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Kinetics Studies of the Partially Purified Cellulase Produced During the Degradation of Rice Husk Pre-Treated at Different pHs Using *Mucor indicus*

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ABSTRACT

This study was aimed at the production of cellulase from the degradation of rice husk using a fungi species isolated from rice husk dump site. Rice husk was pre-treated at 120°C in a mineral nutrient medium at different pH 2, 7 and 12. After the pre-treatment the pH of the different media was adjusted to 7 which was suitable for microbial growth. *Mucor indicus* isolated from the rice husk dump site was inoculated into the pre-treated husk media and the set-up was allowed to stand for 28 days with periodic shaking. At the expiration of the 28 days, the broth was filtered and centrifuged at 4000 rpm for 20 min to remove all cell suspension. Cellulase was isolated using 90% ammonium sulfate saturation. The yield of cellulase produced were 14.81, 13.43 and 26.74% with specific activity 1.415, 23.237 and 15.524 m mg^{-1} for pH 2, 7 and 12, respectively. After gel filtration the yield of cellulase produced was 0.71, 6.01 and 12.10% with specific activity of 0.114, 22.365 and 19.320 m mg^{-1} for pH 2, 7 and 12, respectively. The V_{max} of crude cellulase produced were 0.81, 2.10 and 2.84 μmol min^{-1} while the K_m were 46.30, 42.21 and 47.72 mg mL^{-1} for pH 2, 7 and 12, respectively. The V_{max}: K_m ratio were 0.0176, 0.0497 and 0.0594 mL mg^{-1} for pH 2, 7 and 12, respectively, which showed that the cellulase produced at pH 12 will be more efficient in the hydrolysis of cellulose to glucose. This was also demonstrated by the catalytic efficiency of the enzyme at pH 12 (3.57×10^{-4}). The result also showed that cellulase extracted from rice husk pretreated at pH 12 possesses high catalytic efficiency when compared with other pHs.

Key words: Cellulase, pretreatment, *Mucor indicus*, gel filtration and kinetics

INTRODUCTION

Agricultural wastes and lingo cellulosics material can be converted into products that are of commercial interest such as bioethanol, biosurfactants, etc (Abou-Taleb *et al*., 2009). Cellulase are used for the bioconversion of lignocellulosics to useful products (Ariffin *et al*., 2006). The activity and production are often affected by temperature, substrate concentration (that might act as inducers or repressors), the presence of inhibitors, pH of the environment, cell density and growth rate, etc (Shahriarinour *et al*., 2011).

Lignocellulosics are abundant sources of carbohydrate, which are continually replenished by photosynthetic reduction of carbon dioxide by sunlight energy (Sadhu and Maiti, 2013). Thus, they are the most promising feedstock for the production of energy, food and chemical. The bioconversion of cellulosic materials has been receiving attention in recent years, it is now a subject of intensive research as a contribution to the development of a large-scale conversion process beneficial to mankind (Bhat, 2000). Such process would help alleviate the problem of shortage of food and animal feed, solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose (Maki *et al*., 2011).

Some features of natural cellulosic materials are known to inhibit their degradation or bioconversion of these materials (Immanuel *et al.,* 2006). These features include the degree of crystallinity, lignification and the capillary structure of cellulose. The crystallinity and lignification limit the accessibility and susceptibility of cellulose to cellulolytic enzymes and other hydrolytic agents (Shankar *et al*., 2011). However, many physical, chemical and microbial pre-treatment methods that enhances the bioconversion of cellulosic materials have been reported (Irfan *et al*., 2012). Pre-treatment of cellulose opens up the structure and removes the secondary interaction between glucose chains (Niranjane *et al*., 2007). Since the production of cellulase is a major factor in the hydrolysis of cellulosic materials, it is important to make the process economically viable. Although much work has been done on the production of cellulase from lignocellulosics, there are no much research in utilizing rice husk for this same process.

