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FULL LENGTH ARTICLE

Optimization of production of anti-tumor L-asparaginase by free and immobilized marine *Aspergillus terreus*

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KEYWORDS

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Aspergillus terreus;
Immobilization;
Characterization

Abstract L-asparaginase plays a vital role in medical application, particularly in treatment of acute lymphoblastic leukemia as an effective anti-tumor agent. In the present study, twenty-one fungal strains were isolated from marine environment of the Red Sea coasts of Egypt. Screening for fungal L-asparaginase production was done, and only five fungal strains were selected and identified as *Aspergillus*, *Penicillium* and *Fusarium*. The most potent fungal isolate was *Aspergillus terreus* which yielded the highest L-asparaginase specific activity (4.81 U/mg protein). The highest enzyme productivity was observed on the 5th day and the optimized fermentation parameters were pH 6.0, temperature 35 °C. The yield was also high up on using dextrose and asparagine (8.26 U/mg protein) as carbon and nitrogen sources. The cultural conditions were studied using the Plackett–Burman experimental design. Immobilization using *A. terreus* adsorbed on sponge enhanced the enzyme production by 1.33-fold compared to the conventional free-cells. Repeated reuse of the adsorbed cells achieved the maximum enzyme specific activity after three cycles (33.86 U/mg protein).

Introduction

For over 30 years, L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1) has been applied as a chemotherapeutic agent for factually lymphoblastic leukemia treatment in a sense that converts L-asparagine to L-aspartic acid and ammonia. (Savitri et al., 2003). Current researches have been in progress to reduce the carcinogenicity effect of acrylamide on human by reducing its level in foods (Mottram et al., 2002). Elimination of acrylamide formation having minor effect on general formation of Maillard products in baked goods has been achieved by applying L-asparaginase (Hendriksen et al., 2009; Anese et al., 2011).

A great deal of interest has emerged in studying the possibilities of harnessing potential microorganisms that produce this enzyme. Different prokaryotic microorganisms such as *Pseudomonas stutzeri* (Mannan et al., 1995), *Pseudomonas*

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aeruginosa (Abdel-Fattah and Olama, 2002), *Escherichia coli* (Qin and Zhao, 2003) and *Erwinia carotovora* (Warangkar and Khobragade, 2009) are producers of L-asparaginase enzyme. The production of L-asparaginase enzyme from other sources like eucaryotic microorganisms could have minor effects. Fungi and yeasts proved their importance in different fields, particularly in industry, textile, bioremediation, agriculture, beside their vital role in natural cycling as biofertilizer. Fungi genera such as *Aspergillus, Penicillium*, and *Fusarium* have been investigated as L-asparaginase producers (Wade et al., 1971; Sarquis et al., 2004; Soniyamby et al., 2011; Elshafei1 et al., 2012; Dange and Peshwe, 2015 and Vijay1 and Jaya Raju, 2015).

Cell immobilization is considered a promising approach for enhancing the fermentation processes, enzymes production and bioremediation of toxic substances (Beshay, 2003; Kar and Ray, 2008). Advantages of using immobilized cells have been reported in such a way that facilitate continuous operation over a prolonged period, offer possible recycling of immobilized beads and simple way for harvesting the products, reactor productivity, ensures higher efficiency of catalysis (Kar and Ray, 2008) and development of economical methods focusing on lowering the cost of industrial process. Present investigation deals with isolation of L-asparaginase producing marine fungi as a novel source of enzymes in addition to optimization of physical and chemical factors for L-asparaginase production plus using different immobilization techniques.

Materials and methods

Sample collection

Water samples were gathered from different sites of Red Sea coasts, Egypt, in a sterile screw capped bottles, transferred to laboratory in ice box, and stored at 4 °C till analysis. Different dilutions were prepared, 100 µl of each dilution was spread plated on to sterile plates containing modified glucose Czapek's-Dox medium (MGCD). The composition of the medium (g/l): Glucose 2.0; L-asparagine, 10.0; KH_2PO_4, 1.52; KCL, 0.52; $MgSO_4.7H_2O$, 0.52; NaCL, 20.0 traces of $Cu(NO_3)_2.3H_2O$; $ZnSO_4.7H_20$; $FeSO_4.7H_2O$; Agar, 18.0, initial pH 6.0 and supplemented with 0.009% (v/v) of phenol red dye (Saxena and Sinha, 1981). The plates were incubated at 30 °C for 5 days to obtain colonies with pink color. The appeared colonies obtained were further purified by streaking on MGCD medium.

