Purification and immobilization of L-asparaginase from *Citrobacter freundii* EGY-NE1

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Research

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Abstract

L-asparaginase is used as an antileukemic drug and as a food additive to reduce the risk of acrylamide formation. New L-asparaginases are required to reduce costs and avoid clinical side effects. *Citrobacter freundii* EGY-NE1represents a potential source of new L-asparaginase. We purified extracellular L-asparaginase from *Citrobacter freundii* EGY-NE1 (through ammonium sulfate precipitation, dialysis, Sephadex-G50 and DEAE-cellulose columns) to 5.83 fold and 25.76 % recovery. The purified L-asparaginase was a low molecular weight enzyme of 19 kDa. It was optimally active at 37 ˚C, pH 8 and 40 mM asparagine. The K_m and V_{max} of the enzyme were 0.0179 M and 2.66 U/ml, respectively. Ca^{2+}, Mg^{2+}, K^+ and Ba^{+2} activated the enzyme, while Na^+, Zn^{+2}, EDTA, azide, tartrate and $HgCl_2$ inhibited it. The crude and purified enzymes were immobilized by encapsulation in Ca-alginate; this improved their stability and reusability. The entrapped L-asparaginases on Ca-alginate were examined by scanning electron microscope; their protein diameters ranged from 42.17 to 47.37 nm and from 46.78 to 71.97 nm for the immobilized crude and purified enzymes, respectively. Both the immobilized enzymes kept their maximal activity for 10 minutes at 40 ˚C. After the 5th cycle of repeated use, the immobilized purified and crude enzymes kept 91% and 89 % of their activities, respectively.

Introduction

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) is widely used in the clinical and food industries for converting L-asparagine to aspartate. As a food additive, it reduces the risk of acrylamide formation (Pedreschi et al. 2008). As an antileukemic drug, it consumes asparagine; hence, kills tumor cells (Benchamin and Roshan 2019). It is also used as a biosensor of asparagine in leukemia and food industries (Verma et al. 2012). For these applications, researchers explore L-asparaginases from several microorganisms (Izadpanah Qeshmi et al. 2014).

Bacterial L-asparaginases are classified into two types; L-asparaginase I is constitutively secreted intracellular and L-asparaginase II is extracellularly secreted in response to nitrogen starvation (Izadpanah et al. 2018). L-asparaginase II represents the applicable enzyme because it has more affinity than type I for L-asparagine (Castro et al. 2021) and accumulates in broth cultures, which facilitating downstream processing (Amena et al. 2010).

The downstream processing of the enzyme includes its purification and immobilization. The pure enzyme allows studying its molecular, physiological and Kinetic properties (Nadeem et al. 2009, 2015). Different immobilization methods (including adsorption onto solid carriers, entrapment in polymeric gels, encapsulation in membranes, cross-linking with bifunctional or multifunctional reagents and linking to an insoluble carrier (Klibanov 1983)) limits the mobility of the enzyme, so it facilitates the control of the reaction endpoint and increases the operational stability (Robert DiCosimo et al. 2013; Ahmad and Sardar 2015); hence, guarantees low costs for food and clinical industries.

Although several commercial L-asparaginases are available (Batool et al. 2016), industries still require stable L-asparaginases possessing a high affinity to asparagine (Chand et al. 2020). Since 1966, type II L-asparaginase from *E. coli* was clinically used as an anticancer drug (Borek and Jaskólski 2001). However, it causes hypersensitivity side effects (Pochedly 1977) due to its glutaminase activity against normal cells (Howard and Carpenter 1972). In 2011, FDA approved *Erwinia chrysanthemi*-derived asparaginase for patients with hypersensitivity to *E. coli*-derived asparaginase (Salzer et al. 2014); nevertheless, this is also limited by clinical side effects (Moola et al. 1994).

Thus, researchers still explore and characterize L-asparaginases from new sources to reduce production costs and avoid clinical side effects. *Citrobacter* represents a potential new source of L-asparaginases that was rarely studied; to our knowledge, only intracellular rather than extracellular L-asparaginases from *Citrobacter* were only twice studied (Bascomb et al. 1975; Davidson et al. 1977). This study aims to purify and characterize extracellular type II L-asparaginases from *Citrobacter freundii* EGY-NE1; then, immobilize it via encapsulation in Ca-alginate to improve its stability and reusability.

Materials And Methods

The bacterial strain

Citrobacter freundii EGY-NE1 was isolated from Egyptian clay soil (Damietta) and identified according to its phenotypic and molecular characters (Frederiksen 2005) by Rizk (2019).

Growth medium and conditions

Citrobacter freundii EGY-NE1 was cultured on tryptone-glucose-yeast extract broth medium (tryptone 5.0 g, yeast extract 5.0 g, asparagine 10 g, glucose 1.0 g, K_2HPO_4 1.0 g; pH 6.0) at 37 °C and 75 rpm for 48 hours. The supernatants of grown cultures were separated by centrifugation at 7,000 rpm for 10 minutes.

L-asparaginase assay

We determined the L-asparaginase activity by assaying the released ammonia during the hydrolysis reaction of asparagine by the enzyme extracts (Prakasham et al. 2009). A mixture of the enzyme extract (0.1 ml), Tris-HCl buffer (0.2 ml, 0.05M, pH 8.6) and L-asparagine solution (1.7 ml, 0.01M) was incubated for 10 minutes at 37°C. The reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid followed by centrifugation at 1000 rpm; 0.5 ml of the supernatant was diluted to 7 ml with distilled water and 1 ml of Nessler's reagent was added. After 10 minutes, the absorbance of the developed color was measured at 480 nm by a spectrophotometer (model UV1100, Shanghai Yoke Instrument Company, China). One unit of L-asparaginase was defined as the amount of enzyme that liberates one µmole of ammonia per minute (Prakasham et al. 2009).

Protein Estimation

Total protein was estimated according to the method of Bradford (1976).

Purification of L-asparaginase from *Citrobacter freundii*

Proteins in the crude enzyme preparations were precipitated under cold conditions by using different concentrations of ammonium sulfate (40, 50, 60, 70, and 80%) with stirring for 20 minutes; then, we left it overnight at 4°C for complete precipitation. The precipitated proteins were separated at 5000 rpm for 20 minutes then suspended in sodium phosphate buffer (0.1M, pH 7). These enzyme preparations were put in dialysis tubes against 0.05 M sodium phosphate buffer (pH 7) overnight at 4˚C to get rid of salts.

Gel filtration chromatography followed by anion exchange chromatography was used to purify the enzyme. The enzyme preparation was applied to a gel filtration Sephadex G-50 column (55 × 1.5 cm) equilibrated by Tris-HCl buffer (0.05 M, pH 8), then eluted by the same buffer. The eluted fractions (2 ml) were assayed for L-asparaginase activity and protein content. Active fractions were pooled for further purification by anion exchange chromatography.

The active fractions were applied to an anion exchange column of DEAE-cellulose (30 × 1.5 cm) equilibrated with Tris-HCl buffer (0.05 M, pH 8). The proteins were eluted with a linear gradient of 0.0 to 1.0 M NaCl in the same buffer; each fraction (2 ml) was assayed for L-asparaginase activity and protein content.

Sodium dodecyl sulfate poly-acrylamide gel electrophoresis

Sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) was used for qualifying and comparing protein samples according to the method of Laemmli (1970). Protein samples were boiled for 2 minutes in the sample-loading buffer. The molecular weight of protein bands was referenced to a pre-stained protein marker (# SM 0671, Fermentas). Electrophoresis was carried out by Bio-Rad Mini-Protein II cell gel apparatus at 120 volts.

The separated proteins on the gel were stained in Coomassie brilliant blue R-250 for one hour; then, soaked in a de-staining solution (H_2O: methanol: acetic acid 50:40:10) for two hours until the protein bands appeared.

Activity staining of L-asparaginase

Activity staining was performed in a Petri dish containing L-asparagine gel (1% L-asparagine, 1% agar dissolved in 25 ml of 0.05 M Tris-HCl buffer; pH 8.0). We added the enzyme solution (20 µl) to a formed well in the gel; then, incubated it at 37°C. After 30 minutes, the gel was stained with Nessler's reagent; the formation of a brown zone indicates L-asparaginase activity (Lincoln et al. 2019).

Biochemical and kinetic properties of the purified L-asparaginase

We studied the enzyme activity at various temperatures by incubating the assay reaction mixtures at different temperatures (31, 34, 37, 40, 43 and 46 °C). To study the effect of pH on L-asparaginase activity,

we adjusted pH of Tris-HCl buffer (the standard buffer of the assay) to different pH values (3, 4, 5, 6, 7, 8, 9 and 10). The activities were expressed as a relative percent to the maximal activity.

The effects of metal salts (NaCl, KCl, $CaCl_2$, $ZnCl_2$, $BaCl_2$, $MgCl_2$ and $CaSO_4$) and inhibitors (sodium azide, sodium tartrate, mercuric chloride, EDTA and sodium EDTA) on enzymatic activity were investigated. The purified enzyme (0.2 ml) was mixed with the salt or inhibitor solutions at 10 mM final concentration; then, incubated at room temperature for 30 minutes before the enzyme assay. The activities were expressed as a relative percent to the control.

