Ilorin Journal of Science
Volume 2, Number 1, 2015, pp.1–13 (Printed in Nigeria)
ISSN: 2408 – 4840 © 2015 Faculty of Physical Sciences, University of Ilorin
https://doi.org/10.54908/iljs.2015.02.01.001

ILORIN
JOURNAL OF SCEINCE

ILJS-15-025

Cellulolytic Activity of Wild-Type Fungi on Rice Bran (*Oryza glaberrima* Linn) using Submerged and Solid State Fermentation.

Omojasola[1]*, P.F. and Jimoh[1], O.M.

[1]Department of Microbiology, University of Ilorin, P.M.B 1515, Ilorin, Nigeria.

Abstract

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer on earth, cellulose, to smaller sugar components including glucose subunits. Therefore they have enormous industrial potential. This study was aimed at screening for the cellulolytic ability of fungi from an agro-based waste; rice bran (RB) (*Oryza glaberrima*). Cellulolytic fungi were isolated from RB and screened for cellulase activity on Mandel's Mineral medium supplemented with 1% Carboxyl methylcellulose (CMC). *Aspergillus niger* (ATCC 16404) served as the control organism. Total cellulase activity assay (FPase) was carried out to screen for the most efficient cellulase producers among the isolated fungi using submerged (SmF) and Solid-state (SSF) fermentations at 28± 2^0C for 120 h using CMC as control substrate. Ten fungal isolates were obtained, out of which eight were found to possess cellulose degrading ability as indicated by the diameter of clear, hydrolyzed zones on the medium. These cellulolytic fungi belonged to five genera: *Aspergillus*, *Fusarium*, *Penicillium*, *Saccharomyces* and *Rhizopus.* Among the isolates, strains of *Penicillium citrinum*, *Penicillium chrysogenum*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Fusarium oxysporium*, *Aspergillus flavus* and *A.niger*.were considered as potent producers of cellulase. Cellulase activity was generally higher with SSF than SmF. Crude enzyme filtrates of *P. citrinum*, produced the highest cellulase activity of 0.070 $FPUml^{-1}$ and 0.080 $FPUml^{-1}$, while *F. oxysporium* had the lowest cellulase activity of 0.047 $FPUml^{-1}$ and 0.051 $FPUml^{-1}$ in SmF and SSF respectively. *Penicillium. citrinum* was found to be a good prospect in cellulase production, in which case rice bran a suitable inexpensive substrate for its production.

Keywords: wild-type fungi, cellulase, rice bran, solid state fermentation, agro-waste, submerged fermentation

*Corresponding Author: Omojasola, P.F.
Email: jasola@unilorin.edu.ng

1. Introduction

Increasing demand and the rising cost of fuels, emission of greenhouse gases and air pollution by incomplete combustion of fossils fuels have shifted global efforts to the utilization of renewable resources for the production of "greener" energy replacement (Maki *et al.,* 2011). A large proportion of agricultural, industrial and urban wastes consist of lignocellulosic materials. Lignocellulosic biomass is a renewable, abundant and inexpensive resource for bioconversion to biofuels and bio-products (Raji and Balogun, 2003). It is comprised mainly of cellulose which is the most abundant organic polymer on earth, hemicellulose and lignin.

Cellulose is the principal constituent of the cell wall of most terrestrial plants. It is found in the microfibrils which form the structurally strong framework within the cell walls (Sethi *et al.,* 2013). It is a linear, unbranched polysaccharide consisting of glucose subunits joined together via β-1,4 glycosidic linkages. Individual cellulose molecules vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2002). Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently. Cellulose containing wastes include agricultural residues which pile up to become a nuisance in the environment, used as animal feed or are burned.

Cellulases are enzymes capable of degrading cellulose and represent a multienzyme system composed of several enzymes with numerous isoenzymes which act in synergy (Jahangeer *et al.,* 2005). The basic enzymatic process for the depolymerization of cellulose requires three types of enzymes: endoglucanase which hydrolyzes internal β-1,4 glucan chains of cellulose; exoglucanase which removes cellobiose from the non-reducing end of the cello-oligosaccharide and acid or alkali treated cellulose; and cellobiase or β-glucosidase which hydrolyzes cellobiose to yield molecules of glucose which completes the depolymerization of cellulose (Lynd *et al.,* 2002). This enzyme is produced by several microorganisms, both bacteria and fungi. Cellulases are used in the textile industry for cotton softening; in laundry detergents for colour care and cleaning; in the food industry for mashing; and in the pulp and paper industry for drainage improvement and fibre modification (Cherry and Fidants, 2003; Sethi *et al.,* 2013).