Growth conditions for fungal culture

The fungal isolated were grown on MGCD medium slants for five days at 30 °C. After incubation, conidia were scraped with 5.0 ml of sterile distilled water and the spores were obtained and counted using Hamocytometer. Then, 1 ml (1×10^6 spores/ml) aliquots were used to inoculate 50 ml of sterilized MGCD medium dispensed in 250 ml Erlenmeyer flasks. Thereafter, the flasks were incubated for 5 days at 30 °C under static condition.

Preparation of cell-free extracts

The fungal growth was separated by centrifugation at 6000 rpm for 15 min in a cooling centrifuge and the supernatant was used as the source of the crude enzyme.

Qualitative assay of L-asparaginase

Modified Glucose Czapek's-Dox medium (MGCD) (pH 6.0) was used as an assay medium and the qualitative assay was applied using diffusion technique. Sterilized medium was distributed in the pre-sterilized Petridishes. On the surface of solidified medium each fungal isolate was streaked, and incubated at 30 °C for 48–120 h. Non-inoculated petridish was taken as a negative control. Changing the medium color from yellowish to pink indicated a positive L-asparaginase production. All experiments were done in triplicate.

Quantitative assay of L-asparaginase

L-asparaginase activity was quantitatively estimated by the method of Mashburn and Wriston (1964) and Imada et al. (1973) using Nessler's reagent. The reaction mixture containing 0.5 ml of crude sample, 0.5 ml of 0.5 M buffer (acetate buffer pH 5.4), 0.5 ml of 0.04 M asparagine and 0.5 ml of distilled water was added to make the volume up to 2.0 ml and incubate for 30 min. Thereafter, the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). The mixture was then centrifuged at 10,000 rpm for 5 min, and 0.1 ml of the supernatant was taken and 3.7 ml of distilled water was added; 0.2 ml of Nessler's reagent (Himedia) was added to the reaction tube. The color reaction was developed through 10 min and the absorbance was measured at 500 nm using spectrophotometer. The ammonia liberated was extrapolated from a curve derived with ammonia sulfate as standard curve. One IU of L-asparaginase is the amount of enzyme which liberates 1 µmol of ammonia per ml per minute [µmole/ml/min].

Estimation of protein

Protein content was determined according to the method described by Lowry et al. (1951) using Bovine Serum Albumin as a standard.

Fungal identification

The fungal isolates were identified according to morphological and cultural features (Watanabe, 2002; CBS, 2006). The isolates belonged to three genera (*Aspergillus, Pencillium and Fusarium*). The identification of fungi was confirmed by Mycological Center, Faculty of Science, Assiut University, Egypt.

Effect of temperature and pH on the L-asparaginase production

A. terreus was cultivated in MGCD broth at different pH values ranging from 4.0 to 9.0 at 30 °C under static conditions. For optimum temperature, *A. terreus* was incubated at different temperatures (25, 30, 35, 40, 45, 50, 55, and 60 °C) under static conditions for the optimum incubation time.

Effect of different carbon and nitrogen sources

Different carbon sources were added to MGCD broth at equivalent weight. Thereafter, L-asparaginase production was investigated. Carbon compounds, including glucose, lactose, fructose, sucrose, dextran, maltose, galactose and soluble

starch, were supplied to MGCD medium supplemented with L-asparagine (0.3%) as nitrogen source. The mycelia of *A. terreus* was inoculated in the medium and incubated at 35 °C for 5 days under static conditions. Different nitrogen sources were added at equivalent weight, including asparagine, glutamine, proline, yeast extract, peptone, ammonium sulfate, ammonium chloride, potassium nitrate and sodium nitrate to MGCD medium containing the best carbon source.

Optimization of nutritional factors

Plackett–Burman experimental design was used for optimization of the medium (Plackett and Burman, 1946). Multifactorial design of seven independent variables was established. High (+) and Low (−) levels of each independent factors were examined. All trials were done in triplicates, and the mean of obtaining data was processed as the response of L-asparagine hydrolysis. The main effect of each variable was calculated according to the following equation:

$$\mathrm{Exi} = \left(\sum \mathrm{pi} + - \sum \mathrm{pi}-\right)/N$$

where Exi is the variable main effect, Σpi + and Σpi– are L-asparaginase production responses in trials where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. All trials were performed in triplicates and the responses were reported based on the average of L-asparaginase production percentages. In a comparative test between Plackett–Burman optimum medium, basal medium and Plackett–Burman reverse medium, verification of validity medium was experimentally applied.

