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Production of Extracellular Anti-leukemic Enzyme L-asparaginase from *Fusarium solani* AUMC 8615 Grown under Solid-State Fermentation conditions: Purification and Characterization of The Free and Immobilized Enzyme

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POTENTIALITIES of twenty four fungal isolates were investigated for their ability to produce L-asparaginase using modified Czapek's Dox medium. *Fusarium solani* AUMC 8615 was selected as the most potent fungal strain for the enzyme production under solid state fermentation (SSF) using agro-industrial residues. Wheat bran supported maximum enzyme production followed by rice bran and corn cob. The optimum conditions for maximum production of L-asparaginase under SSF occurred on the fifth day of incubation at 30°C, pH 8.0, 60% of initial moisture content and supplementation with 0.2% (w/v) glucose and 0.5% (w/v) ammonium sulphate. L-asparaginase was purified to homogeneity by ammonium sulphate precipitation, gel filtration and ion exchange chromatography giving 299.58 purification fold. SDS-PAGE showed that the purified enzyme consists of two subunits of molecular weights 70 and 80 kDa, respectively. The enzyme was efficiently immobilized by covalent binding with activated charcoal giving an immobilization yield of 80.40%. Optimum pH values were 9.0 and 8.0 for free and immobilized enzyme, respectively. While, Optimum reaction temperatures were 37°C and 45°C for free and immobilized enzyme, respectively. After incubation for 2 hr at 37°C, the relative activity of free enzyme decreases to 35% whereas for the immobilized enzyme decreased only to 57% at 45°C.

Keywords: L-asparaginase, Solid-State Fermentation, *Fusarium solani*, Purification, Immobilization.

L-Asparaginase (L-Asparagine amidohydrolase E.C.3.5.1.1) is an enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonia (Theantana *et al*., 2009). In cancer treatment, L-asparaginase removes L-asparagine in the serum, depriving tumor cells from large amounts of asparagine required for growth (Verma *et al*., 2007). Its antileukemic effect is attributed to the inability of neoplastic blast cells to synthesize L-asparagine from aspartic acid as they lack L-asparagine synthetase. Lymphatic tumor cells need large amounts of asparagine in order to achieve rapid malignant growth. Therefore, the

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commonest therapeutic practice to treat this condition is to intravenously administer L-asparaginase in order to deplete the blood L-asparagine level and exhaust its supply to selectively affect the neoplastic cells (Theantana *et al*., 2009 and Warangkar and Khobragade, 2010).

In addition, L-asparaginase plays a central role in the amino acid metabolism and utilization, where, in human body, it acts as a precursor of ornithine in the urea cycle and in transamination reactions forming oxaloacetate in the gluconeogenic pathway leading to glucose formation (Hosamani and Kaliwal, 2011). In fact, L-asparaginase is a clinically acceptable anticancer agent for the treatment of Acute Lymphoblastic Leukemia (Verma *et al*., 2007).

L-Asparaginase was conventionally derived from bacteria, typically from *Escherichia coli* and *Erwinia carotovora* due to their cost-effective nature (Theantana *et al*., 2009). The therapeutic effect of L-asparaginase from these two bacterial species is accompanied by side effects that might include anaphylaxis, diabetes, leucopoenia pancreatitis, neurological seizures and coagulation abnormalities which may further lead to intracranial thrombosis or haemorrhage. These side effects are partially attributed to the presence of L-glutaminase activity obtained from these sources (Kotzia and Labrou, 2005). However, L-asparaginase from prokaryotic sources causes has now been found to have many side effects, primarily hypersensitivity which leads to allergic reactions and anaphylaxis. Therefore, it is desirable to search for other L-asparaginase producing microorganisms with novel properties that can produce an enzyme with less adverse effects. Thus, in the recent years, eukaryotic fungi have been investigated as sources of L-asparaginase (Chow and Ching, 2015 and Lincoln *et al*., 2015).

Commercially, L-asparaginase is produced throughout the world by submerged fermentation (SmF). This methodology has many disadvantages such as the low concentration product formation and consequent handling, reduction and disposal of large volumes of water during the downstream processing (Bessoumy *et al*., 2004). Therefore, the SmF methodology is a cost intensive, highly problematic and poorly understood unit operation. An alternative solution to SmF is solid state fermentation (SSF) which is offering a wide range of advantages compared to SmF. SSF offers many advantages over SmF such as lower energy requirements, less risk of bacterial contamination, less waste water generation and less environmental concerns regarding the disposal of solid waste (Doelle *et al*., 1992). L-Asparaginase production in SSF has been reported earlier on wheat bran (Kumar *et al*., 2013), soya bean meal (Bessoumy *et al*., 2004) and wastes from three leguminous crops-bran of *Cajanus cajan*, *Phaseolus mungo* and *Glycine max* (Mishra, 2006).

There are few reports on the L-asparaginase production by *Fusarium solani* but no studies on are available complete purification and physicochemical properties of the free and immobilized enzyme. Therefore, in this paper, we report the optimization of fermentation conditions for L-asparaginase production

from *F. solani* AUMC 8615 under SSF. The paper also reports the L-asparaginase purification and some characterization studies on the free and immobilized enzyme.

Materials and Methods

Isolation and screening of L-asparaginase producing fungi

Fungi used in this study were isolated from different soil samples collected from Kingdom of Saudi Arabia and Mangrove soil of Ras Mohammed protected area at Sharm El-Sheikh, Egypt. The dilution plate method was employed for the isolation of fungal strains (Palaniswamy *et al*., 2008). The fungal strains were identified by Assiut University Mycological Centre (AUMC), Egypt. All cultures were deposited in AUMC with their accession numbers. These fungi were sub-cultured on Czapek's Dox medium every 4 weeks and incubated at 30°C for 7 days. The obtained fungi were subjected for rapid assay of L-asparaginase production by plate assay method as per Gulati *et al.* (1997). Modified Czapek's Dox (MCD) medium (Saxena and Sinha, 1981) used for fungi containing 0.2% glucose, 1% L-asparagine, 0.152% K_2PO_4, 0.052% KCl, 0.052% $MgSO_4.7H_2O$, 0.003% $CuNO_3$. $3H_2O$, 0.005% $ZnSO_4.7H_2O$, 0.003% $FeSO_4.7H_2O$, 1.8% agar, initial pH 6.2 was supplemented with 0.009% (v/v) phenol red as indicator. Control plates were MCD medium containing $NaNO_3$ as nitrogen source instead of L-asparagine. The plates were incubated for 5 days at 30°C. The development of L-asparaginase producing fungi was indicated by the growth of the organism as well as the appearance of a pink zone around the fungal colonies. The isolate that showed largest pink zone around the colonies indicated L-asparaginase production and was selected for determination of enzyme activity.

