JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

L-ASPARAGINASE AND L-GLUTAMINASE FROM *Pseudomonas aeruginosa* PAO1: PRODUCTION AND SOME PHYSICOCHEMICAL PROPERTIES

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doi: 10.15414/jmbfs.2015.5.2.142-146

ARTICLE INFO

Received 20. 6. 2014
Revised 19. 6. 2015
Accepted 30. 6. 2015
Published 1. 10. 2015

Regular article

OPEN ACCESS

ABSTRACT

L- asparaginase (EC 3.5.1.1) is an enzyme which converts L- asparagine to L-aspartic acid and ammonia; L- glutaminase (EC 3.5.1.2) is an enzyme which catalyzes the conversion of L-glutamine to L- glutamic acid and ammonia. Both of these enzymes have been used in as chemotherapeutic agents. Among different sources of L- asparaginase and L-glutaminase enzymes producers, microbial strains possess an elevated edge over other enzyme producers; these enzymes produced by different microbial strains differ in some physiological, biochemical, catalytic and immunological properties. This led to the continuous screening program for isolation of novel microbial strains that could produce an effective enzyme with few limitations at use. A bacterial strain isolated from soil and identified as *Pseudomonas aeruginosa* PAO1 had been found to be capable of producing both extracellular L- Asparaginase and L- Glutaminase enzymes. The enzymes were produced under solid state fermentation. Effects of different fermentation parameters for production of these enzymes were determined. Some physicochemical properties of both of these enzymes were determined. The results obtained in this study revealed the potential of *Pseudomonas aeruginosa* PAO1 as a source of both L-Asparaginase and L- Glutaminase enzymes, which have gained significance in pharmaceutical industry. The uses of inexpensive agro- industrial wastes in this study have important economic advantages over submerged fermentation.

Keywords: Asparaginase; Glutaminase; *Pseudomonas aeruginosa*; Extracellular; Production; Thermostable

INTRODUCTION

L- asparaginase (EC 3.5.1.1) is an enzyme which converts L- asparagine to L-aspartic acid and ammonia has been used as a chemotherapeutic agent. It has received increased attention in recent years for its anti-carcinogenic potential (**Manna *et al.*, 1995**).The clinical action of this enzyme is attributed to the reduction of L- asparagine, since tumor cells unable to synthesize this amino acid are killed by L- asparagine deprivation.

L- glutaminase (EC 3.5.1.2) is an enzyme which catalyzes the conversion of L-glutamine to L- glutamic acid and ammonia. Tumour cells, more specifically lymphatic tumour cells unable to synthesize the L-glutamine unlike the normal cells hence require huge amounts of glutamine to keep up their rapid growth. Hence the clinical action of L-glutaminase lies in the death of L-glutamine-dependent tumour cells by depriving L-glutamine.

Among different sources of L- asparaginase and L-glutaminase producers, microbial strains possess an elevated edge over others due to their growth requirements, easy processing and handling etc (**Satish *et al.*, 2008**). The literature reports suggested that these enzymes produced by different microorganisms differed in some physiological, biochemical, catalytic and immunological properties **(Jeya Prakash *et al*., 2009; Mishra, 2006, El-Bessoumy *et al.*, 2004)**. This led to the continuous screening programme for isolation of novel microbial strains as effective producers of these enzymes with few limitations.In recent times, the bacterial systems are increasingly investigated for the production of enzymes and metabolites by solid-state fermentation (SSF) .The SSF has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, low capital investment, low energy requirement and less water output ,better product recovery and lack of foam build-up (**Carrizales and Jaffe, 1986**).Additionally, the utilization of agro-wastes as a substrate for carbon and energy requirement under SSF makes this approach environment friendly. On the other hand extracellular enzymes are more advantageous than intracellular since they could be produced abundantly in the culture medium under normal conditions and could be purified economically.

The objective of our study was to isolate bacteria from soil of sea coastal region capable of producing both L-asparaginase and L-glutaminase enzymes. In this paper we report the production of both extracellular L-asparaginase and L-glutaminase enzymes by an isolated strain of *Pseudomonas aeruginosa* PAO1 under SSF using Wheat Bran. Attempts were made to study the optimization of both L-asparaginase and L-glutaminase production and preliminary characterization of both of the crude enzymes.

