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ORIGINAL ARTICLE **OPEN ACCESS**

Optimization of Essential Growth Parameters and production of L-asparaginase from Sugarcane rhizosphere soil derived *Aspergillus niger* SA.

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ABSTRACT
L-Asparaginases sourced from molds are being examined for cancer therapy and mitigation of acrylamide formation in food. Filamentous fungi were isolated from rhizosphere soil samples collected from five different farming land of Sivaganga district of Tamil Nadu by conventional method. L-asparaginase production was investigated by rapid plate assay and strain is characterized by 18s rDNA sequencing. Various parameters like pH, temperature, substrate, carbon and nitrogen were evaluated for maximal enzyme production. Out of 17 fungal isolates three genera Aspergillus sp, Fusarium sp and Mucor sp were showed positive enzymatic action. Among them KSFS-3 showed dominant L-asparaginase activity and phylogenetically identified as Aspergillus niger. The optimal growth conditions required for the L-asparaginase production by A. nigerSA were optimized. Maximum enzyme activity recorded at pH 6.0 and less significant at alkaline pH, optimal temperatureis30°C and insignificant at 40°C.Among different carbon glucose found to be ideal source and ammonium sulphate as best nitrogen source. 1.5% of L-asparagine and 1-1.5g/100 mL of rice husk were noted as best inducer. Testing of L-asparagine, Orange peel, Pea nut peel, Cotton seeds, Rice husk and Banana Peel were calculated as 188≥160≥86≥178≥190≥182IU mL$^{-1.}$ Rice husk as natural L-asparagine source gave maximum enzyme activity on 8thday of the incubation and the activity is greater (190IU mL^{-1}) than the synthetic inducer (188IU mL^{-1}).The fungus significantly utilized rice husk than synthetic L-asparagine. The findings would be a sustainable approach to converting organic waste into valuable raw material for the industrial production of the enzyme L-asparaginase.
Keywords: *Rhizosphere soil; Aspergillus niger SA; L-asparaginase; Growth factors; Optimization*

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INTRODUCTION

In the earlier studies, the primary focus is on isolating colonies that generate L-Asparaginase but here we want to identify populations that express L-Asparaginase that is glutaminase-free as well as urease-free enzyme[1].L-Asparaginase has been currently manufactured commercially mostly from prokaryotic microorganisms such as *E. coli* and *Erwiniachrysanthemi.* However, because glutaminase and urease activity coexist, further separation processes are needed to eliminate these two enzymes while maintaining medication effectiveness. L-asparaginase derived from eukaryotic entities such as fungus has indeed been proposed as a less toxic alternative source[2].Most of the cancer cells are dependent on an exogenous source of this amino acid for survival. However, normal cells are able to synthesize L-asparagine and thus are less affected by its rapid depletion due to treatment with L-asparaginase hence used in treatment of leukemia and lymphoma [3–4]. The use of L-asparaginase in anticancer therapy is based on its ability to cleave L-asparagine, an amino acid essential for lymphoblasts' growth, to ammonia and L-aspartic acid in serum and cerebrospinal fluid, since lymphoblasts are unable to produce endogenous L-asparagine which leads to death of these cells [5].L-asparaginase is predominantly found in many organisms, bacteria, plants, fungi, actinomycetes, and serum of certain rodents. Compared to animal and plant enzymes, microbial sources are preferred as they can be produced economically using cheap substrates, can be easily optimized, genetically modified to produce high yield and further they can be extracted and purified. Extended advanced research focuses on eukaryotic fungal L-asparaginase since they have reduced side effects compared to bacterial L-asparaginases [6].Molds of the genera Fusarium, Penicillium and Aspergillus, have been reported to secrete L-asparaginase having anti-lymphoma activity [7]. L-asparaginase produced by *Serratia marcescens*, *Escherichia coli* and *Erwinia chrysanthem* is able to