Rice is the seed of the monocot plants *Oryza sativa* Linn (Asian rice) or *Oryza glaberrima* Linn (African rice). Rice is the most important tropical cereal. After wheat, it is the most

widely cultivated cereal in the world and is the most important food crop for almost half the world's population. It is the agricultural commodity with the third highest worldwide production after sugarcane and maize (FAOSTAT, 2012). The production statistics of the paddy rice has been on increase worldwide. It has been estimated that 738.1 million metric tonnes of paddy rice was produced worldwide in 2012 with Nigeria producing 4.8 million tonnes (FAOSTAT, 2012). For every 100 kg of paddy rice, 18-20 kg of rice bran is generated. The fibrous bran of rice is rich in lipids; 12-14% protein, 19-22% lipids, 50% carbohydrates (21% fiber), the B vitamins; thiamine, riboflavin, and niacin, and the minerals; iron, phosphorus, and potassium (Kaurr *et al.,* 2011). Regarding its constitution, rice bran contains the main nutrients for microbial growth. Therefore, it may serve well as a substrate for microbial fermentation to add value to rice bran.

Microbial utilization of the inexhaustible cellulosic biomass for production of industrial chemicals and production of cellulose polymers will help to meet energy and food demands, and such potential applications could help to solve modern waste disposal problems, alleviate shortage of food, animal feed and reduce man's dependence on fossil fuels (Chandel *et al.,* 2013).

The present study involves isolation and screening of wild types of fungi for cellulase activity using the agro waste rice bran as fermentation substrate and the evaluation of cellulase production by solid state and submerged fermentations.

2. Materials and Methods

Isolation and Screening of Cellulase Producing Fungi

Cellulase producing fungi were isolated from rice bran (RB) samples collected from a local rice mill in Ojagboro market, Ilorin, Kwara State, Nigeria. The RB samples were inoculated on sterile potato dextrose agar (PDA) and incubated for 7 days at 30ºC. Colonies were subcultured to obtain pure cultures. Stock cultures were maintained on PDA at 4ºC. Cellulase-producing fungi were screened on selective carboxymethyl cellulose agar containing Mandel's mineral salt medium g/L solution: $NaNO_3$ 2.0, KH_2 PO_4 1.0, $MgSO_4$ $.7H_2O$ 0.5, KCl 0.5, carboxymethyl cellulose sodium salt 10.0, peptone 0.2, agar 17.0. Plates were spot inoculated with spore suspension of the pure cultures and incubated at 30ºC. After 7 days, plates were flooded with 1% Congo red dye for 30 minutes, followed by destaining with 1 M NaCl for 20 minutes. The diameter of zone of decolourization around each colony

was measured. The fungal colonies showing largest zone of decolourization were selected for cellulase production. *Aspergillus niger* (ATCC 16404) collected from Federal Institute of Industrial Research Oshodi (FIIRO) Lagos was used as the control organism

Fungal Identification

The isolated fungi were presumptively identified by means of colonial and morphological examination and characterization. Hyphae were stained with lactophenol cotton blue stain and viewed under the microscope (Olympus, England). Identification was done with the aid of a mycological atlas (Onions *et al.*, 1981).

Preparation of Medium for Enzyme Assay

Ground rice bran served the source of carbon and was added to Mary Mandels' Mineral Salts Medium which was used for inocula development and served as the fermentation medium. The medium was sterilized at 121^0 C for 15 min in an autoclave. The final pH was adjusted to 4.8 using a pH meter (Denver Instruments Model 20 pH/Conductivity meter).

Solid State Fermentation (SSF)

Three grams of RB was placed separately in 250 ml flasks and Mandel Mineral Medium (Peptone,1g ; $(NH_4)_2SO_4$, 1.4g ; KH_2PO_4, 2g ; CaCl2, 0.3g ; $MgSO_4.7H_20$, 0.3g ; Urea, 0.3g ; *trace element solution (2.5g $FeSO_4$; 0.98g $MnSO_4.H_2O$; 1.76g $ZnSO_4.H_2O$; 1.83g $CoCl_2.6H_2O$ dissolved in 495 ml of distilled water and 5ml of conc. HCl), 1ml ; pH 4.8 (Jeffries, 1996).

