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Comparison of Free and Immobilized L-asparaginase Synthesized by Gamma-Irradiated *Penicillium cyclopium*

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Abstract

Gamma irradiation is used on *Penicillium cyclopium* in order to obtain mutant cells of high L-asparaginase productivity. Using gamma irradiation dose of 4 KGy, *P. cyclopium* cells yielded L-asparaginase with extracellular enzyme activity of 210.8 ± 3 U/ml, and specific activity of 752.5 ± 1.5 U/mg protein, which are 1.75 and 1.53 times, respectively, the activity of the wild strain. The enzyme was partially purified by 40–60% acetone precipitation. L-asparaginase was immobilized onto Amberlite IR-120 by ionic binding. Both free and immobilized enzymes exhibited maximum activity at pH 8 and 40°C. The immobilization process improved the enzyme thermal stability significantly. The immobilized enzyme remained 100% active at temperatures up to 60°C, while the free asparaginase was less tolerant to high temperatures. The immobilized enzyme was more stable at pH 9.0 for 50 min, retaining 70% of its relative activity. The maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) of the free form were significantly changed after immobilization. The K_m value for immobilized L-asparaginase was about 1.3 times higher than that of free enzyme. The ions K^+, Ba^{2+} and Na^+ showed stimulatory effect on enzyme activity with percentages of 110%, 109% and 106% respectively.

Key words: *Penicillium cyclopium*, Amberlite IR-120, gamma irradiation, ionic binding immobilization, L-asparaginase

Introduction

The enzyme L-asparaginase (ASN) (EC.3.5.1.1; L-asparagine aminohydrolase) catalyzes the deamination of L-asparagine (Asn) to L-aspartate and ammonia. The enzyme is isolated from several sources: animal and plant cells, yeast, fungi, and bacteria, with a wide variety of microbial strains producing it (Gulati *et al*., 1997). L-asparaginase received increased attention in recent years as a food processing aid which reduces the formation of acrylamide in starch-based food that is baked, roasted or fried (Tareke *et al*., 2002).

It has been identified as an effective agent in the treatment of certain types of lymphoma and leukemia. Its use in anti-cancer therapy is based on its ability to cleave L-asparagine, an amino acid essential for the growth of lymphoblasts, to ammonia and L-aspartic acid in serum and cerebrospinal fluid. Since lymphoblasts are unable to produce endogenous L-asparagine, starvation for this amino acid leads to the death of these cells (Kotzia and Labrou, 2007).

The enzyme produced by *Escherichia coli* and *Erwinia carotovora* was clinically used to treat patients suffering from asparaginase-dependent leukemia (acute lymphoblastic leukemia) and lymphomas (Keating *et al*., 1993). A number of undesirable side effects were observed in cases of acute lymphoblastic leukemia, which were attributed to the contamination of enzyme preparations with bacterial endotoxins. The search for other asparginase sources such as eukaryotes, can lead to an enzyme with less adverse effects. It has been observed that eukaryote microorganisms such as yeast and filamentous fungi *e.g. Aspergillus*, *Penicilliumm* and *Fusarium* have a potential for asparaginase production (Sarquis *et al*., 2004).

Immobilization of enzymes is one of the important trends in biotechnology. The use of immobilized enzymes lowers production costs as these can be readily separated from the reaction mixture and can hence be used repeatedly and continuously (Maysa *et al*., 2010). Native L-asparaginase is often chemically modified and physically embedded with various kinds of soluble

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and insoluble biocompatible polymers in order to produce immobilized L-asparaginase. It has been reported that the immobilized enzyme not only reduces toxicity, but also greatly improved resistance to proteolysis compared to native L-asparaginase (Zhang *et al.*, 2004; Ghosha *et al.*, 2011). Attempts were made for the preparation of insoluble matrix supports such as collagen (Jefferies *et al.*, 1977), carboxy methyl cellulose (Hasselberger *et al.*, 1970), polyacrylamide and poly (2-hydroxyethyl methacrylate) gels (O'Driscoll *et al.*, 1975) derivatives bioconjucated with L-asparaginase for use in cancer therapy. That said, enzyme immobilization has attracted great interest by chemists and biochemists for its wide application in academic research and industrial processes (Mahmoud and Helmy, 2009; Shafei *et al.*, 2015).