hydrolase 5% of L-glutamine when compared with L-asparaginase hydrolysis. The largest part of microbial L-asparagine presents cross glutaminase activity hydrolysis L-glutamine and L-asparagine due to the structural similarity from both amino acids [8-9].The industrial utility of fungi has been well known since antiquity. Industrial fungal enzymes are high-molecular-weight proteins that are catalysts. Currently, more than half of the industrial enzymes are of fungal origin and are being used successfully in diverse industrial processes and products [10].The most widely used enzymes of occupational importance are derived from the genus Aspergillus and include α-amylase, xylanase, and cellulase. According to Baur[11], more than 186 commercial enzymes were produced in the European Union in 2001, and many of these were produced by recombinant technology or had been genetically engineered.To date, close to 200 fungal enzymes have been purified from fungal cultures and the biochemical and catalytic properties characterized[12]

MATERIAL AND METHODS

Collection and processing of the soil samples

The rhizosphere soil samples were collected from five different farming land of Sivaganga district of Tamil Nadu. Collected soil samples were air dried, ground and passed through a 2mm pore size sieve.

Isolation and identification of fungi

Ten grams of each sample was suspended in sterile water (1000ml) and the suspension was serially diluted. One ml of sample suspension were taken from dilution, 10^{-3} and pipetted out into sterile petriplates and Potato Dextrose agar medium was dispensed over in the petriplates and gently rotated to enhance even distribution. The plates were then allowed to solidify and incubated at 28°C temperature for 3 days.The isolated soil fungal colonies were identified byLacto phenol cotton blue staining and the preparation was observed under the light microscope.

Screening of fungi for L-Asparaginase production[13]

The fungal isolates were subjected to rapid screening for L-asparaginase production by rapid-plate assay. A loop full of mycelial fragments of each predominant fungal colonies from well incubated PDA plates was inoculated on freshly prepared and sterilized CzapekDox agar medium (100 mL) supplemented with 1% L-asparagine and0.009% (v/v) phenol red as indicator. The inoculated plates in triplicates were incubated for 96 h at 28 ± 2°C. The zone of L-asparaginase production and colony with pink zone was considered as positive L-asparaginase producers. Isolates with maximum zone diameter were selected for further study.

Molecular characterization of test fungal isolate

Active enzyme producing fungal isolate genomic DNA was isolated and used for Molecular characterization of the fungal isolate was performed. The 18s rRNA gene sequence of the fungal isolate carried out by Sangers method and the sequence is Blasted at NCBI. Further it was submitted to Gen Bank (National Centre for Biotechnological Information, USA) for accession number.

Optimization of L-Asparaginase production [14]

The fermentation medium was adjusted with differentpH (5.0, 5.5, 6.0, 6.5, and 7.0), temperature,25ºC, 30ºC, 35ºC, 40ºC, and 45 ºC carbon source (glucose, maltose, sucrose, starch, and fructose), nitrogen sources(soybean meal, yeast extract, urea, ammonium nitrate, and ammonium sulphate) , synthetic L-Asparagine (0.5, 1.0, 1.5, 2.0, and 2.5) , and natural (organic) L- Asparagine (orange peel, peanut peel, cotton seeds, rice husk, and banana peel) were used for the determining the influence on enzyme production by *A. niger* SA. The culture inoculated flasks (in triplicates) were incubated in an orbital shaker with 120 rpm for 8 days. After incubation, L-Asparaginase activity was calculated as per Nessler's Reagent (ammonia formation) based method.

Determination of L-Asparaginase Activity

This L-Asparaginase activity of crude extra-cellular enzyme derived from filamentous test fungal strain using Nessler's Reagent to quantify the ammonia formation by measuring the reading at 340 nm using UV-visible spectrophotometer (UV-1900i- Shimadzu's) analysis. Furthermore, the standard Lowry's method was performed to determine the protein content of the supernatant and BSA was used as standard.