Solid-State Fermentation (SSF)

Seven agro-industrial residues namely corn cob, ground nut shell, rice bran, soya bean meal, sugar cane bagasse, wheat bran and wheat straw were evaluated for their potential as substrate in SSF for L-asparaginase production. Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 5 g substrates moistened with 10 ml of MCD containing 0.152% K_2HPO_4, 0.052% KCl, 0.052% $MgSO_4.7H_2O$, 0.003% $CuNO_3.3H_2O$, 0.005% $ZnSO_4.7H_2O$, and 0.003% $FeSO_4.7H_2O$. All flasks were sterilized at 121°C for 20 min. The flasks were inoculated with 1ml spore suspension of *F. solani* containing 10^6-10^7 spores/ml and then incubated at 30°C for 5 days.

Crude Enzyme Extraction

The crude enzyme was extracted at the end of fermentation period by adding 50 ml of distilled water, shaking for 30 min followed by centrifugation at 8,000 rpm for 20 min. The cell free supernatant was used as the crude enzyme preparation. All experiments were carried out in triplicates.

L-asparaginase Assay

L-asparaginase activity was measured following the method of Imada *et al*. (1973). This method utilizes the determination of ammonia liberated from L-asparagine in the enzyme reaction by the Nessler's reaction. Reaction was started by adding 0.5 ml enzyme extract into 0.5 ml 0.04 M L-asparagine and 0.5ml 0.05 N Tris-HCl buffer, pH 7.0 and incubated at 37ºC for 30 min. The reaction was stopped by the addition of 0.5 ml of 1.5M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined by adding 0.2 ml Nessler's reagent into tubes containing 0.1 ml supernatant and 3.75 ml distilled water and incubated at room temperature for 10 min, and absorbance of the supernatant was measured at wavelength of 450 nm. The blank was prepared by adding enzyme preparation after the addition of TCA. The amount of ammonia liberated by the test sample was calculated using the ammonium sulfate standard curve. One unit of L-asparaginase activity is defined as that amount of enzyme which catalyzes the formation of 1µmol of ammonia per min under the assay conditions.

Protein determination

The protein content of the enzyme preparations was determined by the method of Lowry *et al*. (1951). All determinations were carried out in triplicates and the mean values are presented.

Optimization of fermentation parameters for maximum production of L-asparaginase by F. solani AUMC 8615 under SSF

The *F. solani* which showed highest production was studied for the optimal conditions of L-asparaginase production under SSF conditions. Various process parameters were optimized one factor at a time, keeping all other variables constant except one. Once the optimization has been done for a factor, it was incorporated into the experiment for the optimization of the next parameter. These parameters were incubation period (2~9 days), incubation temperature (25~45°C), initial pH of the medium (6~9; pH was adjusted using 1N NaOH or 1N HCl) and initial moisture content of the substrate (40~90%). The effect of different carbon sources by supplementation of salt basal medium with 0.2% (w/v) galactose, glucose, fructose, maltose, lactose, sucrose and starch was studied. The effect of different nitrogen sources on enzyme production by supplementation of salt basal medium with 0.5% (w/v) peptone, beef extract, yeast extract, urea, ammonium sulphate, ammonium chloride, ammonium nitrate and sodium nitrate was also investigated. The L-asparaginase activity was determined as previously mentioned.

L-asparaginase Purification

The optimized fermentation medium was inoculated and incubated at 30°C for 6 days. The crude enzyme was centrifuged at 8,000 rpm for 20 min and the supernatant served as the cell free filtrate (CFF). The purification was carried out at 4ºC on the CFF, according to the modified method of Distasio *et al*. (1976). The CFF was treated with finely powdered ammonium sulphate saturation ranging from 50% to 90% with continuous overnight stirring and the precipitates

were collected by centrifugation at 8,000 rpm for 20 min. The precipitate was dissolved in a 0.01 M phosphate buffer (pH 8.5) and dialyzed overnight against the same buffer at 4ºC.

The dialyzed ammonium sulphate fraction was applied to a Sephadex G-100 column (2.5 x 82 cm) of Fraction Collector (Fra100, Pharmacia-Fin Chemicals) that was pre-equilibrated with a 0.01 M phosphate buffer pH (8.5). The protein elution was done with the same buffer at a flow rate of 2 ml/min. The fractions were collected and the active fractions were pooled, dialyzed against the 0.01 M phosphate buffer (pH 8.5) and concentrated.

The concentrated enzyme solution was applied to the column of DEAE cellulose chromatography (Diethyl amino ethyl cellulose, fast flow, fibrous form-Sigma product) that was pre-equilibrated with a 0.01 M phosphate buffer (pH 8.5). It was eluted with the NaCl gradient (0.1-0.5 M) prepared in 0.01 M phosphate buffer (pH 8.5). The active fractions were collected, dialyzed once again against 0.01 M phosphate buffer (pH 8.5) to remove Na^+ and Cl^- ions, concentrated, and lyophilized. This preparation was stored at 0°C for further investigations.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to Laemmli (1970) using 10% polyacrylamide. Proteins were detected by Coomassie Brilliant Blue R250 staining.

