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Production of *Aspergillus niger* Biomass from Rice Bran

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Abstract: Growth of *Aspergillus niger* on rice bran medium in submerged fermentation was studied using standard methods for determinations of dry biomass, total crude protein and final pH values. It was observed that *A. niger* biomass cropped on rice bran medium supplemented with glucose, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$ and $ZnSO_4.7H_2O$ designated as Glucose supplemented rice bran (GRB) medium was highest at 2.03±0.02g/l while the unsupplemented rice bran medium (RBM) has the lowest value of 0.97±0.00g/l. The effect of various nitrogen sources on the final biomass yield was compared. Ammonium sulphate [$(NH_4)_2SO_4$] gave the highest biomass yield of 1.95±0.03g/l and sodium nitrate ($NaNO_3$) had the lowest yield of 0.97±0.02g/l. Glucose and nitrogen supplements increased biomass yield.

Key words: Rice bran, *Aspergillus niger*, biomass yield, crude protein

Introduction

Rice (*Oryza sativa* and *Oryza glaberrima*) is the most important tropical cereal and supplies a quarter of the entire caloric intake of human race. It is the staple food of more people than any other crop and 90% is grown and consumed in South and Southeast Asia (Catling, 1999). Rice is, after wheat, the most widely cultivated cereal in the world and is the most important food crop for almost half the world's population. Rice is commonly used as staple food for human consumption, animal feed, source of industrial starch, for production of glucose, fermented and unfermented alcohol and beer. The production statistics of the paddy rice has been on increase worldwide. It has been estimated that 588 million tones of paddy rice was produced worldwide in 1999 with Nigeria producing 3.397 million tones. And, for every one hundred kilograms of paddy rice 18-20 kg rice bran is generated (Schalbroeck, 2001). It is thus estimated that about 6.1-6.8 x 10^8 kilogram of rice bran was produced in Nigeria in 1999 .

Most of the developing countries of the world has been facing malnutrition problems due to deficiency of protein in human food and animal feed and the rapid growth of population. It is therefore, important to increase protein production by utilizing all the available ways and means. The increasing world demand for food and feed protein led to the search for non-conventional protein sources to supplement the conventional protein sources. A great deal of interest has been focused on the potential of converting agricultural wastes to microbial protein or single cell protein. The impetus behind single cell protein production lies partly in the need for more protein and partly in the commercial increase in the economic advantages gained by substitution of microbial protein for the conventional protein supplements used in livestock feeding (Khan *et al*., 1992). Substrate fermentation has been exploited for production of feed and food. Fungi have been used as sources of protein in specialty food for centuries and *Aspergillus* sp has been widely used foe single cell protein production (Ravinder *et al*., 2003). The ever increasing protein demands and high prices of soyabean meal have further necessitated the search for an alternative means of cheap and economic agroresidue such as rice bran for protein enrichment. Therefore, the use of rice bran as a fermentation medium was taken into consideration as an alternative raw material for the production of *Aspergillus niger* biomass in this investigation . The effect of various nitrogen sources on biomass production was also studied.

Materials and Methods

Sample collection and preparation: Rice bran was obtained from a small scale rice milling industry in Auchi, Edo State, Nigeria. Samples were collected in 4.5l plastic container previously cleaned and rinsed with 70% ethanol. The samples were ground into fine powder with a blender. Aqueous extracts of the ground rice bran were prepared as previously described by Akpan *et al.* (1996). In this rice bran suspension (2%w/v) was autoclaved at 121°C for 15 minutes. The autoclaved suspension was passed through muslin cloth to remove solids leaving rice bran broth designated rice bran medium (RBM) which was used in this investigation.

Microorganism and inoculum preparation: An isolate of *Aspergillus niger* from peeled orange (*Citrus sinensis*) fruit was used. Identification of isolate was carried out based on cultural characteristics and microscopic morphology with reference to the manuals of Rohde and Hartman (1980) and Barnett and Hunter (1972). The *A. niger* was isolated and maintained on Potato Dextrose Agar (PDA) medium. *A. niger* inoculum was prepared as previously described by Ikenebomeh and Chikwendu

(1997) from a 7 days old culture of the organism on PDA slant.