Immobilization of A. terreus by entrapment

Entrapment in Ca-alginate

Entrapment was done in 3% Sodium alginate as described by Eikmeier and Rhem (1987). A twenty ml sodium alginate solution was prepared by dissolving 0.3 g in 16 ml distilled water and autoclaved at 121 °C for 10 min. Four ml of *A. terreus* suspension was added to the sterilized alginate solution. 10 ml of alginate-fungus combination was introduced into a cross linking solution (sterile 2% $CaCl_2$) using sterilized syringe to obtain spherical beads of calcium alginate beads entrapping the fungus. The beads were left in the solution for two hours, washed several times and transferred to 50 ml optimized medium.

Entrapment in K-carrageenan

Immobilization by entrapment in K-carrageenan was done using the method detailed by Wada et al., 1980. Four milliliter of fungus suspension (1×10^6 spores/ml) was added to 16 ml sterile 3% K-carrageenan. The solution was mixed well and then 10 ml fraction was introduced drop by drop with a sterile syringe into a 20 ml sterilized 2% KCl, left for 2 h to harden, washed several times with sterile distilled water, then transferred aseptically to 50 ml of the sterile cultivation medium.

Entrapment in agar and agarose

0.6 gm agar-agar or agarose was dissolved in 16 ml water, and stirred well before sterilization. Four ml *A. terreus* suspension (1×10^6 spores/ml) was added and mixed well, the formed mixture was aseptically poured into sterile Petridish and left to solidify. The solidified agar (50 ml) was cut into equal size cubes (4 mm), refrigerator (1 h) for curing. Afterward, phosphate buffer was decanted and the cubes were washed with sterile distilled water 3–4 times.

Immobilization by adsorption

Two ml of fungal suspension was added to 50 ml medium dispensed in Erlenmeyer flasks (250 ml capacity) containing 50 ml sterilized culture medium and support materials such as sponge cubes, clay, ceramic, luffa pulp, pumice and art pumice. The flasks were incubated under static condition at 35 °C for 5-days (Vassilev et al., 1993).

Recycling of adsorbed A. terreus

The fermentation medium containing 20 cubes of sponge was supplemented with 1.5 ml of fungal spores, the pH adjusted to 6 and incubated at 35 °C for 5-days. The reuse of the adsorbed cells was carried out by removing the medium after 5-days, and then a new sterilized medium (50 ml) was added, and new cycle was run. This process was repeated several times. At the end of each cycle the protein content, L-asparaginase activity and cell-Leakage were determined.

Results and discussion

Isolation and screening marine fungi producing L-asparaginase

In the present study a total of twenty-one fungal strains were isolated from different water samples from Red Sea coasts, Egypt. The isolated fungi were identified according to physiological and morphological characters, to the genera *Aspergillus, Penicillium, Fusarium*. The isolated fungal species were screened for their capabilities to produce L-asparaginase using MGCD agar medium. Out of 21 fungal isolates, only 5 isolates showed pink zone around the colonies on MGCD agar medium containing phenol red as indicator that changes from yellow (acidic condition) to pink (alkaline condition) where L-asparaginase enzyme hydrolyze the asparagine to L-aspartic acid and ammonia. The liberation of ammonia results in the pH change from acidic to alkaline.

The strains *A. flavus* (isolate from Safaga) *A. terreus* (isolated from Safaga and QuseirNorth), *Fusarium* sp. (isolated from MarsaAlam) and *Penicillium* sp. (isolated from Safaga, Quseir south and MersaAlam) were selected for quantitative screening (Fig. 1). Among these only one strain showed the highest L-asparaginase specific activity of 4.81 U/mg protein, which was selected for further study. The selected strain was identified as *Aspergillus terreus*.