This research applied partial purification and immobilization of gamma-irradiated *Penicillium cyclopium* in order to improve the stability of L-asparginase for the effective mitigation of acrylamide formation in industrially processed and home-cooked high heat-treated potato products. The work was extended to compare between the kinetic parameters such as pH, thermal stability, K_m and V_{max} of L-asparginase in its native and immobilized forms.

Experimental

Materials and Methods

Chemicals. All the chemicals used in this study are of analytical grade unless otherwise stated. Dowex, duolite, chitosan, cellulose, silica gel were purchased from Fluka company, Switzerland; Amberlite IR-120 from Fine-Chem Ltd. Boisar; and L-asparagine from Merck Company.

Microorganism. *P. cyclopium* was obtained from the culture collection of the National Research Center, Dokki, Cairo, Egypt. The stock culture was maintained on agar slopes that contained potato dextrose agar medium (PDA) at 30°C and preserved at –80°C in 50% (v/v) glycerol with regular monthly transfer.

Enzyme production. *P. cyclopium* was incubated at 30°C for 3 days on a rotary shaker (200 × g) using a medium of the following composition (g/l): sucrose, 2; L-asparagine, 10.0; $NH_4(SO_4)_2$, 8.77; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; and $Cu(NO_3)_2 \cdot 3H_2O$, $ZnSO_4 7H_2O$ and $Fe SO_4 \cdot 7H_2O$ as trace elements (El Refai *et al.*, 2014). Fresh fungal spores were used as inoculums and 1 ml spore suspension (containing around 10^6 spores/ml) was added to the sterilized medium and incubated at 30°C containing 100 ml of the sterile medium. The pH of the medium was initially adjusted at pH 6.2 in reciprocal shaker. At the end of the incubation period, the mycelia were removed by centrifugation (5000 × g) for 20 min at 4°C. Proteins in the filtrate were precipitated by 60% acetone. This partially purified enzyme (specific activity 2120.8 U/mg protein) was used for the determination of L-asparaginase activity.

Enzyme assay. L-asparaginase activity of culture filtrate was determined by quantifying the ammonia formation using Nessler's reagent (Usha *et al.*, 2011). One unit (IU) of L-asparaginase activity is defined as the amount of enzyme which liberates 1 μmol of ammonia per minute (μmole/ml/min) under the standard assay conditions.

Protein determination. The protein content was determined according to Lowry method (1951) using bovine serum albumin as standard.

Mutagenesis of *P. cyclopium* isolates by gamma irradiation

Effect of γ-irradiation on *P. cyclopium*. *P. cyclopium* was cultivated on potato dextrose agar plates and incubated at 30°C for 7 days. The developed colonies were scraped off and suspended in sterile saline solution. Spore suspension in triplicates was irradiated using Co^{60} gamma source according to Iftikhar *et al.* (2010), and radiation doses of 0.5–6.0 KGy in 0.5 KGy intervals were applied. As control, the number of colony-forming unit (CFU/ml) prior to irradiation was determined upon culturing on potato dextrose agar plates, as well as the number of survivors after exposure to different radiation doses.

Viable count determination. Ten-fold serial dilutions of irradiated spore suspension of *P. cyclopium* along with the control (non-irradiated) were prepared, and 0.1 ml of each appropriate dilution was plated onto sterile potato dextrose agar media. The plates were incubated at 30°C for 24 h, and the count of survived colonies as well as the initial one were determined. A dose response curve was plotted using the resulting counts. The sublethal dose and the D_{10} value for the strain were also calculated.

Effect of γ-irradiation on L-asparaginase activity. After exposure of spore suspension of *P. cyclopium* to gamma radiation, each test tube was inoculated in the basal medium and the flasks were incubated at 30°C for 3 days. L-asparaginase activity for each irradiated dose was estimated as explained above, and the results were compared to those of the control (irradiated).

Immobilization methods

Physical adsorption. Enzyme immobilization using physical adsorption was done using alumina, polyvinyl alcohol and silica gel, prepared according to Abdel-Naby *et al.* (1998). One hundred milligrams of the carriers were incubated with 2.545 U of 60% acetone enzyme fraction

dissolved in 1 ml of 0.1 M Tris-HCL buffer (pH 8.0) at 4°C overnight. The unbound enzyme was removed by washing with Tris-HCl buffer (0.1 M, pH 8.0).