***Trace element stock solution (1ml is used per L).**

Mineral salts medium was prepared, sterilized at 121°C for 15 min and cooled to room temperature (28 ± 2^0 C). Each inoculum consisted of 2 ml of spore suspension containing approximately (10^6 spores/mL) was added separately, mixed well and incubated at 28± 2 °C in a humidified incubator for 120 h. The flasks were periodically mixed by gentle shaking.

Submerged Fermentation (SmF)

Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of fermentation medium. Three grams of the RB was added to the medium which was sterilized by autoclaving at 121ºC for 15 min. Each flask was inoculated separately with 2ml of spore

suspension containing approximately (10^6 spores/mL) of the inoculum. The cultures were incubated on a rotary shaker (60 rpm) at 28± 2ºC for 120 h.

Enzyme Extraction

At the end of the fermentation, the culture from submerged fermentation was centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant was used as a source of extracellular enzyme. In solid state fermentation, the enzyme was extracted by mixing homogenously the entire waste with 30 ml of 0.2M acetate buffer at pH 4.8 and agitated on a rotary shaker (120 rpm) at 28± 2 ºC with a contact time of 30 min. Whatman No1 filter paper was used to filter the extract and pooled extracts were centrifuged at 4000 rpm for 10 minutes and to remove fungal cells, using a table top high speed refrigerated centrifuge H1850R at 4°C .The cell free supernatant was then used for further analysis.

Total Cellulase assay: Filter Paper Assay

Cellulase activity was evaluated by the method of Ghose (1987). An aliquot of 0.5 ml of cell-free culture supernatant was transferred to a clean test tube and 1 ml of sodium citrate buffer (pH 4.8) was added. Whatman no. 1 filter paper strip (6cm x 1cm) was added to each tube. Tubes were centrifuged and incubated in a water bath at 50^0C for 60 min followed by the addition of the DNS reagent. Mixture was placed in boiling water for 5 min then cooled in an ice-bath. Thereafter 15 ml of distilled water was added to each tube and absorbance taken at 550nm. Cellulase activity was expressed in terms of filter paper unit (FPU) per ml of undiluted culture filtrate. A filter paper unit (FPU) is defined as mg of reducing sugar liberated in one hour and calculated by comparing the absorbance at 550nm with that of a standard curve.

1.0 mg glucose = 1.0/0.18x0.5x60 mo1 mm ^{ml} 1 substrate cleavage

= 0.185 units min^{-1}

FPU was then calculated: FPU = mg glucose released x 0.185

Statistical analysis

Analysis of variance (ANOVA) was performed on all data with Statistical package, SPSS 15.0. The mean values were compared by the least significant difference (LSD) test at 5% level of confidence. All experiments were performed in triplicate.

3. Results and Discussion

Isolation of Fungi

A total of 10 fungi belonging to five genera were isolated from the rice bran waste. The isolates were strains of *Aspergillus, Penicillum, Saccharomyces*, *Rhizopus* and *Fusarium*.

Screening of Fungal Isolates for Cellulase Activity

The absence of clear zones in Plate A indicated absence of cellulase activity; whereas presence of clear zones as shown in Plates B and C around the fungal colonies indicated cellulase activity. Seven fungal isolates which showed zones of clearance greater than the control organism (36.0 mm) were selected for further study (Table1).

Characterization of Fungal Isolates

The identification of cellulase producing fungi was performed by studying their morphological and colonial characters. Morphological characters of cellulase positive isolate with zones of clearance greater than the control are shown as Table 2.

Table 1: Cellulase activities of fungi isolated from rice bran

Fungal Isolates	Isolate code	Presence of clearance zone	Mean Diameter of clearance zone (mm)
Aspergillus spp	RB_3	+	37.5±2.50
	RB_5	+	31.0±1.0
	RB_7	+	44.0±1.0
	RB_8	+	41.0±8.0
Penicillium spp	RB_1	+	37.5±2.50
	RB_2	+	43.5±8.50
Saccharomyces spp	RB_{10}	_	0.0
	RB_4	+	61.0±1.0
Rhizopus spp	RB_9	_	0.0
Fusarium spp	RB_6	+	39.5±0.5
Control	Cp	+	36.0±4.0

Key: + positive; - negative Values represent means of 4 replicates

Table 2: Colonial and cellular morphology of selected isolated cellulolytic fungi