Rice husk on the other hand is one of the most widely available agricultural wastes in many rice producing countries around the world. Globally, approximately 600 million t of rice paddy are produced each year. On average, 20% of the rice paddy is husked giving an annual total production of 120 million t (Rabelo *et al*., 2011). In majority of rice producing countries, much of the husk produced from processing of rice is either burnt or dumped as waste. Burning of rice husk in ambient atmosphere leaves a residue called rice husk ash. For every 1000 kg of paddy milled, about 220 kg (22%) of husk is produced and when this husk is burnt in the boilers, about 55 kg (i.e., 25%) of rice husk ash are generated. Rice husk ash causes a lot of environmental problems resulting in the damage to the land and the surrounding area where it is dumped (Yoo *et al*., 2011). Rice husk could be applied as a fuel in power plant (Saha and Cotta, 2007) formation of activated carbon (Rabelo *et al*., 2011) and as a source of silica and silicon compounds (Mosier *et al*., 2005). This study is therefore aimed at isolating and partially purify cellulase produced during the fermentation of rice husk pretreated at pH 12 using Mucor isolated from rice husk dumps and also to determine the kinetics of the cellulase.

MATERIALS AND METHODS

Rice husk: The rice husk used in this study was collected from a rice dump site located at Adani in Uzo Uwani local government Enugu State. The 2 kg of rice husk was collected from the dump site and put in a polyethylene bag before transporting to the laboratory where it was stored at 4°C before analysis.

Preparation of agar for isolation of fungi: A modified czapek agar medium was prepared as follows, agar (15.0 g), $NaNO_3$ (3.0 g), K_2HPO_4 (1.0 g), KCl (0.5 g), $MgSO_4.7H_2O$ (0.5 g), $FeSO_4.7H_2O$ (0.01 g), rice husk (10.0 g) and chloramphenicol (0.1 g) were put in a 1 L conical flask and 400 mL of distilled was added to dissolve compound and then made to mark with distilled water. The pH of the mixture was adjusted to 7.0 and the mixture was autoclaved at 100 and 120°C at a pressure of 10 psi for 20 min. The autoclaved mixture was allowed to cool to about 5°C before aseptically dispensed into petri dishes.

Isolation of fungi: One gram of rice husk from the dump site was put in a test tube and 10 mL of distilled water was added. The mixture was vortex for 10 min and allow to stand for 10 min. This process was repeated 3 times. The vortex mixture (0.5 mL) was aseptically inoculated into the petri dishes containing the modified Czapek agar media using the spread plate technique. The petri dishes were incubated at 28°C for 4 days, then the organism with the highest visible growth were isolated.

Identification of organism: The isolated organism was smeared on a slide glass and lacto phenol staining blue was dropped on it and a cover glass was used to place on it. The prepared organism was viewed in the microscope for morphological appearances. The image was used to match the one on color atlas of diagnostic microbiology.

Preparation of media for pretreatment/fermentation of rice husk: The modified Czapek broth was prepared as follows: $NaNO_3$ (3.0 g), K_2HPO_4 (1.0 g), KCl (0.5 g), $MgSO_4.7H_2O$ (0.5 g), $FeSO_4.7H_2O$ (0.01 g), rice husk (50.0 g) and chloramphenicol (0.1 g) were put in a 1 L conical flask and 400 mL of distilled was added to dissolve compound and was made to mark with distilled water. The pH of the mixture was adjusted to 12.0 and the mixture was autoclaved at 100 and 120°C at a pressure of 10 psi for 1 h. The autoclaved mixture was allowed to cool.

Determination of protein standard curve: Bovine serum albumin was used in preparing the protein standard curve and this involves dissolving 0.2 g of bovine serum albumin in 100 mL of water and making a serial dilution of bovine serum albumin in the order: 0.0, 0.1.0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0. The individual volumes were made up to 1.0 mL using distilled water after which 5 mL of solution D (Freshly prepared alkaline solution was made by mixing 50 mL of solution A, 1 mL of solution B and 1 mL of solution C) was added to the diluents. The mixtures were allowed to stand for 10 min, after which 0.5 mL of solution E (Folin-ciocauteau phenol reagent was made by diluting the commercial reagent with water in a ratio of 1:1 freshly prepared) was added to the test tubes and allowed for 30 min. Finally, the absorbance was taken at a wavelength of 740 nm using spectrophotometer and were used to plot the standard curve.