Ionic binding. Ionic binding was used to achieve enzyme immobilization using 0.2 g of the cation (Dowex-50w, Amberlite IR-120) or anion exchanger (DEAE-cellulose) equilibrated with 0.1 M acetate buffer (pH 8) or 0.1 M Tris-HCL buffer (pH 8) respectively, and incubated with 5 ml partially purified enzyme containing 2545 U in the same buffer at 40°C for 24 h. The unbound enzyme was removed by washing with the same buffer.

Covalent binding. For enzyme immobilization using covalent binding, 0.4 g of chitosan was shaken in 5 ml of Tris-HCL and 0.1 M buffer (pH 8) containing 2.5% (V/V) glutaraldehyde (GA) for 24 h at 30°C. The solubilized chitosan was precipitated by the addition of 1 ml of 0.1 M NaOH. The precipitate was collected by filtration and washed with distilled water to remove excess GA. The wet chitosan was mixed with 1 ml partially purified enzyme (2545 U). After being shaken for 1 h at 30°C, the unbound enzyme was removed by washing with distilled water.

Immobilization yield (U/g carrier). Immobilization yield was calculated according to the following equation:

Properties of the free and immobilized L-asparaginase

Optimum pH. The effect of pH on the free and immobilized L-asparaginase was studied using citrate-phosphate buffer (0.1 M pH 3.0–7.0), and Tris-HCL buffer (0.1 M, pH 8.0–10.0).

Optimum temperature. The effect of temperature was studied by incubating both the free and immobilized enzymes in their respective optimum pH at different temperatures (ranging from 25 to 70°C), with different controls, for 20 min using 2.0% L-asparagine as substrate.

Thermal and pH stability of L-asparaginase. The thermal stability of L-asparaginase was investigated by incubating the free and immobilized enzyme at various temperatures (30–80°C) with different incubation periods (15, 30, 45 and 60 min) in absence of the substrate. The relative activities were then determined. For pH stability, the free and immobilized enzymes were incubated using different pH buffers for different time intervals (30 and 60 min) after which the residual enzyme activity was determined.

Activation energy (Ea). The activation energy was determined using the slope of a linear plot of the log of the enzyme activity (v) versus 1/T. The enzyme activity (v) was expressed in U (μg protein)$^{-1}$, the temperature (T) in Kelvin (K), the gas constant (R) = 1.987 cal K^{-1} mol^{-1} and the activation energy (Ea) in kcal mol^{-1}.

Substrate concentration and determination of K_m and V_{max}. The effect of incubating different concentrations of L-asparagine (0.02–0.12 mM) with the free or immobilized enzyme at 40°C for 30 min, was investigated by estimating the residual enzyme activity. The initial velocity was measured as a function of substrate concentration and plotted as double reciprocals in accordance with the line-weaver-Burk analysis (Lineweaver and Burk, 1934). The K_m and V_{max} values were 0.0259 mM and 757.6 U/mg protein for the free enzyme, and 0.033 mM and 581 U/mg protein for the immobilized enzyme.

Effect of metal ions and EDTA on L-asparaginase activity. For determining the effect of some metal ions on L-asparaginase activity, the partially purified and immobilized enzyme were preincubated with 10^{-3} M of Na^+, K^+, Ba^{2+}, Ca^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+} and EDTA for 2 h at 30°C and the residual activity was determined.

Statistical analysis. All experiments were repeated three times. Data provided in the corresponding tables and figures represent the mean values of the results obtained, along with the relative standard deviations.

Results and Discussion

Effect of γ-radiation on the survival of *P. cyclopium*. *P. cyclopium* strain was exposed to different doses of γ-radiation ranging from 0.5 to 6.0 KGy at 0.5 KGy interval. The number of viable cells decreased exponentially with increasing radiation dose. The D_{10} value was 2.5 KGy and the sublethal dose was found to be 6.0 KGy.