For the kinetic properties, the enzyme activity was assayed by using reaction mixtures containing different L-asparagine concentrations (9, 10, 20, 30, 40, 50 and 60 mM). The Km and Vmax values were calculated from Lineweaver–Burk plots.

Immobilization of the crude and purified L-asparaginase by encapsulation in Ca-alginate

The enzyme was encapsulated in Ca-alginate beads as described by Ulu and Ates (2017). We mixed the crude or purified L-asparaginase (10 ml) with Na-alginate solution (40 ml, 2%); this mixture was extruded (from 3 cm controlled distance) in 2% $CaCl_2$ solution using a micro-pipette. After 30 minutes, the formed beads were filtrated; then, washed with a sterile NaCl solution (0.9%) followed by sterile distilled water (three times). The protein content and enzymes activity were assayed after immobilization.

Characterization of the immobilized enzymes morphology

Morphology of immobilized crude and purified L-asparaginase was examined by scanning electron microscope (SEM, JEOL, JSM IT200, Japan).

Effect of temperature and pH on the immobilized crude and purified L-asparaginase

To determine the effect of temperature on the immobilized crude and purified L-asparaginase, the assay reaction mixture was incubated at different temperatures (31, 34, 37, 40, 43 and 46 °C). The effect of different pH was also studied by adjusting pH of the standard assay buffer to different values (3, 4, 5, 6, 7, 8, 9 and 10). The thermal stability of the immobilized crude and purified L-asparaginase was studied at 40˚C. The enzyme beads were incubated at 40˚C for 60 miutes, without a substrate; the enzyme activity was assayed at 10 minutes intervals. The activities of the enzyme were expressed as a percent of the maximal activity.

Reusability of the immobilized enzyme

The reusability of the immobilized crude and purified enzyme were tested up to 5 rounds; the enzyme beads were incubated with the reaction mixture at optimum conditions for the assay; at the end of the reaction, the beads were removed from the reaction mixture and washed with distilled water; these beads were repeatedly used in the subsequent four assay rounds. The activities of the enzyme were expressed as a percent of its initial activity.

Results

Purification of L-asparaginase from *Citrobacter freundii*

Extracellular L-asparaginase from *Citrobacter freundii* was precipitated using ammonium sulfate followed by purification by chromatographic techniques. The optimum ammonium sulfate concentration for L-asparaginase precipitation was 50-60% (Table 1). The precipitated enzyme was then dialyzed and concentrated on a sucrose bead. When the concentrated enzyme was applied to the sephadex G-50 column, three large active peaks were detected (Figure 1). L-asparaginase specific activity (in these active peaks) increased from 28.12 U/mg protein to 37.73 U/mg protein; that step recovered 26.28% of the activity and purified the enzyme by 3.75 fold (Table 2).

The active fractions obtained from gel filtration were further purified using anion exchange chromatography (DEAE-cellulose column); active fractions of the large peak (Figure 2) showed specific enzyme activity of 98.617 U/mg proteins; this finally purified the enzyme to 5.83 fold and recovered 25.76% of L-asparaginase from *Citrobacter freundii* (Table 2).

SDS-PAGE of the purified L-asparaginase

Figure 3 shows the protein profiles of the crude and the purified enzyme by Sephadex G-50 and DEAE-cellulose columns. Two protein bands of approximate molecular weight 19 kDa were detected in the pure enzyme sample.

Activity staining of L-asparaginase

The L-asparaginase activity of the purified protein was confirmed by the active staining; a brownish-yellow spot (indicating L-asparagine degradation by L-asparaginase) was developed only in the purified and crude sample (Figure 4).

Biochemical and kinetic properties of the purified L-asparaginase

The enzyme exhibited its maximal activity at 37 °C (Figure 5). While, the activity declined at temperatures below or above this optimal value; the enzyme relative activities were 48.73 % and 54.05 % at 34 °C and 40 °C, respectively. The enzyme activity peaked at pH 8 (Figure 6); however, the enzyme retained only 75.27 % and 53.86 % of its activity at pH 7 and pH 9, respectively. The enzyme kept more than 50 % of its activity through the range from pH 5 to pH 9.

The activity of the purified enzyme increased gradually with increasing L-asparagine concentration until saturation at 40 mM. The lineweaver-Burk plot (Figure 7) shows the affinity of the enzyme to L-asparagine. The K_m and V_{max} of the purified enzyme were 0.0179 M and 2.66 U/ml, respectively.

The cations Ca^{2+}, Mg^{2+}, K^{+} and Ba^{+2} activated L-asparaginase from *C. freundii*. The Zn^{+2} cation slightly inhibited the enzyme, while Na^{+} moderately inhibited it (Table 3). EDTA and mercuric chloride represented

the strongest inhibitors among the tested inhibitors (Table 3). Table 3 also shows the moderate inhibition of azide and tartrate to the enzyme.

Characterization of the immobilized crude and purified enzyme

Morphology of the entrapped L-asparaginase on Ca-alginate was examined by scanning electron microscope; the diameters of the entrapped proteins ranged from 42.17 to 47.37 nm and from 46.78 to 71.97 nm for the immobilized crude and purified enzymes, respectively (Figure 8.).

At the temperature range from 31 °C to 43 °C, both the crude and purified immobilized L-asparaginase retained more than 60 % of their activity (Figure. 9). The crude exhibited its maximal activity at 34 °C to 37 °C, while the purified exhibited its maximal activity only at 37 °C; the relative activity of the purified immobilized enzyme at 34 °C was 87 %. The crude and purified immobilized L-asparaginase kept their maximal activity for 10 minutes at 40 ˚C (Figure. 10); after 60 minutes, they retained 76 % and 61 % of their activity, respectively.

Figure 11 shows that the activities of the crude and purified immobilized L-asparaginase increased gradually over a pH range of 3.0 to 8.0 (an optimum pH of 8.0); above pH 8 to pH 10, the activities slightly declined.

Regarding the reusability of the enzyme, both the immobilized enzymes kept their activities over the tested 5 cycles (Figure. 12); at the end of the 5^{th} cycle, the immobilized purified and crude enzymes kept 91% and 89 % of their initial activities, respectively.

Discussion

L-asparaginase from *C. freundii* EGY-NE1 represents a promising candidate for biotechnological applications; its purification is the first step for its downstream processing. The purification approach used by this research (precipitation by ammonium sulfate followed by chromatographic techniques) was adopted by several studies (Batool et al. 2016); the precipitation step is usually followed by dialysis to remove excess salts (Makky et al. 2014). In comparing with those studies, our purification process recovered a moderate amount of the enzyme (25.8%) with a satisfied fold (5.8) (Batool et al. 2016); that fold and recovery agree those of the purification of L-asparaginase from *Bacillus licheniformis* (Alrumman et al. 2019). The purified L-asparaginase from *C. freundii* EGY-NE1 appeared as a single band of 19 kDa, which confirmed its purity.

L-asparaginase from *C. freundii* EGY-NE1 would be considered as a low molecular weight enzyme (19 kDa). The molecular weight of microbial L-asparaginase varies from one source to another. This usually falls in a moderate range from 40 to 80 kDa (Batool et al. 2016); L-asparaginase from *Bacillus* sp. (Moorthy et al. 2010), *Streptomyces gulbargensis* (Amena et al. 2010) and *Streptomyces fradiae* NEAE-82 (El-Naggar et al. 2016) exhibited molecular weights of 45, 85 and 53 kDa, respectively. Some have high molecular weights up to 120–170 kDa such as those from *Pseudomonas aeruginosa* 50071 (El-

Bessoumy et al. 2004) and *Fusarium tricinctum* (Scheetz et al. 1971). But, few L-asparaginases exhibited low molecular weights below 20 kDa; L-asparaginase from *Streptobacillus* sp. KK2S4 (Makky et al. 2014) and *Flammulina velutipes* (Eisele et al. 2011) were reported at 11.2 and 13 kDa, respectively.

The optimum temperature of the purified L-asparaginase from *C. freundii* EGY-NE1 (37°C) is the same human body temperature; this allows elimination of the body-asparagine upon in vivo treatment (Elshafei 2012); this also increases the half-life time of the enzyme in the serum; hence, avoid the need to multiple-dose causing hypersensitivity (Krishnapura et al. 2015). L-asparaginase's optimum temperatures differ from one species to another; however, those often range between 25°C and 45°C (Chand et al. 2020). In agreement with our results, L-asparaginase from the marine bacterium *Halomonas elongate* was optimally active at 37 ˚C (Ghasemi et al. 2017); while L-asparaginases from *Bacillus licheniformis* (Alrumman et al. 2019) and *Streptobacillus* sp. KK2S4 (Makky et al. 2014) were optimally active at 40˚C and 35°C, respectively.

