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Stimulatory Effect of Acid Hydrolysed Okara Fortified with *L-Asparagine* on *Fungal L-Asparaginase* Production by *Aspergillus Terreus* MTCC 1782

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ABSTRACT

The insoluble fraction of soy bean wet processing namely okara was employed to investigate the suitability of okara or associated fractions of okara preparation or the hydrolysate made from okara, for their ability as substrate for L-Asparaginase production using *A. terreus*. Un-supplemented okara, hydrolysate of okara &soybean batter did not produce appreciable enzyme titters. Okara cake managed to give a maximum of 0.51 U/ml of L- Asparaginase activity on day 6. Fortification of okara or associated fractions (okara hydrolysate & soybean batter) with ammonium sulphate did not produce rise in enzyme titters. When okara cake was supplemented with 1.5% (w/v) ofL- Asparagine, theenzyme yield was increased by 23.63 folds in comparison with that of the unsupplemented control. For okara hydrolysate & soybean batter too induced levels of enzyme were observedupon supplementation with L-asparagine. For soybean batter this was 42.6 fold increase in activity and okara hydrolysate fortified with L-asparagine gave 63.89 U/ml of enzyme activity on day five as against 0.06U/ml for the unsupplemented control. The positive influence of L- asparagine fortification to okara hydrolysate was more pronounced till the percentage supplementation was up to 1.5%. Though additional supplementation of specific nitrogen source beyond 1.5% resulted in enhanced enzyme titters, the level of enhancement was not well pronounced.

Keywords: *A. terreus*, L-asparaginase, SSF and Okara

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INTRODUCTION

Therapeutic enzymes constitute a significant and reliable domain of today's pharmaceutical industry. L-Asparaginase (EC 3.5.1.1) the tetramericoncolytic enzyme has been used as an anti-tumor agent for the effective treatment of acute lymphoblastic leukemia and lymphosarcoma[1, 2]. It catalyses the hydrolytic deamination of L-asparagine to yield aspartic acid and ammonium ion [3, 4].

Cancer cells differentiate themselves from normal cells in reduced expression of L-asparagine [5, 6]. Hence, they are not proficient of generating L-asparagine and mainly rest on the L-asparagine from circulating plasma pools [6]. This clinical action is recognised as decline of L-asparagine and since tumour cells unable to synthesize this amino acid, are selectively killed by L-asparagine deprivation [7].Bacterial L-asparaginase leading to hypersensitivity especially with excessive use of L-asparaginase is a serious problem. Search for new L-asparaginase without side effects led the researchers to bang on eukaryotic microorganisms like yeast and filamentous fungi such as *Aspergillus, Penicillium*and*Fusarium* etc. [8, 9].Recently, L-asparaginase was also used for the production acrylamide free food products [10]. L-asparaginasescan effectively reduce the level of acrylamide up to 90% in a range of starchy foods without changing the taste and appearance of the end product [11].

The residue left from ground soy beans after extraction of the water extractable fraction, used to produce soy milk or tofu, is called okara. About 1.1 kg of fresh okara is produced from every kilogram of soybean processed into soymilk or tofu [12]. Annually about 0.7 million tons of okara is produced as by-product of tofu manufacture and most of it has been destroyed as waste [13]. Okara is rich in water-insoluble ingredients (protein (4.8%), fat (3.6%), starch and sugar (6.4%), fiber (3.3%), ash (0.8%) and water (81%)) making it a potentially suitable substrate for microbial fermentation [14].

Hitherto, L-asparaginase was produced by the technique of submerged fermentation. Though solid-state fermentation (SSF) could be the preferred format for the effective production of various enzymes, by dint of the yield in SSF remains several times higher than that of submerged fermentation (SmF), the former is less exploited for L-asparaginase production. Okara being an ideal substrate for SSF, the same was exploited in our study to find the suitability of the substrate in supporting L- asparaginase production. It also offers many other advantages including resistance to contamination, ease of product extraction and simpler methods for treating the fermented residue [15]. Our study was envisaged with the idea of finding out the suitability of okara based bioprocess forL-asparaginase production in tandem with supplementation by specific and general nitrogen sources.

