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Effect of Rice Bran Husk Substrate on the Production of Asparginase by *Streptomyces* sp. Derived from Termite Mound

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ABSTRACT

An important criterion for the potency assessment is that the source of cheap substrate for L-asparaginase production is much needed. Isolation of novel actinobacteria from a termite mound was performed by conventional pretreatment isolation and enzyme screening by plate assay method to compare the effect of carbon and substrate over L-asparaginase produced by submerged fermentation state with minimal medium. The enzyme production was studied by the Nesslerization method. Following heat treatment, the results indicated that the isolate decreased the eubacterial colony-forming unit. Out of the ten pre-heated samples, one soil tested positive for actinobacteria and nine tested negative. The isolate detected as asparaginase-positive was annotated as TS5 morphologically showed aerial and substrate mycelia formation and was identified as *Streptomyces* sp. The production of L-asparaginase among four different carbons revealed that lactose was an inducer. Further the cheap substrate such as rice bran is evaluated under substrate state fermentation and a good result on enzyme production is noted on both defatted and raw rice bran. The highest peak activity of the enzyme production (183 ± 9.11 U/mL) was attained at 10% raw rice bran with 66.3 ± 0.26 mg/mL protein. These results provide evidence that agro-waste substrate within the investigated parameters increased the LA generation. These findings are expected to promote the industrial utilization of important actinomycetes from termite mound.

Key words: agro-waste; substrate fermentation; rice bran; asparaginase; cancer

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for over half of the world's population. The rice bran that is produced during the rice milling process contains a sizable quantity of bioactive chemicals (Tan et al., 2023). Deoiled rice bran (DRB) possesses more stability, water, fat, and foaming resources than full-fat rice bran (Sudha et al., 2011). Globally, 1.9 billion tons of bran, husk, and other fibrous material remnants are produced, and this number is expected to rise to 2.2 billion tons by 2025. In the last few years, agricultural residues have become an important cause of environmental damage, threatening ecological viability and human health (Liu et al., 2015). Large volumes, low quality, low cost, and high risk are the four attributes of waste from agricultural pollutants (Rangabhashiyam et al., 2013). Waste disposal is still a relatively new topic. Accordingly, application of biotechnology has enabled the efficient utilization of these residues and promoted the production of diverse goods with additional benefits. L-asparaginase has reached economic usage in the food and pharmaceutical industries due to its ability to catalyze the conversion of L-asparagine to L-aspartic acid and ammonia (Juluri et al., 2022). Researchers are interested in how microorganisms can produce this enzyme industrially using less costly substrates (Beckett and Gervais, 2019). Many variables have been investigated for their potential to commercially produce L-asparginase in a cost-effective and high-yielding manner (Faret et al., 2019). Another promising approach for manufacturing asparginase is solid-state fermentation, which uses agroindustrial wastes as substrate or support (Lopes et al., 2015). Studies have been conducted to optimize the culture conditions for the generation of L-asparginase, both continuously and in batches (Narta et al., 2007). The asparaginase yield is highest in a medium that contains organic nitrogen, and optimal cell development necessitates a high production cost (Van den Berg, 2011). L-glutamic acid, L-glutamine, and monosodium glutamate also reduce or inhibit enzyme activity (Vidhya et al., 2010). To develop strategies for turning organic matter into enzymes and other beneficial chemicals, significant research has been conducted (Nguyen et al., 2010). This study investigated the usage of rice bran to produce L-asparaginase.

MATERIALS AND METHODS

Isolation of Actinomycetes

Samples of termite mounds were gathered from ten distinct locations in Tiruchirappalli, Tamilnadu, India. After being separated from the termite mound

(Scheme 1), the soil was pretreated for 15 min at 55 °C. The sample was subjected to serial dilution and 1 mL of 10^7 dilutions was plated on screening actinomycetes agar medium and incubated for 24 h under 25 °C for 15 days. Suspected colonies were selected and the morphology of spore and type of mycelium were examined (Pandey et al., 2011).