Fermentation process: Submerged fermentations were carried out in shake flasks with three trial media. One of these designated supplemented rice bran (SRB) medium had the following composition $(NH_4)_2SO_4$ (2.0g/l), KH_2PO_4 (1.0g/l), $MgSO_4.7H_2O$ (0.5g/l) and $ZnSO_4.7H_2O$(0.1g/l) made up to 1 litre with RBM. The second medium designated glucose supplemented rice bran (GRB) had all the composition of SRB medium and glucose (2.0g/l). The third had the rice bran medium (RBM) only.

In studying the effects of various nitrogen supplements and input on biomass cropped, $(NH_4)_2SO_4$ (2.0g/l) in the SRB medium was substituted with each of the following nitrogen sources: NH_4Cl, $NaNO_3$, NH_4NO_3 and KNO_3 in the concentration of 1.6, 2.6, 1.2 and 3.0g/l respectively for each to supply 0.42 gram of nitrogen per litre of medium.

In all the media, initial pH was adjusted to 3.5 using 1 N H_2SO_4 and/or 1N NaOH. Each medium (98.0ml) was transferred into 250ml Erlenmeyer flask and sterilized at 121°C for 15 minutes. Inoculum size of 2.0ml from homogenate of *A. niger* culture was aseptically transferred into each medium. Fermentation was at a temperature of 28±2°C on an orbital shaker at a speed of 120 rpm followed by determinations of biomass and other parameters at 24 hours interval for 8 days.

Analytical methods: Dry mycelia biomass was determined in the following manner: The growth medium was pasteurized at 65°C for 30 minutes in a water bath after each fermentation period. The mycelia were removed from the flasks by passing through a dried and preweighed Whatman No 1 filter paper and washed twice with 50ml of sterile distilled water. The biomass on filter paper was dried at 90-100°C, in a Genlab hot air oven YIA110 model from England, until a constant weight was obtained.

Protein content of dry biomass was determined by a modified biuret method as earlier described by Herbert *et al.* (1971) and Tietz (1986). The initial and final pH values of culture media were determined using pH meter 3305 supplied by Jenway England. Analyses of variance and Duncan's Multiple Range test, were carried out on data obtained from the different experimental results.

Results

The isolated fungus culture obtained from Orange (*Citrus sinensis*) fruit was related mainly to the generic nomenclature *Aspergillus* known as *Aspergillus niger*.

The rice bran medium was supplemented with minerals for the growth of *A. niger* in single cell protein production. Shake flask fermentation results with *A. niger* as inoculum in the SRB, GRB and RBM are shown in Fig. 1. The dry biomass increased steadily and was highest in the range day six and seven at 1.96±0.01g/l for SRB medium, 2.00±0.01g/l for GRB medium and 0.97±0.00g/l for RBM. GRB medium had its biomass peak on day seven (2.03±0.02g/l) and decreased to 1.96±0.02g/l on day eight. Biomass in SRB medium and RBM decreased on day eight with values of 1.73±0.01 and 0.79±0.01g/l respectively (Fig. 1). The crude protein content of biomass shown in Fig. 2 peaked on day six with values of 0.65±0.00g/l for SRB medium, 0.69±0.01g/l for GRB medium and 0.33±0.00g/l for RBM. Among all the nitrogen sources investigated, supplementation of the medium with ammonium sulphate [$(NH_4)_2SO_4$) gave the highest biomass yield of 1.95±0.03g/l followed by 1.83±0.04g/l when supplemented with ammonium nitrate (NH_4NO_3). But, the biomass yield was lower when media were supplemented with potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl) and control (with no nitrogen supplement).Values of biomass obtained are shown in Fig. 3 with 1.26+0.03g/l, 0.97+0.02g/l, 1.09+0.02g/l and 1.01+0.01g/l respectively. The protein content of dry biomass cropped in supplemented rice bran containing $(NH_4)_2SO_4$, KNO_3, $NaNO_3$, control (no nitrogen supplement), NH_4NO_3 and NH_4Cl in g/l were 0.67±0.01, 0.60±0.01, 0.54±0.01, 0.43±0.03, 0.68±0.02 and 0.62±0.00 respectively (Table 1). The pH values of media (Table 1) were high when $NaNO_3$, KNO_3 and control (no nitrogen supplement) were employed as nitrogen sources at 4.51±0.01, 4.00±0.03 and 3.97±0.02 respectively. Lower values were recorded when $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 were employed at 3.36±0.06, 3.43±0.01 and 3.35±0.02 respectively.