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MATERIALS AND METHOD

Materials

All the chemicals used were of analytical grade and procured from Renkem, New Delhi, Himedia, Mumbai and Merck, Mumbai, India. Soybean was obtained from the local market, Sathyamangalam.

Microorganism

Aspergillus terreus (MTCC-1782 Chandigarh, India) was used for the present study. The culture was grown and maintained on Sabouraud dextrose agar slants. The slants were stored at 4°C and sub culturing was carried out as per standard procedures. The pre-cultures and main cultures (fermentation) for the production of enzyme were grown in Sabouraud dextrose broth (pH 7.0) for 36 hours and 8 days respectively on a rotary shaker at 120 rpm and at 30^0C. Fraction of the centrifuged mycelia (5000 rpm for 10 min and 10°C) was stored in 40% (v/v) glycerol stock at -20° C.

Substrate Used

Preparation of Okara

500g of soybean was soaked in 1 L of distilled water and allowed to stand for overnight swelling. The grains were filtered off hulls and were homogenized by pouring equal quantity of water. This was filtered using nylon cloth to retain the insoluble fraction of okara.

Media Used for Study

Three basic media materials were obtained while preparing okara(Table1).

Okara – the residual cake obtained after filtration of the aqueous extract of soybean grains as stated above

a) Soy bean batter – The batter (filtrate) obtained during the filtration step in okara preparation was referred to here as soy bean batter
b) Acid hydrolysed okara: 300g of okara cake was dissolved in 500ml of distilled water, 0.75% of concentrated H_2SO_4 was added and heated in water bath at 70° C for about 4 hours with regular stirring. This was then filtered using nylon cloth. The filtrate was used for fermentation after neutralizing the pH to 7.0 using 2M NaOH

Inoculum

The spore suspension *A. terreus* made using sterile tween water (tween 60; 0.01% (v/v)) through stirring the same using inoculation loop in fungal slant culture aseptically was used as

the inoculum. 1 mL of the spore suspension was added to 50 ml of Sabouraud dextrose broth (pH 7) in a rotary shaker at 120 rpm for 48 hours. The aforesaid culture at 5 % (v/v) was used as inoculum for the fermentation flasks.

Table 1: Various media formulations made from different fractions of wet soybean grains

S.No	Flask label	Fraction of soy bean extract used	Quantity used	Supplementary chemicals	pH	Nature of medium after sterilization
1.	A1	Okara cake	15 g	-	7	Solid
2.	A2	Okara cake	15 g	1.5% NH_4SO_4 + 0.75% phosphate	7	Solid
3.	A3	Okara cake	15 g	1.5% L-asparagine + 0.75% Phosphate	7	Solid
4.	B1	Acid hydrolysed okara	50 ml	-	7	Liquid
5.	B2	Acid hydrolysed okara	50 ml	1.5% NH_4SO_4 + 0.75% phosphate	7	Liquid
6.	B3	Acid hydrolysed okara	50 ml	1.5% L-asparagine + 0.75% Phosphate	7	Liquid
7.	C1	Soy bean batter	50ml	-	7	Semi solid
8.	C2	Soy bean batter	50 ml	1.5% NH_4SO_4 + 0.75% phosphate	7	Semi solid
9.	C3	Soy bean batter	50 ml	1.5% L-asparagine + 0.75% Phosphate	7	Semi solid

Fermentation

SmF:

5 % (v/v) each of inoculum was added to 50 ml each of acid hydrolyzed okara (with and without supplementary chemicals) and the flasks were kept in a rotary shaker at 120 rpm for 8 days. 2 ml of samples were collected for every 24 hrs from third day, and centrifuged at 6000 rpm for 10 mins. Supernatant was used for enzyme assay. All the experiments were performed independently in triplicates.