Effect of pH and temperature on L-asparaginase production

The pH of the fermentation medium plays a vital role in enhancement of L-asparaginase production and a pivotal role in transportation of various components across the cell membrane and in managing the metabolic activities of the cell. Effect of initial pH on culture conditions and enzyme production were studied in the pH range from 4.0 to 10.0. L-asparaginase activity of *A. terreus* was observed along the

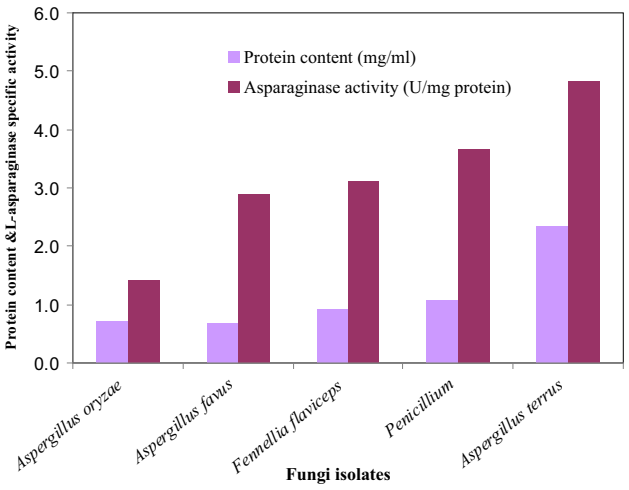

Figure 1 Quantitative screening for production of L-asparaginase using different marine fungal isolates.

tested pH range, but the highest L-asparaginase specific activity (6.59 U/mg protein) was observed at pH 6.0 (Fig. 2). Further production of the enzyme decreased subsequently at all higher pH values attaining activity 1.45 U/mg protein at pH 10.0. This result revealed the acidophilic nature of this fungus. Our results are in agreement with other investigations (Gulati et al., 1997; Sarquis et al., 2004) with rare exceptions, where asparaginase production by filamentous fungi occurs in cultures with an initial pH under 7.0 (Hosamani and Kaliwal, 2011; Chandrasekhar,2012; Monica et al. 2014).

A. terreus was able to grow and produce L-asparaginase at temperature ranging from 25 °C to 35 °C (Fig. 3). The L-asparaginase specific activity reached its maximum value (6.59 U/mg protein) at 35 °C. A reduction in enzyme activity was observed with incubation temperatures lower and higher than the optimum temperature. At 25 °C and 60 °C activity of L-asparaginase specific activity was 2.30 U/mg protein and 1.52 U/mg protein, respectively. The optimum temperature 30 or 37 °C was reported in most of the L-asparaginase producing fungal species (Gulati et al., 1997; Lapmakm et al., 2010; Hosamani and Kaliwal, 2011; Rani et al., 2011).

Effect of carbon and nitrogen sources on L-asparaginase production

In this experiment different carbon sources were used to substitute glucose in the fermentation medium. Various sources of carbon such as soluble starch, fructose, maltose dextrose, lactose and sucrose were added in growth media. The results given in Fig. 4 revealed that, dextrose brought the highest L-asparaginase production (8.26 U/mg protein) compared to other carbon sources at 5-days incubation of *A. terreus* culture, while the lowest L-asparaginase production was recorded when using soluble starch (1.98 U/mg protein). The results of the present investigation are also in agreement with other authors concerning the production of L-asparaginase enzyme (Dange and Peshwe, 2015; Kalyanasundaram et al., 2015).

As a fact, the nitrogen sources considering the secondary energy source after carbon sources and they play a vital role in the growth of organisms and enzyme production In the present experiment, effect of providing nitrogen sources on L-asparaginase production showed that L-asparagine was found to be the best nitrogen source for this isolate realizing enzyme specific activity up to 8.26 U/mg protein as shown in Fig. 5. These results are in good agreement with those reported for the production of L-asparaginase by other microorganisms (Thirunavukkarasu et al., 2011). In contrast, Kalyanasundaram et al., 2015, used ammonium sulfate as a nitrogen source for the maximum production of L-asparaginase from *A. terreus*.

