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Isolation and Partial Purification of Cellulase from Rhizopus Stolonifer

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ABSTRACT

Cellulase produced by Rhizopus stolonifer isolated from decayed wood was partially purified using ammonium sulphate precipitation and dialysis. Sawdust and millet stalk were the feed substrates and served as carbon sources for the growth of the organism and induction of the enzyme. Cellulase activity was determined at 24 hour interval and lasted for 192 hours using 1% standard cellulose powder as the enzyme substrate at room temperature 28±1^{o}C and pH 5. The optimum cellulase induction/secretion was achieved at 72 hour incubation period in both feed substrates (millet stalk had 41.8 U/ml and sawdust had 25.3 U/ml cellulase activity respectively). Ammonium sulphate precipitation of the 72 hour culture gave cellulase activity of 33.00U/ml at 80% saturation for millet stalk and 28.00U/ml at 50% saturation in sawdust feed substrate which indicated increase in activity in sawdust and a decrease in millet stalk. In the dialysis step with acetate buffer pH 4.0, there was a general decrease in activity in the two substrates (millet stalk 31.00U/ml and sawdust 21.67U/ml). The dialysis may have caused the loss of some cofactors essential for the activity of the cellulase. From the result obtained, it was obvious that Rhizopus stolonifer has the ability of secreting cellulase in culture media containing the two carbon sources (sawdust and millet stalk). The study also revealed that proper pretreatment methods of millet stalk feed substrate may support higher yield of the enzyme. As with most enzyme purifications, dialysis reduced the activity of the enzyme.

Keywords: *Cellulase, Rhizopus stolonifer, Sawdust and Millet stalk*

1. INTRODUCTION

Cellulose is the most abundant and important polymers on earth and as a result about 90% of solid waste disposable materials are made up of cellulose (Bisaria, 1991). In view of this, the world attention in the field of scientific development has shifted to environmental biotechnology, because of the great challenge posed by accumulation of these wastes, cellulosic material have become difficult to handle. These solid waste materials can be hydrolysed efficiently by some class of fungi known as filamentous fungi (Wong et al., 1988, Howard et al., 2003, Subramaniyan and Prema, 2004). Among the filamentous fungi of environmental importance are Trichoderma sp. Penicillium sp., Aspergillus sp., Fusarium sp., Rhizopus sp. and so on. Their hydrolytic efficiency is as a result of secretion of extracellular enzymes such as cellulases, hemicellulases and ligninases. Of these, cellulase is the most important and a complex enzyme that acts synergistically though often described as contrasting faces of a single enzymatic capability. It exists in two forms whether as membrane bound or free in the medium depending on the type of organism in the culture media.

The features of natural cellulosic materials are known to inhibit their bioconversion or degradation (Bisaria, 1991), such as the degree of crystallinity and lignification of cellulose to cellulolytic enzymes and other hydrolytic agents (Gharpuray et al., 1983). However, many physical, chemical and microbial pretreatment improves significantly bioconversion of cellulosic materials. Bacterial and fungal degradation of cellulose has been extensively studied (Eriksson et al., 1990). Production of cellulase enzyme is a major factor in the hydrolysis of cellulosic materials, it is important to make the process economically viable and its purification increases its effectiveness and implying that a few milligram quantity of the enzyme can catalyze the production of huge products.

Although much work has been done on the production of cellulase using lignocellulose materials, the emphasis here is on cellulase production by Rhizopus stolonifer with sawdust and millet as carbon sources.

The objective of the study was to evaluate the cellulase activity of Rhizopus stolonifer and partially purify the enzyme with sawdust and millet stalk as carbon sources under submerged fermentation conditions.

2. CHEMICALS

All chemicals and reagents used in the study were of analytical grade.

3. MATERIALS AND METHODS

3.1 Source of the Fungi

The pure isolate of Rhizopus stolonifer was obtained from the Plant Pathology Laboratory, Crop Protection Department, University of Maiduguri, Borno state, Nigeria. It was maintained on potato-dextrose agar (PDA) at 4^{o} C.