SSF

5 % (v/v) each of inoculum was added to 15 g of okara and 50 ml of soy bean batter (with and without supplementary chemicals) separately. The inoculated flasks were kept in room temperature for 8 days. 2g of samples were collected for every 24 hrs from third day. They were added to 10 ml each of distilled water and stirred for half an hour. These were then filtered using wattman No.1 filter paper and centrifuged at 6000 rpm for 10 mins. Supernatants were used for enzyme assay.

Enzyme Assay

Assay of enzyme was carried out as per Imad*et al.*[16]. The enzyme activity was determined by calculating the amount of ammonia liberated by Nesslerization method. A reaction mixture containing 0.5ml of 0.04M L-asparagine, 0.5 ml of 0.5M buffer (acetate buffer pH 5.4), 0.5 ml each of enzyme preparation and distilled water to a total volume of 2.0 ml was incubated at 30°C for 30 min. After the incubation period, the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). From the above mixture 0.1 ml was transferred into a clean test tube. To this 3.7 ml of distilled water and 0.2 ml of Nesslers reagent were added, and the mixture was again incubated at 30°C for 20 min. The OD was measured at 450 nm. The blanks were made by adding enzyme preparation after the addition of TCA. One unit of L-asparaginase is the amount of enzyme which liberates 1µmole of ammonia per min per ml [µ mole/ml/min].

Effect of Nitrogen Source on L-Asparaginase Production

Fermentation was carried out in Erlenmeyer flasks (A2, B2, and C2) holding the three major fractions of okara preparation separately. They were all supplemented with 1.5% of ammonium sulphate & 0.75% of phosphate each. The flasks were kept in rotary shaker at 120 rpm for 8 days. 2 ml of samples were collected for every 24 hrs from third day, and centrifuged at 6000 rpm for 10 mins. The cell free supernatants were used for enzyme assays. A set of un-supplemented controls (A1, B1 and C1) were also maintained for the sake of comparison.

Effect of L-asparagine in Inducing the Production of L-Asparaginase

Fermentation was carried out in Erlenmeyer flasks (A3, B3, and C3) holding the three major fractions of okara preparation as stated above. They were all supplemented with 1.5% of Asparagine & 0.75% of phosphate each. The flasks were kept in rotary shaker at 120 rpm for 8 days. 2 ml of samples were collected for every 24 hrs from third day, and centrifuged at 6000 rpm for 10 mins. The cell free supernatants were used for enzyme assays. A set of un-supplemented controls (A1, B1 and C1) were also maintained for the sake of comparison.

Suitability of Different Percentage of L-asparagine for L-asparaginase Production by *A.terreus*

Fermentation was carried out in Erlenmeyer flasks holding 50ml okara acid hydrolysate. They were all supplemented with different percentage of L- asparagine (0.5, 1.0, 1.5, 2.0, 3.0%) & 0.75% of phosphate each. The flasks were kept in rotary shaker at 120 rpm for 8 days. 2 ml of samples were collected for every 24 hrs from third day, and processed as stated above.

RESULTS

Suitability of different fraction of soybean wet processing as substrates for L-asparaginase production by *A. terreus*

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The enzyme activity assay findings clearly indicated lack of appreciable levels of enzyme titers released during growth of the fungal culture on all the three types of substrates tested. Though okara cake and soybean batter managed to produce some detectable enzyme activity (0.51U/ml on 6th day for the former & 0.65 U/ml on the day 3 by the later (Table.2)). These were commercially significant level of enzyme activity.