Sch. 1. Termite mound sample.

Screening of L-asparginase by Plate Assay

A qualitative plate assay was used to determine isolates that could produce extracellular enzymes. Nutrient agar amended with 0.05% L-asparagine as an inducer and phenol red as an indicator. Suspected colonies streaked on agar plates and incubated for 7 days. The pink region around the colony, which results from ammonia being released, indicates the development of L-asparaginase.

Effect of Carbon on L-asparginase

A semi-qualitative assay was employed to identify bacteria with L-asparaginase activity. The isolates were streaked to the mineral media supplemented with L-asparagine. The composition of M-9 medium is (g/L): sodium mono phosphate, 6; potassium dihydro phosphate, 3; NaCl 0.5; L-asparagine, 5; $MgSO_4$.0.005 g, $CaCl_2$ 0.001 g. different carbon sources, such as 2% of Glucose, lactose, sucrose and starch were incorporated. Medium with phenol red as pH indicator and incubated at 37 °C for 24 h.

Submerged Fermentation (SF) Method

Yeast extract broth (production media, pH 7.0) comprising of NaCl 0.5 g, K_2HPO_4, 0.1 g, yeast extract, 0.5 g; water 100 mL was prepared in a 250 ml Erlenmeyer flask, and enriched with 0.5, 1, 2.5 and 5 g of lactose. all the flask were autoclaved at 121 °C/15 lbs/15 min. 5 mL of the active strains 72 old was introduced into production medium and kept incubation under 150 rpm/37 °C for 72 h. A cell free culture filtrate was taken by centrifugation at 10,000 rpm for 20 min (Rabelo et al., 2025).

Effect of Rice Bran on Enzyme Production

100 mL of production medium Yeast extract broth containing 1, 5, 10% of defatted husk and raw husk were introduced to replace the lactose and sterilized. Production medium inoculated with active strain and was incubated at 30 °C, 150 rpm. The seed culture was transferred to the fermentation medium and was cultivated for 3 days. A medium without husk was used as a control to evaluate the effect of the substrate. Samples were withdrawn on the 3rd day for isolation of the protein, purified with sepharose column and subjected to the L-asparaginase assay. The total protein was calculated using the Lowry et al. technique with standard bovine serum albumin (Kumar et al., 2022).

L-asparaginase Activity

The reaction mixture contained 0.5 mL of the samples and 0.5 mL of 0.04 mL L-asparagine solution in 0.5 mL of 0.05 mM Tris HCL buffer pH 8, which were mixed to begin the reaction. The mixture was incubated at 37 °C for 30 min. By adding 0.5 mL of 1.5 M TCA (trichloroacetic acid), the process was terminated. Precipitated proteins were removed through centrifugation and the ammonia that was released was calculated spectrometerically at a wavelength of 425 nm using Nessler's reagent. Based on the ammonia liberation computed using a standard curve of ammonium sulphate, enzyme activity was ascertained (Shakambari et al., 2016).

RESULT AND DISCUSSION

Isolation of L-asparaginase Producer

Out of 10 processed Termite soil (TS1-TS10) one sample (TS5) showed positive L-asparginase producing bacteria isolated from termite mound soil identified as *Streptomyces* sp. and CFU were 08×10^{-6}. Out of 10 samples, the formation of CFU of actinomycetes among 9 samples found to be absent but showed bacterial colonies at least number due to pre-heat treatment (Table 1). Though the heat-treated samples showed low CFU, most failed to develop actinobacterial colonies unlike sample TS5. A previous study on termite's mound identified several bacteria in the soil of mounds (Manjula et al., 2014). Both closed and open termite mound soil have been reported to have larger bacterial populations compared with regular soil (Kumar et al., (2018)). Novel antibacterial-producing bacteria were previously isolated and identified as *Micromonospora* sp by heat treatment (Zhang et al. (2024) Baskaran et al. (2011)) following heating at 60 °C.