3.2 Source of the Lignocellulose Materials

Millet stalk and the sawdust (composite) were obtained on farm lands and sawmill around Dass local government area of Bauchi state and Maiduguri, Borno state respectively.

3.3 Pretreatment of the lignocellulose Materials

The materials were subjected to mechanical communition which was a combination of chopping,

grinding and milling to reduce the cellulose crystallinity, increase surface area for chemical pretreatment as reported by Millet et al., (1976).

The ground carbon sources were pretreated by a modification of Ali et al.,(1991) method. The substrates 5g/100ml in separate conical flasks (250ml) were soaked in 5% (w/v) NaOH solution in a ratio of 1:20 to delignify them. They were then autoclaved at 121^o C for 1 hr. The pretreated carbon sources were filtered with muslin cloth and residues were neutralized with 1N HCl and washed with distilled water. This was then oven-dried at 65^o C.

3.4 Fermentation Media

The fermentation media or mineral salt medium (MSM) was prepared as described by Ali et al., (1991) by dissolving the following salts in distilled water (g/ml): $ZnSO_4.7H_2O$ 0.04, KH_2PO_4 10.5, $MgSO_4.7H_2O$ 0.5, and $CaCl_2$ 0.5, $FeSO_4.7H_2O$ 0.13, $MnCl_2.4H_2O$ 0.05, K_2HPO_4 0.5, $NaMbO_4$ 0.5 and $(NH_4)2SO_4$. This was then autoclaved at 121^o C for 1hr. Five gram of the pretreated carbon sources each were added separately to 100ml of MSM and sterilized again at 121^o C for 15min. This medium provides nitrogen and mineral element requirements of the fungus. The pretreated carbon sources both served as energy sources and inducers of the cellulase.

3.5 Inoculation

Spores of the 4-day old Rhizopus stolonifer maintained on potato-dextrose-agar at 4^o C were washed with 2ml of 0.2% tween 80 solution into the 250ml conical flasks. The flasks were incubated at 30^o C in orbital shaker at 200rpm for a period of 10 days.

3.6 Enzyme assay

The cellulase activity was assayed by estimating the amount of glucose released upon hydrolysis of cellulose from the culture medium by the R. stolonifer. Glucose released was determined at 24 hour intervals.

One unit of cellulase activity was defined as the amount of the enzyme which released one micromole of glucose equivalents per minute in 30 minutes under the specified assay conditions. Therefore,

Glucose concentration (mg/dL) = Absorbance of Test/Absorbance of standard X100.

Values obtained were converted to international unit.

Estimation of glucose released was by the use of glucose oxidase kits obtained from Randox Laboratories ltd UK.

3.7 Protein Estimation

Total protein was estimated by Biuret method using egg albumin as the standard protein.

3.8 Precipitation of Protein

Ammonium sulphate salt was used to precipitate out proteins from the cell-free culture from 20-90% saturation and was kept overnight at 4^{o}C.

4. RESULTS

As the period of fermentation increased, cellulase activities also increased. Peak levels were obtained on the 3rd day (72hr) for both feed substrates with activities of 25.3IU and 41.8IU (Table 1) for sawdust and millet stalk respectively. Peak specific activities of 9.56U/mg protein and 11.15U/mg protein for sawdust and millet stalk in that order was obtained on the 72 hr fermentation period.

Ammonium sulphate precipitation of the 72 hr sample was between 30-80% saturation. As presented in table 2, the maximum activity of 28.00IU for sawdust substrate was obtained with 50% ammonium sulphate saturation, while the activity for millet stalk substrate of 33.0IU was obtained with 80% ammonium sulphate saturation.

Dialysates of the 50% and 80% ammonium sulphate precipitation of both substrates gave activities of 21.67IU for sawdust and 31.00IU for millet stalk respectively as presented in table 4. The partial purification profile data gave folds of 1.58 and 1.28 for sawdust and 1.56 and 1.46 for millet stalk after ammonium sulphate precipitation and dialysis steps respectively. The yields for the two substrates in the steps were 89.3% and 84.0%, 78.9% and 74.2% respectively as presented in table 3.