However, the extracted biosurfactants were prepared in the same way as the bovine serum albumin used for standard curve and their protein composition determined using spectrophotometer at a wavelength of 740 nm.

Cellulase activity assay: The cellulase assay was conducted by the method described by Eveleigh *et al.* (2009). Filter paper strip (50 mg Whatman No. 1 filter paper strip (1.0×6.0 cm) was put into 13×100 test tubes and 1.0 mL 0.05 M Na-citrate, pH 4.8 to the tube was added (the buffer should saturate the filter paper strip). Equilibrate tubes with buffer and substrate to 50°C. Then 0.5 mL enzyme was added. It was Incubated at 50°C for exactly 60 min and at the end of the incubation period, the test-tubes were removed from the 50°C bath and the enzyme reaction was stopped immediately by the addition of 3.0 mL DNS reagent and mixing. The blank was prepared with 1.5 mL citrate buffer without the enzyme. Boil all tubes for exactly 5.0 min in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. Determine color formation by measuring absorbance against the reagent blank at 540 nm. The amount of glucose formed was a measure of enzyme activity.

Ammonium sulfate saturation and centrifugation: A total of 1 L of each of the crude enzyme filtrate was subjected to 90% ammonium sulfate saturation by dissolving 663.44 g of the salt in the

cold filtrate until the salt became completely dissolved. The solution was then stored at 4°C. Centrifugation was carried out at 3,000 rpm for 30 min and the precipitate was re-dissolved in the sodium acetate buffer pH 5.5 and stored at 4°C.

Gel filtration chromatography: The gel filtration chromatography technique was used in the purification of cellulase produced by *Mucor indicus* by size exclusion chromatography technique. A volume of 200 mL of crude cellulase was applied on Sephadex G-100 column, which previously equilibrated with 0.1 M potassium phosphate buffer (pH 5.5). The 5 mL of the eluent were in different tube and cellulase activity was determined in each tube. The graph of cellulase activity and tube number was plotted as presented in the results.

Statistical analysis: The result was analyses statistically using the analysis of variance (ANOVA). It was presented as mean±standard deviation. The difference in means was considered significant at $p<0.05$ and the Graphpad prism v6 was used for the analysis.

RESULTS

Isolation and characterization of organs: The organism that was used in this study was isolated from the rice husk dump site and after characterization, it was identified as *Mucor indicus*.

Determination of percentage of cellulose in rice husk: The result of the percentage cellulose in the rice husk and filtrate before and after fermentation were determined. Figure 1 showed the colors of the broth after fermentation filtration and centrifugation. The result showed that pH 2 produced a very faint color, pH 7 showed a deeper pale color and the pH 12 showed a very dark solution. From the colors observed, it shows that as the pH increases, more components of the rice husk are being broken down.

The result of the percentages of cellulose, lignin and hemicellulose in the rice husk and its filtrate before and after fermentation. Table 1 showed that there was an increase in the quantity of in the quantity of cellulose in the filtrate after fermentation. This is because of heat pretreatment

Fig. 1: Color of the broth after pretreatment and fermentation

of cellulosic materials release some quantity of cellulose but during fermentation, the organism still attacks the material thereby releasing more of the cellulose into the broth. Although the organisms still depend on the cellulose that is a release of energy and growth. The lignin and hemicellulose component were also found to decrease after fermentation. The difference observed were all statistically significant.

In Fig. 2 the ammonium sulfate precipitation graph shows the highest precipitation at 90%. Therefore, the protein was precipitated at 90% ammonium precipitate. It was observed that the precipitation was more for the set up at pH 7 and 12 when compared to pH 2.