Production of L-asparaginase by mutant gamma-irradiated *P. cyclopium*. The potential improvement of L-asparaginase production in γ-irradiated *P. cyclopium* was studied. Table I shows that L-asparaginase activity

Table I
Influence of Co^{60} gamma irradiation at various doses on L-asparaginase activity

Recovered activity (%)	Specific activity (U/mg protein)	L-asparaginase activity (U/ml)	Dose (KGy)
100	400 ± 0.6	160 ± 1	Control
33 ± 1.5	294.4 ± 3	53 ± 2.5	0.5
46 ± 1	389.5 ± 0.5	74 ± 2.6	1
49.7 ± 0.9	397.5 ± 4	79.5 ± 1.6	1.5
57.9 ± 0.9	421.4 ± 2.5	92.7 ± 2.5	2
62.9 ± 1	437.8 ± 2	100.7 ± 0.5	2.5
93.7 ± 1.5	441.4 ± 3.3	150 ± 2.9	3
122.2 ± 0.8	651.7 ± 1.3	195.5 ± 1.4	3.5
131.6 ± 0.9	752 ± 1.5	210.8 ± 3	4
65.9 ± 0.4	363.4 ± 1.8	105.4 ± 2.8	4.5
34.8 ± 0.5	289.5 ± 2.4	55 ± 2.9	5

and specific activity increased with increasing the dose of gamma radiation. They both reached a maximum 210.8 ± 3 U/ml and 752.5 ± 1.5 U/mg protein at dose level 4 KGy. This was 1.75 times more for asparaginase activity, and 1.53 times more for specific activity of the wild strain. Similar results were obtained by El-Batal *et al.* (2000) and Fadel and El-Batal (2000). This enhancement by gamma radiation may be either due to an increase in gene copy number or gene expression or both (Rajoka *et al.*, 1998). Several studies recorded that low doses of gamma radiation may stimulate microbial growth and metabolic activities. Meanwhile, high doses of gamma radiation were proved to be inhibitory for both growth and enzymatic activities of microorganisms. The exposure of cells to ionizing radiation sets off a chain of reactions giving rise to chemical and then to metabolic or physiological changes. The irradiation presents an additional stress to the cells which tends to disturb their organization. Irradiation effects have been shown to occur with proteins, enzymes, nucleic acid, lipids and carbohydrates, all of which may have marked effects on the cell (Ismail *et al.*, 2010).

Partial purification of *P. cyclopium* L-asparaginase. The crude enzyme of the γ-irradiated *P. cyclopium* was subjected to purification (Table II). Both the enzyme activity and the specific activity of the partially purified asparaginase were increased to 3.0 fold and 2120.8 U/mg protein respectively.

Immobilization of *P. cyclopium* L-asparaginase using different carriers. The partially purified enzyme was dialysized, lyophilized and immobilized. Different immobilization techniques were investigated: physical adsorption, covalent binding and ionic binding. Results are presented in Table III. The highest loading efficiency (1200 ± 1.1 U/g carrier) and immobilization yield (80 ± 0.2%) were detected with ionic binding technique using Amberlite IR-120 which is a strongly acidic cation exchange resin suitable for a wide variety of chemical process applications. The least enzyme activity (200 U/g carrier) and immobilization yield (11%) were detected with covalent binding using Duolite 147 as a carrier. Therefore, Amberlite IR-120 proved to be the most appropriate carrier and was used in the succeeding work. Abdel-Naby *et al.* (1998) found that Amberlite IR-120 was a good carrier for *Bacillus mycoides* alkaline protease ionic binding. Also, Spinelli *et al.* (2013) stated that the use of low-cost matrices such as Amberlite IR-120 for enzyme immobilization represents a promising product for enzymatic industrial applications. On the contrary, Sundaramoorthi *et al.* (2012) showed that the highest enzyme immobilization activity and highest immobilization yield were achieved by the cross-linking technique using silica gel.

Optimum pH for free and immobilized enzymes. Both free and immobilized *P. cyclopium* L-asparaginase preparations exhibited maximum relative activity at pH 8.0 (Fig. 1). The immobilized form showed better relative activities across different pH levels ranging from 3.0 to 10.0. Dramatic decrease in relative activity (55–45%) was observed for the free enzyme at pHs 9

Table II
A profile of partial purified *P. cyclopium* L-asparaginase.

Purification steps	Protein of fraction (mg/F)	Recovered protein (%)	Total activity (U/ml)	Recovered activity (%)	Specific activity (U/mg protein)	Purification Fold
Culture filtrate	28 ± 0.3	100.00	210.8 ± 3.7	100.0	750 ± 2.8	1.0
Precipitation by 40–60% acetone	1.2 ± 0.2	4.3 ± 0.7	2545 ± 4.5	12 ± 0.05	2120.8 ± 0.9	3 ± 0.1

Table III
Different immobilization methods of *P. cyclopium* L-asparaginase.