Discussion

Rice bran is one of the most abundant and locally available agricultural waste which contains variable ingredients such as carbohydrate that maybe used as a carbon and energy source for the growth of fungi in the production of single cell protein (Khan *et al.*, 1992). Rice bran was converted to fermentable sugar by heat treatment. These fermentable sugars were supplemented with minerals for the growth of *A. niger* to produce single cell protein. Ikenebomeh and Chikwendu (1997) observed high amylolytic activity of *A niger* in biomass production while Yigitoglu (1992) found that *A. niger* was superior to other species of *Aspergillus* and strains of fungi in biomass yield from agricultural waste. Appreciable biomass were cropped although not all fermentation carried out gave good yields. The mean dry biomass harvested at the end of fermentation (Fig. 1) from the SRB medium, GRB medium and RBM were 1. 44 ± 0. 01 g/1, 1. 56 ± 0.0g/l and 0.83 ± 0.01 g/l respective. This gave a mean total protein content of

Table 1: Effects of various nitrogen supplements on the final ph of growth medium and total protein content using *A. niger* after 6 days fermentation period on a shaker at 120 rpm and 28±2°C

Nitrogen sources	Final pH[(b)]	Total Protein Content[(b)] (g/l)
Control(a)	3.97 ± 0.02	0.43 ± 0.03
$NaNO_3$	4.51 ± 0.01	0.54 ± 0.01
$(NH_4)_2SO_4$	3.36 ± 0.01	0.67 ± 0.01
NH_4Cl	3.47 ± 0.01	0.62 ± 0.00
NH_4NO_3	3.35 ± 0.02	0.68 ± 0.02
KNO_3	4.00 ± 0.03	0.60 ± 0.01

a- Control had no nitrogen supplement added.
b- The values were the mean of five fermentations.

Table 2: Analysis of variance to test for significant difference in the dry biomass yield (g/l) using different nitrogen supplements in rice bran

S.O.V	DF	SS	MS	F	$F_{0.05\,(1)}$
Total	29	4.63			
Treatment	5	4.61	0.922	922	2.62
Error	24	0.02	0.001		

F-Calculated > F-critical, Ho is rejected (P<0.05), Ho - No significant difference of the various nitrogen supplements in dry biomass yield.

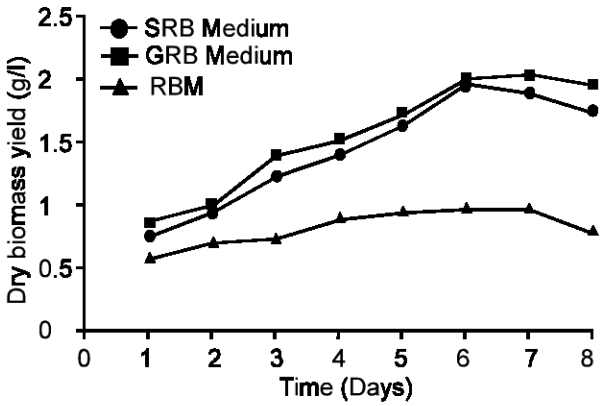

Fig. 1: *A. niger* dry biomass produced in supplemented (SRB and GRB) and unsupplemented (RBM) rice bran medium in shake flask fermentor at 120rpm on a time course basis.

0.46 ± 0.01g/l and 0.23± 0.01g/l of medium for the SRB medium, GRB medium and RBM respectively (Fig. 2). However, the total protein percentage on dry weight basis was 32% crude protein content for SRB medium 33% for GRB medium and 28% for RBM. In contrast to other works, Ravinder *et al.* (2003) recorded 43% protein content when *A. oryzae* was cultivated in de-oiled rice bran. Protein content of 18-25% was observed when *Penicillin javanicum* was grown on rice husk (Khan *et al.*, 1992). *A. niger* cultivated on cassava whey recorded protein percentage of 34% (Ikenebomeh and