Isolates	Colonial Characteristics				Morphological Characteristics			Identity
	Appearance	Shape	Colour	Colour of Reverse	Hyphae	Spore Shape	Spore Texture	
RB_1	Powdery /compact	Round	Green	Yellowish Blue	Septate	Elliptical	Smooth	*Penicillum citrinum*
RB_2	Powdery /compact	Round	Green	Yellow	Septate	Elliptical	Rough	*Penicillium chrysogenum*
RB_3	Floccose /compact	Round	Black	Cream	Septate	Globose	Rough	*Aspergillus niger*
RB_4	Dull	Round	White	Whitish	Large globose	Blastoconidia	-	*Saccharomyces cerevisiae*
RB_6	Floccose /compact	Round	White	Cream	Septate	Globose	Rough	*Fusarium oxysporium*
RB_7	Velvety	Round	Yellowish Green	Yellow	Septate	Globose	Rough	*Aspergillus flavus*
RB_8	Floccose /compact	Round	Black	Cream	Septate	Globose	Rough	*Aspergillus niger*

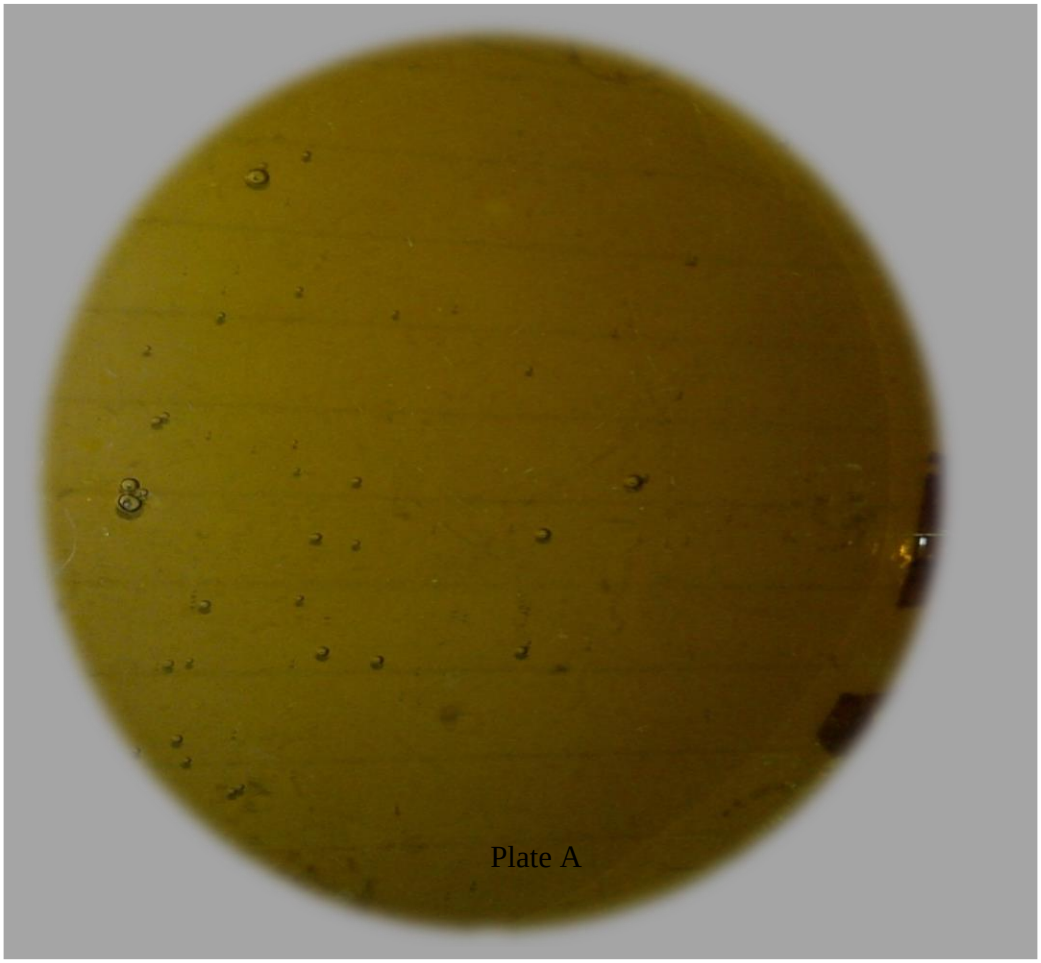
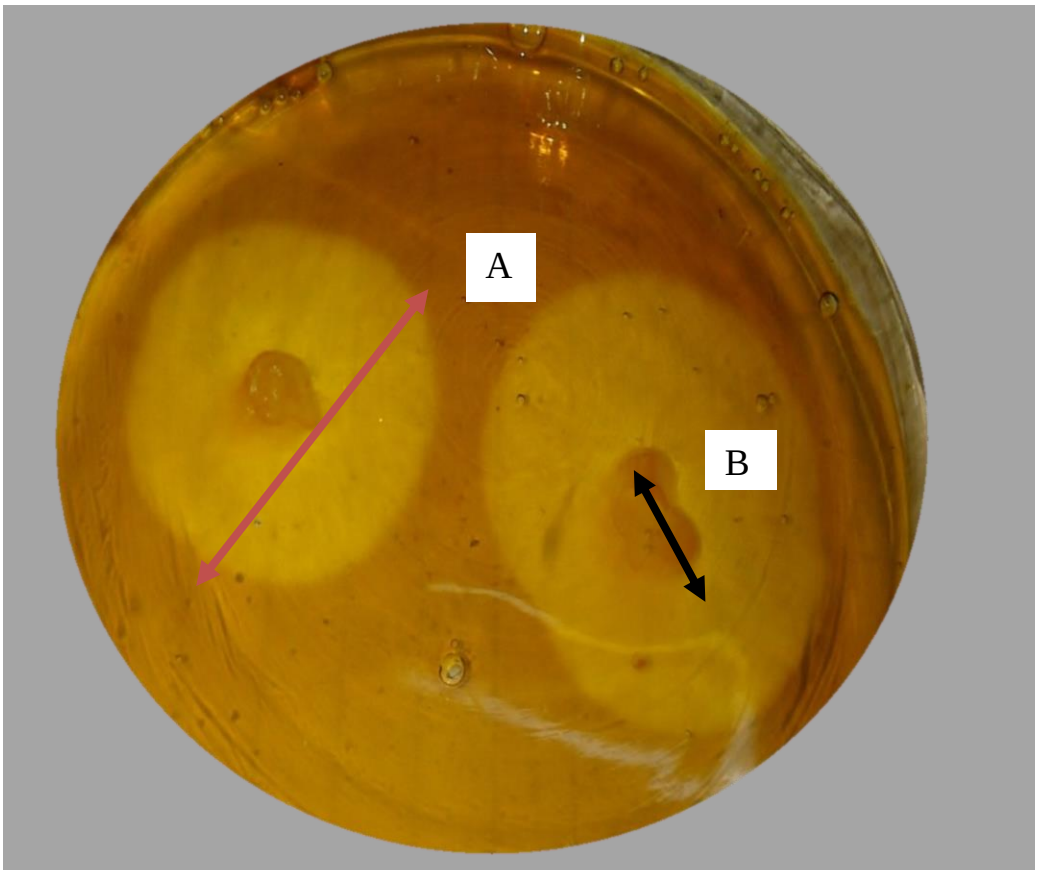