Immobilization of L-asparaginase

Different supports namely activated charcoal, agarose, carboxymethyl cellulose, chitin, bentonite and silica gel were employed for L-asparaginase immobilization according to Abdel Naby (1993). Experimentally, 200 mg of each support was shaked in 5 ml Tris-HCl buffer (0.01 M, pH 8.5) containing 2.5% glutaraldehyde at room temperature for 2hr. The carriers were filtered off and washed with distilled water to remove the excess of glutaraldehyde then each treated carrier was incubated with 5 ml of Tris-HCl buffer containing 1 ml of enzyme. After being shaken for 2 hr at 30°C, the unbounded enzyme was removed by washing with distilled water until no protein or activity was detected in the wash. The immobilization yield was calculated by the following equation:

% yield = immobilized enzyme (I) / Added enzyme (A) - Unbounded enzyme (B)

Catalytic properties of the free and immobilized L-asparaginase

In order to validate the efficiency of the carrier for immobilization of L-asparaginase for use, the following experiments were conducted.

pH and pH stability

To determine the optimum pH for the activities of free and immobilized L-asparaginase, the reaction mixture was incubated at various pHs at room temperature for 30 min. pH stability for the free and immobilized enzyme was

investigated under the same conditions as described above, with the residual activity measured after incubating the reaction mixture for 1 hr at room temperature.

Temperature and thermal stability

The optimum temperatures for the free and immobilized L-asparaginase were determined with assay reaction mixture incubated at different temperatures ranging from 25 to 70°C for 30 min. The optimum temperature outcome was then taken as 100% activity and the relative activity at each temperature was expressed as a percentage of that activity. To determine the stability of the free and immobilized enzyme at high temperatures, the purified enzymes were incubated in buffer solution for a period of 2 h at 37 and 45°C, respectively and then they were examined for enzyme activity as above. 1 ml of the mixture was removed each time at different time intervals (15, 30, 45, 60, 75, 90, 105 and 120 min) and assayed. The optimum temperature outcome was again taken as 100% activity and the relative activity after incubation at each temperature was expressed as a percentage of that activity.

Statistical analysis

The obtained data were statistically analyzed with SPSS (Scientific Package for Scientific Social Studies, version 20), in which the equations of the hypothesis tests, including the mean, standard deviation, T-statistics value and probabilities (p) were used. The levels of significance were expressed as highly significant ($p \leq 0.01$), significant ($p \leq 0.05$) and non-significant ($p > 0.05$) and represented by ***, **, and *, respectively.

Results and Discussion

Screening of fungal isolates for L-asparaginase production using Plate assay method

Potentialities of twenty four fungal isolates were investigated for their ability to produce L-asparaginase belonged to nine genera, namely, *Aspergillus*, *Cunninghamella*, *Emericella*, *Fusarium*, *Humicola*, *Paecilomyces*, *Penicillium*, *Rhizopus* and *Trichoderma* by using plate assay method. L-asparagine in CDM also served as an enzyme inducer. The formation of a pink zone around the microorganism was an indication of the L-asparaginase production. The change of the indicator color from yellow to pink resulted from the increase in pH due to ammonia release. The ammonia along with L-aspartic acid is formed by the deamination reaction of the substrate L-asparagine by L-asparaginase (Imada *et al*., 1973 and Gulati *et al*., 1997). Thus, the potential strain was selected on the basis of the appearance of a pink zone around the fungal colonies as well as growth of the organism. The results in Table 1 revealed that only *F. solani*, *Paecilomyces lilacinus, A. ochraceus, Penicillium citrinum, Penicillium chrysogenum* and *A. niger* showed strong L-asparaginase production as compared with other fungal strains. It was obvious that *F. solani* exhibited maximum growth (3.9 cm) with higher zone diameter of 2.7 cm. Hence this isolate was selected for further investigation. Our results were in accordance with Gupta *et al.* (2009) who recorded that the following genera; *Aspergillus*,

Penicillium, *Fusarium* and *Paecilomyces* were found to be good source of L-asparaginase enzyme. Also, the obtained results confirmed that there is no relation between the fungal growth and enzyme production (Yadav and Sarkar, 2014).

TABLE 1. Screening for L-Asparaginase production by investigated fungal species.

Region of isolation	Fungal species	AUMC No.	Colony diameter (Cm)	Zone diameter (Cm)
Kingdom of Saudi Arabia	*Aspergillus flavus* Link	8653	1.5±0.1***	0.9±0.3***
	Aspergillus fumigatus Fresenius	8594	1.1±0.9***	0.4±0.1***
	Aspergillus niger Van Tieghem	8593	2.5±0.1***	1.5±0.4***
	Aspergillus ochraceus Wilhelm	8670	1.8±0.7***	2.2±0.9***
	Aspergillus sydowii (Bainier &Sartory) Thom & Church	8660	1.6±0.5***	-
	Aspergillus terreus Thom	8605	1.9±0.4***	1.4±0.7***
	Cunninghamella phaeospora Boedijn	8662	1.4±0.6***	-
	Emericella nidulans (Eidam) Vuillemin	8644	1.0±0.2***	0.5±0.2***
	Emericella quadrilineata (Thom &Raper) Benjamin	8636	1.2±0.1***	-
	Fusarium proliferatum (Matsush.) Nirenberg	8617	2.8±0.3***	-
	Fusarium solani (Martius) Saccardo	8615	3.9±0.8●	2.7±0.4●
	Humicola grisea Traaen	8598	1.7±0.2***	0.5±0.1***
	Humicola insolens Cooney &Emerson	8607	1.3±0.5***	0.3±0.1***
	Penicillium chrysogenum Thom	8656	1.5±0.4***	1.8±0.4***
	Penicillium corylophilum Diercks	8601	1.3±0.9***	-
	Penicillium duclauxii Delacroix	8667	1.1±0.2***	-
	Rhizopus stolonifer (Ehrenberg) Vuillemin	8671	3.0±0.9**	-
Ras Mohammed (Egypt)	*Aspergillus flavus* Link	10150	1.8±0.6***	1.3±0.3***
	Aspergillus niger Van Tieghem	10146	2.2±0.1***	2.1±0.4**
	Aspergillus ustus (Brinier) Thom & Church	10151	2.5±0.3***	1.3±0.5***
	Penicillium citrinum Thom	10147	1.2±0.7***	2.0±0.8***
	Penicillium crustosun Thom	10148	0.7±0.1***	1.5±0.3***
	Paecilomyces lilacinus (Thom) Samson	10149	1.8±0.5***	2.3±0.6***
	Trichoderma longibrachiatum Rifai	10155	0.8±0.2***	1.3±0.4***

Data represent the mean of 3 different readings ± standard deviation.
*** Highly significant, $p \leq 0.01$ and ** Significant, $p \leq 0.05$. Data was statistically compared using t-test (N = 3). The obtained data were statistically compared to the highest value obtained which is marked as ●.