RESULTS AND DISCUSSION

A total of 17 fungal isolates were obtained from the rhizosphere soil samples collected from different farming land of Sivagangai district of Tamil Nadu and designated as KSFS-1 to 4, PBFS- 1 to 3; KPFS-1 to 4;KGAS-1 to 3;KCFS-1 to 3 respectively from Sugarcane field, Brinjal cultivation field, Paddy field, Garden Area and Corn field (table 1).Isolates were designates based on site of sample and number of strains. *Aspergillus spp* is most frequently isolated followed by*Fusariumsp and Mucor sp.*

Table 1. Sources of Fungi isolated

S.No	Soil Type	Place	No. of Isolates
1	Sugarcane field	Karuviappatti	4 (KSFS-1 to 4)
2	Brinjal cultivation field	Periyakottai	3 (PBFS- 1 to 3)
3	Paddy field	Kothari	4 (KPFS-1 to 4)
4	Garden Area	Karaikudi	3 (KGAS-1 to 3)
5	Corn field	Kalloor	3 (KCFS-1 to 3)
	Total		17

The rapid plate assay for L-Asparaginase activity reveals that five strains designated as KSFS-3, KPFS-2, KGAS-3, KGAS-1, PBFS-4 were identified with L-Asparaginase producer and maximum apargine hydrolysis was noted on KSFS-3(table 2). The 18S rRNA sequencing analysis showed 98% and 96% similarity with *A. niger*ANN4 and *A. niger*CBS 554.65 and KSFS-3 strain phylogenetic tree analysis (Figure1) identified as *Aspergillus niger (*genebank accession MG897307 *)*). *A. nige r*mediated asparaginase production finding was similar to Doria and Santhosh Kumar[15]

Table 2. Screening of L-Asparaginase producing Isolates

Positive Fungal Isolate Number	Name of the fungi	Zone diameter (cm)
KSFS-3	*Aspergillus sp.*	5.8
KPFS-2	*Aspergillussp*	3.4
KGAS-3	*Fusarium sp.*	3.2
KGAS-1	*Aspergillussp*	1.7
PBFS-4	*Mucor sp.*	1.4

The media optimization is an important aspect to be considered in the development of fermentation considered in the development of fermentation technology.Hence, in this study, the essential growth conditions required for *A. niger* were studied with various growth parameters to obtain maximum L-Asparaginase synthesis by standardizing pH, temperature, carbon, nitrogen and rice husk substrate.In our study the data revealed that the pH of 6.0 was found as suitable for maximum production 178 IU mL^{-1}L-asparaginaseby*A.niger*. (Fig. 2a) and the maximum production of L-asparaginase by *A. niger* was found to increase with temperature upto 30°C , as 176 IU mL^{-1} (Fig. 2b). Increasing the temperature of a growth condition can enhance the metabolic reaction, while reducing the temperature slows down the metabolic activity and enzyme secretion. However, the exceptionally high temperatures, on the other hand, can denature the enzyme and lose its activity[16]. The optimal temperature for L-Asparaginase secretion by other species of *Aspergillus* was reported as for *A. oryzae* CCT 3940: 40 and 50 °C, *A. oryzae* LBA 01: 40 °C at these optimal temperatures, the L-Asparaginase can be stable (100%) for up to 60 min[17-18].The physical factors pH and temperature plays essential role in the growth and metabolic activities of microbes [19]

The enzyme secretion and their activity may directly or indirectly relate to the availability of suitable carbon, nitrogen, and other essential factors during the fermentation process. Figure 3a represent effect of carbon and Figure3bcorresponding to impact of nitrogen on enzyme activity produced by *A.niger*. Data reveal that the L-Asparaginase activity of *A. niger* SA was found effective at 1% of glucose (194 IU mL^{-1}) and less significant at sucrose (86IU mL^{-1}). It was statistically significant at $P<0.03$ compared to other carbon sources. Among the tested nitrogen sources (fig 3b) ammonium sulphate is found to be best (188IU mL^{-1}) and insignificant at urea (72IU mL^{-1}). As glucose is a simple carbon source, the fungus prefer choosing them as the sole carbon source for active metabolisms. According to this statement, *A. niger* isolated from soil sample effectively utilized the glucose (1%) as sole carbon source and produced more L-asparaginase in short duration of submerged fermentation process [20-21].Nitrogen is a significant factor that regulates the production process. Baskar and Renganathan reported that 1.2% of L-asparagine induced extend L-asparaginase activity in *A. niger* as up to 30.35 IU mL^{-1} on 4 days of fermentation[22]. The Baskar, and Renganathan[23] reported that the *Aspergillus terreus* preferably utilize the ammonium chloride as a suitable nitrogen source and reported significant L-asparaginase activity as up to 26.47 IU mL^{-1}.