Plate A

Plate A: Fungal isolate with no clearance zones



Key: A: Clearance zone ; B: Fungal colony

Plate B: *Aspergillus niger* showing zones of clearance

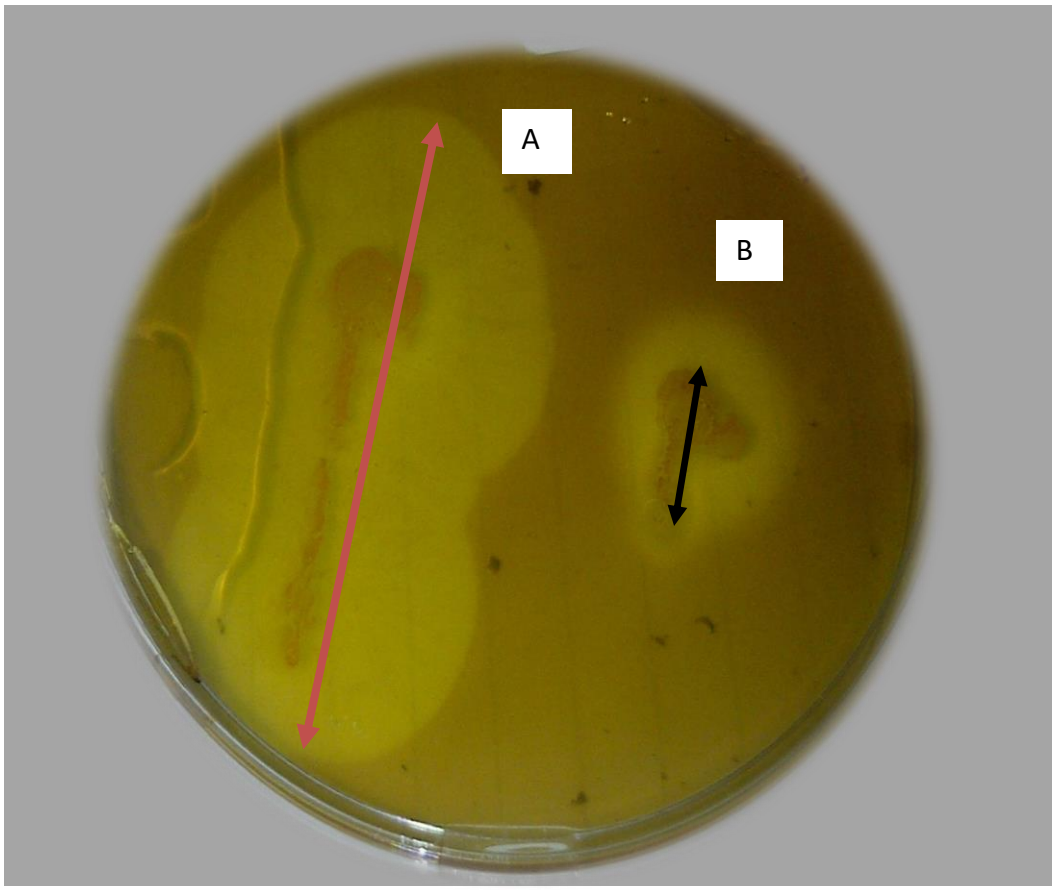


Plate C: *Saccharomyces cerevisae* showing zones of clearance

Key: A: Clearance zone ; B: Fungal colony

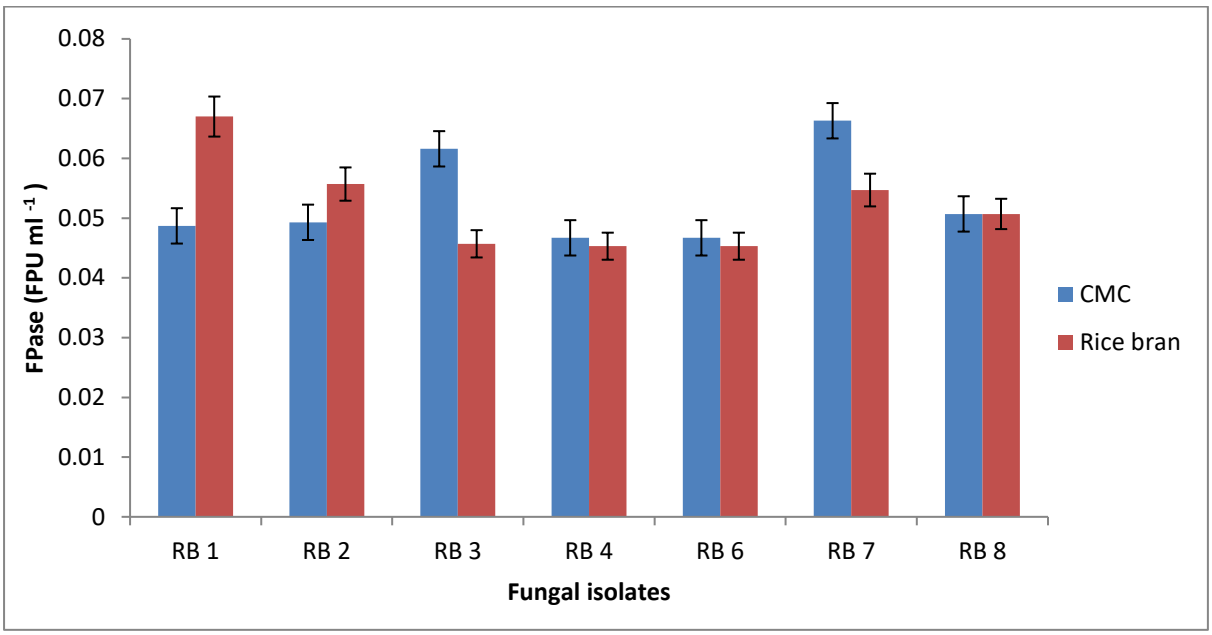


Figure 1: Cellulase activity of selected fungal isolates using CMC and rice bran as carbon source in submerged fermentation
Key: RB_1 : *P. citrinum*; RB_2: *P. chrysogenum*; RB_3: *A. niger*; RB_4: *S. cerevisiae*; RB_6: *F. oxysporium*, RB_7, *A. flavus*; RB_8, *A. niger*

