombinant *Ec*L-ASP was examined, a single peak was observed in the obtained graph and the optimum pH value was determined as 7.0. In the literature, optimum pH 7.0 in *Corynebacterium glutamicum*, *Flammulina velutipes* L-asparaginases (Mesas et al., 1990; Eisele et al., 2011); *Erwina carotovora* L-asparaginase pH 8.6 (Kamble et al., 2006) and *Pseudomonas stutzeri* L-asparaginase has been reported as the optimum pH 9.0 (Manna et al., 1995).

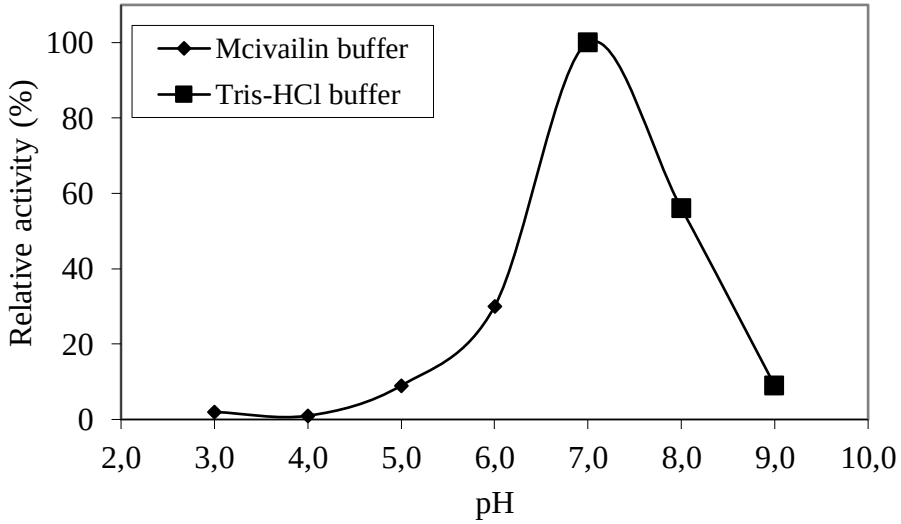

Figure 3. pH effect on recombinant *Ec*L-ASP activity

Effect of Temperature on *Ec*L-ASP

The change in *Ec*L-ASP activity with respect to temperature was tested between 20 °C and 50 °C in increments of 10 °C and the temperature-% relative activity graph is shown in Figure 4. It was observed that the enzyme activity reached an optimum at 37 °C and maintained its activity at approximately 20% at 40 °C. When similar studies were examined in the literature, pure L-asparaginases obtained from *Corynebacterium glutamicum* and *Flammulina velutipes* showed optimum activity values at 40 °C (Mesas et al., 1990; Eisele et al., 2011), *Erwina carotovora* L-asparaginase at 35 °C (Kamble et al., 2006) and *Pseudomonas stutzeri* L-asparaginase showed optimum activity at 37 °C (Manna et al., 1995).