The role of inducer such as aspargine at different concentration and different substrate was evaluated and the data is given in figure 4. Figure 4a shows L-asparagine induced L-asparaginase activity effectively at 1.5% and estimated activity was 152 IU mL^{-1}(Fig 4a).likewise the productivity of enzyme under

Orange peel, Pea nut peel ,Cotton seeds, Rice husk and Banana Peel were calculated as 160≥86≥178≥190≥182IU mL^{-1}.The *A. niger* SA secreted maximum quantity of L-asparaginase using 1% of rice husk (190 IU mL^{-1}), and it was statistically significant at P<0.05 and it followed by banana peel (182 IU mL^{-1}) . Organic wastes might be a valuable source for L-asparaginase production as a sustainable approach. Using natural L-asparagine enriched organic sources can minimize the cost of production. These organic wastes also provide most essential minerals and sugars that may chelate the enzymatic activity and metabolic process [24]. A report states that the *Fusarium culmorum* enumerated from tropical soil effectively utilizes the combined (1:1 ratio w/w) form of soybean meal and wheat bran and produced L-asparaginase up to 0.5 fold higher than earlier [25]

The fermentation of rice husk and optimal incubation days for *A.niger* fungal strains is given in table 3 The optimal rice husk concentration was 1.5 and temperature 30° C and maximum productivity was noted on 8th day of incubation (196IU mL^{-1}) Statistically significance at *P*< 0.03.In general, the optimal incubation days for *Aspergillus* species were reported as 4 to 5 days of incubation, and *Fusarium* sp. required 5 to 7 days of incubation to produce maximum quantity of L-asparaginase [26].

Table 3. L- Asparaginase production using Rice husk by *Aspergillus niger*

Day	OD	OD	OD	Enzyme activity (u /ml)
2	0.423	0.421	0.424	94
4	0.387	0.386	0.388	122
6	0.708	0.709	0.710	162
8	0.916	0.918	0.916	196
10	0.691	0.687	0.689	178

Author contribution statement

Anandaraj B conceived and planned the experiments. Sudha N has performed the experiment and wrote the paper