Screening of different agro-industrial residues for L- asparaginase production under SSF

In recent years, the production of L- asparaginase enzyme based on using SSF technique. Selection of natural substrate is one critical role for the L-asparaginase production because they mimic the conditions under which the microbe grows natured (Yadav *et al*., 2014). In the present study, SSF was carried out on different agro-industrial residue substrates for enzyme production. Among seven natural substrates employed for the production of L-asparaginase, wheat bran was found to be the most potential substrate for the L-asparaginase production using *F. solani* giving maximum enzyme activity 187.9 U/ml (Table 2). Wheat bran was considered as the universal substrate among various substrates because it acts as a complete nutritious feed for microorganisms having all the ingredients and remains loose even under moist conditions providing a large surface area (Archana and Satyanarayana, 1997). Moreover the biochemical composition of wheat bran indicates that it contains various soluble sugars like glucose, xylose, arabinose, galactose, etc. which are helpful for the initiation of growth and replication of microorganisms (Lequart *et al*., 1999). In addition, higher L-asparaginase production on wheat bran may be possibly due to low lignin content and more amount of protein as compared to other substrates (Lequart *et al*., 1999).

TABLE 2. Screening of different agro-industrial residues on L-asparaginase production by *Fusarium solani* AUMC 8615 under Solid State Fermentation.

Substrate	L-asparaginase activity (U/ml)
Corn cob	97.1±3.5***
Ground nut shell	82.2±7.3***
Rice bran	105±9.2***
Soya bean meal	46.3±7.5***
Sugar cane bagasse	20.2±1.2***
Wheat bran	187.9±6.7●
Wheat straw	59.7±2.4***

Data represent the mean of 3 different readings ± standard deviation.
*** Highly significant, $p \leq 0.01$ and ** Significant, $p \leq 0.05$. Data was statistically compared using t-test (N = 3). The obtained data were statistically compared to the highest value obtained which is marked as ●.

Optimization of fermentation parameters for maximum L-asparaginase production

In order to achieve optimal activity under SSF conditions a detailed investigation was carried out to find out the effect of various physio-chemical parameters on the production of L-asparaginase by *F. solani*.

Effect of different incubation periods

Incubation period is one of the most critical parameter in SSF conditions. Maximum enzyme production could be obtained only after a certain incubation period which allows the culture to grow at a steady state (Pandey *et al*., 2000). Five days was the ideal incubation period, giving L-asparaginase activity of 192.3 U/ml (Table 3). Similar findings were reported earlier by Nair *et al*. (2013)

and Kalyanasundaram *et al*. (2015). At longer incubation periods, the enzyme activity decreased which might be due to depletion of nutrients, accumulation of toxic end products and change in the pH of the medium or loss of moisture content (Stanbury *et al*., 1997).

Effect of incubation temperature

The incubation temperature is a critical environmental factor for L-asparaginase production by microbes because it regulates microbial growth and consequently enzyme secretion. In the current study, a maximum enzyme activity of 190.9 U/ml was obtained by *F. solani* at 30°C (Table 3). Similar results were obtained by Thakur *et al*. (2014) and Kalyanasundaram *et al*. (2015) who recorded that the optimum temperature for L-asparaginase production from *Mucor hiemalis* and *A. terreus* in SSF was 30°C, respectively.

Effect of initial pH

The initial pH of the production medium is an important parameter affecting the enzyme production since it can indirectly act on the fungal growth by affecting the availability of medium nutrients. The maximum L-asparaginase production was obtained at an initial pH of 8.0; giving 302 U/ml, thereafter a decline in enzyme production was seen (Table 3). This finding was in accord with observations made by Varalakshmi and Raju (2013) who recorded that the optimum pH of L-asparaginase production by *A. terreus* MTCC 1782 was 8.0 under SSF conditions.

Effect of moisture level

Generally the moisture level of substrates in SSF processes varies between 30-85% has a marked effect on growth kinetics of fermenting organism (Raimbault, 1998). In the current study, the highest L-asparaginase production 400.5 U/ml was obtained at 60% initial moisture content (Table 3). Similar results were obtained by Pallem *et al*. (2011) and Kumar *et al*. (2013) who found that 60% and 58% initial moisture content was the optimum for L-asparaginase production from *F. oxysporum* and *Cladosporium* sp., respectively.

Effect of different carbon sources

The carbon source has a positive effect on L-asparaginase production and high titers can be obtained in a medium rich of carbon source. Among the tested carbon sources, glucose was found to be the most suitable carbon source to maximize the production of L-asparaginase giving 510.7 U/ml (Table 3). Glucose, is an excellent carbon source for growth and interferes with the biosynthesis of many secondary metabolites (Thakur *et al*., 2014). Our results were in accordance with observations made by Pallem *et al* (2011); Thakur *et al*. (2014) and Kalyanasundaram *et al*. (2015).

TABLE 3. Effect of various parameters on L-asparaginase production by *F. solani* AUMC 8615 under Solid State Fermentation.