Method of immobilization	Carrier	Added Enzyme (U/g carrier) A	Unbound Enzyme (U/g carrier) B	Immobilized enzyme (U/g carrier) I	Immobilization yield (I/A-B) x100
Physical adsorption	Alumina	2545	603.8 ± 0.9	700 ± 2	36.1 ± 0.2
	P.V.A	2545	241.5 ± 1.3	328.1 ± 2	14.2 ± 0.1
	silica gel	2545	633.9 ± 2.1	800 ± 3	41.8 ± 1.2
Covalent binding	Chitosan	2545	682.5 ± 1	500 ± 1.5	26.8 ± 3
	duolite-147	2545	301.9 ± 1	200 ± 2	11 ± 0.25
	duolie-C280	2545	300 ± 2.5	450 ± 2.6	36.1 ± 0.3
Ionic binding	Amberlite 120	2545	1045 ± 2.6	1200 ± 1.1	80 ± 0.2
	Dowex 40–50	2545	656.3 ± 2.4	350 ± 2.8	18.5 ± 1.5
	Dowex 50	2545	780 ± 0.6	250 ± 0.6	14 ± 0.2
	Cellulose	2545	600 ± 1.5	360 ± 2.8	18.5 ± 0.3

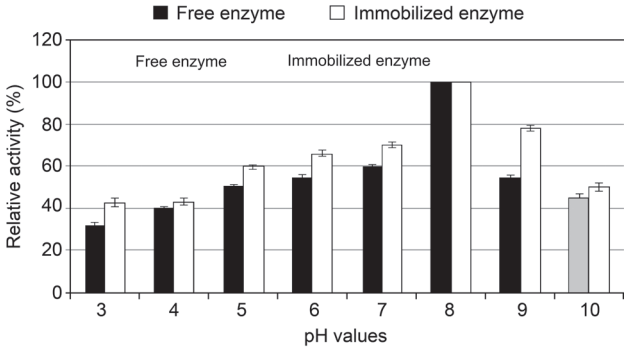

Fig. 1. Effect of pH values on the activities of the free and immobilized *P. cyclopium* L-asparaginase.

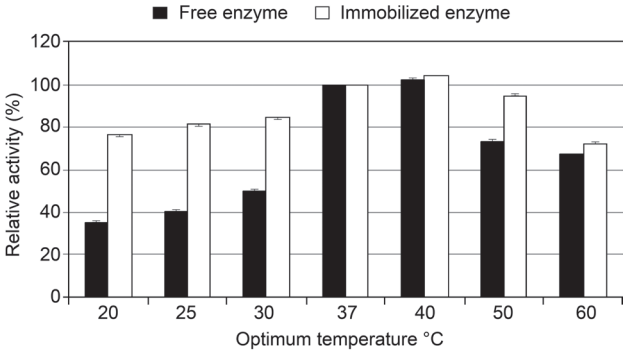

Fig. 2. Effect of temperature on the activities of the free and immobilized *P. cyclopium* L-asparaginase.

and 10. This clearly reflects the suitability of Amberlite IR-120 as a carrier for this enzyme as pH varies. Tabandeh and Aminlari (2009) reported that the optimum activity of L-asparaginase is at the alkaline pH of 8. This is probably due to the production of L-aspartic acid which acts as a competitive inhibitor for the enzyme under acidic conditions. Changes in pH activity behavior may be due to the immobilization of the enzyme, which could be explained by the unequal distribution of hydrogen and hydroxyl ions between the polyelectrolyte phase on which the enzyme is immobilized and the external solution (Kojima and Shimizu, 2003).

pH stability of free and immobilized L-asparaginase activity. The profile of pH stability (Table IV) showed that the immobilized L-asparaginase was more stable in a wider range of pH (3.0–6.0) during 30 and 60 min incubation compared to the free enzyme (pH 3.0–4.0), where it retained 100% of its relative activity. This result means that immobilized L-asparaginase would be more resistant to pH changes, hence could be used at the industrial level. The ionic binding technique using Amberlite IR-120 stabilizes the enzyme in acidic pH, making it suitable for a wide variety of chemical process applications.