The application of Plackett–Burman statistical design

In Table 1, the multi-factorial design aimed at screening the most effective factors by low or high level to produce L-asparaginase as a response taking into consideration the *t*-value and main effect values for each factor, while in Table 2, the multi-factorial design matrix showed the high

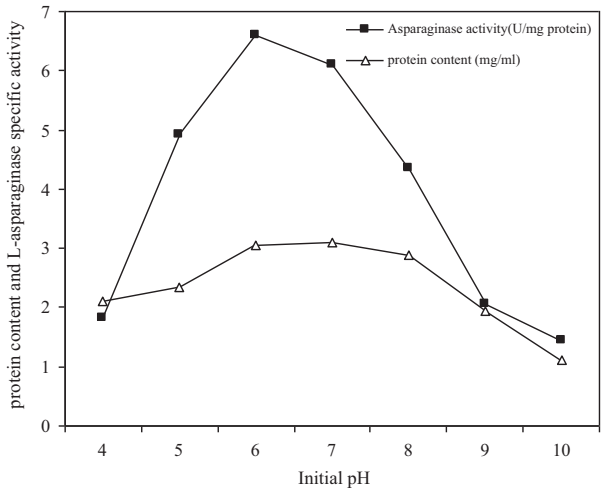

Figure 2 Effect of different pH on of L-asparaginase production by *A. terreus*.

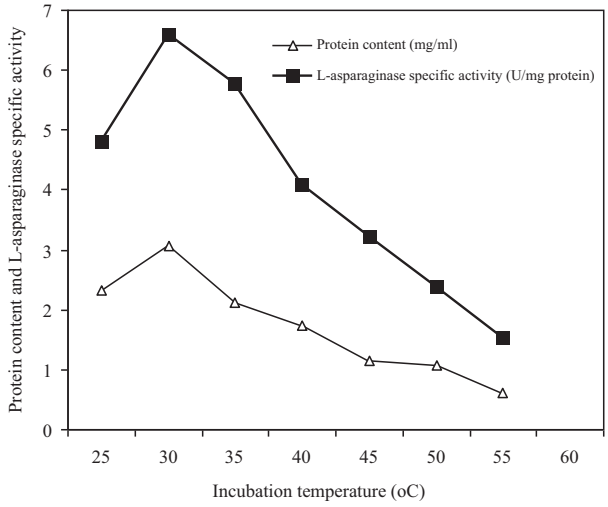

Figure 3 Effect of different temperature on L-asparaginase production by *A. terreus*.

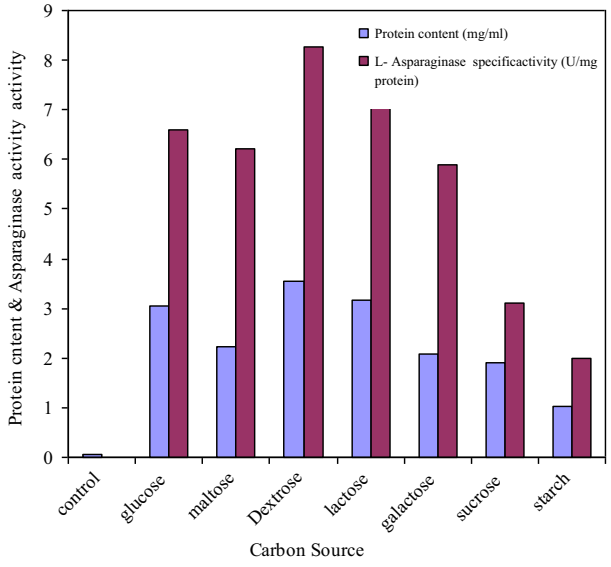

Figure 4 Effect of different carbon sources on L-asparaginase production by *A. terreus*. * Control without carbon source.

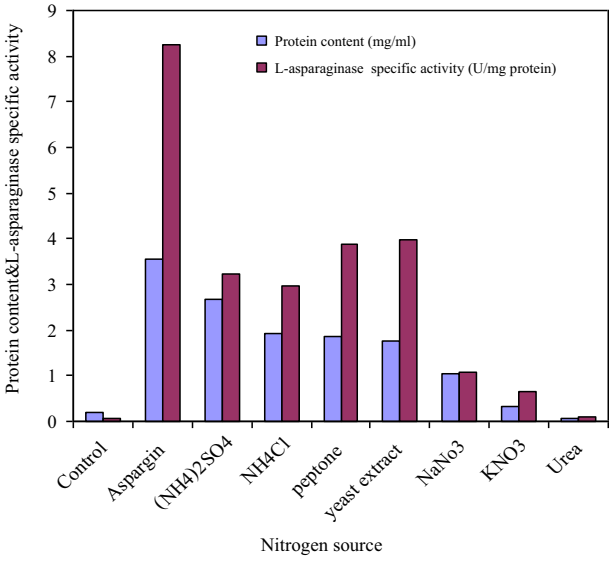

Figure 5 Effect of different nitrogen sources on L-asparaginase production by *A. terreus*. *Control without nitrogen source.

and low level for each factor and the obtaining results of L-asparaginase production and biomass as the response.