The L-asparaginase positive among isolates obtained using the phenol red qualitative method. The agar plate turns from yellow to pink on the third day of incubation. The results indicated that the TS3 strain had the brightest pink hue among the five cultures examined. The isolated *Streptomyces* were found to

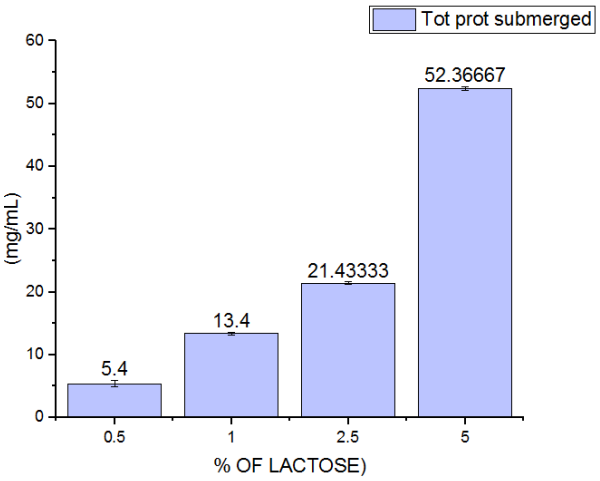
be asparginase-producers based as indicated by the its pink coloration on agar. The gram-positive filamentous bacteria TS3 produced a spiral chain of spores on the substrate mycelium. These isolates generate filamentous, heavily branched mycelium that resembles a net.

Table 1. Colony morphology and preliminary screening.

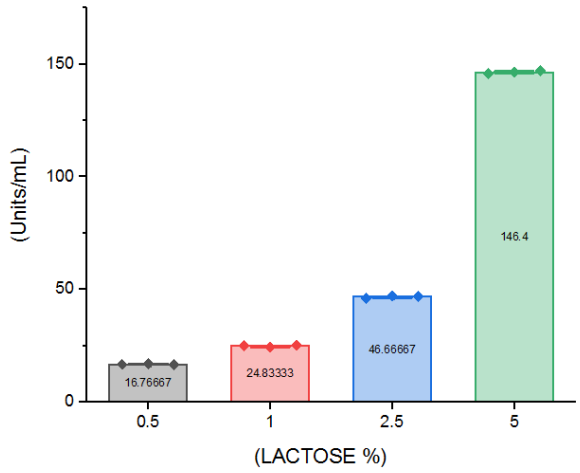
Strain code	Heterotrophic population CFU $\times 10^{-6}$	Actinomycetes CFU and colony morphology
TS1	6	-
TS2	8	-
TS3	11	-
TS4	10	-
TS5	12	8 Ash powder, Gram positive filamentous
TS6	10	-
TS7	11	-
TS8	7	-
TS9	8	-
TS10	6	-

The formation of a distinctive aerial mycelium on top of the substrate growth was leveraged to identify the *Streptomyces* sp. It was found that the aerial hyphae exhibited diverse lengths. Similar studies by experts found that *Streptomyces* members accounted for over 90% of the actinomycetes isolated from rhizospheric soil samples (Dastager et al., 2006). The simplest plating test is a quick, simple, and indirect method of identifying the synthesis of L-asparaginase from actinomycetes by looking at plates immediately. A previous study reported that *Streptomyces* isolates produced the enzymes gelatinase, amylase, and protease; however, no research has explored the capacity of actinomycetes to produce L-asparaginase (Gautham et al., 2011).