Table IV
The pH stability of the free and immobilized *P. cyclopium* L-asparaginase.

pH value	Relative activity (%)			
	Time of exposure			
	Free		Time of exposure	
	30 min	60 min	30 min	60 min
control	100	100	100	100
3	100	100	100	100
4	100	100	100	100
5	100	80 ± 0.5	100	100
6	80 ± 0.7	75 ± 1	100	100
7	72 ± 0.5	67.8 ± 1.4	95.7 ± 0.3	90 ± 0.5
8	70 ± 0.3	60 ± 1.1	88 ± 1.1	80 ± 1
9	60 ± 0.5	40 ± 0.6	86 ± 1.5	70 ± 1.7
10	30 ± 1.5	20.5 ± 0.5	45 ± 1.3	28 ± 0.5

Optimum temperature. When profiling relative activity vs. reaction temperature, the optimum reaction temperature for maximal activity was apparently shifted to 40°C for the free and immobilized enzymes. Even though the reaction temperature rose to 60°C, the relative activity of the immobilized enzyme was still above 72%, while that of free L-asparaginase was 66.7% (Fig. 2). The temperature data were replotted in the form of Arrhenius plots. The plots of the immobilized and free enzymes were found to be linear, and the calculated values of Ea were 1.75 and 3.46 Kcal/mol, respectively. The E_a of asparaginase binding to Amberlite IR-120 was much lower than that of the free one, suggesting that the enzyme had significantly higher affinity to the Amberlite IR-120 active sites (Su *et al.*, 2010). That said, the immobilization of the enzyme widened the optimum reaction temperature range (Zhang *el al.*, 2004). Also, Youssef and Al-Omair (2008) reported that the optimum reaction temperature of immobilized asparaginase produced by *E. coli* was 60°C while that of free L-asparaginase was 50°C.

Thermal stability for the free and immobilized *P. cyclopium* L-asparaginase. Heat stability of the free and immobilized L-asparaginase in terms of the residual activities was compared (Table V). The immobilized enzyme remained 100% active at temperatures up to 60°C for 30 min while the free asparaginase was 100% active up to 50°C for 30 min. On the other hand, the effect of high temperatures (50–80°C) was more pronounced in case of the free enzyme than the immobilized one, the later was more resistant to high temperatures compared to the free one. On the contrary, Zhang *et al.* (2004) reported that the thermostability of the immobilized L-asparaginase was very similar to that of the native enzyme, and that there were no obvious changes in the activities. On plotting log of the relative activity against time at different tested temperatures (60, 70 and 80°C), both the free and the immobilized enzyme preparations gave straight lines (Fig. 3 and

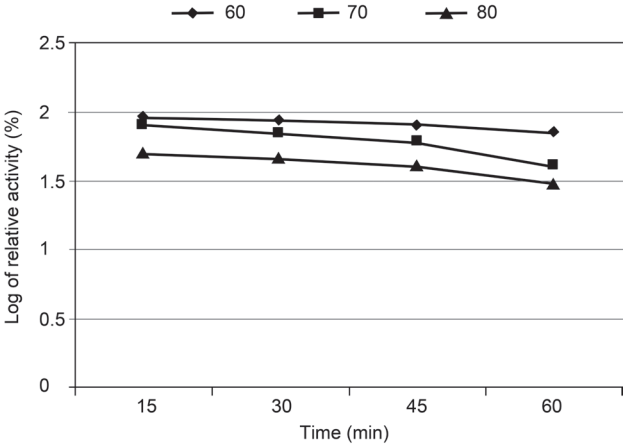

Fig. 3. First-order plots of thermal inactivation for the free *P. cyclopium* L-asparaginase activity.

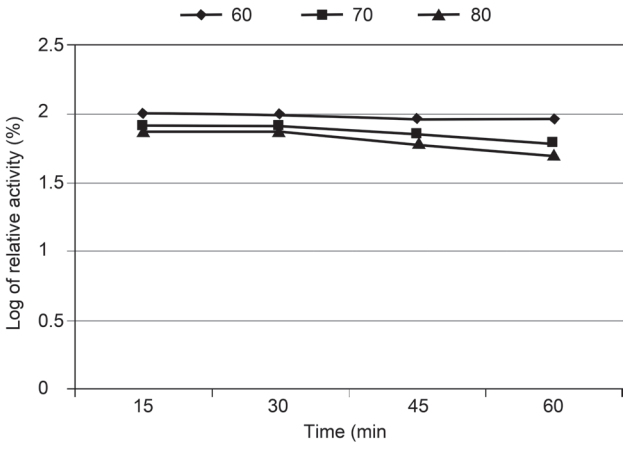

Fig. 4. First -order plots of thermal inactivation for the immobilized *P. cyclopium* L-asparaginase activity.