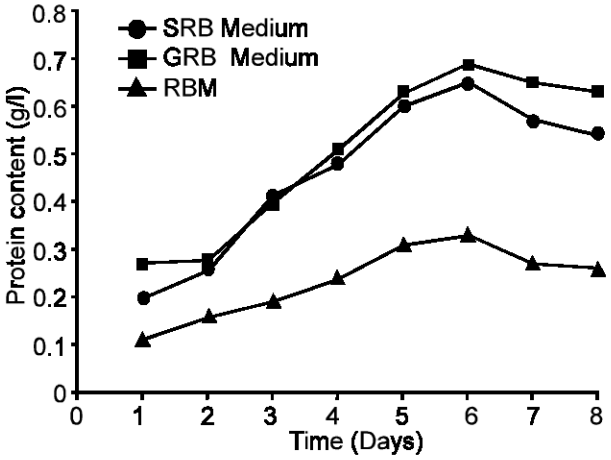

Fig. 2: Protein content (g/l) of *A. niger* biomass cropped in supplemented (SRB and GRB) and unsupplemented (RBM) rice bran medium in shake flask fermentor at 120 rpm on time course basis

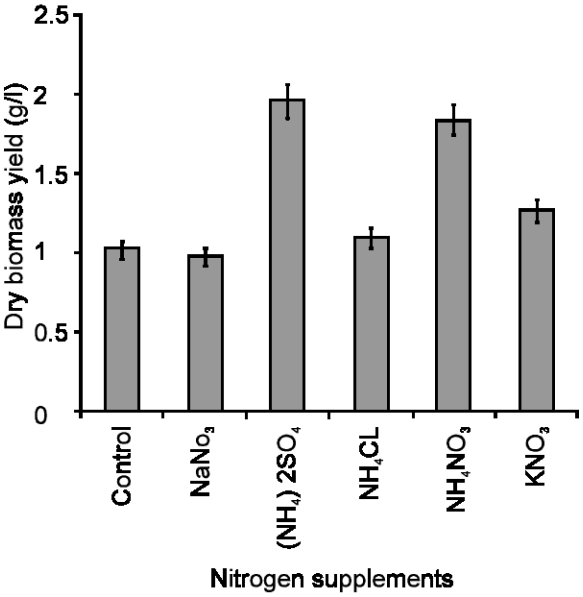

Fig. 3: Effects of various nitrogen supplements on biomass yield of *A. niger* after 6 days fermentation period on a shaker at 120 rpm and 28±2°C.

Chikwendu, 1997). *Candida rugosa* cultivated on palm oil had total protein percentage of 17-40% (Montet *et al.*, 1993).

From the results, the supplemented media (SRB and GRB) gave more biomass yields than the unsupplemented medium (RBM).This highlights the importance of supplementation to increase biomass yield. Also ,glucose supplemented rice bran medium gave higher biomass yields than the supplemented rice

Table 3: Duncan multiple range test for the mean of dry biomass yield (g/l) using different nitrogen supplements in rice bran

Means arranged in ascending order						
	A	B	C	D	E	F
Nitrogen supplement	$NaNO_3$	Control	NH_4Cl	KNO_3	NH_4NO_3	$(NH_4)_2SO_4$
Mean	0.974	1.01	1.092	1.26	1.83	1.95
Work Table						
P	2	3	4	5	6	
Significant studentised range	2.885	3.085	3.131	3.199	3.250	
Least square range	0.040	0.043	0.044	0.045	0.46	

Comparison of means with critical values of appropriate p level

Comparison	Difference	P	LRS	Significance
F - A	0.976	6	0.046	Reject Ho
F - B	0.940	5	0.045	Reject Ho
F - C	0.858	4	0.044	Reject Ho
F - D	0.690	3	0.043	Reject Ho
F - E	0.120	2	0.040	Reject Ho
E - A	0.856	5	0.045	Reject Ho
E - B	0.820	4	0.044	Reject Ho
E - C	0.738	3	0.043	Reject Ho
E - D	0.570	2	0.040	Reject Ho
D - A	0.280	4	0.044	Reject Ho
D - B	0.2005	3	0.043	Reject Ho
D - C	0.168	2	0.040	Reject Ho
C - A	0.1108	3	0.043	Reject Ho
C - B	0.082	2	0.040	Reject Ho
B - A	0.036	2	0.040	Accept Ho

The pairing of means are all higher than the critical values except A and B.