Table 2: Production of L-asparaginase by *A. terreus* on various fractions of soybean wet processing (Okara cake, Hydrolysate of okara and soybean batter) with no additional nitrogen supplementation

Duration	(L-asparaginase activity (U/ml))		
Days	A1(Okara cake)	B1 (Hydrolysate okara)	C1 (Soybean batter)
3	0.19	0.07	0.65
4	0.31	0.08	0.64
5	0.42	0.06	0.06
6	0.51	0.05	0.05
7	0.11	0.04	0.02
8	0.01	0.03	0.01

Influence of nitrogen supplementation on L-asparaginase production by *A. terreus* on okara and okara associated fractions

Supplementation of okara cake with Ammonium sulphate or L-asparagine (corresponding enzyme substrate)

Okara fortified with L-asparagine influenced the enzyme yield such that the un-supplemented control and readily accessible ammonium salts when present in the medium did not produce any significant levels of L-asparaginase. This is depicted in figure 01 where A3 showed 12 U/ml of enzyme activity on 6th day. Since presence of L-asparagine is favouring the stimulated levels of enzyme titters, L- asparaginase production by *A. terreus* is induced one.

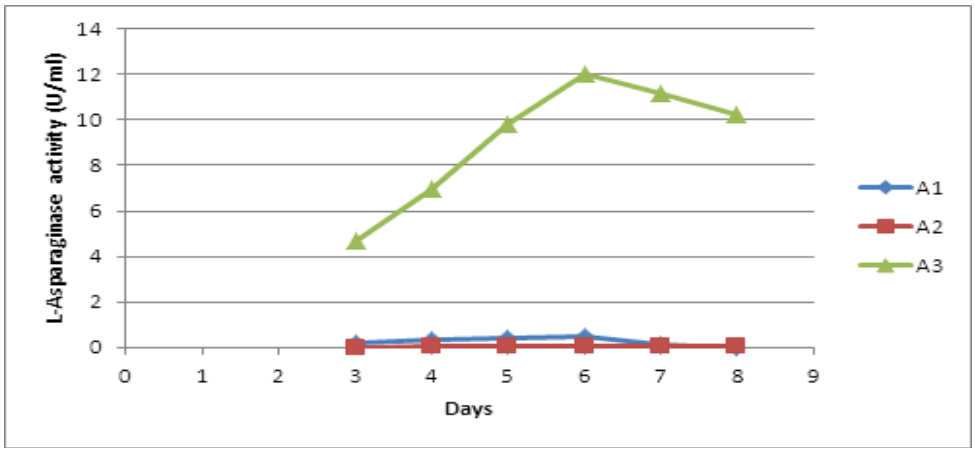

Fig. 1 Influence of supplemented nitrogen source on the production of L-asparaginase by *A. terreus* on okara cake

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Supplementation of acid hydrolysate of okara with ammonium salt or L-asparagine

Addition of L-asparagine in hydrolysate of okara also induced the *A. terreus* for the better production of L- asparaginase and gave maximum yield of about 64 U/ml on 5th day. This further confirms the inducing effect of L-asparagine on *A. terreus* for the production of L-asparaginase (Figure 2).

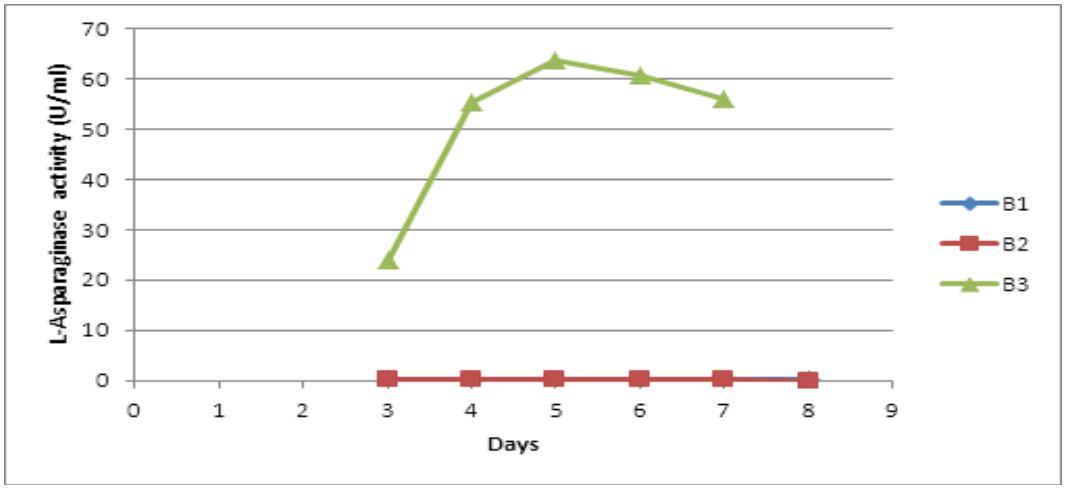

Fig. 2 Influence of supplemented nitrogen source on the production of L-asparaginase by *A. terreus* on hydrolysate of okara