Fig. 4 respectively). This means that the thermal inactivation process of both enzyme forms corresponded to the theoretical curves of the first order reaction. The results showed that the immobilized enzyme was more thermostable than the free one. For example, the calculated half-lives of the free enzyme at 60, 70 and 80°C were 5.2, 3.6 and 2.47 hrs, respectively, proving to be lower than those of the immobilized enzyme, which were 10.4, 4.26 and 3.06 hr, respectively. The values of the deactivation rate constant (the slope of the relative activity) at increasing temperatures showed that the immobilized enzyme is highly stable compared to the free one. The calculated deactivation rate constants at 60, 70 and 80°C for the free enzyme were 2.2×10^{-3}, 3.7×10^{-3} and 5.3×10^{-3}, compared to 1.1×10^{-3}, 2.7×10^{-3} and 3.8×10^{-3}, respectively, for the immobilized enzyme.

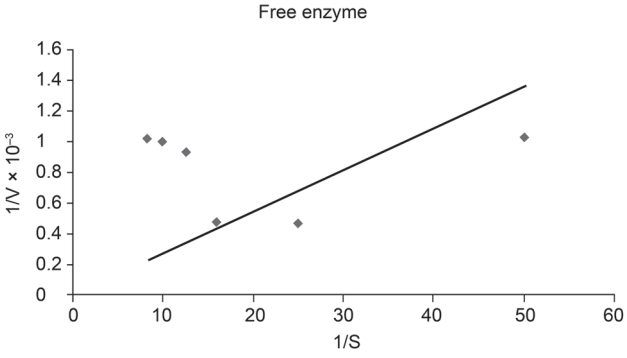

Fig. 5. Lineweaver – Burk plots for the free *P. cyclopium* L-asparaginase activity.

Kinetics and hydrolysis. The Michaelis-Menten Kinetics of the hydrolytic activity of the free and immobilized L-asparaginase were investigated using varying initial concentrations of asparagine as a substrate. There was a parallel increase in the relative enzyme activities of the free and immobilized treatment and the substrate concentrations (0.02–0.04 mM/ml). The initial velocity was measured as a function of substrate concentration for both free and immobilized enzyme and plotted (Fig. 5 and Fig. 6 respectively) as double reciprocals in accordance with the line-weaver-Burk analysis (Lineweaver and Burk, 1934). The K_m and V_{max} values were 0.0259 mM and 757.6 U/mg protein, respectively, for the free enzyme, and 0.033 mM and

Table V
Thermal stability of the free and immobilized *P. cyclopium* L-asparaginase activity.

Temperature °C	Residual activity (%)							
	Free				Immobilized			
	Time of exposure (min)							
	15	30	45	60	15	30	45	60
Control	100	100	100	100	100	100	100	100
30	100	100	100	100	100	100	100	100
40	100	100	100	100	100	100	100	100
50	100	100	90 ± 1.2	80 ± 1	100	100	100	100
60	90 ± 1	85.5 ± 0.8	80 ± 1.2	70 ± 1.5	100	100	90 ± 0.5	90 ± 0.5
70	80 ± 1.1	70 ± 0.6	60 ± 1	40 ± 0.5	80 ± 0.6	80 ± 0.6	70 ± 0.6	60 ± 1.7
80	50 ± 1.1	45.9 ± 0.5	39.9 ± 0.5	30 ± 2.3	73.9 ± 1	73.7 ± 0.9	60 ± 1.7	50.0 ± 0.5

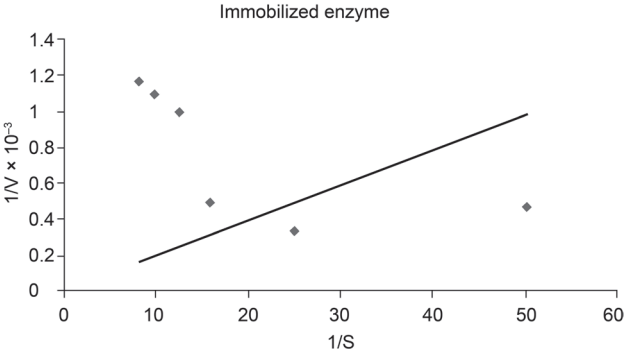

Fig. 6. Lineweaver – Burk plots for the immobilized *P. cyclopium* L-asparaginase activity.