L-asparaginases are generally active from acidic to alkaline pH (Amena et al. 2010; Chand et al. 2020). L-asparaginase from *C. freundii* EGY-NE1 was optimally active at pH 8 like those from *Streptomyces* sp. PDK7 (Dhevagi and Poorani 2006), *B. subtillis* (Jeyaraj et al. 2020) and *Streptomyces fradiae* NEAE-82 (El-Naggar et al. 2016); however, it kept more than half of its activity even at pH 5 and pH 9. The pH affects both the enzyme structure and affinity for the substrate. Therapeutic uses prefer optimally active enzymes at neutral pH, while food industries prefer those keeping enough activity at acidic pH (Krishnapura et al. 2015).

The potential applications of L-asparaginase are evaluated based on its kinetics parameters; the Michaelis-Menten constant K_m reflects the affinity of the enzyme for its substrate; the V_{max} measures the substrate turnover and is expressed in units of product formed per unit of time (Mangamuri et al. 2016). L-asparaginase of low K_m is preferred for therapy as safe doses of it would remove the L-asparagine (Beckett and Gervais 2019). L-asparaginase from *C. freundii* EGY-NE1 exhibited a high affinity to L-asparagine with K_m of 17.9 mM and V_{max} of 2.66 U/ml/min. These parameters may depend on the enzyme source and the assay conditions (Farag et al. 2015); the values of K_m and V_{max} for L-asparaginase from *Bacillus licheniformis* were 0.42 mM and 2778.9 µmol/ml/min, respectively (Sudhir et al. 2016); while those values were 0.014 mM and 4.03 IU, respectively for another *Bacillus licheniformis* (Mahajan et al. 2014); L-asparaginase from *Streptomyces fradiae* NEAE-82 showed K_m of 10.07 mM and V_{max} of 95.08 U/ml/min (El-Naggar et al. 2016).

Metal ions interface with proteins as electron donors or acceptors (Buchholz et al. 2012); hence, they can inhibit or activate enzymes by regulating the multimeric structure of the enzyme or the enzyme reaction intermediates (Krishnapura et al. 2015). However, the same metal ion or metal chelator can differently influence the L-asparaginases from different sources. While Ca^{2+}, Mg^{2+}, K^{+} and Ba^{2+} activated L-asparaginase from *C. freundii* EGY-NE1, all these ions except K + inhibited L-asparaginase from *Bacillus aryabhattai* ITHBHU02 (Singh et al. 2013); Mg^{2+} also inhibited L-asparaginase from *Cladosporium* sp. (Mohan Kumar and Manonmani 2012). In contrast, Na + inhibited L-asparaginase from *C. freundii* EGY-

NE1and activated that enzyme from *Bacillus aryabhattai* (Singh et al. 2013). L-asparaginase from *C. freundii* EGY-NE1, similar to that from *Pseudomonas stutzeri* MB-405 (Manna et al. 1995), was moderately inhibited by Na^{+}, Hg^{2+} Zn^{2+} and EDTA. Thus, data from the inhibition study help to improve the enzyme catalytic efficiency (Krishnapura et al. 2015); for L-asparaginase from *C. freundii* EGY-NE1, the presence of Ca^{2+}, Mg^{2+}, K^{+} or Ba^{2+} improves the enzyme catalytic efficiency.

Immobilization of L-asparaginases can enhance their thermal and pH stabilities in addition to their selectivity (Nunes et al. 2020); several immobilization methods and carriers have been developed. In this study, L-asparaginase was entrapped in Ca alginate beads; this generally enhanced the enzyme thermal stability and pH range. The properties of immobilized enzymes are governed by the characters of the enzyme, the carrier material and the specific interaction of the immobilization method. Immobilization of the recombinant L-asparaginase from *Erwinia chrysanthemi 3937* on epoxy activated Sepharose CL-6B enhanced its stability it at 4°C (Kotzia and Labrou 2011). The L-asparaginase fatty acid bio-conjugates improved the kinetic properties, the biological half-life, hence, the in-vivo antitumor activity of the immobilized L-asparaginase (Ashrafı et al. 2013). We firstly confirmed the efficiency of entrapment of L-asparaginase from *C. freundii* EGY-NE1 (both crude and purified enzymes) on Ca-alginate by examining their morphology by SEM; then we characterized their temperature and pH ranges.

The immobilized L-asparaginase from *C. freundii* EGY-NE1 kept the same optimum temperature (37 ˚C) of the free purified enzyme. But, the immobilized enzyme retained more activity than the free one at different temperatures along the tested range; this may be attributed to protection of the tertiary structure of the enzyme by the support material (Martinek et al. 1977). The immobilization of L-asparaginase from *C. freundii* EGY-NE1 on Ca alginate also increased the enzyme half life time at 40 ˚C; this is an expected advantage since immobilization by entrapment method protects enzymes against thermal denaturation by increasing their rigidity (Abdel-Naby 1993; Chang and Juang 2005).

The optimum pH of the immobilized L-asparaginase from C. freundii EGY-NE1 was pH 8.0, the same for the free enzyme. As a comparison, the immobilized L-asparaginase retained higher activities than the free purified enzyme along the pH range; both the immobilized purified and free purified L-asparaginase recorded their minimum relative activities at pH 3 as 75 % and 34.11 %, respectively. The solid matrix may modify the pH in the enzyme micro-environment, as compared to the bulk environment, through its surface and residual charges (Abdel-Naby 1993). Interactions between the enzyme and its carrier may also affect the intra-molecular forces maintaining the enzyme conformation, which would affect the enzyme activity (Talekar et al. 2010).

The enzyme immobilization guarantees the reusability of enzyme preparation. Thus, it permits a low cost for industrial use. Immobilization of L-asparaginase from *C. freundii* EGY-NE1 on Ca alginate beads would be useful for its application; these beads can be further packed in a column to hydrolyze asparagine. However, repeated reactions may reduce the enzyme's catalytic efficiency by weakening its binding to the carrier or distorting its active site (Abdel-Naby 1993). The immobilized L-asparaginase, in this study, kept 91 % of its catalytic efficiency even after the 5th cycle of reusability.

Conclusions

This study offers a promising new microbial L-asparaginase for applications. This extracellular type II L-asparaginase from *Citrobacter freundii* EGY-NE1 possess a low molecular weight, and well activity at temperature and pH of the human body; further in-vivo studies are needed to confirm its efficacy and safety as an anticancer agent for clinical application. The enzyme encapsulation in Ca-alginate beads improved its stability and reusability allowing an economic application in industries.

Abbreviations

SDS-PAGE : Sodium dodecyl sulfate poly-acrylamide gel electrophoresis

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics declarations

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the

corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Authors' contributions

N.R. and M.I.A. isolated and identified the bacterial strain. M.M.N. and M.I.A. conducted the experiments. M.M., N.R. and M.M.N. performed the enzyme analysis. N.R., M.I.A. and M.M. presented data in figures

and tables. M.M.N., M.I.A. and M.M. wrote the draft of the manuscript. All authors read and approved the manuscript.

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Tables

Table 1. Activities of L-asparaginase from *Citrobacter freundii* in the supernatants and precipitates during its precipitation by different concentrations of ammonium sulfate.

Ammonium sulfate concentrations (%)	Supernatants		Precipitates	
	Activity (U/ml)	Specific activity (U/mg protein)	Activity (U/ml)	Specific activity (U/mg protein)
40	1.33	49.2	2.26	52.8
50	1.39	43.3	3.06	70.2
60	0.72	21.0	4.18	71.7
70	1.40	43.6	1.87	34.2
80	1.61	49.2	1.88	34.4

Table 2. Summary of purification steps of the extracellular L-asparaginase from *Citrobacter freundii* through precipitation by ammonium sulfate, dialysis, gel filteration column (Sephadex G-50) and anion exchange column (DEAE-cellulose).

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude enzyme	3004	177.6	16.914	100	1
Concentrated crude enzyme	2775.6	153.36	18.099	92.40	1.07
Precipitation by ammonium sulfate	1187.8	35.6	33.365	39.54	1.97
Dialysis	992.73	27.06	36.686	33.05	2.17
Sephadex G-50	789.314	12.439	63.455	26.28	3.75
DEAE- Cellulose	773.85	7.847	98.617	25.76	5.83

Table 3. Effect of metal salts and inhibitors on the activity of the purified L-asparaginase from *Citrobacter freundii.* The enzyme activities are expressed relative to a control activity.