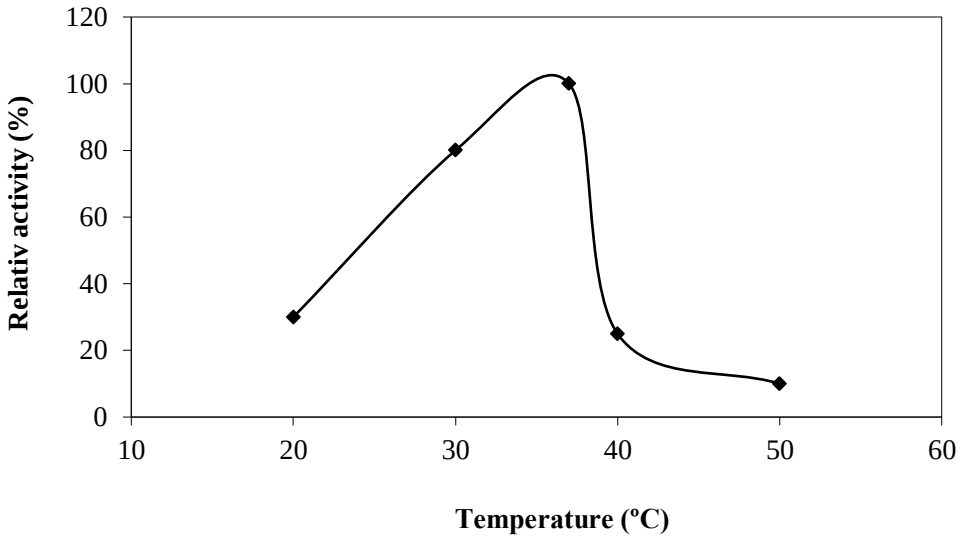

Figure 4. Effect of temperature on *Ec*L-ASP activity

Effect of Enzyme Concentration on Activity

In order to investigate the dependence of *Ec*L-ASP activity on the amount of protein, the variation of the activities depending on the amount of protein in the reaction mixture was examined. Activity determinations were made using enzyme solutions to contain protein at the final concentration of 0.01-1 mg mL^{-1} in the reaction mixture. Optimal enzyme concentration was determined as 0.4 mg mL^{-1} from hyperbolic curve obtained by graphing activity values against protein concentration.

Effect of Substrate Concentration on *Ec*L-ASP

In order to examine the effect of substrate concentration on *Ec*L-ASP activity, activity determinations were performed in the presence of L-asparagine at the final concentration of 0.005-30 mM in the reaction mixture while keeping the enzyme concentration constant. Lineweaver-Burk (Figure 5) graphs were drawn with the help of the data obtained after the activity measurements performed. The enzyme showed the highest activity in the presence of 10 mM L-asparagine; On the Lineweaver-Burk graph, it was calculated that K_m value was 0.06 mM and V_{max} was 666.7 U mg^{-1} protein.

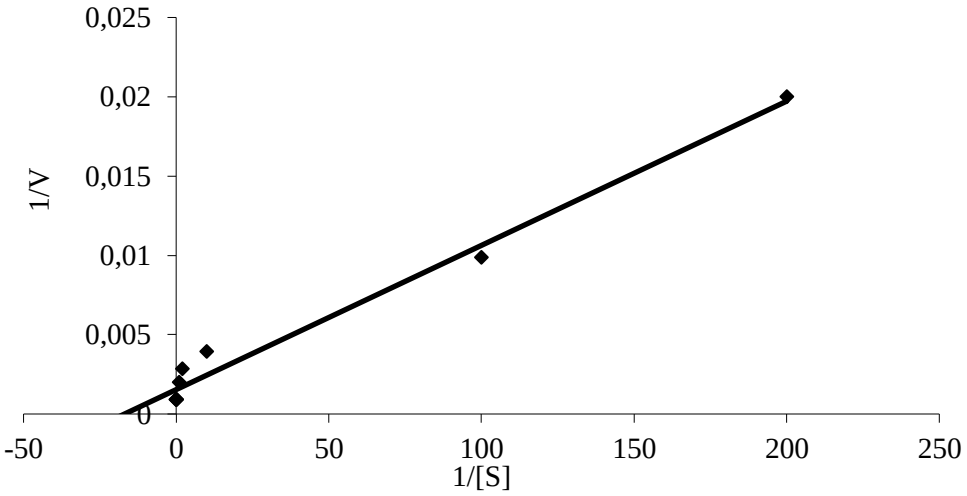

Figure 5. Lineweaver-Burk plot for *Ec*L-ASP

CONCLUSION

Considering all these data, It was determined that the enzyme which was cloned from *E. carcerogenus* and transferred to *E. coli* BL21 (DE3) pLysS strain, expressing and purified with six His tails at the head, was L-asparaginase, an enzyme that breaks down L-asparagine. It has been found that this partially characterized enzyme has an optimum temperature at 37 °C, which is desirable human metabolism temperature. The optimum pH value of 7.0 was found with a physiological pH. However, at acidic pH (5.0-6.0) where cancer cells live, activity is observed to be around 40%. As a result, it is contemplated that the resulting recombinant enzyme may be used in the bakery sector for pre-treatment with dough to reduce the formation of acrylamide or to treat ALL by shifting the optimum pH to acidic pH with mutation.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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