Incubation period	**Days**	**2**	**3**	**4**	**5**	**6**	7		
	Enzyme activity (U/ml)	95.7±6.5	125.5±4.8	148.6±7.9	192.3±7.2	144.7±9.1	102.9±3.6		
Temperature	(°C)	25	30	35	40	45			
	Enzyme activity (U/ml)	98.4±6.3	190.9±8.8	172.6±4.7	99.5±5.5	40±2.1			
Initial pH		6.0	6.5	7.0	7.5	8.0	8.5	9.0	
	Enzyme activity (U/ml)	190.1±7.3	205.4±2.9	227.3±9.0	259.5±8.2	302±3.0	278.6±5.2	176.8±4.4	
Moisture content	(%)	40	50	60	70	80	90		
	Enzyme activity (U/ml)	201.6±4.8	295.9±2.1	400.5±6.3	320±8.8	280±9.2	210±11.0		
Carbon source	(0.2%, w/v)	Glucose	Galactose	Fructose	Maltose	Lactose	Sucrose	Starch	
	Enzyme activity (U/ml)	510.7±5.2	249.5±1.3	462.4±5.5	438.4±6.2	350.4±2.4	480.8±2.8	300.7±7.3	
Nitrogen source	(0.5%, w/v)	Peptone	Beef extract	Yeast extract	Urea	Ammonium sulphate	Ammonium Chloride	Ammonium nitrate	Sodium nitrate
	Enzyme activity (U/ml)	382.3±5.9	401.3±2.4	520.8±6.3	377.9±1.1	572.6±3.5	411.9±8.8	550.4±1.3	230.5±4.8

Effect of different nitrogen sources

When the effect of organic and inorganic nitrogen sources on L-asparaginase production was investigated, it was observed that ammonium sulphate was the best nitrogen source for the production of L-asparaginase, giving 572.6 U/ml (Table 3). These results were also reported by Sreenivasulu *et al*. (2009) and Kalyanasundaram *et al*. (2015) who reported that ammonium sulphate exhibited maximum L-asparaginase activity by the isolated fungus VS-26 and *A. terreus*, respectively.

Enzyme purification

Summary of the results concerning the purification of L-asparaginase produced by *F. solani* are summarized in Table 4. After the final purification step, the enzyme was purified 299.58 fold with a recovery of 6.10% and specific activity of 4.314 U/mg protein. Different specific activities and yields have been reported for various fungal species. For example a specific activity of 69.43 U/mg with 18.46% recovery and 4.59-purification fold was noted for the L-asparaginase produced by *Mucor hiemalis* (Thakur *et al*., 2014). A recovery of 30.6% and 13 fold with 78.2 U/mg was recorded for L-asparaginase of *Trichoderma viride* (Lincoln *et al*., 2015). The present study showed that *F. solani* has produced L-asparaginase with 299.58 fold purity with 6.10% yield which is better compared to *Fusarium* sp. which has shown 242.65 fold purity with 1.5% yield (Asha and Pallavi, 2012).

TABLE 4. Purification summary of *F. solani* AUMC 8615 L-asparaginase.

Purification step	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Cell free filtrate (CFF)	85890	5964583	0.0144	100	1.0
Ammonium sulphate (80% saturation) precipitation	32495	28858	1.126	37.80	79.30
Gel filtration Sephadex G-100	8110	4284	1.893	9.44	133.31
Ion exchange DEAE-Cellulose	5238	1214	4.314	6.10	299.58

SDS-PAGE and Molecular weight estimation

The purified L-asparaginase appeared as two protein bands in SDS-PAGE with apparent molecular weight of 70 and 80 kDa, respectively (Fig. 1). Similar observations were made by Asha and Pallavi (2012). Different molecular masses that ranged from 40-99 kDa have been reported for other fungal L-asparaginases (Thakur *et al*., 2014; Lincoln *et al*., 2015; Kalyanasundaram *et al*., 2015).

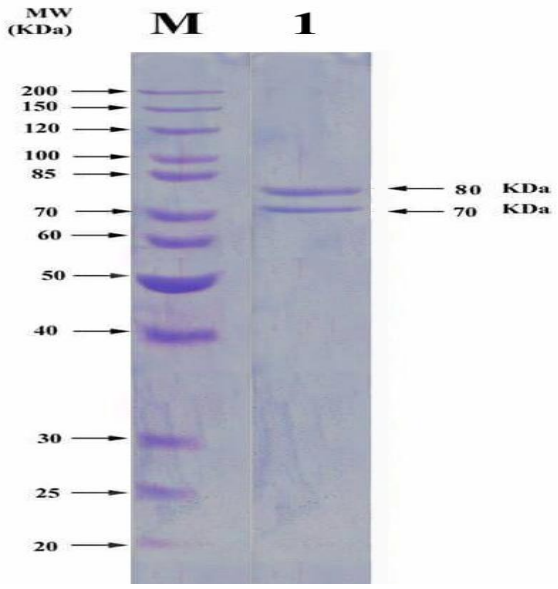

Fig. 1. SDS-PAGE analysis of the purified L-asparaginase by *F. solani* AUMC 8615. Lane 1, L-asparaginase sample. Lane M, molecular weight standards (Marker).

Immobilization of L-asparaginase

Six supports, pre-activated with glutaraldehyde, react with different degrees with the terminal amino residues of the enzyme protein. Immobilization of *F. solani* L-asparaginase by covalent binding was achieved by cross linking between the enzyme and tested carriers throughout glutaraldehyde. The amount of enzyme used for immobilization was 1146 U/g carrier. The results (Table 5) indicated that the highest immobilized activity (833 U/g carrier) and immobilization yield (80.40%) carrier was detected with activated charcoal as a carrier. Activated carbon is a form of carbon that has been processed to make it extremely porous and thus to have a very large surface area available for chemical reactions (Ahmad *et al*., 2013). These results are in agreement with Moharam *et al*. (2010) and Ahmad *et al*. (2013).

TABLE 5. Immobilization of *F. solani* AUMC 8615 L-asparaginase by covalent binding with different carriers.

Carrier	Added enzyme (U/g) (A)	Unbounded enzyme (U/g) (B)	Immobilized enzyme (I)	Yield I / (A-B)%
Activated charcoal	1146	110	833	80.40
Agarose	1146	48	290	26.41
caboxymethyl cellulose	1146	166	567	57.85
Chitin	1146	150	330	33.13
Bentonite	1146	137	651	64.51
Silica gel	1146	92	748	71.10

Characterization of L-Asparaginase Enzyme

Some characteristics of purified L-asparaginase are performed on the free and immobilized form as pH, pH stability, temperature and thermal stability.

pH and pH stability

Immobilized L-asparaginase showed its maximum activity at pH 8.0, whereas the optimum pH of the free enzyme was shifted to a more alkaline value of pH 9.0 (Fig. 2).