As shown in Table 1 and graphically presented in Fig. 6, the main effect of each variable on L-asparaginase production and biomass estimation plus *t*-values was calculated for each independent variable. Results indicated that the production of L-asparaginase by *A. terreus* was positively affected by asparagine, sodium chloride, size of inoculum and magnesium sulfate while it was negatively affected by dipotassium hydrogen phosphate, dihydrogen potassium phosphate and dextrose, i.e. increasing the concentrations of asparagine, sodium chloride, magnesium sulfate and size of inoculum in the culture

Table 1 Main effect and *t*-value results of multi-factorial independent variables affecting the production of L-asparaginase.

Factors	Symbols	Levels			Main effect	*t*-value
		(−1)	(0)	(+1)		
Dextrose	Dex	5	10	15	−0.194	−0.045
Asparagine	Asp	1.5	3	4.5	6.804	+2.065
K_2HPO_4	K_2	0.5	1	1.5	−0.876	−0.204
KH_2PO_4	KH	1.5	3	4.5	−0.147	−0.344
$MgSO_4.7H_2O$	Mg	0.25	0.5	0.75	0.764	+0.178
NaCl	Na	12.5	25	37.5	4.568	+1.176
Inoculum size	Is	1.0	2.0	3.0	6.384	1.86

medium causes an increase in the L-asparaginase production while increasing the concentrations of dipotassium hydrogen phosphate, dihydrogen potassium phosphate and dextrose in the culture medium causes a decrease in the L-asparaginase production. The calculated *t*-test (Table 1), revealed that asparagine, sodium chloride, and inoculums size were the most significant variables affecting L-asparaginase production. Many authors used placket–Burman experimental design for enhancement of L-asparaginase production (Hymavathi et al., 2010 and El-Naggar et al., 2014) (see Table 3).

Verification experiment

The predicted optimum levels of independent variables were subjected for verification and compared with the anti optimized levels and the basal condition results in order to evaluate the efficacy of the design. Data exposed that the L-asparaginase production raised by 1.33-fold for *A. terreus* when growing in optimized medium (Table 3). Accordingly, the predicted medium composition that offers high L-asparaginase production should be as follows (g/l): asparagen, 4.5; KH_2PO_4, 1.5; K_2HPO_4, 0.5; $MgSO_4.7$ H2O, 0.75; NaCl, 37.5; dextrose, 5 and inoculum size, 3 ml/50 ml.

L-asparaginase production by immobilized A. terreus culture using different techniques

The biosynthesis of L-asparaginase by *A. terreus* entrapped in different gel materials was studied. The biocatalysts formed of ca-alginate beads proved to be superior to all other biocatalysts producing the highest L-asparaginase activity (12.32 U/mg protein) which is also higher than free culture (10.97 U/mg protein) as shown in Fig. 7. The higher production of L-asparaginase in case of entrapped cells could be due to the minimal growth of cells in the matrix and also due to enzyme activities being retained at higher levels than that of the free cells (Kar and Ray, 2008). The alginate and gelatin-immobilized cells were previously used for enhancement the L-asparaginase production (Amena et al., 2008; Amena et al., 2010).

Adsorption of *A. terreus* on different solid porous supports such as clay particles, pumice, ceramic, Art pumice, luffa pulp and sponge cubes for L-asparaginase production was investigated. The results graphically illustrated in Fig. 8 showed a significant adsorption of fungal cells on sponge, clay and pumice

Table 2 The Plackett–Burman experimental design matrix for 7 factors.

Trials	Independent variable							(Response) L- asparaginase activity (U/mg protein)	Protein content (mg/ml)
	Dex	Asp	K2	KH	Mg	Na	Is		
1	−1	−1	−1	1	1	1	−1	7.17	3.14
2	1	−1	−1	−1	−1	1	1	7.38	2.44
3	−1	1	−1	−1	1	−1	1	7.47	2.63
4	1	1	−1	1	−1	−1	−1	6.78	2.43
5	−1	−1	1	1	−1	−1	1	10.49	3.23
6	1	−1	1	−1	1	−1	−1	7.89	3.45
7	−1	1	1	−1	−1	1	−1	7.17	3.02
8	1	1	1	1	1	1	1	10.05	3.45
9	0	0	0	0	0	0	0	8.26	3.55