MATERIALS AND METHODS

Chemicals

Various carbon sources like glucose, sucrose, lactose, soluble starch and nitrogen sources like sodium nitrate, yeast extract, ammonium sulphate, peptone and other chemicals like magnesium sulphate sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, sodium acetate, boric acid, sodium metaborate, hydrochloric acid, acetic acid and calcium chloride dihydrate were procured from E. Merck Pvt Limited (Kolkata, India). Other chemicals like L-Asparagine and L-Glutamine, EDTA, Tris buffer were obtained from SRL Pvt Limited (Mumbai, India).

Microorganism and Media for maintenance and inoculation

Pseudomonas aeruginosa PAO1 producing L-asparaginase and L-glutaminase was isolated from the soil of coastal region of Andaman(India). The bacteria was maintained in the modified M_9 medium (**Gulati *et al.*, 1997**) containing the following constituents (g/L): $NaH_2PO_4.2H_2O$ - 6; KH_2PO_4- 3; NaCl - 0.5; L-asparagine/L-glutamine- 5; 1(M) $MgSO_4$, $7H_2O$ - 2ml; 0.1 M $CaCl_2$,$2H_2O$ - 1ml; 20% glucose stock solution - 10ml; Agar - 25. pH7.0±0.2. The strain was subcultured at regular interval of time of 15 days at 37° C. The composition of inoculum medium was the same except using agar to solidify the medium.

Fermentation medium

Different agro-wastes like rice husk, wheat bran, maize dust, bengal gram husk and husk of yellow lentil were used as a substrate. Ten grams of each substrate

was measured into 250ml Erlenmeyer flask into which a supplemental salt solution was added properly to get the desired moisture level. The salt solution was composed of $Na_2HPO_4.2H_2O$ - 6g/L, KH_2PO_4- 3g/L, $MgSO_4.7H_2O$- 0.5 g/L and NaCl-0.5 g/L. L-asparagine /L-glutamine were supplemented to induce synthesis of enzymes L-asparaginase and L-glutaminase, respectively (**Gulati *et al.*, 1997**).

Screening of Asparaginase/Glutaminase Producing Organism

L-asparaginase and L-glutaminase producing bacteria (antifungal agents Fluconazole -75µg/ml was added to control fungal contamination) were isolated separately from soil using plate and dilution technique (**Salle, A. J., 1974**). Each isolate was tested for its extracellular L-asparaginase and L-glutaminase activity (**Hymavathi *et al.*, 2009**).The L- asparagine/ L- glutamine containing media were supplemented with 0.135µl of 2.5 % of phenol red as an indicator. Two control plates were also prepared for both asparagine and glutamine – one was without dye while the other was without asparagine/ glutamine (using $NaNO_3$ as a nitrogen source). L-asparaginase and L-glutaminase activity was identified separately by formation of a pink zone around colonies. Zone diameters were measured after 24 h of incubation at 37°C. Based on the zone diameter colonies were picked up and broth studies were also carried out in order to compare the results obtained with the plate assay. Based on the zone diameter and broth studies a few colonies were selected and maintained on the asparagine / glutamine slants at 4°C.

Solid state fermentation and preparation of Crude Enzyme

Inoculum was prepared by growing the organism in 50 ml medium in 250-ml Erlenmeyer flasks incubated at 37°C for 24 h in a rotary shaker (120 rpm).The sterilized fermentation media was inoculated with 2ml of inoculums, mixed thoroughly and incubated at 37°C for 4 days in a stationary condition. The recovery of crude L-asparaginase from the fermented material was done by simple extraction method. For this, the fermented substrate was mixed thoroughly with 50 ml of 0.1 M Tris-HCl buffer (pH 7.2) and the contents were agitated for 1 h at room temperature in a rotary shaker at 150 rpm. At the end of extraction, the liquid was filtered off through Whatman No. 1 filter paper and the final volume was made upto 50 ml, then the resulting clear filtrate was used for L-asparaginase / L-glutaminase assay.

Assay Method

Extracellular L- asparaginase / L- glutaminase activity was determined using L-asparagine / L-glutamine as a substrate and the product, ammonia, released during the catalysis was measured by using Nesseler's reagent (**Imada *et al.*, 1973**). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µmol of ammonia under standard assay conditions.