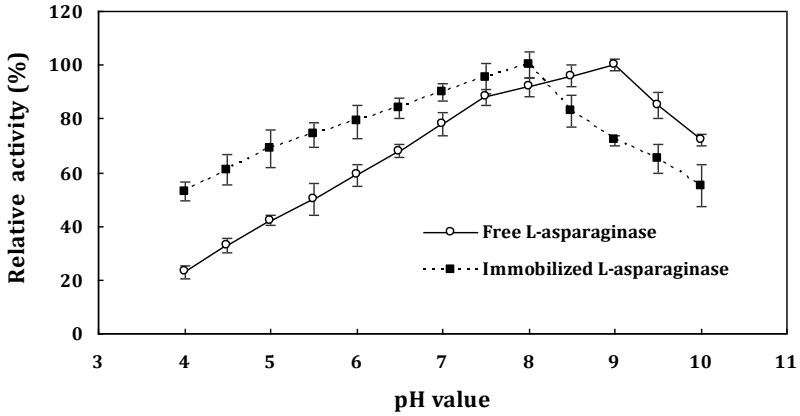

Fig. 2. Optimum pH profile of free and immobilized L-asparaginase by *F. solani* AUMC 8615.

This pH shift appears partly because there was a micro-environmental difference such as surface charge of the carrier material and the molecules capacity of enzyme protein and its matrix carrier (Tor *et al*., 1989). Free enzyme was quite stable in the pH ranges from 7.0 to 9.0 for 1 h (Fig. 3), while the immobilized one was stable from 6.0 to 9.5. Similar results are obtained by Siddalingeshwara and Lingappa (2010); Dange and Peshwe (2011) and Asha and Pallavi (2012). The L-asparaginase activity below pH 8.0 would not be expected to be effective for the treatment of the tumor patient (Yadav *et al*., 2014), so our results suggested that both free and purified L-asparaginase from *F. solani* can be used as a better source of L-asparaginase for the treatment of leukemia.

Temperature and thermal stability

The optimum temperature for the immobilized enzyme increased to 45°C compared to 37°C for the free enzyme (Fig. 4). This temperature shift could be due to the formation of a molecular cage around the enzyme, which protected the enzyme molecules from the temperature (Tor *et al*., 1989). The optimum temperature 37°C was reported for L-asparaginase of various fungal species (Dange and Peshwe, 2011; Asha and Pallavi, 2012; Thakur *et al*., 2014 and Lincoln *et al*., 2015).

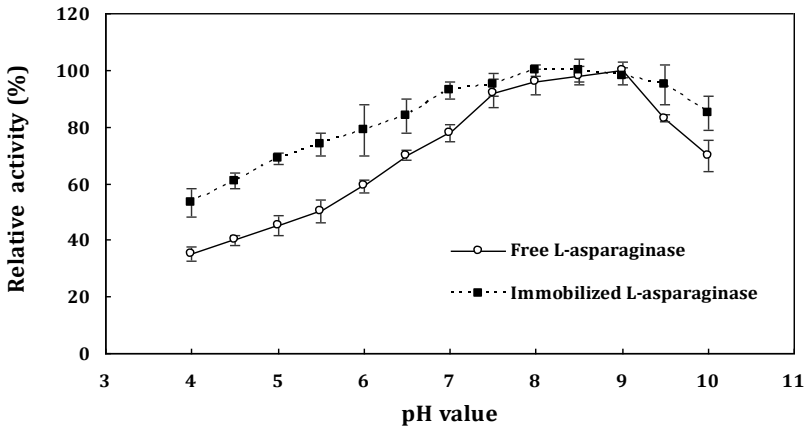

Fig. 3. pH stability profile of free and immobilized L-asparaginase by *F. solani* AUMC 8615.

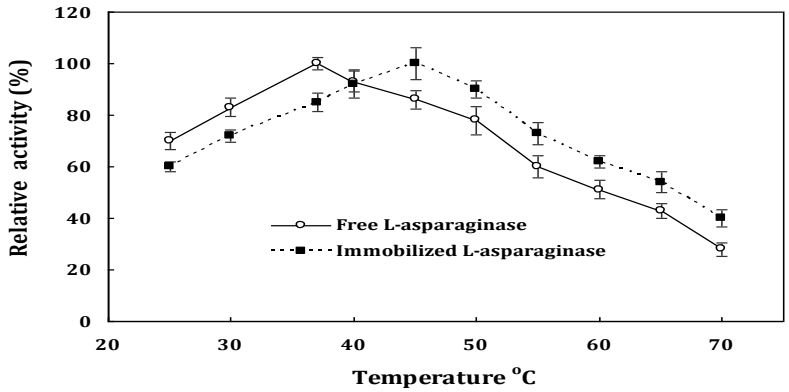

Fig. 4. Optimum temperature profile of free and immobilized L-asparaginase by *F. solani* AUMC 8615.

Figure 5 showed the stability of the free L-asparaginase to pre-incubation at optimum temperature (37°C). Pre-incubation up to 60 min displayed 89% of its relative activity and declined at the 90 minutes and 120 min pre-incubation with relative activity of 61% and 35%, respectively. The stability of immobilized L-asparaginase to the pre-incubation at optimum temperature (45°C) showed the relative activity of 83% at the 60 min pre-incubation, and a decline in relative activity at the 90 min and 120 min pre-incubation with relative activity of 68 and 57%, respectively. L-asparaginase of *Penicillium brevicompactum* NRC 829 maintained stability up to 1 hr at 37°C (Elshafei *et al.*, 2012) while the enzyme of *A. aculeatus* remained stable for 2 hr at 30°C (Dange and Peshwe, 2011). Our

results make immobilized L-asparaginase of the present study more suitable for complete elimination of ammonia by the hydrolysis of L-asparagine in the presence of L-asparaginase and can be applied as a therapeutic enzyme due to its thermostability and temperature-activity profiles.