Gel filtration chromatography: In Fig. 3 the graph shows gel filtration between tube 7-14 was collected in pH 12, tube 7-28 was collected in pH 7 while tube were collected to pH 2 because these are the point where maximum absorbance was observed 13-50.

Table 1: Determination of percentage of cellulose, lignin and hemicellulose in rice husk

	pH	Cellulose (%)	Lignin (%)	Hemicellulose
Normal rice husk		89.247±8.21	1.071±0.025	1.542±0.033
Pretreated rice husk filtrate	pH 2	21.428±3.24	0.043±0.010	0.100±0.013
Fermented filtrate		40.000±3.65	0.018±0.001	0.071±0.015
Pretreated rice husk filtrate	pH 7	18.181±1.23	0.017±0.004	0.157±0.005
Fermented filtrate		57.142±5.43	0.028±0.006	0.100±0.022
Pretreated rice husk filtrate	pH 12	24.440±3.28	0.014±0.001	0.128±0.013
Fermented filtrate		56.667±7.34	0.011±0.002	0.086±0.029

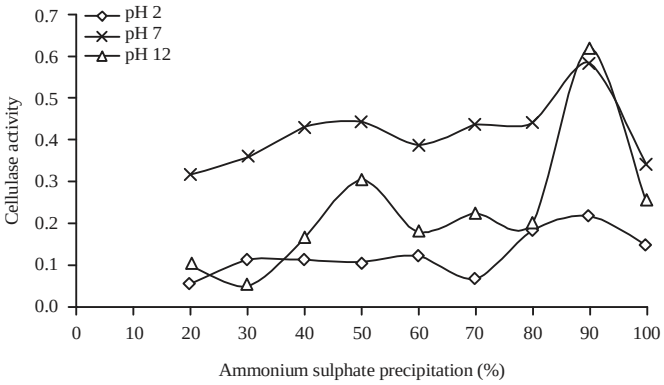

Fig. 2: Ammonium sulfate precipitation

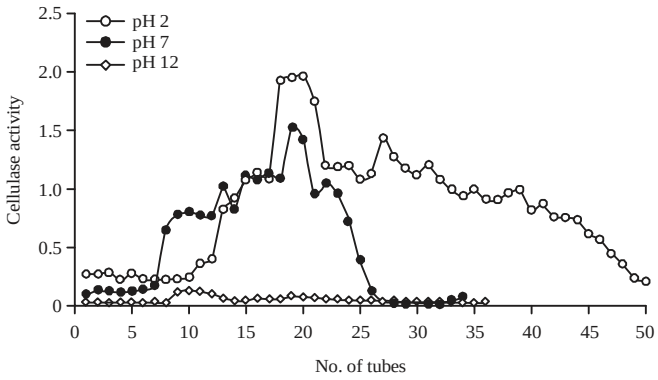

Fig. 3: Gel filtration

The purification fold table in the Table 2 above, reveals that the specific activity of the crude enzyme was 1.641, 39.443 and 14.677 U mg^{-1} for pH 2, 7 and 12, respectively, after 90% ammonium sulfate precipitation, it was observed that the specific activity of pH 12 increases to 15.54 U mg^{-1}. While that of the pH 2 and 7 decreases to 1.415 and 23.237 U mg^{-1}. It was observed that percentage yield decreased after ammonium sulfate precipitation and gel filtration.

Effect of substrate concentration: The result of the primary plot activity against substrate concentration (Fig. 4) showed that the maximum activity of cellulase produced after pretreatment at pH 12 (1.753 µmol h^{-1}) was found to be higher when compared with cellulase produced at pH 2 (0.493 µmol h^{-1}) and pH 7 (1.357 µmol h^{-1}). The result also showed that as the pH of pretreatment increases, the maximum activity of the cellulase produced also increases (Fig. 4).