The maximum production of L-asparaginase was determined by experimenting with several carbon sources and how they combined with a L-asparagine inducer. Table 2 shows the effect of carbon on the amount of L-asparginase produced upon the addition of 2% glucose, lactose, sucrose, and starch. Steptomyces tested positively for L-asparginase only when lactose was present. The production of the lactose-dependent L-asparaginase enzyme at varying doses was discovered to be correlated with the amount of protein. The total protein content increased when the lactose concentration was higher and recorded as 5.6, 13.6, 21.2, 52.5 mg/mL (Table 3). Maximum total protein was recorded at 5% lactose and its asparaginase activity was recorded as 16.7 ± 0.20, 24.8 ± 0.4, 46.66 ± 0.4 and 146.4 ± 0.6 Units (Table 4). The concentration of purified protein increased as the lactose increased (Figure 1a). Strains grown on high lactose had more enzyme activity than low concentration (Figure 1b), indicating that the lactose act as inducer. Estefanía Arévalo et al. (2019) studied the production of asparaginase by under liquid culture with lactose as substrate has been reported to be higher enzyme activity in *Streptomyces* sp., Release of fermentable sugars from rice bran (Siepmann et al., 2018) followed by cellulolytic activity of bacteria leads to enhanced enzyme production.



(a)



(b)

Fig. 1. Effect of submerged fermentation on (a) total protein (b) Asparaginase activity.

Table 2. Effect of carbon on L-asparginase.

Carbon source	L-asparginase production
Glucose	-
Lactose	+
Sucrose	-
Starch	-

Table 3. Total protein estimated from submerged state.

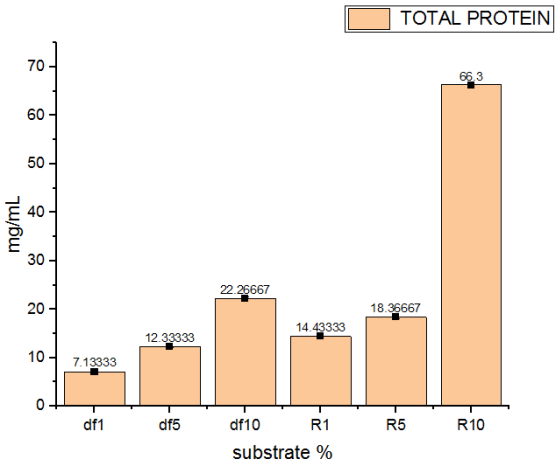
% of Lactose	T1	T2	T3	Mean total protein mg/mL	SD
0.5	4.8	5.8	5.6	5.4	0.52915026
1	13.2	13.4	13.6	13.4	0.2
2.5	21.6	21.5	21.2	21.43333333	0.2081666
10	52	52.6	52.5	52.36666667	0.32145503

Table 4. L-asparginase enzyme assay on submerged state with lactose.

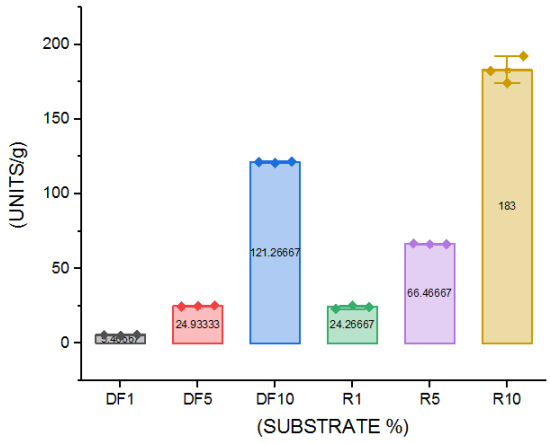
Trails	Con of lactose in %			
	0.5	1	2.5	5
T1	17	24.3	47	146.4
T2	16.7	25	46.2	145.8
T3	16.6	25.2	46.8	147
Mean	16.76667	24.83333	46.66666667	146.4
SD	0.208167	0.472582	0.4163332	0.6
P	0.001183	0.000211	2.90433×10^{-6}	4.59518×10^{-6}