RESULTS AND DISCUSSION

Screening of L-asparaginase/L-glutaminase Producing Organism

Fifteen strains having extracellular L-glutaminase and twelve strains having L-asparaginase activity were isolated from soil. Among these one isolate (ANG2) was selected for further studies as it showed the highest L-glutaminase activity (activity 191±1.02 IU/ml).The organism ANG2 was allowed to grow in L-asparagine slant in an attempt to test whether it has any L-asparaginase activity or not and after that it's extracellular L- asparaginase activity was examined. The isolate ANG2 was also found to be a good producer of L-asparaginase enzyme and in fact the one with highest L-asparaginase activity (activity 170.33±1.05 IU/ml) among the L-asparaginase producing strains. Hence the isolate ANG2 was taken for further studies as it showed the highest activities for both L-asparaginase and L-glutaminase enzymes.

Taxonomical Studies

The organism was identified on the basis of cultural and biochemical characteristics (Tab 1) following Bergey's Manual of Determinative Bacteriology (Holt et al, 1995). 16S rRNA sequence analysis was done by GCC Biotech, India and The Phylogenic tree is shown in figure 1. On the basis of 16S rRNA sequence analysis, figure 1, the isolate ANG2 has been identified as *Pseudomonas aeruginosa* PAO1 strain.

Table 1 Taxonomical characteristics of the selected strain ANG2

	PARAMETERS	CHARACTERISTICS	
A) CULTURAL CHARACTERISTICS	Morphology	Coccus occurring singly or in pairs, Non-motile	
	Staining Characteristics	Gram negative, Endospore formation	
B) CULTURAL CHARACTERISTICS	Nutrient Broth (Stationary Condition) 48 hrs	Moderate Growth, Flocculant Sedimentation, No ring and pellicle formation	
	Nutrient Broth (Shaking Condition) 48 hrs	Turbidity, Abundant Growth, No ring and pellicle formtion, Bluish green colour of growth medium	
C) PHYSICO-CHEMICAL CHARACTERISTICS	Growth at different Temperature	45ºC – Moderate	
		30ºC – Abundant	
		10ºC – Scanty	
	Growth at different pH	5.4 – Scanty	
		6.8 – Abundant	
		9.6 – Scanty	
D) BIOCHEMICAL CHARACTERISTICS	NH_3 from Arginine	+	
	Catalase Test	+	
	Carbohydrate Fermentation		
	Carbohydrate Source	Acid formation	Gas formation
	1) Sucrose	+	-
	2) Dextrose	+	-
	3) Mannitol	+	-
	4) Lactose	+	-
	5) Starch	+	-
	Indole Synthesis	-	