5. DISCUSSION

The crude cellulase activity of Rhizopus stolonifer using sawdust and millet stalk as cellulosic feed substrates during fermentation period of 192 hours (8 days) showed the highest levels of cellulase activity were at 72 hr in both substrates and this was accompanied by corresponding increase in activity from 0-72 hr. This is in contrast with the findings of Milala et al; (2009) who reported peak levels of cellulase activities on the 7th day (168) for rice husk, millet stalk and sawdust using Aspergillus candidus. Ojumu et al., (2003) reported the peak levels at 72hr period and the enzyme could be harvested at this point when Aspergillus flavus linn isolate NSPR 101 was used for cellulase production on sawdust substrate.

The activity of cellulase began to decline after the peak period of fermentation, this may be attributed to feedback inhibition of the enzyme (cellulase) by the reaction products mainly glucose and cellobiose, which is in line with the work of Howell (1978) who reported that accumulative effects of cellobiose inhibit both endoglucanases and beta-glycosidase. Biochemical explanation to this is based on the mechanism of switching on and off of genes make up of the organism. Reaction products induced production of repressor protein and this in turn switched off the expression of

cellulase producing gene. Another factor for the decline in cellulase activity could be due to depletion of carbon and nitrogen source which might have caused starvation, and hence the organism cannot grow because enzyme activity is growth related (Duff and Murray 1996). The submerged culture method used in the study could also have affected the activity. It has been shown compared to other methods such as the solid state fermentation, that submerged fermentation has shearing forces which rupture mycelia cells and deactivate enzyme, thus enzyme activity is decreased (Wase et al, 1985). Submerged condition is not close to the natural habitat of this organism which is known to grow at relatively low water activities.

However this decline was only observed in 92 and 120hrs in both the feed substrates (millet stalk and sawdust). The activity increased sharply in both the culture media containing millet stalk and sawdust at 144 hr. The explanation may be as the available carbon sources in the media are utilized by the organism in buildup of biomass during the switch off period, now begun to deplete and the concentration of the reaction product in the media decreased. With availability of feed substrate in the media and coupled with the decrease in the reaction product, the cellulase producing genes are activated or switched on in order to replenish the carbon lost in the culture media and that probably explains the fluctuation in the cellulase activity day after day. When the cellulase activity in the two substrates are compared, the peak activity in millet stalk was 41.8U/ml and that of sawdust was 25.8U/ml. The difference in the activity may be attributed to several factors; some of which includes, sawdust may be difficult to preterit compared millet stalk as applied in this study due to its high lignin content and higher intermolecular ester bonds cross linking lignin and hemicelluloses which makes the cellulose content inaccessible to the enzyme (cellulase). This is because the porosity of the lignocelluloses materials increases with the removal of the cross links (Park et al., 1992). From the above, it could be that the sawdust used in the study contained higher amount of soft woods and that may result in lower cellulase activity. Millet et al., (1976) reported soft woods contain higher content of lignin.

The structure of lignin contains a polyphenolic compounds that bind irreversibly to cellulase and inhibit its action, therefore the higher the lignin content in a particular substrate is equally signifying that the polyphenolic compounds are also present in higher amount and hence much of the enzyme produced by the organism were irreversibly bound to the cellulase and inhibited its activity. Oxalates may be produced by the organism in response to peroxidase. These actions inhibit the peroxidase which is the potent lignin degrading enzyme and this could also be responsible for the differences in cellulase activity between the substrates (Kirk et al., 1980).

When cellulase was precipitated with ammonium sulphate, the activity in millet stalk decreased from 41.8 U/ml to 33.0U/ml, while that from sawdust increased from 25.3U/ml to 28.00U/ml at 80% and 50% purification factor. The principle of ammonium precipitation is basically the saturation of the protein solution with an increase in protein-protein interactions and decrease in the protein to water interaction. However, as the salt dissociate in the solution, it loads more ions in the solution. Some enzymes in the presence of certain ions in relatively high concentration are inhibited (Kulkarni 1996), this could be reasons why the cellulase activity in millet stalk decreased. The nature of substrate my induce secretion of different cellulase units in different substrates, probably the cellulase units secreted in millet stalk medium may differ from one secreted in the sawdust medium and that explain why they are precipitated at different percentage saturation with an increase in activity in sawdust medium.