Supplementation of okara batter i.e., the filtrate formed during okara preparation from soybean with ammonium or L-asparagine

When the okara batter was used as substrate, supplementation with ammonium sulphate and phosphate did not influence production of L-asparaginase whereas L-asparagine incorporation gave higher enzyme titers as given below in figure 03.

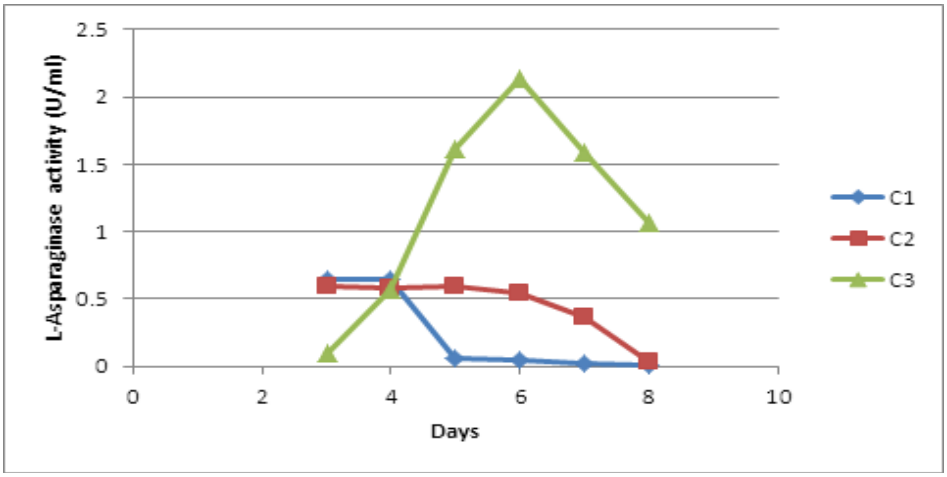

Fig. 3Influence of supplemented nitrogen source on the production of L-asparaginase by *A. terreus* on soybean batter

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Production of L-asparaginase by *A. terreus* on okara and associated fractions of okara in the presence of L-asparagine the corresponding substrate A3, B3, C3

The study aimed at finding out the comparative ability of three fractions of okara when supplemented with the corresponding enzyme substrate indicated that the L-asparagine induces the enzyme production by the *A. terreus* in all three instances. Acid hydrolysed okara turned out to be the promising substrate for the enzyme production as the yield was as high as 64 U/ml against 12 U/mL and 2.2 U/mL (Figure 4) recorded for okara and okara batter respectively.