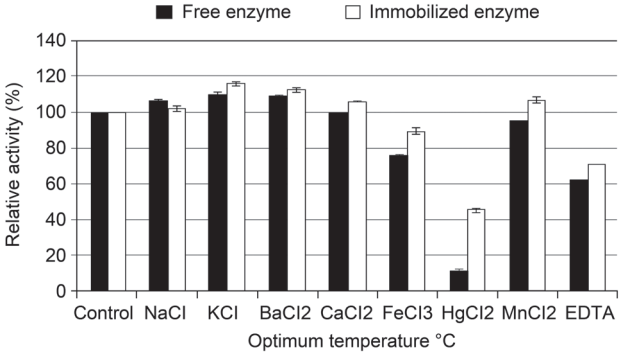

Fig. 7. Effect of different additives on activity of L-asparaginase of *P. cyclopium.*

581 U/mg protein, respectively, for the immobilized enzyme. The K_m value for immobilized L-asparaginase was about 1.3 times higher than that of the free enzyme, indicating a lower affinity towards the substrate. This increase in K_m might be either due to the structural changes in the enzyme induced by the immobilization method, or due to the lower accessibility of the substrate to the active sites (Su *et al*., 2010). On the other hand, fixation of the enzyme on the immobilization matrix could lead to a decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in the catalytic activity (Cao, 2005). Consequently, the maximum rate of the reaction V_{max} catalyzed by the immobilized enzymes was lower than that of the free enzyme, which was in agreement with Su *et al.* (2010). The ratio V_{max}/K_m is a measurement of the catalytic efficiency of an enzyme-substrate pair. The catalytic efficiency of L-asparaginase was decreased by about 40% upon immobilization. Another important aspect to take into consideration in order to evaluate the immobilization process is the efficiency factor. This factor can be calculated from the higher reaction rates of the immobilized enzyme divided by that of the free counterpart. That said, the efficiency factor = $v_{immobilized}/v_{free}$, where $v_{immobilized}$ is the reaction rate of the immobilized enzyme, and v_{free} is that of the free enzyme. For L-asparaginase, the efficiency factor was calculated to be 0.766.

Effect of metal ions on L-asparaginase activity. In this experiment, both the free and immobilized enzymes were incubated with different metal ions in their salt solutions at room temperature for 30 minutes. Following this, the relative activity was measured at optimum conditions. The results show that K+, Ba2+ and Na+ enhanced the enzyme activity with 110%, 109% and 106% respectively (Fig. 7). Considerable loss of activity was observed with Mn^{2+} and Fe^{3+}. Inhibition of enzyme activity with EDTA by 61.9% possibly suggest that the purified L-asparaginase might be a metaloenzyme (Elshafei *et al*., 2012). However, the highest inhibition value was recorded with Hg^{2+} which inhibited the enzyme to 11%. This might be indicative of essential vicinal sulfhydryl groups (SH-group) of the enzyme for productive catalysis (Elshafei *et al*., 2012). Similar results were reported by Basha *et al.* (2009) and Moorthy *et al.* (2010).

Conclusions. Our aim in this study was using gamma irradiation, purification, immobilization and characterization of L-asparaginase from *P. cyclopium* and to do a comparative study based on temperature, pH, inhibitor and activation concentration between free and immobilized enzyme. Gamma irradiation proved to be effective in the production of L-asparaginase with a total increase in enzyme activity of 1.75 folds and specific activity of 1.53 folds over the wild strain. L-asparaginase was partially purified then immobilized onto Amberlite IR-120. The immobilized enzyme showed optimal activity over a wide range of temperature and pH values. The immobilized L-asparaginase widened the optimum reactive temperature range and showed higher thermostability as well as higher affinity to the substrate. Considering all these characteristics, the production of L-asparaginase from gamma irradiated immobilized *P. cyclopium* may be recommended for industrial production.

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