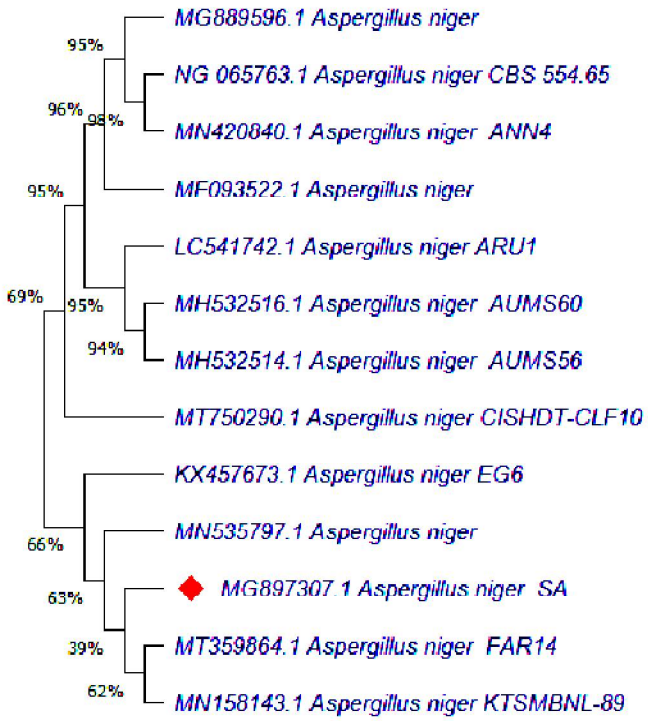

Figure 1.Phylogenetic tree analysis of *Aspergillusniger* SA

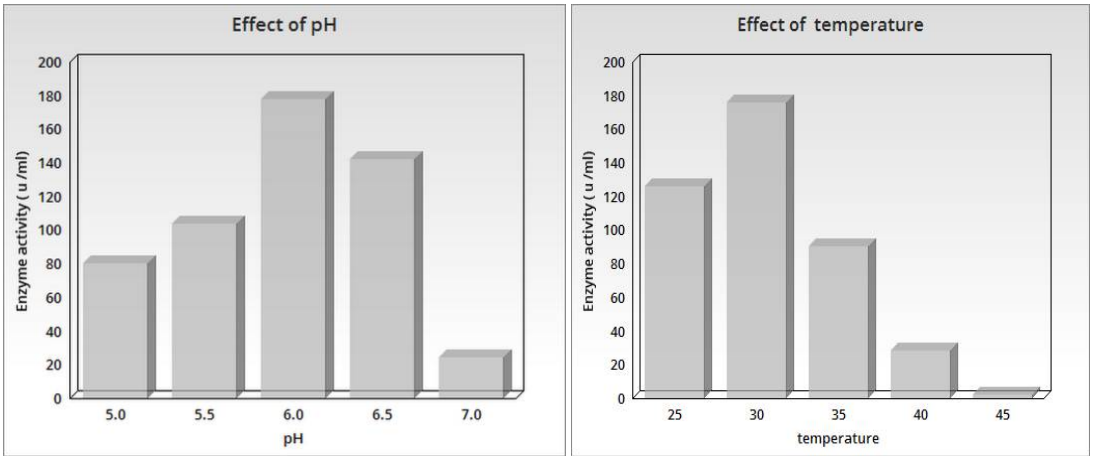

Figure 2(a). Effect of pH on enzyme production (b): Effect of Temperature on enzyme production

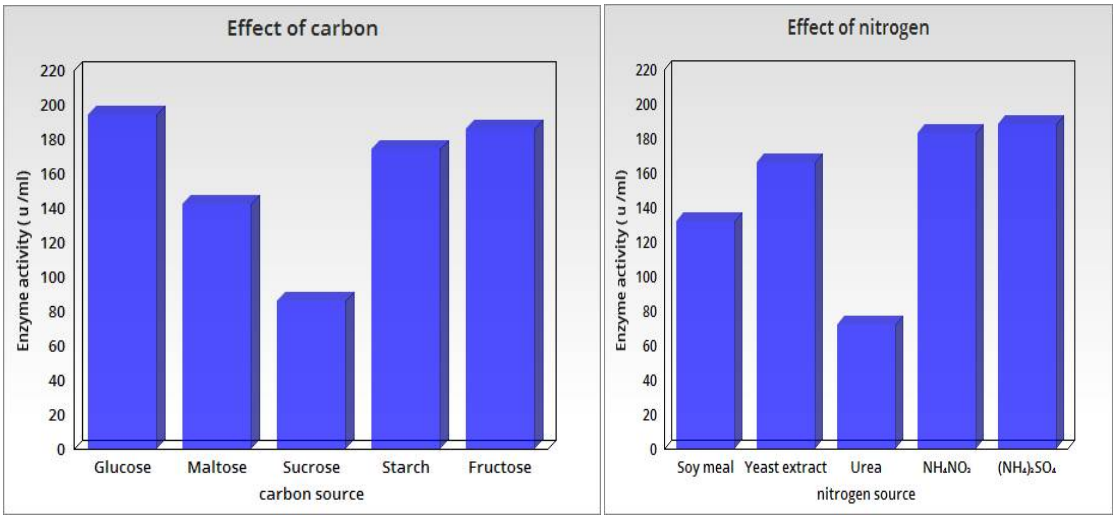

Figure 3 (a). Effect ofcarbon on enzyme production (b): Effect of nitrogen on enzyme production