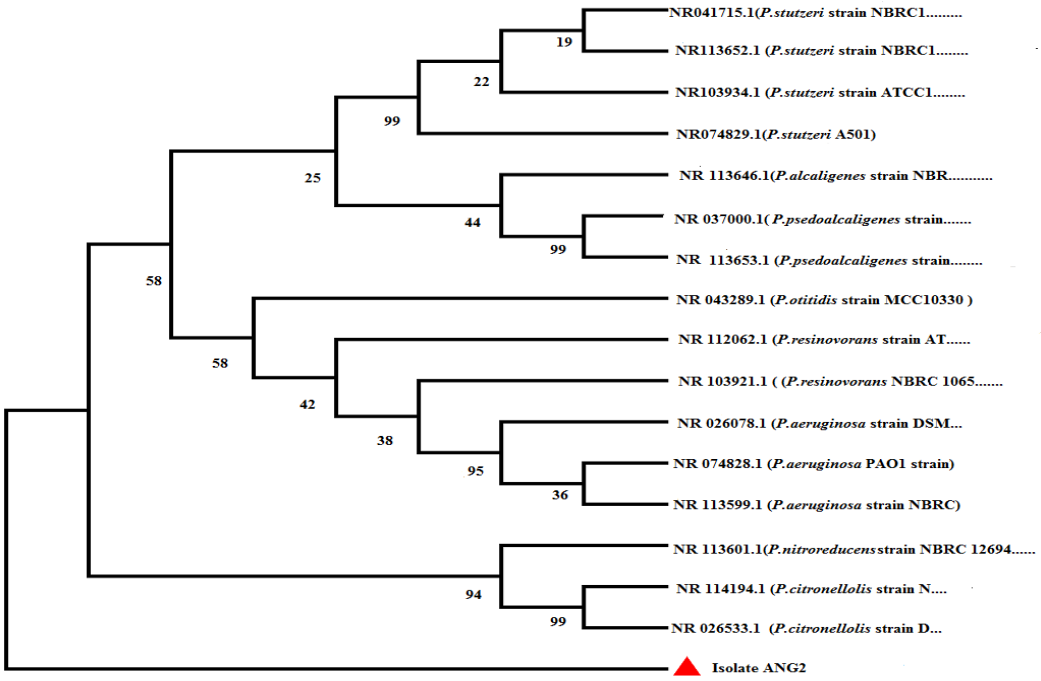

Figure 1 Phylogenic Tree

Fermentative Parameters for the Production of L-asparaginase and L-glutaminase

Evaluation of different agro-residual substrates for L-asparaginase and L-glutaminase production

The screening of the best agro-waste as a substrate for maximum enzyme production in SSF process mainly depends on its easier degradation into nutrients and uptake by the bacteria to synthesize the targeted metabolite, its cost effectiveness and availability in nature. The present study revealed that production of extracellular L- asparaginase and L- glutaminase varied with the type agro-residual substrates. Maximal production of both enzymes by *Pseudomonas aeruginosa* PAO1was observed in the medium with wheat bran (170.33±1.05 IU/ml for L-asparaginase and 191±1.02 IU/ml for L-glutaminase) (figure 2). Wheat bran contains approximately 18% protein, 5% fat and 62% carbohydrate (**Madruga, and Camara, 2000**)) and is rather complete source of nutrients for microorganisms (**Elliah et *al.*, 2004; Beg *et al.*, 2000**).

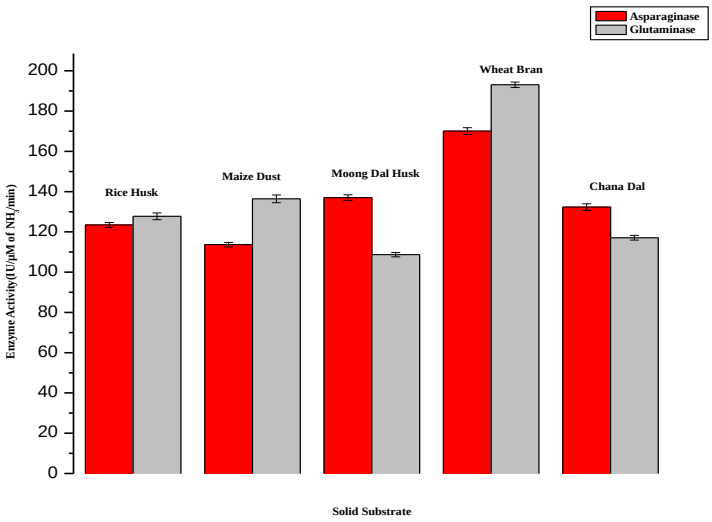

Figure 2 Evaluation of different agro-residual substrates for L-asparaginase/L-glutaminase production

Effect of fermentation time

Production of both extracellular L-asparaginase and L-glutaminase by *Pseudomonas aeruginosa* PAO1 were low initially and increased over time up to 96 h and then decreased. Upto 96h, the enzyme might be excreted in the fermentation medium due to lysis of the cell thus causing increase in extracellular enzyme production, figure 3.

Effect of moisture content

Moisture content is one of the most significant factors under SSF using a specific substrate as it plays a key role in microbial growth and enzyme production. A maximum production of L-asparaginase (242±0.42IU/ml) and L-glutaminase (269.67± 0.69 IU/ml) was observed at 90% moisture level, figure 4. A linear correlation between moisture content was observed until 90% and the enzyme activity declined sharply at further increase in moisture content.