The dialysis generally has not favored the enzyme activity as there were general loss of activity, this may be as a result of loss of some important ions which could serve as cofactors during the step. These ions may be essential to the functions of cellulase from R.stolonifer.

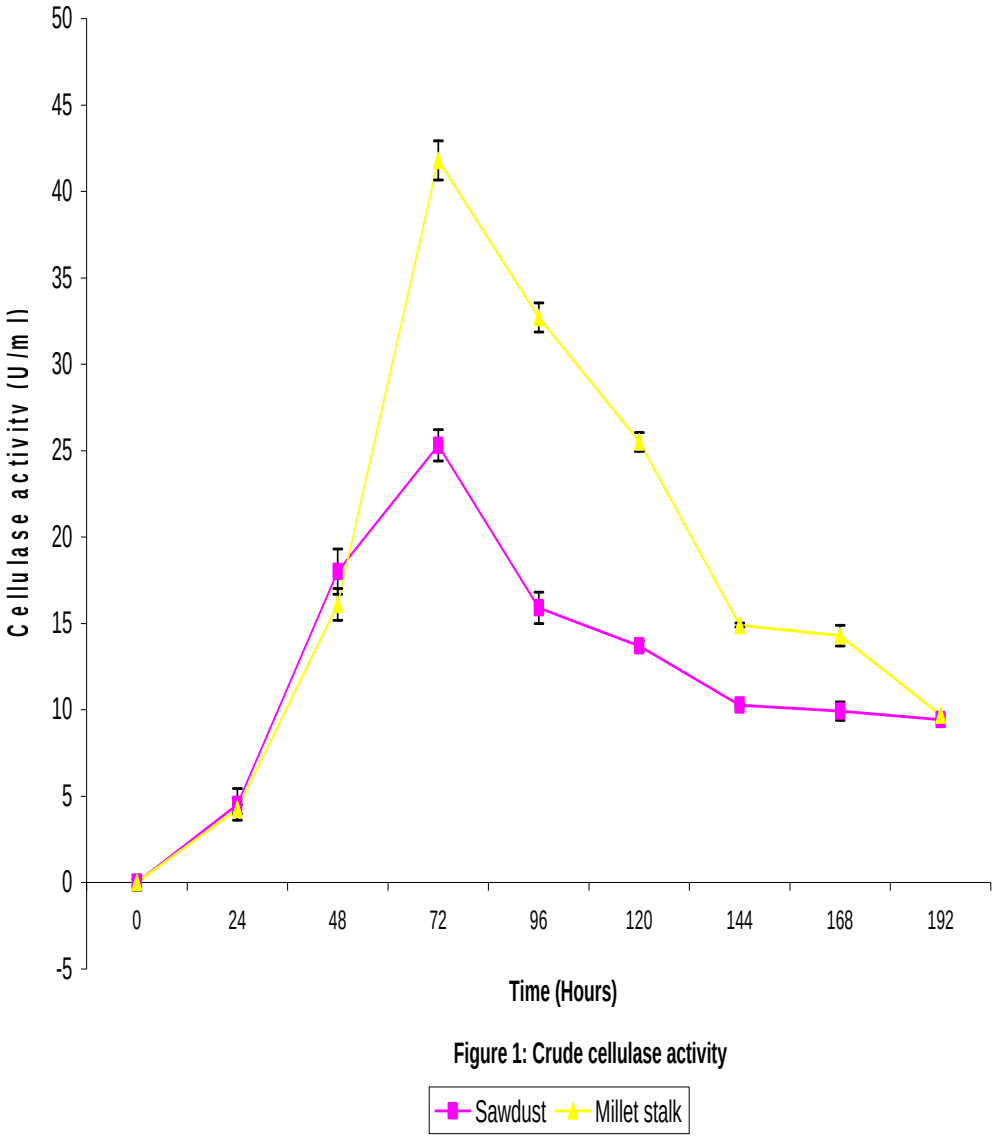

Figure 1: Crude cellulase activity

Fig 1: Crude cellulose activity

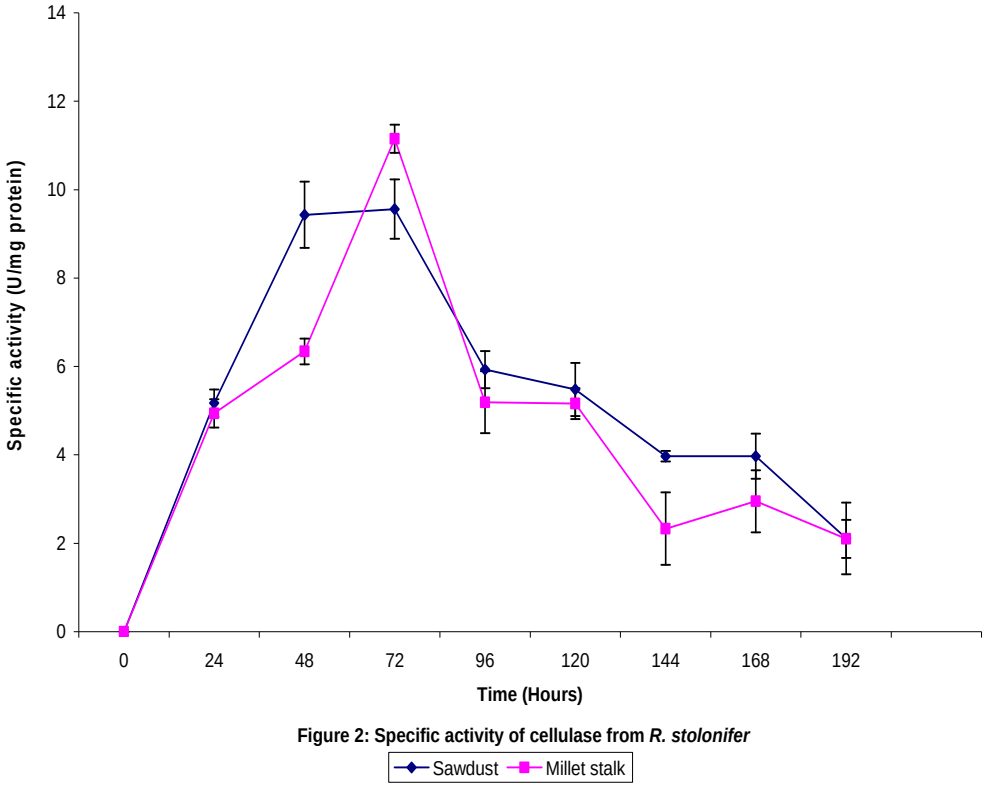

Fig 2: Specific activity of cellulose from R. Stolonifer

Table 1: Cellulase activity before and after dialysis purification step

Ammonium sulphate (%)	Feed substrate	Cellulase activity (undialyzed) U/ml	Cellulase activity (dialyzed) U/ml
50	Sawdust	28.00±2.63	21.67±0.65
80	Millet stalk	33.00±2.89	31.01±0.27

Table 2: Purification profile of cellulase from Rhizopus stolonifer

Purification step	Total protein (mg/ml)		Total activity (U/ml)		Specific activity (U/mg)		Purification fold		Yield (%)	
	Sawdust	Millet stalk	Sawdust	Millet stalk	Sawdust	Millet stalk	Sawdust	Millet stalk	Sawdust	Millet stalk
Crude	2.67	3.75	25.3	41.8	9.56	11.5	1	1	100	100
Ammonium precipitation	1.90	1.90	28.0	33.0	14.7	17.3	1.58	1.56	89.3	78.9
Dialysis	1.77	1.18	21.67	31.01	12.3	16.4	1.28	1.46	84.0	74.2

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