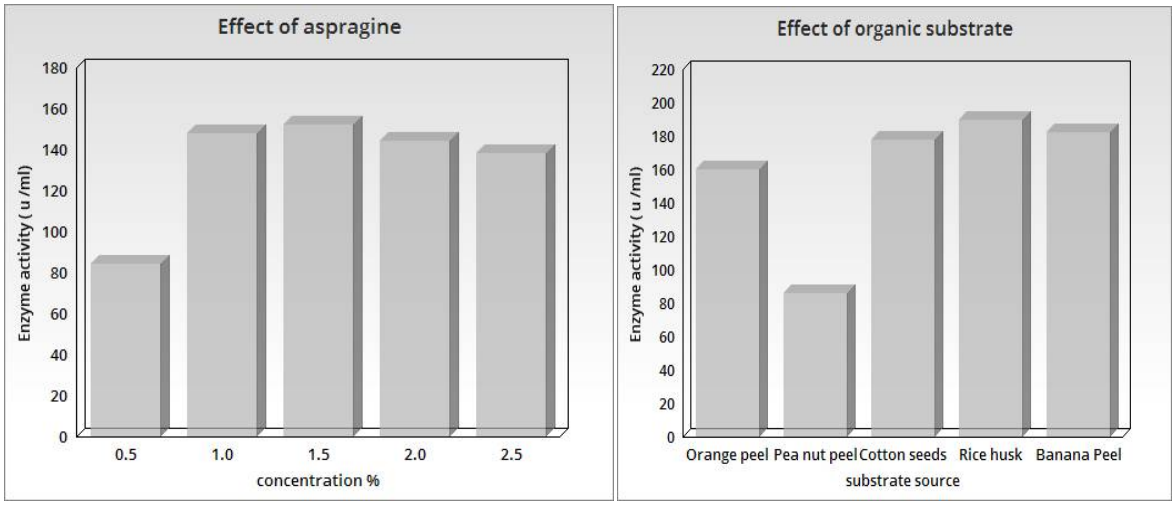

Figure 4. Effect of aspargine on enzyme production (b): Effect of substrate on enzyme production

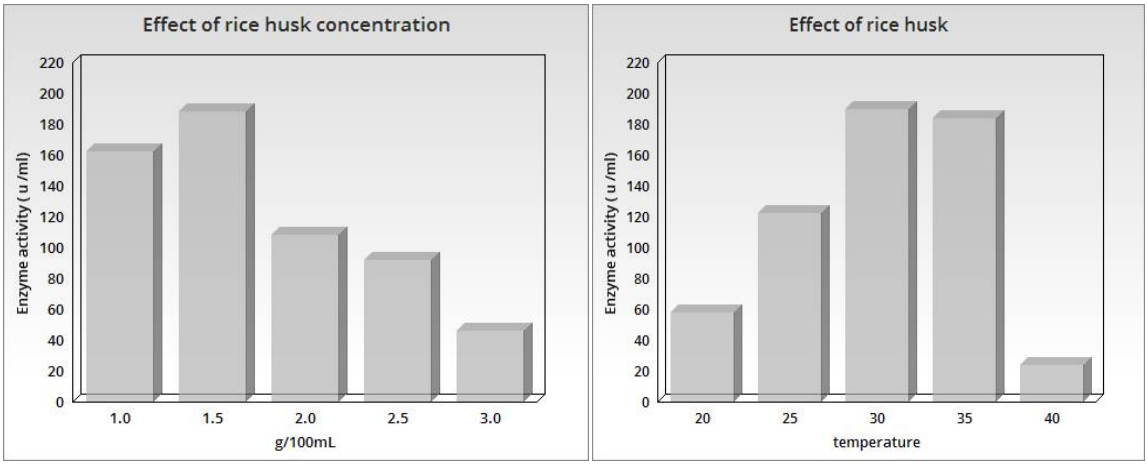

Figure 5(a). Effect of rice husk enzyme production (b) Effect of Temperature on husk fermentation

CONCLUSION

In this study, A fungal isolate *A. niger* from the rhizosphere soil sample of sugarcane filed was isolated and optimal conditions for maximum L-asparaginase activity is evaluated. The data confirms that the rice husk is effectively utilized by *A. niger*as cheapest substrate and gave maximum enzyme production.

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