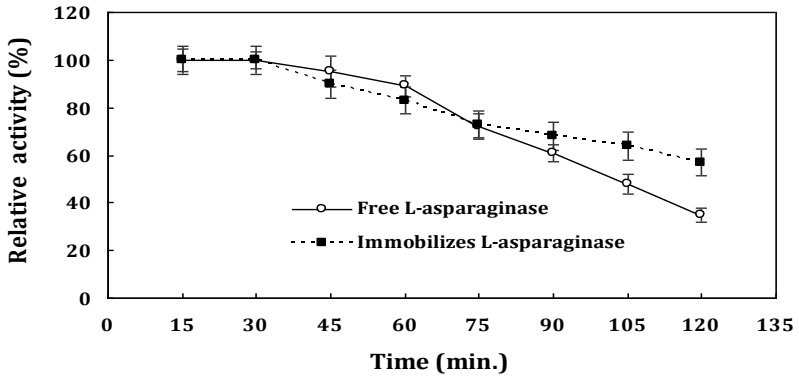

Fig. 5. Temperature stability profile of free and immobilized L-asparaginase by *F. solani* AUMC 8615.

References

Abdel Naby, A.M. (1993) Immobilization of *Aspergillus niger* NRC-107 xylanase and â-xylosidase and properties of immobilized enzyme. *Appl. Biochem. Biotech.,* **38**, 69-81.

Ahmad, A., Patta, A.M. and Natsir, H. (2013) Purification and immobilization of L-Asparaginase enzyme from thermophilic bacteria *Bacillus licheniformis* strain HSA3-1a. *Int. J. Pharm. Bio. Sci.*, **4** (4), 274-280.

Archana, S. and Satyanarayana, T. (1997) Xylanase production by thermophilic *Bacillus lichenformis* A99 in solid-state fermentation. *Enzyme. Microb. Technol*., **21**, 12-17.

Asha, A. and Pallavi, B. (2012) Production, purification and characterization of Extracellular L-asparaginase having antineoplastic activity from *Fusarium* sp. *J. adv. Res. Biol. Sci*., **4**, 293-301.

Bessoumy, A.A., Sarhan, M.J. and Mansour, J. (2004) Production, isolation and purification of l-asparaginase from *Pseudomonas aeruginosa* 50071 using solid-state fermentation. *J. Biochem. Mol. Biol*., **37**, 387-393.

Chow, Y. and Ching, A.S.Y. (2015) Endophytic L-asparaginase-producing fungi from plants associated with anticancer proper. *J. Adv. Res*., **6** (6), 869-876.

Dange, V.U. and Peshwe SA (2011) Production, purification and characterization of fungal L-asparaginase. *Bionano. Frontier*., **4**, 162–167.

Distasio, J.A., Nredrerman, R.A., Kafkewitz, D. and Goodman, D. (1976) Purification and characterization of L-asparaginase with antilymphoma activity from *Vibroi succinogenes*. *J. Biolog. Chem*., **251**, 6929-6933.

Doelle, H.W., Mitchell, D.R. and Rolz, C.E. (1992) Solid substrate cultivation. In: "*Applied Science*". Elsevier, London, pp. 7-16.

Elshafei, A.M., Hassan, M.M., Abouzeid, M.A., Mahmoud, D.A. and Elghonemy, D.H. (2012) Purification, characterization and antitumor activity of L-asparaginase from *Penicillium brevicompactum* NRC 829. *Br. Microbiol. Res. J*., **2**, 158–174.

Gulati, R., Saxena, R.K. and Gupta, R. (1997) A rapid plate assay for screening L-asparaginase producing micro-organisms. *Lett. Appl. Microbiol*., **24**, 23-26.

Gupta, N., Dash, S.J. and Basak, U.CH. (2009) L- asparaginases from fungi of Bhitarkanika mangrove ecosystem. *Ass. Pac. J. Mol. Biol. Biotechnol*., **17**, 27-30.

Hosamani, R. and Kaliwal, B.B. (2011) L-Asparaginase–an antitumor agent production by *Fusarium equiseti* under solid state fermentation. *Int. J. Drug. Disc.*, **3**, 88-99.

Imada, A., Igarasi, S., Nakahama, K. and Isono, M. (1973) Asparaginase and glutaminase activities of microorganisms. *J .Gen. Microbiol.*, **76**, 85-99.

Kalyanasundaram, I., Nagamuthu, J., Srinivasan, B., Pachayappan, A. and Muthukumarasamy, S. (2015) Production, purification and characterization of extracellular L-asparaginase from salt marsh fungal endophytes. *World. J. Pharm. Sci*., **4**, 663-677.

Kotzia, G.A. and Labrou, N.E. (2005) Cloning, expression and characterisation of *Erwinia carotovora* L-asparaginase. *J. Biotechnol.*, **119**, 309-323.

Kumar, M.N.S., Ramasamy, R. and Manonmani, H.K. (2013) Production and optimization of l-asparaginase from *Cladosporium* sp. using agricultural residues in solid state fermentation. *Ind. Crops. Prod*., **43**, 150-158.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lequart, C., Nuzillard, J.M., Kurek, B. and Debeire, P. (1999) Hydrolysis of wheat bran and straw by an endoxylanase: production and structural characterization of cinnamoyl-oligosaccharides. *Carbohydr. Res*., **319**, 102–111.

Lincoln, L., Niyonzima, F.N. and More, S.S. (2015) Purification and properties of a fungal L-Asparaginase from *Trichoderma viride* Peres: SF Gray. *J. microb. Biotech. food. Sci*., **4**, 310-316.

Lowery, O.H., Resenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., **193**, 265-275.

Mishra, A. (2006) Production of l-asparaginase, an anticancer agent, from *Aspergillus niger* using agricultural waste in solid state fermentation. *Appl. Biochem. Biotechnol*., **135**, 33-42.

Moharam, M.E., Gamal-Eldeen, A.M. and El-sayed, S.T. (2010) Production, Immobilization and Anti-tumor Activity of L-Asparaginase of *Bacillus* sp R36. *J Am. Sci*., **6**, 131-140.

Nair, A., Kumar, R., Devi, R.A. and Balakrishnan, K. (2013) Screening of commonly available solid process residues as substrate for L-asparaginase production by *Aspergillus terreus* MTCC 1782. *Res. J. Pharm. Biol. Chem. Sci*., **4**, 1731-73.

Palaniswamy, M., Pradeep, B.V., Sathya, R. and Angayarkanni, J. (2008) Isolation, identification, and screening of potential xylanolytic enzyme from litter degrading fungi. *Afri. J. Biotech*., **7**, 1978-1982.