The result of the secondary plot of the inverse of the activity against the inverse of the substrate concentration (Fig. 5) showed intercepts at 1.229, 0.476 and 0.353 on the 1/v axis for pH 2, 7 and 12 respectively which corresponds to the inverse of the various maximum activity (V_{max}) at these pH of pretreatments. The result also reveals the intercept on the 1/(S) axis for pH 2, 7 and 12 which corresponds to the 0.022, 0.024 and 0.021, respectively, these values also define the inverse of the K_m which is a measure of the binding affinity of enzyme (cellulase) to cellulose.

Table 3 reveals an increase in V_{max} as pH increases but the K_m which is a measure of enzyme affinity to the substrate did not follow the trend but a decrease in pH 7 was observed when

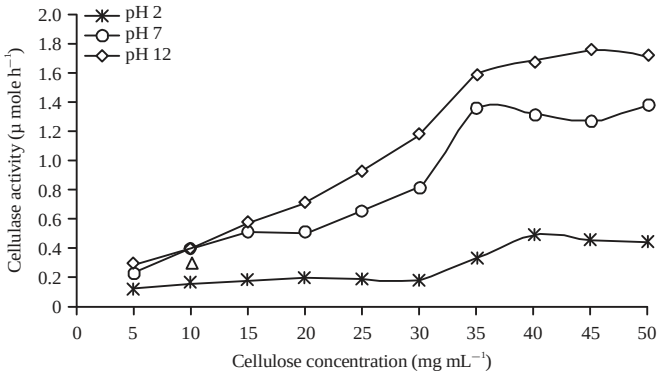

Fig. 4: Effect of substrate concentration 5

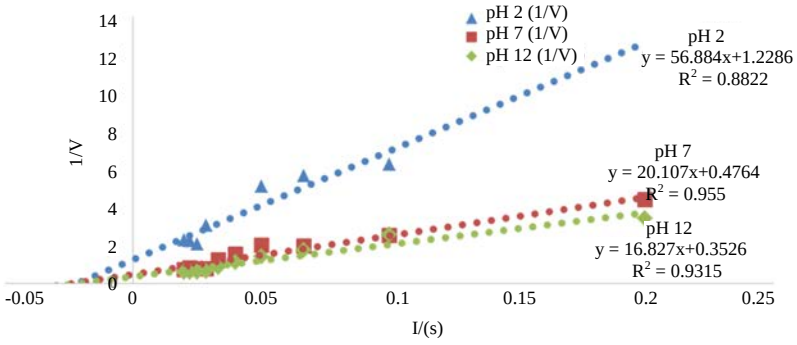

Fig. 5: Graph of the secondary plot of 1/v against 1/(S)

Table 2: Purification fold table the cellulase produce at different pHs from rice husk by *Mucor indicus*

pH	Enzyme sample	Volume (mL)	Protein (mg mL^{-1})	Total protein (mg)	Activity (U)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
pH 2	Crude extract	200	1.426	285.20	2.34	468.00	1.641	1.000	100
	90% $(NH_4)_2SO_4$ precipitation	90	0.544	48.96	0.77	69.30	1.415	0.863	14.808
	Gel filtration	60	0.4843	29.06	0.06	3.60	0.114	0.069	0.705
pH 7	Crude extract	200	0.862	172.40	34.00	6800.00	39.443	1.000	100
	90% $(NH_4)_2SO_4$ precipitation	60	0.655	39.30	15.22	913.20	23.237	0.589	13.429
	Gel filtration	40	0.457	18.28	10.22	408.84	22.365	0.567	6.012
pH 12	Crude extract	200	0.832	166.40	12.21	2442.20	14.677	1.000	100
	90% $(NH_4)_2SO_4$ precipitation	70	0.601	42.07	9.33	653.10	15.524	1.058	26.742
	Gel filtration	35	0.437	15.30	8.44	295.505	19.320	1.316	12.100