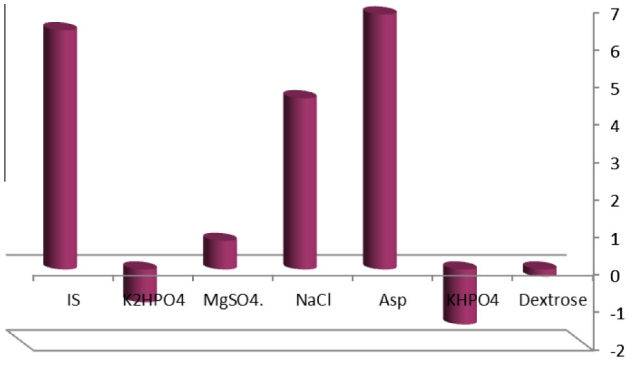

Figure 6 Elucidation of fermentation conditions affecting the production of L-asparaginase by *A. terreus*.

Table 3 A verification experiment of the Plackett–Burman experimental results.

Medium	Protein content (mg/ml)	L-Asparaginase specific activity (U/mg protein)	Dry weight (mg/100 ml)
Basal medium	3.55	8.26	540
Optimized medium	3.45	10.97	588
Anti-optimized medium	1.30	4.88	230

supports in terms of enzyme specific activity. Particularly, sponge cubes showed a slightly higher fungal adsorption when compared to the other supports. Cultures containing adsorbed *A. terreus* on sponge, clay and pumice showed a relatively high L-asparaginase specific activity (14.54, 10.77 and 10.23 U/mg protein, respectively), while luffa pulp showed the lowest activity (3.48 U/mg protein). Thus cultures containing sponge cubes had higher L-asparaginase specific activity (14.54 U/mg protein) than that of free cultures. Therefore, sponge cubes were selected for production of L-asparaginase. Electron microscopic photographs (Fig. 9) showed the adsorption of *A. terreus* on the pores of sponge. Kattimani et al. (2009) used the polyurethane foam as a support material for immobilization of *Streptomyces gulbargensis* mu24 and L-asparaginase yield was increased by 30.2% as compared to free cells.

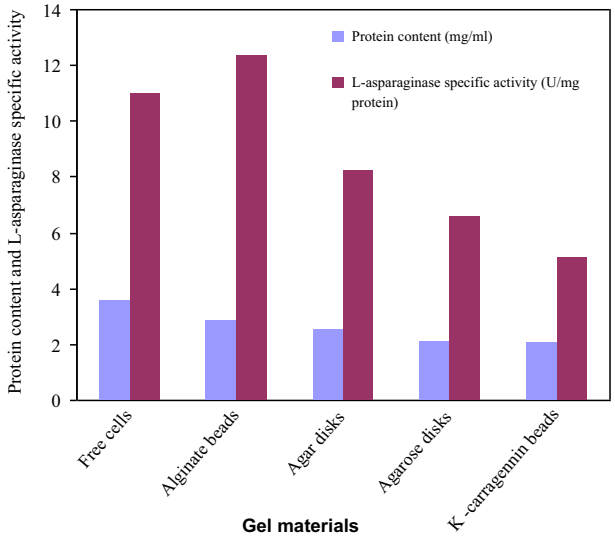

Figure 7 Effect of immobilization using entrapment techniques on L-asparaginase production by *A. terreus*.

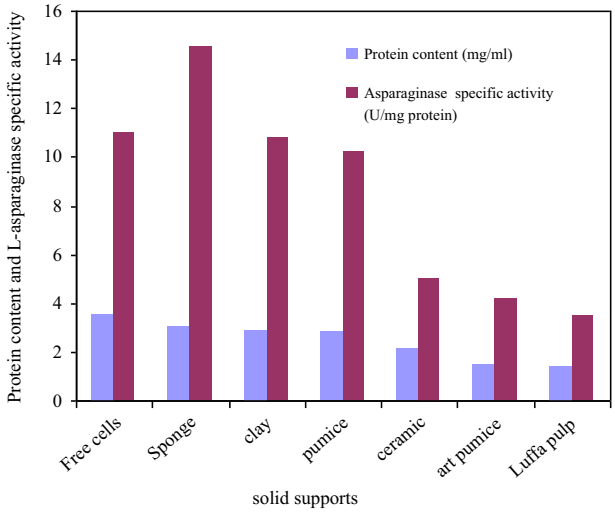

Figure 8 Production of L-asparaginase by *A. terreus* adsorbed on different solid supports.