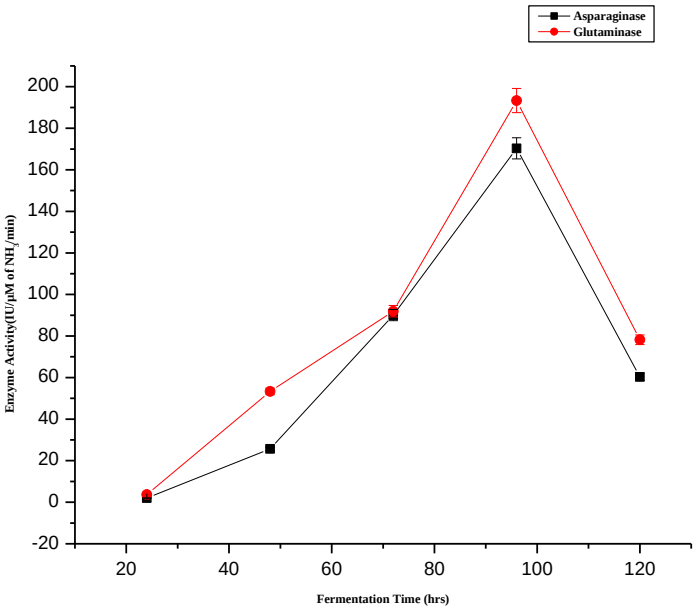

Figure 3 Effect of fermentation time on enzyme production

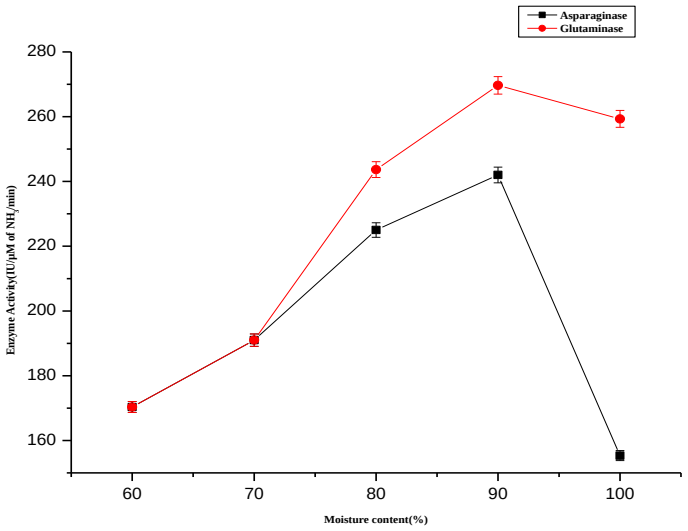

Figure 4 Effect of moisture content (%) on enzyme production

Effect of initial pH and temperature on enzyme production

Influence of initial pH of the fermentation medium on the production of L-asparaginase and L-glutaminase production by *Pseudomonas aeruginosa* PAO1 was studied over pH values 5.0–9.0. The experiment could not be conducted below pH value 5.0 or above 9.0 due to inhibition of growth of the microorganism. Maximum production of both the enzymes was revealed when the initial pH of the production medium was adjusted to 7.0. Fermentation was carried out at different temperatures (from 30–40°C) and pH 7.0. There was a sharp rise in enzyme production with increase in temperature up to 37°C (Fig. 5).

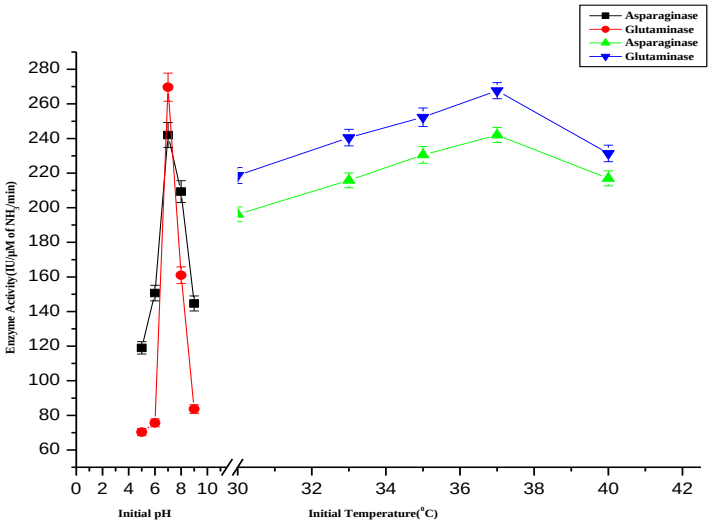

Figure 5 Effect of initial pH and temperature on enzyme production

Effect of carbon and nitrogen sources on enzyme production

In order to scrutinize the optimum carbon and nitrogen sources various carbon source like glucose, sucrose, lactose, soluble starch and nitrogen sources like ammonium sulphate, sodium nitrate, peptone and yeast extract were used as supplements separately (1% w/w) with wheat bran .But no such significant increase or decrease in enzyme activity observed for both the enzymes with such supplementations (Tab 2).