Salts	NaCl	KCl	$CaCl_2$	$ZnCl_2$	$BaCl_2$	$MgCl_2$	$CaSO_4$
Relative activity (%)	85.07	114.68	113.88	96.72	104.65	116.85	125.53
Inhibitors	EDTA	$HgCl_2$	Sodium tartrate	Sodium azide	Sodium EDTA		
Relative activity (%)	75.28	80.17	94.05	88.84	84.20		

Figures

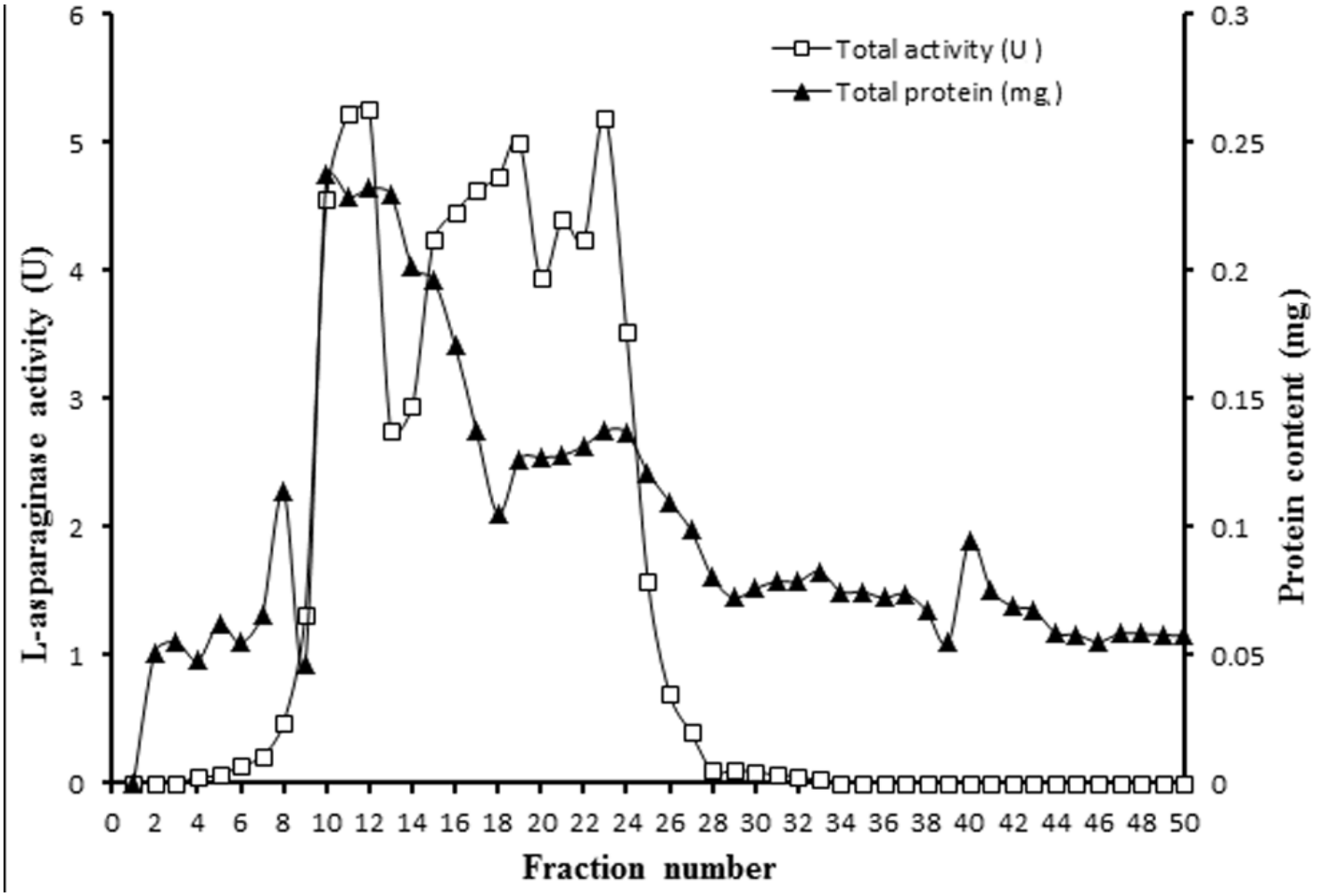

Figure 1

Purification of L-asparaginase from Citrobacter freundii using a gel filtration column of sephadex G-50.

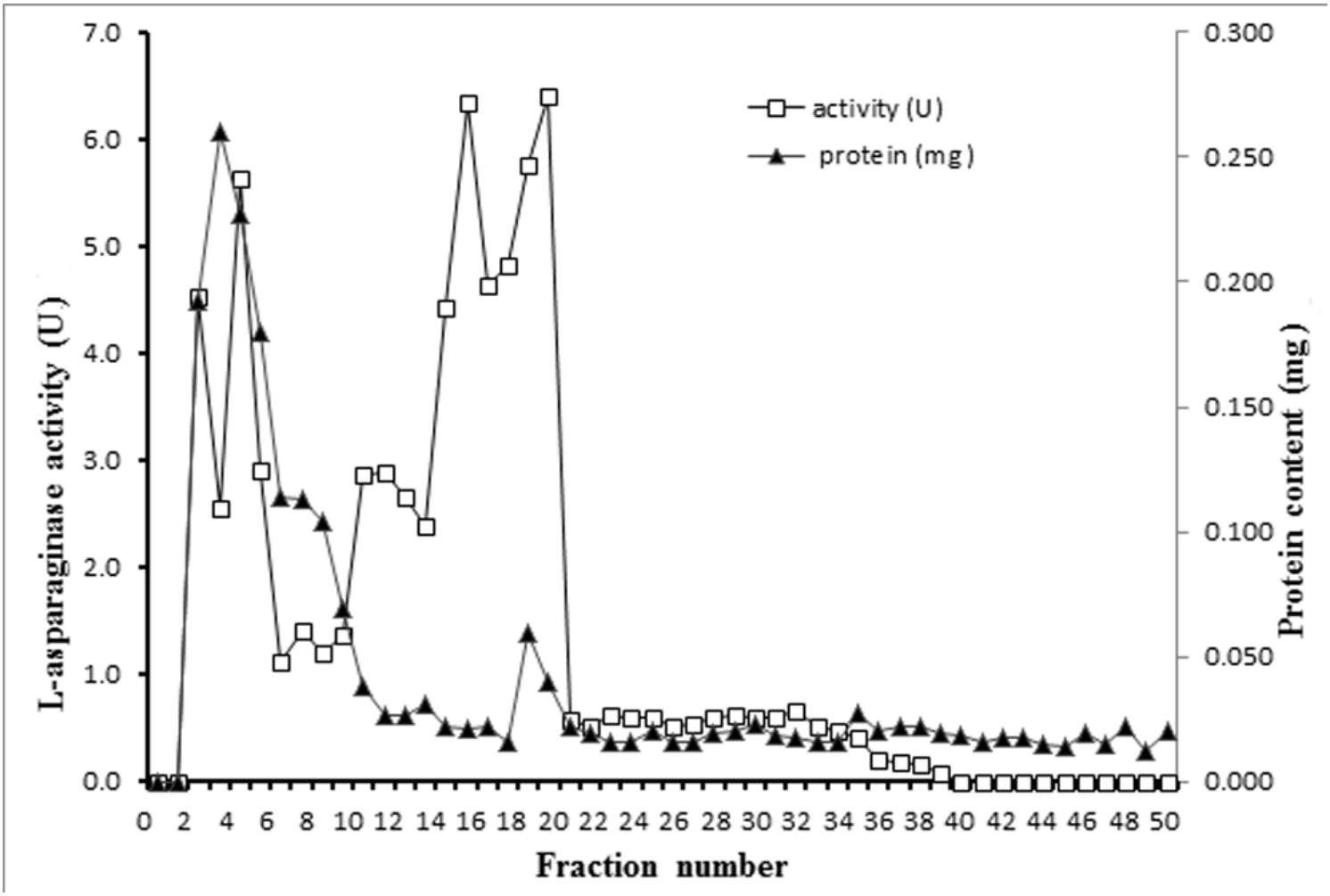

Figure 2

Purification of L-asparaginase from Citrobacter freundii using an anion exchange column of DEAE-cellulose.