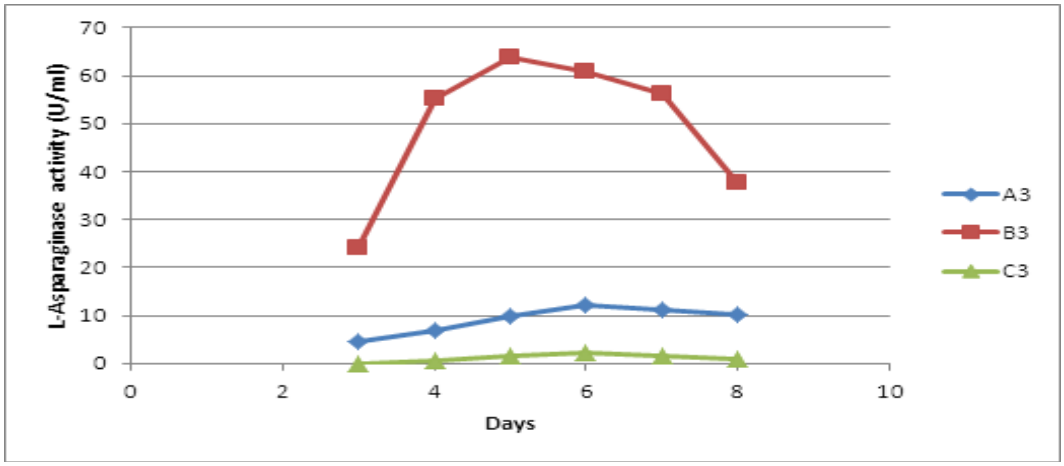

Fig. 4 Comparative yields of enzyme activity produced by *A. terreus* when grown on different fractions of soybean wet processing supplemented with the corresponding enzyme substrate, L-asparagine

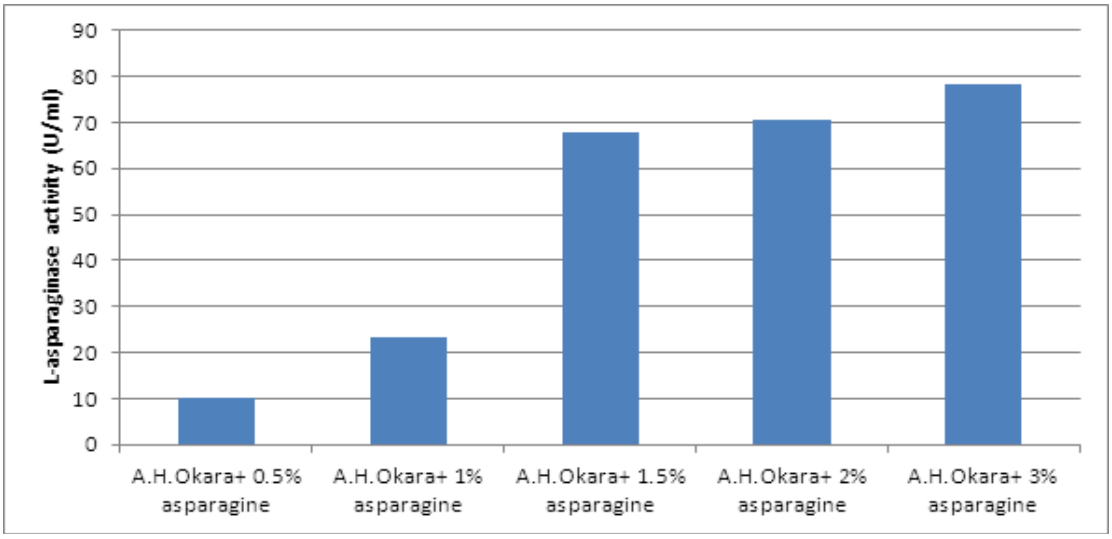

Fig. 5 Influence of L-asparagine fortification to okara hydrolysate for the production of microbial L-asparaginase by *A. terreus*

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Influence of L-asparagine fortification to okara hydrolysate for the production of microbial L-asparaginase by *A. terreus*

The positive influence of L-asparagine fortification to okara hydrolysate was more pronounced till the percent supplementation was up to1.5%. Further increase up to 3% was investigated in this study. Though additional supplementation of specific nitrogen source resulted in enhanced nitrogen titer, the level of enhancement was not well pronounced as depicted in figure5. Further studies incorporating higher levels of L-asparagine as supplemented nitrogen source to the medium are currently underway. The results of these investigations will be important for the development of a desirable bioprocess strategy for optimized production of L-asparaginase from *A. terreus*.