Table 2 Effect of carbon and nitrogen source on enzyme production

Carbon Source (1%)	L-Asparaginase activity (IU/ml)	L-Glutaminase activity (IU/ml)
Control	242.48±4.84	269.67±5.39
Glucose	243.65±0.65	267.34±1.76
Sucrose	241.67±0.83	269.34±2.34
Lactose	239.56 ±2.43	268.11±0.06
Starch	240.43±1.56	265.45±3.45
Nitrogen source (1%)	**L-Asparaginase activity (IU/ml)**	**L-Glutaminase activity (IU/ml)**
Control	242.48±4.84	269.67±5.39
Ammonium sulphate	244.56±0.56	271.34±1.47
Sodium nitrate	241.78±0.45	267.54±2.34
Yeast extract	240.24±3.42	266.45±3.28
Peptone	242.43±1.75	269.34±1.59

Physico-Chemical Properties of the Crude L-asparaginase and L-glutaminase

Study of kinetic parameters

L-asparaginase and L-glutaminase produced by *Pseudomonas aeruginosa* PAO1 exhibited hyperbolic response with increasing concentration of L -asparagine and L- glutamine (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 M), the assay substrates for the enzymes, respectively, figure 6. The K_m and V_{max} calculated for L-asparaginase are 6.62×10^{-2}M and 242mM/min/ml and those for L-glutaminase are 7.3×10^{-2}M and 269 mM/min/ml respectively. The low K_m for both the enzymes suggests that they have high affinity for their respective substrates.

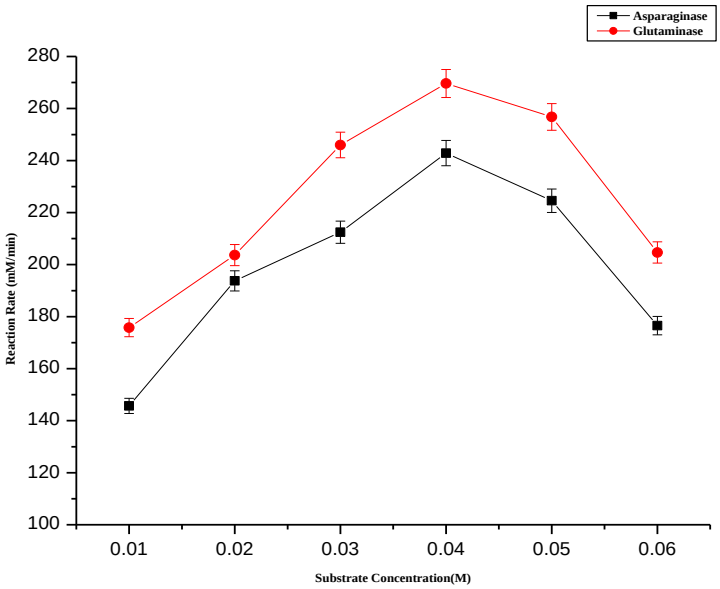

Figure 6 Effect of substrate concentration on enzyme activity

Effect of pH on enzyme activity and stability

Effect of pH on activities of L-asparaginase and L- glutaminase produced by *Pseudomonas aeruginosa* PAO1 were studied over a pH range 5-10 using Acetate buffer (pH 5-6), Tris- HCl buffer (pH 7.2-7.8), Borate buffer (pH 8-10), figure 7. Maximum L-asparaginase activity was observed at pH5.5 (242.34 ±2.34IU/ml),unlike majority of asparaginase reported earlier having alkaine pH optima (**El-Bessoumy *et al.*, 2004; Liboshi *et al.*, 1999; Balcao *et al.*, 2001**); further increase in pH decreased the enzyme activity .L-glutaminase activity revealed pH maxima at 7.6 (267.56±2.54IU/ml) like most microbial glutaminases having alkaline pH optima (pH 7.0-9.0) (**Nandakumar *et al* , 2003;Wakayama *et al*, 2005**).pH stability of enzymes was detected after incubation in the pH range (5-10) for 1 hr at the room temperature . The stability of enzymes showed similar trend like their activity with slightly diminished values, figure7.