Pallem, C.H., Nagarjun, V. and Srikanth, M. (2011) Production of A Tumor Inhibitory Enzyme, L-Asparaginase through Solid State Fermentation using *Fusarium oxysporum*. *Int. J. Pharm. Sci. Rev. Res*., **7**, 189-192.

Pandey, A., Soccol, C.R. and Mitchell, D. (2000) New development in solid state fermentation: 1-Bioprocesses and products. *Process. Biochem*., **35**, 1153-1169.

Raimbault, M. (1998) General and microbiological aspects of solid substrate fermentation. *Electron. J. Biotechnol*., **1**, 1-15.

Saxena, R.K. and Sinha, U. (1981) L-asparaginase and glutaminase activities in culture filtrates of *Aspergillus nidulans*. *Curr. Sci*., **50**, 218-219.

Siddalingeshwara, K.G. and Lingappa, K. (2010) Key fermentation factors for the synthesis of L-asparaginase-An anti tumour agent through SSF methodology. *Int. J. Pharm. Sci*., **1**, 103-112.

Sreenivasulu, V., Jayaveera, K.N. Mallikarjuna Rao, P. (2009) Optimization of process parameters for the production of L-asparaginase from an isolated fungusVS-26. *Res. J. Pharmaco. Phytochem*., **1**, 30-34.

Stanbury, P.F., Whitaker, A. and Hall, S. (1997) "*Principles of Fermentation Technology*", 2nd ed. New Delhi, India. Aditya; pp. 93–105.

Thakur, M., Lincoln, L., Niyonzima, F.N. and More, S.S. (2014) Isolation, purification and characterization of fungal extracellular L-Asparaginase from *Mucor hiemalis*. *J. Biocat. Biotrans*., **2**,1-9.

Theantana, T., Hyde, K.D. and Lumyong, S. (2009) Asparaginase production by endophytic fungi from Thai medicinal plants: cytotoxicity properties. *Int. J. Integr. Biol*., **7**, 1-8.

Tor, R., Dror, Y. and Freeman, A. (1989) Enzyme stabilization by bilayer "encagement". *Enzyme. Microb. Technol*., **11**, 306-312.

Varalakshmi, V. and Raju, J.K. (2013) Optimization of L-asparaginase production by *Aspergillus terreus* MTCC 1782 using bajra seed flour under solid state fermentation. *Int. J. Res. Eng. Technol*., **2**, 121-129.

Verma, N., Kumar, K., Kaur, G. and Anand, S. (2007) L-Asparaginase: a promising chemotherapeutic agent. *Crit. Rev. Biotechnol*., **27**, 45-62.

Warangkar, S.C. and Khobragade, C.N. (2010) Purification, characterization, and effect of thiol compounds on activity of the *Erwinia carotovora* L-asparaginase. *Enz. Res*., **1**, 1-10.

Yadav, C.N. and Sarkar, S. (2014) Production of L-Asparaginase by *Fusarium Oxysporum* using submerged fermentation. *Int. J. Pharm. Sci. Inven*., **3**, 32-40.

Yadav, S., Verma, K.S., Singh, J. and Kumar, A. (2014) Industrial production and clinical application of L-Asparaginase: A chemotherapeutic agent. *Int. J. Med. Health. Biomed. Pharm. Eng*., **8**, 54-60.

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إنتاج إنزيم إل-أسباراجينيز مكافح اللوكيميا المفرز خارجياً من فطر فيوزاريوم سولانى AUMC 8615 عن طريق مزارع الحالة الصلبة: تنقية وتوصيف الإنزيم الحر والمقيد

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أجريت دراسة مسحية على أربعة وعشرين عزلة فطرية محلية لمعرفة قدرتها على إنتاج إنزيم إل-أسباراجينيز باستخدام بيئة دوكس-زبك المعدلة و تحتوى على الحمض الأمينى إل-أسبراجين كمصدر نيتروجينى وحيد. وقد سجل أعلى انتاج للإنزيم لفطر فيوزايم سولانى AUMC 8615 فى مزارع الحالة الصلبة التى تحتوى على ردة القمح يليه ردة الأرز ثم قوالح الذرة. كما وجد أن أقصى إنتاج للإنزيم المنتج من فيوزايم سولانى عند اليوم الخامس لفترة التحضين ، درجة حرارة ٣٠ مئوية ، تركيز أيون الهيدروجين (٨) ، ومحتوى رطوبة (٦٠٪) وقد تم تحسين إنتاج الإنزيم باضافة الجلوكوز بتركيز ٠,٢٪ وكبريتات الأمونيوم ٠,٥٪. تم تنقية الإنزيم بالترسيب بكبريتات الأمونيوم والترشيح الهلامى خلال أعمدة السيفادكس متبوعاً بالتبادل الأيونى فى أعمدة ثنائى ايثيل أمينو ايثيل السيليلوزحيث قدرت نقاوة الانزيم ب ٢٩٩,٥٨ مرة. وقد وجد أن الإنزيم النقى يتكون من تحت وحدتين لكل منهما وزن جزيئى ٧٠، ٨٠ كيلو دالتون على التوالى. تم تقيد الإنزيم المنقى باستخدام الفحم النشط بنسبة تقييد ٨٠,٤ ٪ . ووجد أن تركيز أيون الهيدروجين الأمثل لنشاطه ٨ ودرجة الحرارة المثلى هى٣٧ درجة مئوية للإنزيم الحر ، وبلغت القيم المناظرة للنشاط الأمثل للإنزيم المقيد ٩ و ٤٥ درجة مئوية على الترتيب. وقد انخفض النشاط النوعى للإنزيم الحربنسبة ٣٥٪ بعد فترة تحضينه لمدة ساعتين عند درجة حرارة ٣٧ درجة مئوية ، بينما انخفض النشاط النوعى للإنزيم المقيد إلى ٥٧٪ عند تحضينه لذات الفترة عند درجة ٤٥ درجة مئوية . أظهرت نتائج البحث أن إنزيم إل-أسباراجينيز الحر والمقيد يمكن استخدامهما بكفاءة فى مجال علاج مرض اللوكيميا.