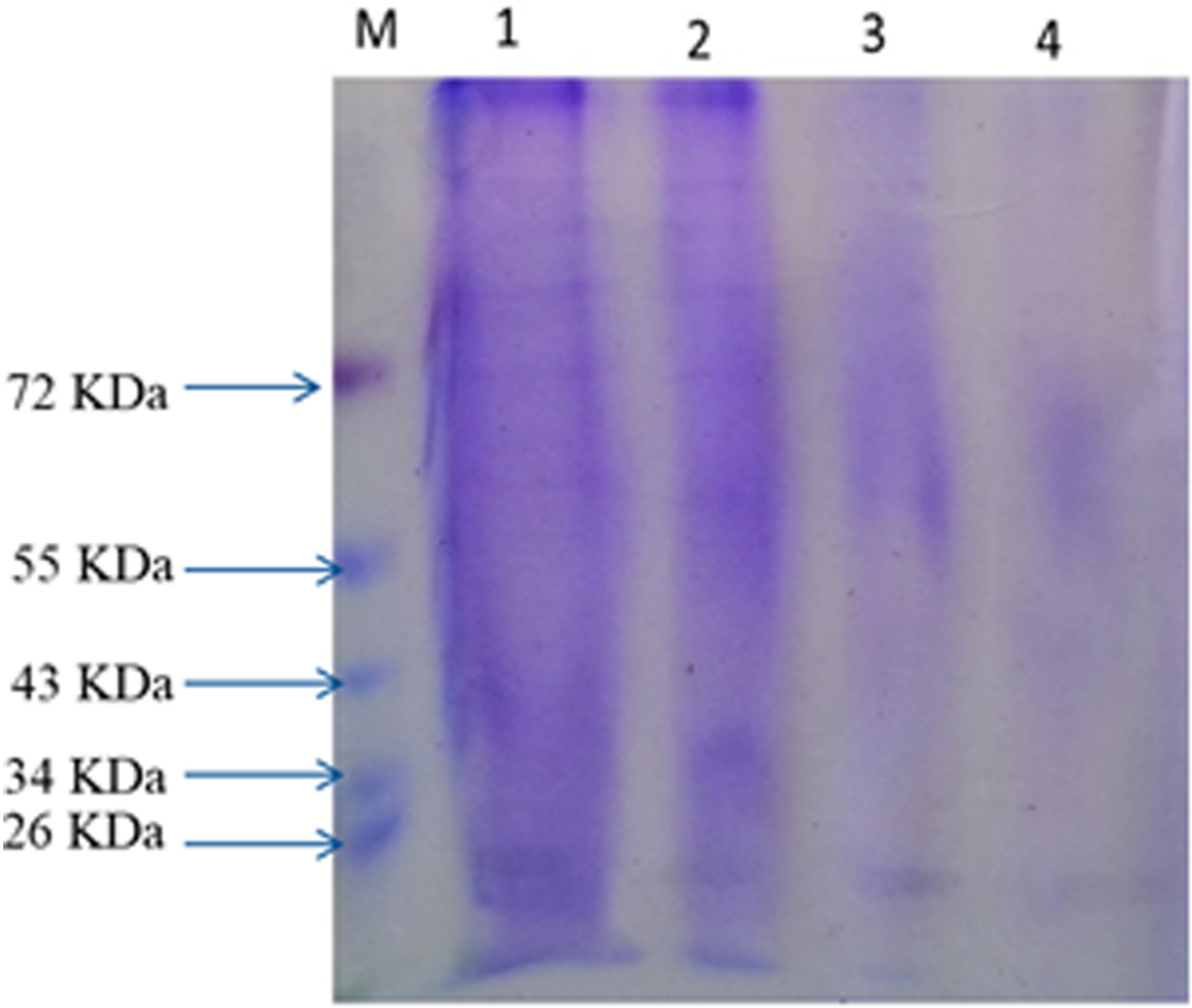

Figure 3

Protein profiles through the purification steps of L-asparaginase from Citrobacter freundii. SDS-PAGE staining Coomassie Brilliant blue R-250 of crude L-asparaginase (1), partially purified L-asparaginase after gel filtration (2), purified L-asparaginase after anion exchange (3), (4) and protein ladder (#SM 0671, Fermentas) (M).

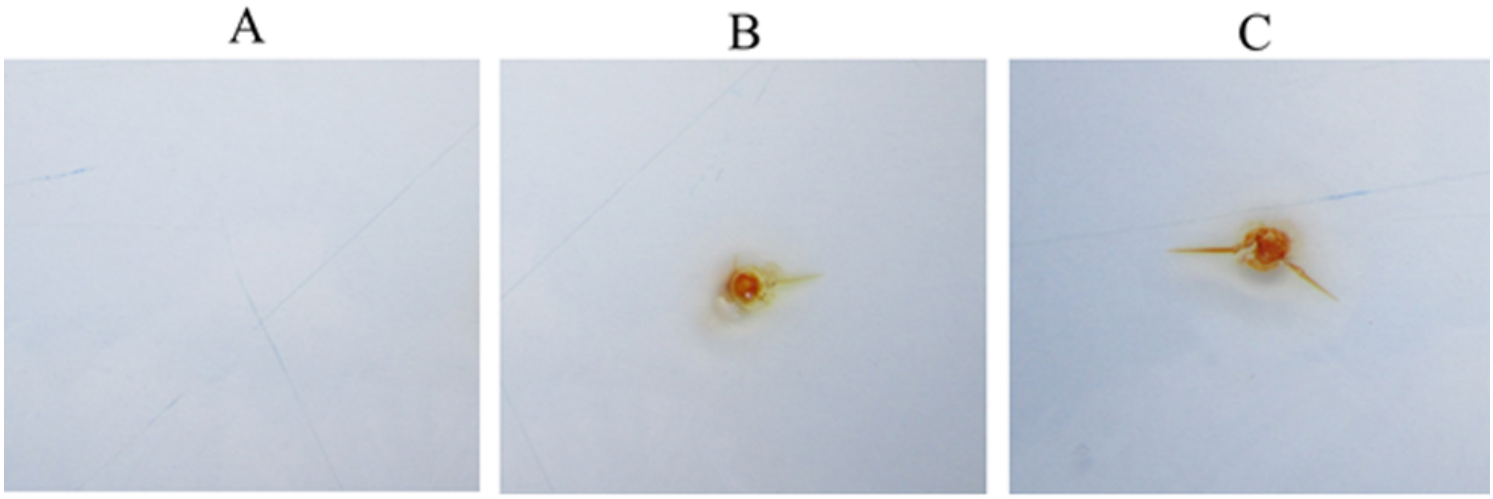

Figure 4

Activity staining of L-asparaginase from Citrobacter freundii; a control without the substrate (A), the crude sample (B) and the purified enzyme sample (C).

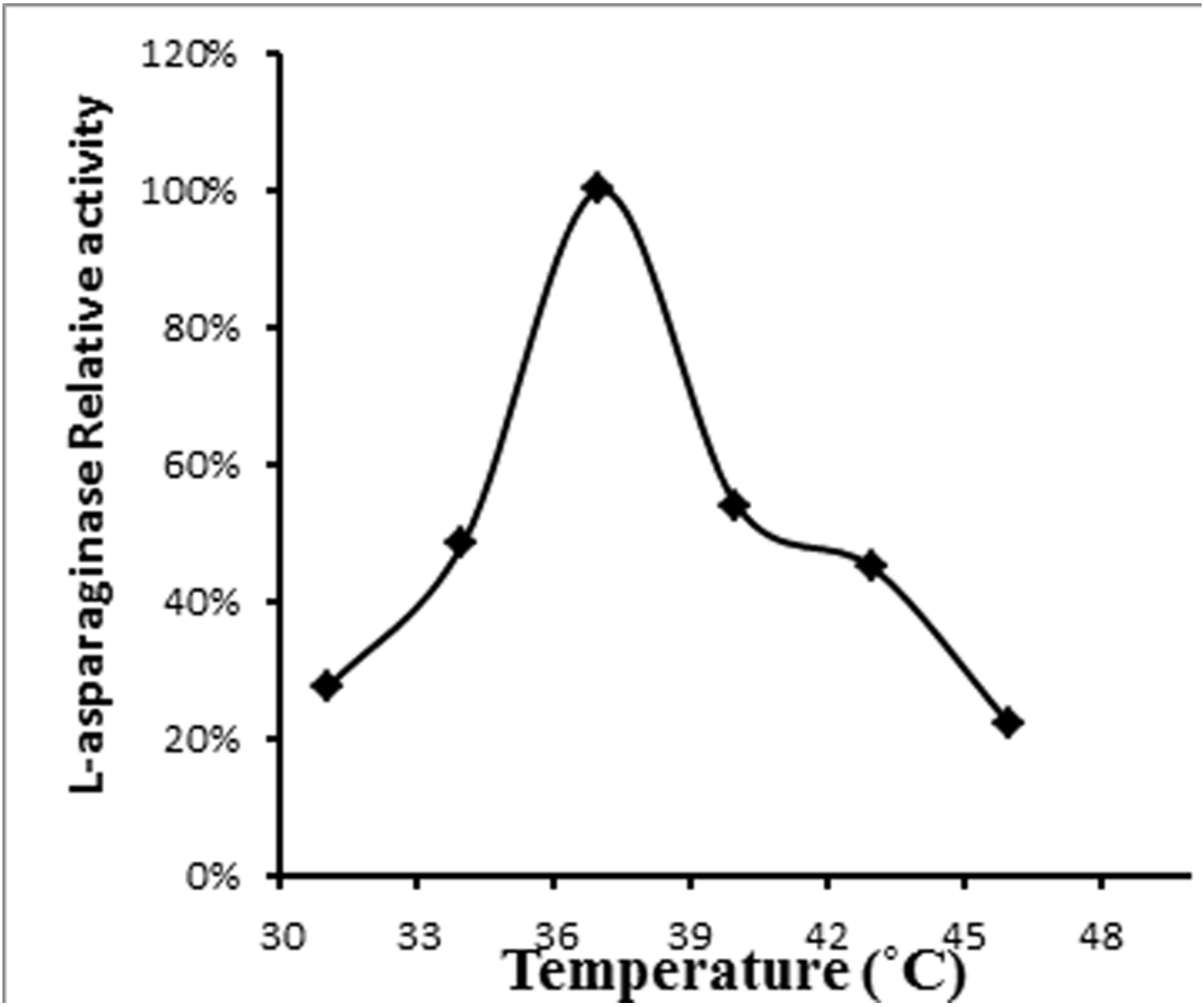

Figure 5

Effect of temperature on the purified L-asparaginase activity of Citrobacter freundii. The enzyme activities are represented relative to the maximal value.