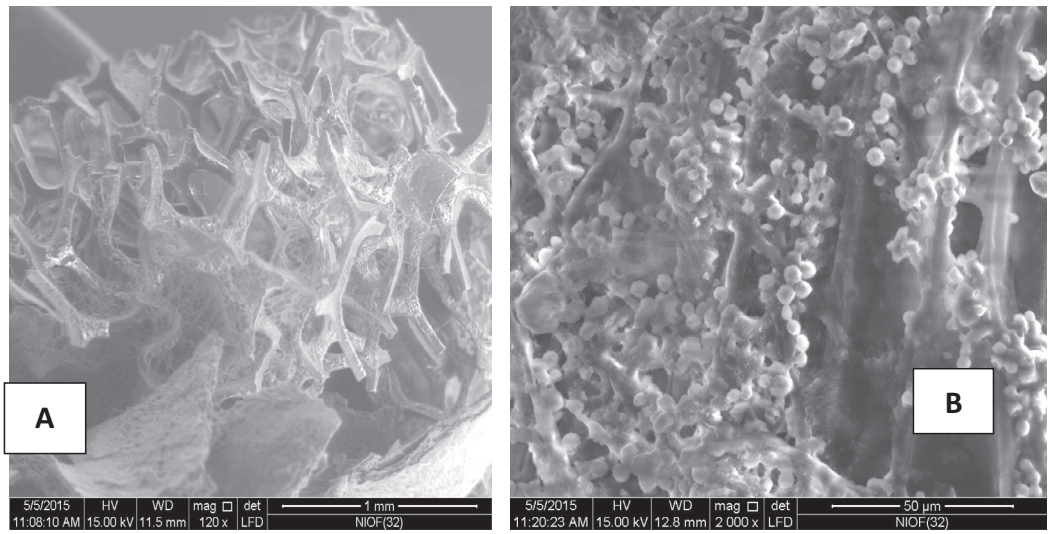

Figure 9 Electron microscope micrographs illustrate (A) sponge cubes and (B) the adsorbed *A. terreus* on sponge cubes.

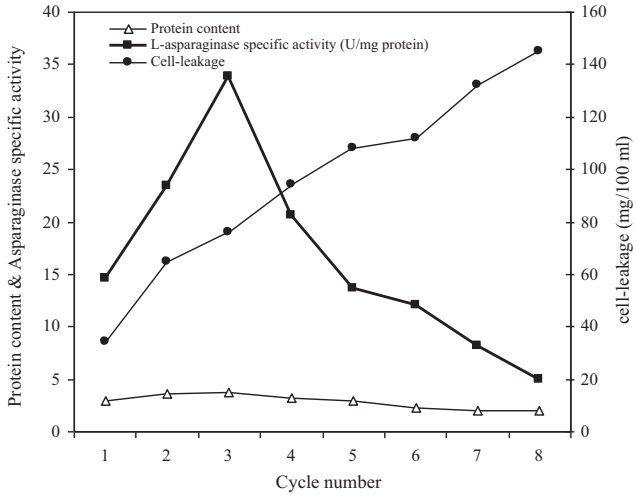

Figure 10 Effect of reused adsorbed *A. terreus* on the production of L-asparaginase enzyme.

The semicontinuous production of L-asparaginase by *A. terreus* adsorbed on sponge was investigated. Semicontinuous production of the L-asparaginase enzyme was conducted using batch wise reuse of the absorbed cells for 8 successive cycles The results in Fig. 10 showed that by reusing the cells adsorbed on sponge as a solid support, the activity increased slightly in the cultures of the second, third and fourth runs (23.46, 33.86 and 20.54, Respectively), followed by a gradual decrease up to the 8th run, then the reused cultures showed a gradual decrease up to the 8th run. The advantage of using immobilized *A. terreus* cells for maximum production of L-asparaginase was achieved by recycling the fungal cells in batch mode using good cell adsorption on low price support material.

Hence, *Aspergillus terreus* appears to produce high amount of L-asparaginase enzyme which may be useful in many applications. The study may confirm the advantage of immobilization of the whole cells and reuse of them to increase the production of the enzyme.

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