DISCUSSION

Though there are many reports on the microbial production of L-asparaginase by fungal cultures on diverse solid substrates, okara has not been studied for its suitability to be used as solid substrate for fungal L-asparaginase production. Invariably submerged fermentations of fungal cultures resulted in relatively lower levels of enzyme activity in the culture broth as reported by Sutthinan Khamna*et al*. [17]&Soniyamby*et al*.,[18]. While the former could detect the enzyme activity at 3.05 µmol ammonia/ml/hourat pH 7.0 and temperature 30°C after 178 hours of fermentation, the latter could detect 3.8U/ml of L- asparaginase activity at 96 hours.*Aspergillus* sp. in submerged fermentation produced L-asparaginase up to 3.8 U/ml at 96 hours of incubation period [18]. *A. terreus* produced maximum L-asparaginase activity at 48 hours in liquid medium [9]& the optimal period for enzyme production was 96 hours in solid medium [19]. Soniyamby*et al*., [18] by using orange peel as a substrate could record maximum L-asparaginase activity of 70.67±1.14 U/g while ground nut shell based media produced only 10.27±0.40 U/g. Our highest enzyme titer (64 U/ml) reported in the present work by growing *A. terreus* on okara acid hydrolysate fortified with L-asparagine was matching with the aforesaid finding. The production increased several fold from the unsupplemented control.

Bhaskar and Renganathan[20] reported production L-asparaginase by *A. terreus* using ground nut oil cake as substrate. The impact of nitrogen source supplementation was examined in shake flask cultures and L- proline was detected as best nitrogen source for maximum enzyme activity. In our experimentation we got matching stimulatory effect on enzyme titers through L- asparagine fortification. Carob pod when usedas substrate[21]a maximum enzyme activity of 6.05 IUthrough solid state fermentation by *A. terreus*. Balasubramanian*et al*., [22] detectedmaximumL-asparaginase production by *A. terreus* in SmF at96 hours with activity of 9.3 IU/ml using modified CzapekDox medium.

Although L-asparagine is known asexcellent nitrogensource for the growth of eukaryotic micro algae, L-asparaginase from algal cultures was reported only fairly recently. Marine *Chlamydomonas sp*. could use L-asparagine for autotrophic growth. Nitrogen deprivation stimulated enzyme synthesis andthe enzyme was repressed in the presence of combined nitrogen. These are characteristics common to extra cellular enzymes or enzymesfound in

periplasmic space wherein the ionic strength, pH and substrate concentration are poorly controlled[23].In our experiments too presence of easily assimilable nitrogen source like ammonium sulphate was found to be in effective as a nitrogen source for getting higher enzyme activities. Quite contrary to this, ammoniumions and fumarate resulted in maximum enzyme production in algal systems [24].

External L-asparaginaseactivity of less than 1 nmol/min per mg of cells would be verydifficult to detect using the Nesslers method. Rajesh *et al.,*[25] indicated the positive role of glutamine & L-asparagine for enzyme production by A. terreus MTCC 1782. Our findings corroborate with the inducing effect of L-asparagine reported for enzyme production by Rajesh *et al.,*[25].Sukumaran*et al.*,[26] used *Serratiamarcescens* to report L-asparaginase synthesis by fortification with L-asparticacid, L-glutamine, L-glutamic acid and L-asparagine to the level of20, 20.7, 19.8 and 25 IU/ 10ml respectively.Suresh and Raju[27] reported that sesame oil cake(SOC) and black gram husk (BH)mixture was used in supplementation with maltose (1.5%), ammonium sulphate (2.0%), and magnesium sulphate (0.1%w/v) and reported maximum L-asparaginase production of 163.34 U/gds through SSF using *A. terreus* MTCC 1782.

CONCLUSIONS

Our studies on L-asparaginase production by *A. terreus*conclusively indicates the stimulatory impact of induced enzyme L-asparaginase by *A. terreus* MTCC 1782. Though okara fractions with supplementation by the corresponding substrate L-asparagine managed to yield appreciable levels of enzyme activity by acid hydrolysate of okara appeared to be best of the three fractions tested. L-asparagine supplementation by 1.5% w/v appeared to be sufficient to generate better stimulatory impact in the fungal culture.

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