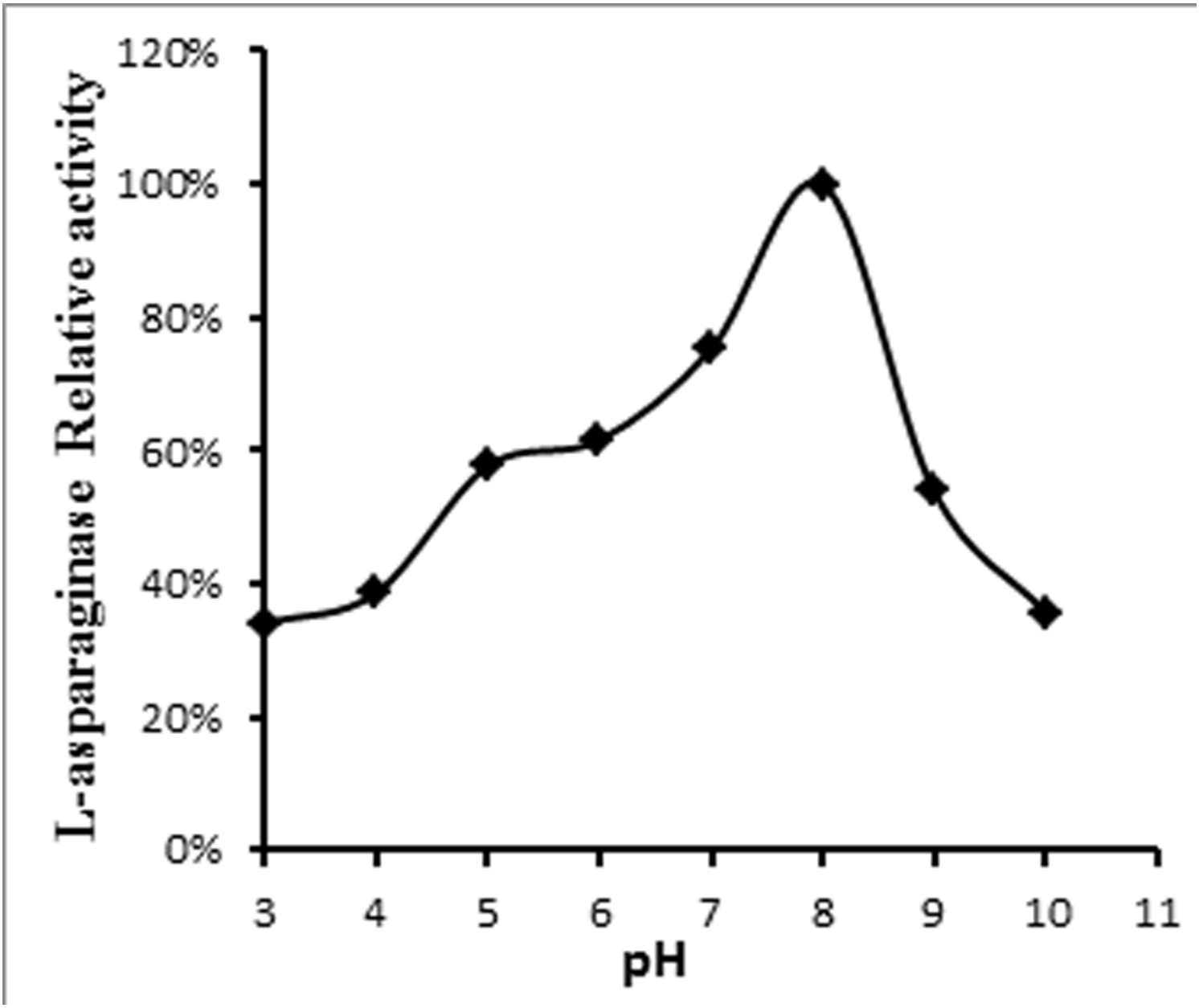

Figure 6

Effect of pH on purified L-asparaginase activity of Citrobacter freundii using Tris-HCl buffer (0.05M, in pH range 3-10). The enzyme activities are represented relative to the maximal value.

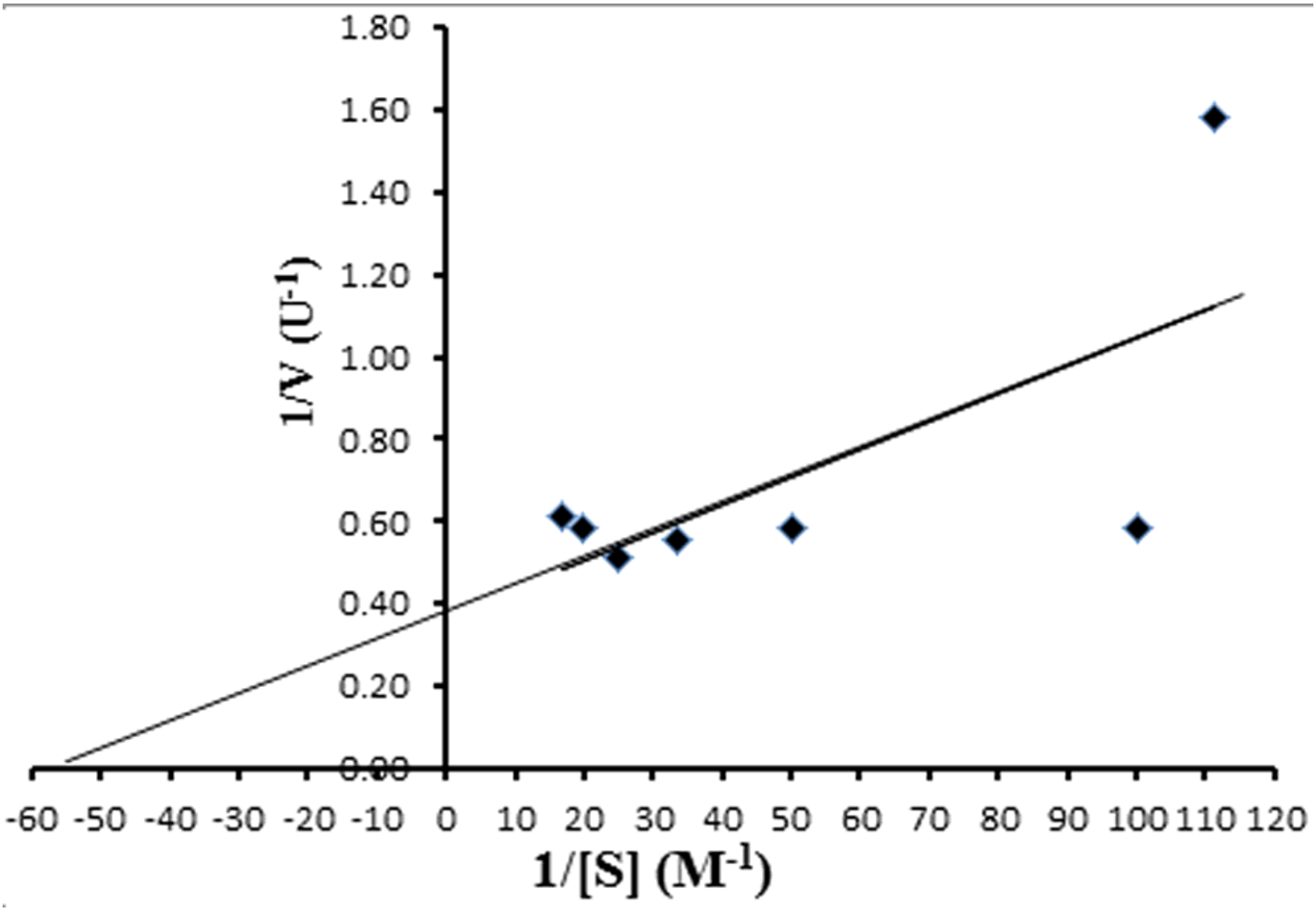

Figure 7

Lineweaver-Burk plot for determining the kinetic parameters of the purified L-asparaginase from Citrobacter freundii.