Effect of Substrate on Enzyme Production

In recent years, cheap substrate-based fermentation techniques have emerged as a valid alternative to SF for the production of extracellular enzymes. Extracellular enzyme assay using rice bran demonstrated that the enzyme released from inside the cell was significantly influenced by the concentration of carbon. The effect of percentage of rice bran on protein production (Figure 2a) was tested at 1, 5, and 10% rice bran and compared with enzyme activity (Figure 2a). The total extracellular protein concentration was 6 ≥ 12 ≥ 22 mg/mL in defatted rice bran and 14 ≥ 18 ≥ 66 mg/L (Table 5). Out of the two tested concentration rice bran extract 10% fatted rice bran showed a positive result on maximum L-asparginase activity. The defatted rice bran had 5, 25 and 120 U while fatted rice bran had a maximum enzyme activity at 25, 66 and 180 U compared with that of defatted. The quantity of enzyme that catalyzed the production of one µmol of ammonia per minute at 37 °C is known as one unit of asparaginase (Shahana Kabeer et al., 2023). The unit of enzyme assay was 120 U in 10% culture filtrate and 5 U at 1% (Table 6). Notably, the enzyme production increased with the concentration of rice bran. The *Streptomyces* sp. grown on raw rice bran had maximum asparaginase activity at 10% recorded as 183 ± 9.11 U. The defatted rice bran sample had a maximum of 121.26 ± 0.611 U at 10% husk. At 1% DFH, the enzyme activity was 5.46 ± 0.41, whereas the raw hush (R1) had 24.266 ± 1.205 U. The activity of enzyme at 5% 24.93 ± 0.30 and 66.46 ± 0.30 units recorded among the DFH and raw substrate(R5). The one-way ANOVA test for the raw husk revealed a p value of 7.1 and a p value of 1.7 for defatted husk, indicating non-significant differences. The results suggested that the changes in concentration altered the extracellular production. Maximum enzyme activity of 136 IU/ml by *Streptomyces* sp. under submerged fermentation in the study by Saleena et al. (2023). Chergui et al. (2023) showed that wheat bran could enhance the production of asparaginase by *Streptomyces Paulus*. This is consistent with the study by Deokar et al. (2010) who observed the impact of this substrate quantity. It should be noted that the culture conditions heavily influenced our strain's production of LA, and the variables chosen for this investigation have a lot of scope to maximize the enzyme yield.



(a)



(b)

Fig. 2. Effect of rice husk substrate fermentation on (**a**) total protein (**b**) Asparaginase activity.

Table 5. Effect of substrate fermentation on total protein.

% of Husk	T1	T2	T3	Total protein (mg/mL)
DF1	6.8	7	7.6	7.13±0.41
DF5	12	12.4	12.6	12.33±0.30
DF10	22	22.3	22.5	22.26±0.25
RAW1	14	14.7	14.6	14.43±0.37
RAW5	18	18.3	18.8	18.36±0.40
RAW10	66	66.4	66.5	66.3±0.26

Table 6. Effect of substrate fermentation on asparginase activity.

Repeats	Defatted husk percentage (DFH)			Raw husk percentage		
	1	5	10	1	5	10
T1	5	25	120.6	25.4	66.4	174.2
T2	5.6	24.6	121.4	23	66.8	182.4
T3	5.8	25.2	121.8	24.4	66.2	192.4
Units/mL	5.46	24.93	121.26	24.266	66.46	183
SD	0.41	0.30	0.611	1.205	0.30	9.11
p	1.7			7.1		

CONCLUSIONS

Our study found that the actinomycete strain TS5, an asparaginase-producing *Streptomyces* sp. isolated from termite mound, can produce asparaginse using rice husk. Raw rice husk with oil showed maximum enzyme productivity compared with the defatted husk and lactose medium. However, the optimal temperature, pH, inducer concentration, and minerals need to be determined to improve the enzyme activity, yield and to explore the potential of actinomycetes to serve as a reservoir of asparaginase.

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