Effect of temperature on enzyme activity

Extracellular *Pseudomonas aeruginosa* PAO1 L-asparaginase and L-glutaminase activities increased progressively with increase of temperature from 30–60°C,

figure 8. The maximal activity for both L-asparaginase (267.6±1.2 IU/ml) and L-glutaminase (306.54±3.57 IU/ml) was observed at 50°C. Glutaminase activity from *Aspergillus sojae* also showed its optimum temperature at 50°C (**Ito *et al.*,2013**). Thermostability of L-asparaginase produced by *Pseudomonas aeruginosa* PAO1 was examined by incubating the enzyme at different temperatures (30-60°C) for 1 h at pH 5.5, while thermostabilty of L-glutaminase was observed at the same temperature range for 1 h at pH 7.6, figure 8. It was revealed that even after incubating at 50°C for 1 h, L- asperaginase and L-glutaminase retained 98.12% and 87.87% of the activity, respectively, but the enzyme activity decreased sharply with further increase of temperature .

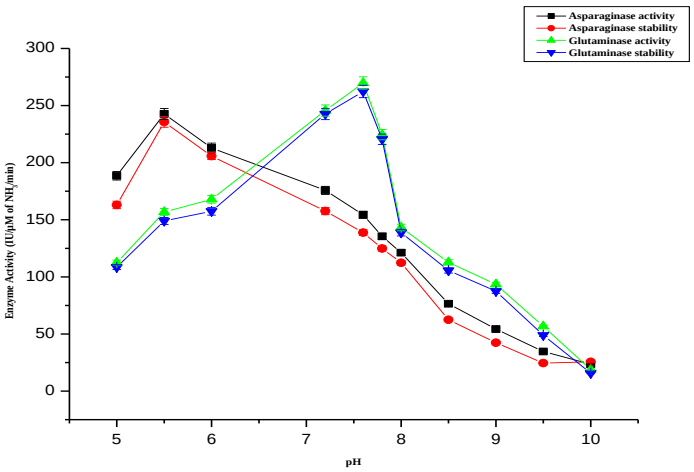

Figure 7 Optimum pH and pH stability of the crude asparaginase and glutaminase enzyme

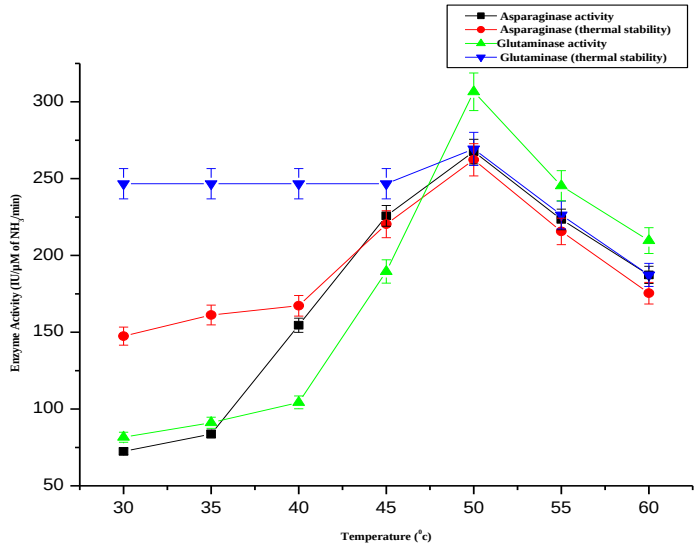

Figure 8 Optimum temperature and thermostability of the crude asparaginase and glutaminase enzyme

CONCLUSION

The study has been taken up with a view of exploring the possibilities of using *Pseudomonas aeruginosa* PAO1 as microbial source for the production of L-asparaginase and L- glutaminase enzymes, which have gained significance in pharmaceutical industry. The results obtained in this study revealed the potential of *Pseudomonas aeruginosa* as a source of both L-asparaginase and L-glutaminase enzymes, as both of these enzymes were found thermostable, with very low K_m values. The use of inexpensive agro- industrial wastes for the production of enzymes has important economic advantages over submerged fermentation. Future work should involve purification of both of these enzymes to homogeneity and study of their antitumor activity on different human cell lines.

Acknowledgement: We thank Techno India Authority for extending financial support to carry out the project.

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