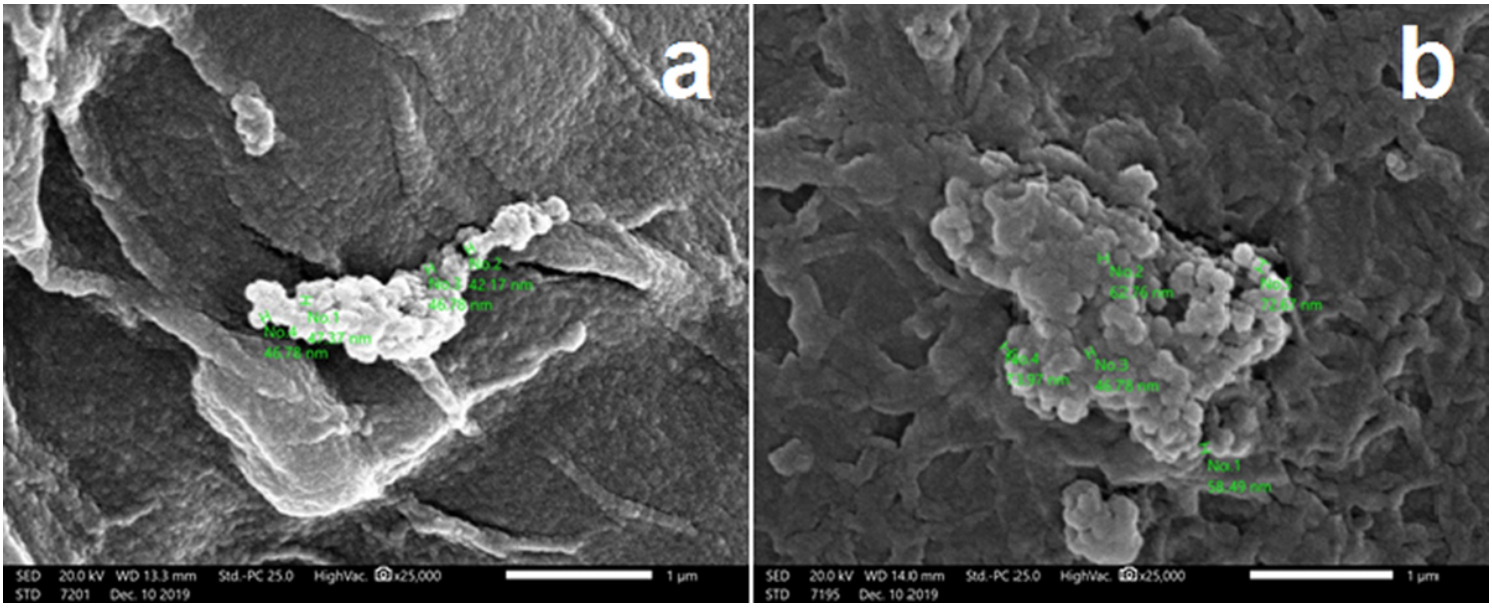

Figure 8

Scanning electron micrographs showing the morphology of the immobilized crude (a) and purified (b) L-asparaginase from Citrobacter freundii by encapsulation in Ca-alginate .

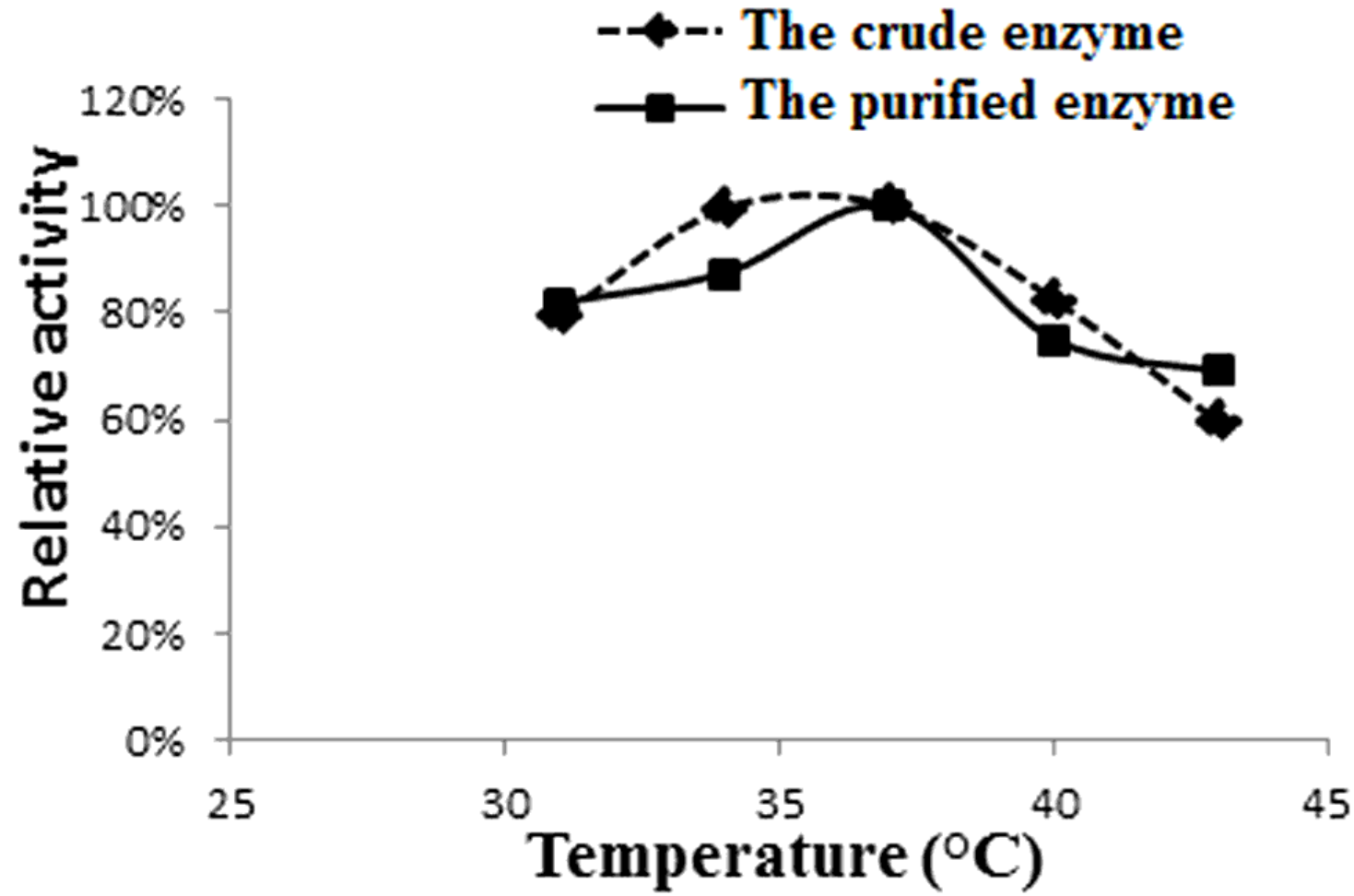

Figure 9

Effect of temperature on the immobilized crude and purified L-asparaginase activities. The activities are represented relative to the maximal value.

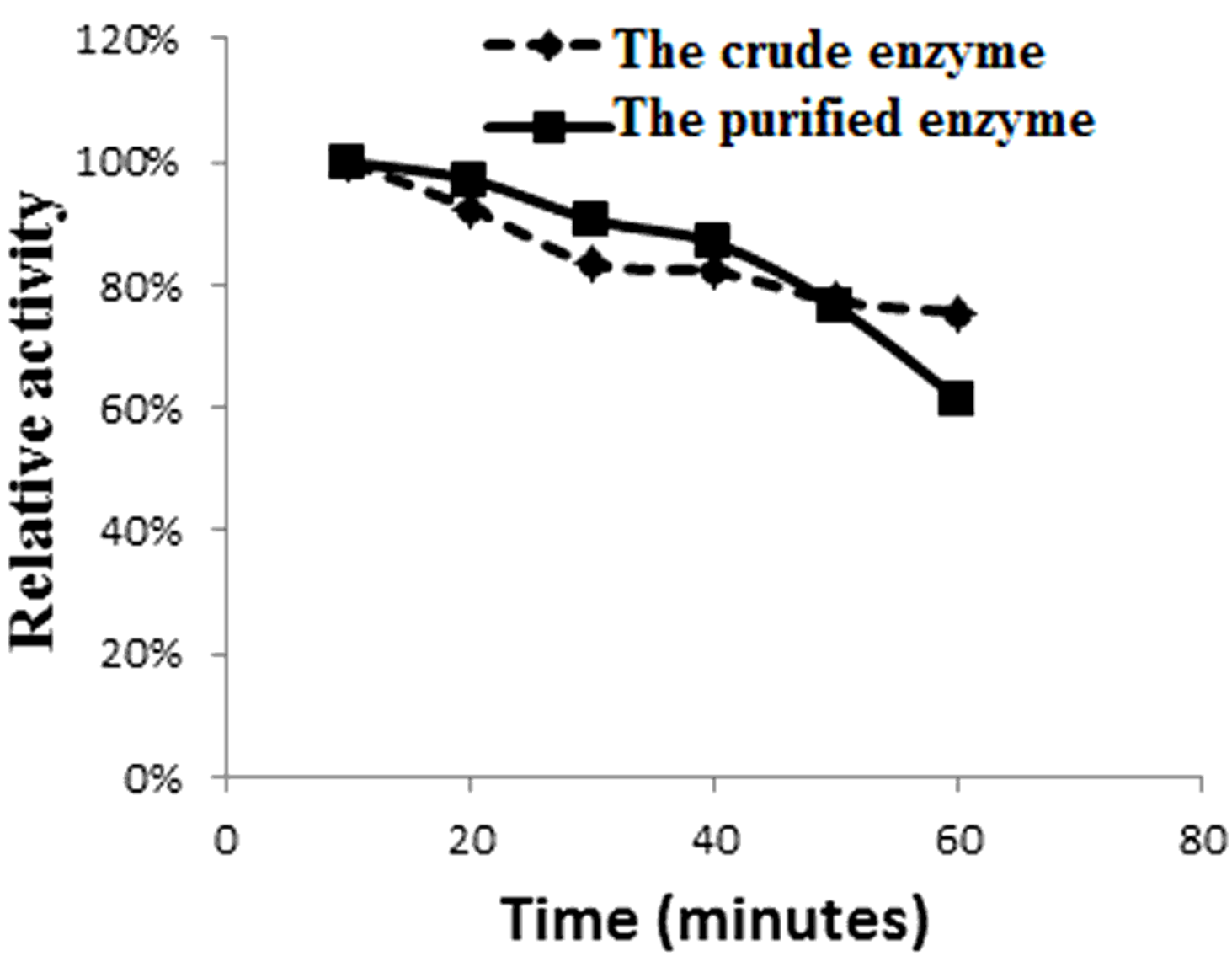

Figure 10

Thermal stability of immobilized crude and purified L-asparaginase activity at 40 ˚C. The enzyme activities are represented relative to the maximal value.