Table 3: Kinetics of cellulase produced from rice husk pretreated at pH 2, 7 and 12

pH	V_{max} mol min^{-1}	K_m (mg mL^{-1})	V_{max} :K_m Ratio	Total enzyme (Et)	$K_{cat} = V_{max}/E_T(sec^{-1})$	Catalytic efficiency ($Umol^{-1}$ sec^{-1})
2	0.81	46.30	0.0176	285.20	0.0029	6.16×10^{-5}
7	2.10	42.21	0.0497	172.40	0.0122	2.88×10^{-4}
12	2.84	47.72	0.0594	166.40	0.0170	3.57×10^{-4}

compared with the other pHs. It was observed that the V_{max}: K_m ratio increased with an increase in the pH of pretreatment. It was also observed that the total Enzyme Et decreased with increase in pH of pretreatment. Table 3 also showed that the catalytic efficiency of cellulase also increased with increase in pH of pretreatment of the rice husk.

DISCUSSION

The effect of acid and alkaline pretreatment on the kinetic properties of cellulase produced during the degradation of rice husk using microorganism isolated from the dump site was assessed. The essence of using microorganisms from dump site is to enable the use of organism that have adapted to the breakdown of rice husk, just as adapted organisms are used in the degradation of crude oil (Sepahi *et al*., 2008). The isolated organism used in this study was morphologically identified as *Mucor indicus*.

In this study, the *Mucor indicus* was used to break down the structural cellulosic material to release cellulose. It was observed that the organism used up some of the cellulose for energy and biomass formation. The result in Table 1 showed that after pretreatment and prior to fermentation, the amount of cellulose found in the media was 21.43, 18.18 and 24.44% for pH 2, 7 and 12, respectively. This showed that heat treatment to some extent, broke down some of the structural carbohydrate in rice husk thereby releasing cellulose (Madorsky *et al*., 1958; Potthast *et al*., 2002; Wang *et al*., 2011). The result also showed that heat has also released some of the lignin and hemicellulose in the rice husk in the media. According to Bobleter (1994) and Garrote *et al*. (1999), lignocellulosic materials when heated to 150-180°C cause some of the hemicellulose and lignin to solubilize.

At the end of the fermentation (after 28 days of the experiment), the percentage of cellulose in the media was increased to 40.00±3.65, 59.14±5.43 and 56.67±7.34% for pH 2, 7 and 12, respectively. This shows that during fermentation, the organism did not only metabolize the cellulose in the media but also released cellulose from the lignocellulosic material in the media as the rice husk degrade. The concentration of the lignin and hemicellulose also reduced during fermentation. Therefore, *Mucor indicus* can metabolize the rice husk to release more cellulose and increase biomass and energy generation. It was also observed that lignin also decreased in the media showing that the organism (*Mucor indicus*) can degrade lignin which shows that the organism can secrete ligninase.

The cellulase produced by *Mucor indicus* during the degradation of rice husk pretreated at different pHs were characterized with a view of determining the optimum pH of pretreatment for

cellulose production. After the experiment the cell free broth was subjected to ammonium sulfate precipitation and the result showed that most of the cellulase will precipitate at 90% ammonium sulfate precipitation. It was observed that the activities of the eluent collected from the partial purification of cellulase produced from pH 12 pretreated rice husk were very low when compared with the others.

Considering the purification table, the yield of crude cellulase from pH 2 pretreated rice husk was found to be 14.81% but after gel chromatography, the yield dropped as low as 0.71%, while the yield of the pH 7 and 12 pretreated rice husk was 6.01 and 12.1%, respectively.

The K_{cat}-K_m ratio, which is a criterion of substrate specificity, catalytic efficiency and kinetic perfection were determined. In other words, the K_{cat}-K_m ratio shows enzyme's substrate preference and enzyme's catalytic efficiency. The catalytic efficiency increases as the pH of pretreatment of rice husk increase (Hendriks and Zeeman, 2009). Therefore, to use *Mucor indicus* in the production cellulase from rice husk, the pH of the pretreatment media should be alkaline as shown by this study.

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