Therefore: $\mu_A = \mu_B \neq \mu_C \neq \mu_D \neq \mu_E \neq \mu_F$

bran medium indicating that biomass yield can be increased when a carbon source like glucose is added to the medium. The low yields obtained from RBM could be as a result of limited concentration of nutrients particularly nitrogen source required for microbial growth and biomass formation.

From observation it is clear that the availability of the nitrogen source is the major controlling factor in the final biomass yield. Rice bran when supplemented with nitrogen sources improved the biomass yield. $(NH_4)_2SO_4$ supplement gave the highest biomass yield of 1.95±0.03g/l. Other nitrogen sources supported the biomass yield to a lesser extent (Fig. 3). This is in agreement with the report of Akpan *et al.* (1999); Anupama and Ravindra (2001), who found that nitrogen supplementation enhances the production of the organism and hence increase in the biomass cropped. However, supplement with $NaNO_3$ had a suppressive effect on *A. niger* growth as the biomass yield (0.97±0.02g/l) was very low in the presence of this compound (Fig. 3). The contributing factor for the low biomass yield of this compound was reported by Anupama and Ravindra (2001) to be the reactivity of sodium ion. Sodium is placed higher than all the metals in the solution such as Zinc, Ferrous, Magnesium and Hydrogen ions in the activity series of metals. Hence, it displaces all these metals from their salts. The nitrate ions released from $NaNO_3$ in the medium (due to the principle of displacement) reacted with the free cations of the mineral solution and formed nitrates. As such, free nitrogen and also minerals solution controlling the pH was not available to support much growth of *A. niger*.

Ammonium Sulphate $(NH_4)_2SO_4$ gave the highest biomass yield of 1.95±0.03g/l followed by NH_4NO_3 (1.83±0.04g/l). The reason for this higher biomass value could be that the activity of mineral solution was not hindered, as ammonium ion formed was weak hence unable to replace the metal ion in the mineral solutions. The work of Ikenebomeh and Chikwendu (1997) found that $(NH_4)_2SO_4$ was the choice of supplement to improve biomass yield in cassava whey. Apart from providing nitrogen it also provides sulphur in the medium; an element known to be required for growth. Ammonium Sulphate [$(NH_4)_2SO_4$] in the fermentation medium of rice bran enhanced amylase production by *Rhizopus* sp (Akpan *et al.*, 1996).

Apparently, the preferred nitrogen source for *A. niger* biomass was $(NH_4)_2SO_4$. Analysis of variance (Table 2) showed that different nitrogen supplements in the rice bran gave significantly different dry biomass ($P<0.05$).

Duncan's Multiple Range test (Table 3) indicated that the nitrogen supplements are significantly different from one another in dry biomass yield. Although, the supplement $NaNO_3$ and control (no nitrogen supplement) had dry biomass yield which are not significantly different in terms of nitrogen source on the biomass yield.

The final pH values when using $(NH_4)_2SO_4$ and NH_4NO_3 supplements (Table 1) were 3.36±0.01 and 3.35±0.02 respectively. This agreed with observations that $(NH_4)_2SO_4$ and NH_4NO_3 increased acid production in the medium (Ali *et al*., 2002; Ikenebomeh and Chikwendu, 1997) which might eliminate the need for pH control equipment, due to their low pH values. This might have created self sterilizing environment; mostly against bacterial contaminants which ensures less competition for the available nutrients in the substrate and promote higher biomass yield.

In conclusion higher yield of single cell protein production from *A. niger* was possible by submerged fermentation of rice bran. Supplementation of rice bran with minerals enhanced fungal utilization of the substrate as well as biomass yield. Glucose supplemented rice bran (GRB) medium gave the highest yield than the supplemented rice bran (SRB) medium. In essence, rice bran was used as a potential source for product with higher protein content by utilizing cellulose present in the rice bran. Overall results indicated that *A. niger* can be used as a potential strain for SCP production. Since rice bran was successfully utilized for the enrichment of protein in product, there is a possibility of converting agroresidue waste to proteinaceous feed and food. Moreover, the result confirmed that enrichment of rice bran with various nitrogen sources enhanced product yield. The biomass yield was best with rice bran based medium with $(NH_4)_2SO_4$ as nitrogen source for SCP.

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