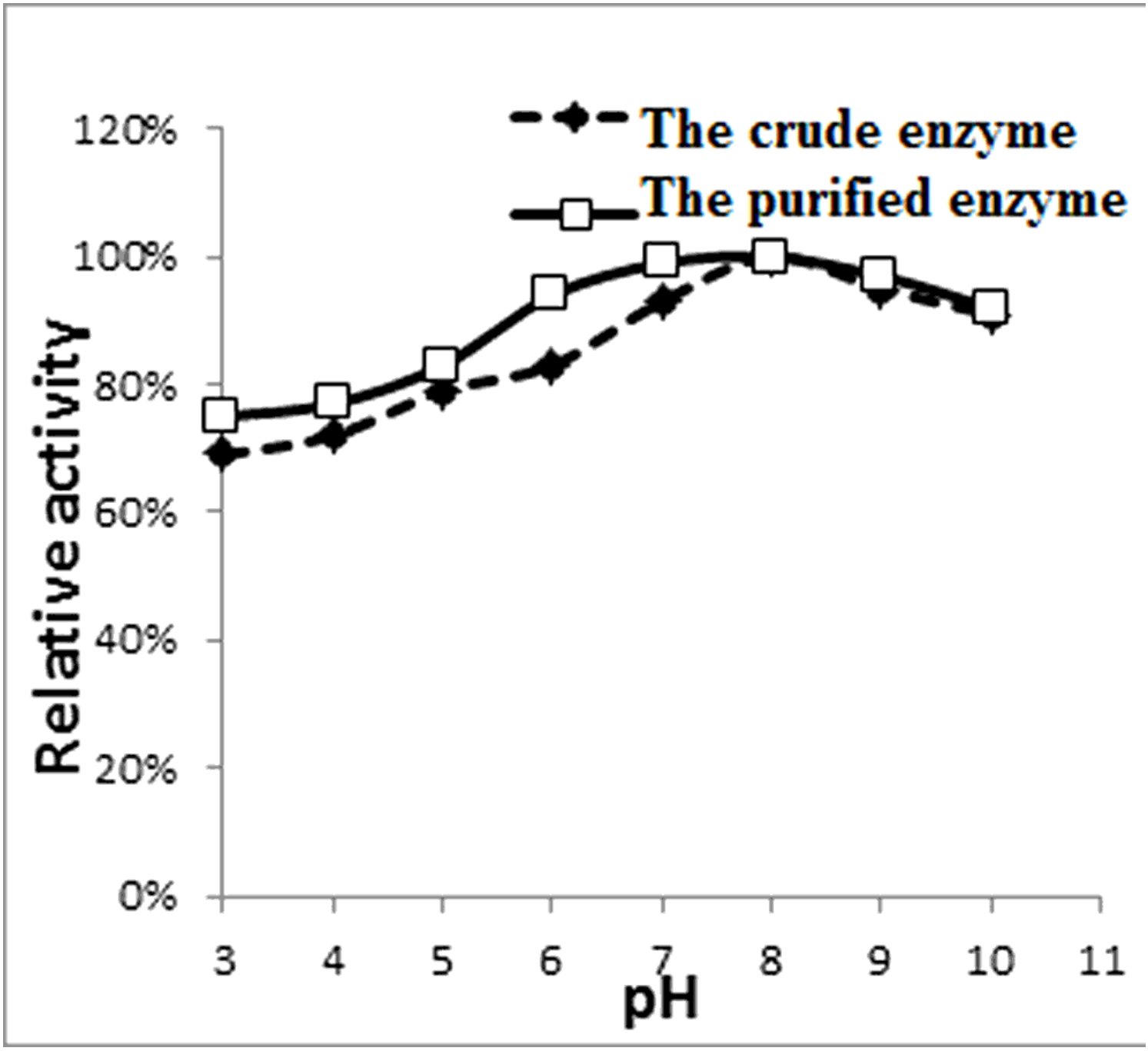

Figure 11

Effect of pH on immobilized crude and purified L-asparaginase activity. The enzyme activities are represented relative to the maximal value.

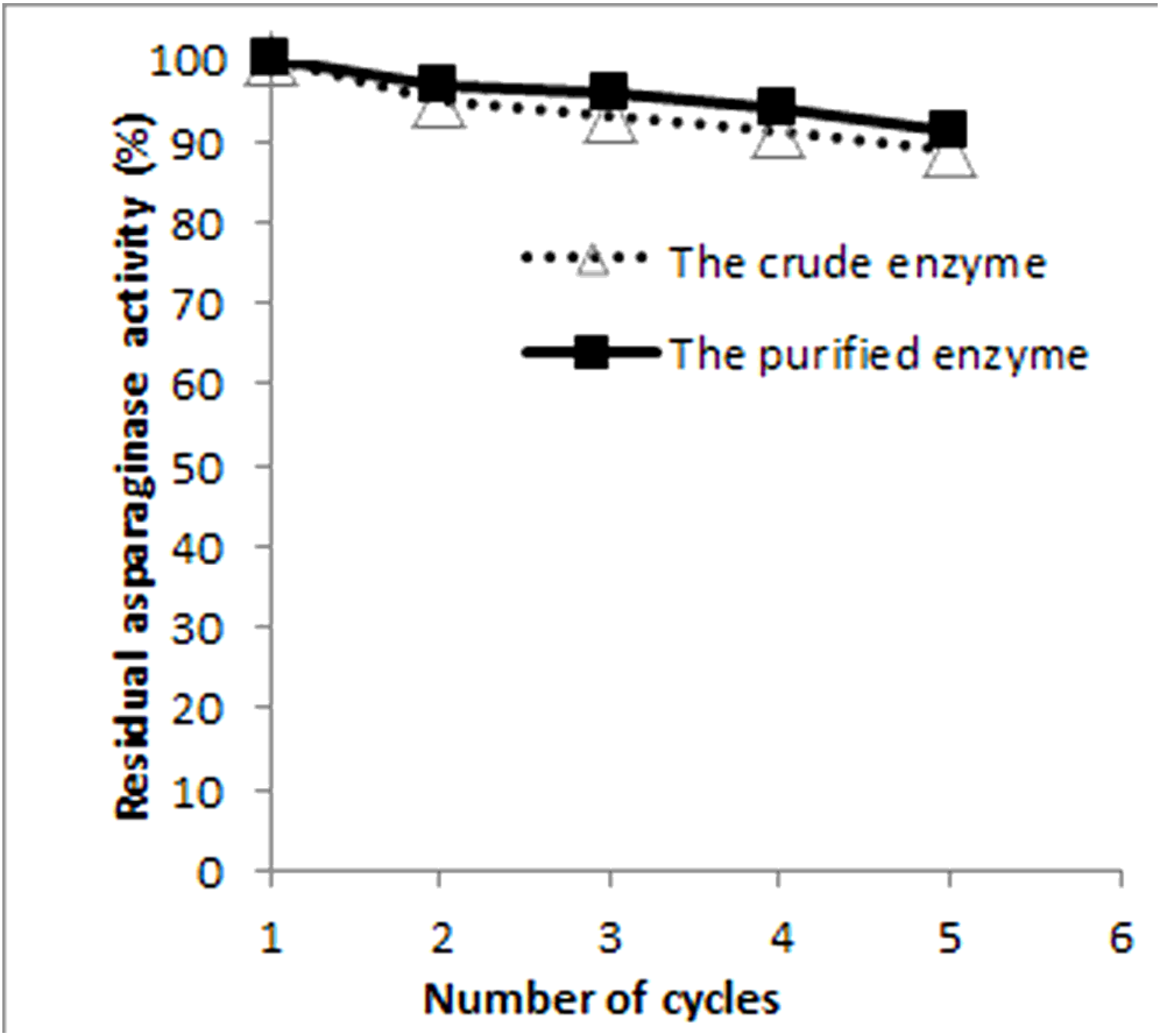

Figure 12

Reusability of the immobilized crude and purified L-asparaginase up to 5 cycles.

Supplementary Files

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- graphicalabstract.jpg