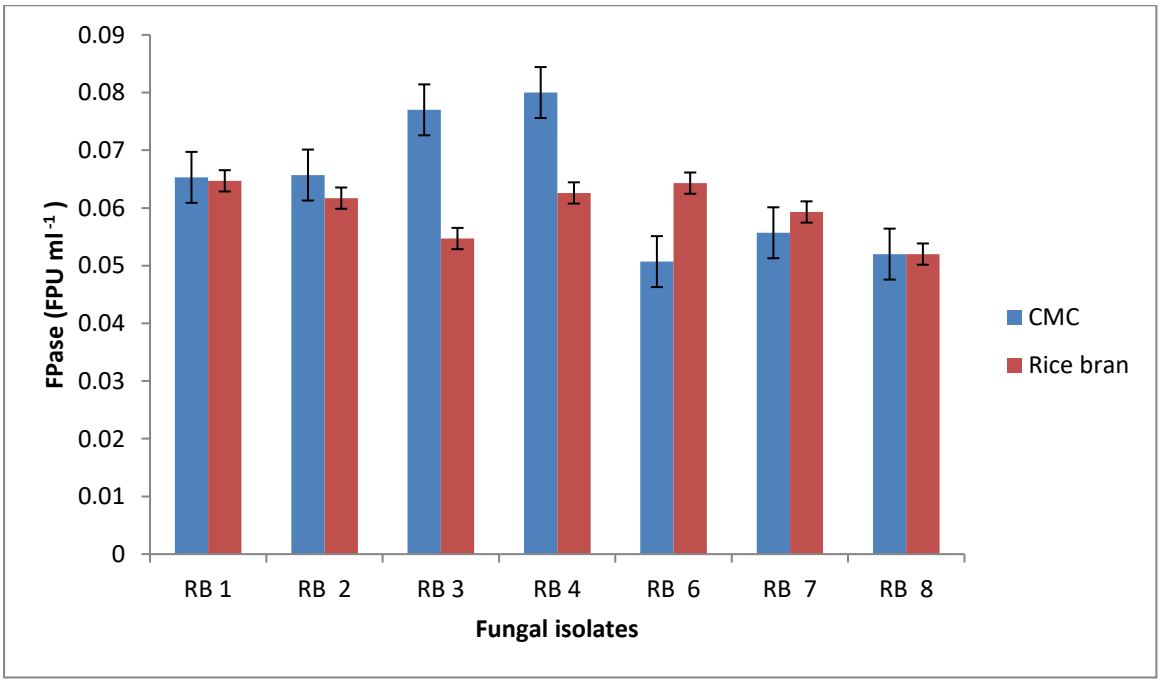


Figure 2: Cellulase activity of selected fungal isolates using CMC and rice bran as carbon source in solid state fermentation
Key: RB_1: *P. citrinum*; RB_2: *P. chrysogenum*; RB_3: *A. niger*; RB_4: *S. cerevisiae*; RB_6: *F. oxysporium*, RB_7, *A. flavus*; RB_8, *A. niger*

Cellulose is the most abundant organic polymer on earth and comprises a major storage form of photosynthesized glucose. Fungi are well known agents of decomposition of organic matter in general and cellulosic substrates in particular. Cellulase producing fungi have been isolated from various biomass wastes such as wheat agro wastes, orange wastes, mangrove leaves, corn cob and mushrooms (Jahangafeer *et al.,* 2005; Omojasola *et al*., 2008; Saliu and Sani, 2012; Chandel *et al.,* 2013). Cellulase producing fungi have also been isolated from the digestive system of termites and some vertebrates. *Fusarium culmorum* and *Penicillium crustosum* isolated from the gut of a wood inhabiting beetle, *Saperda vestita* were found to be highly cellulolytic (Delalibera *et al.,* 2005). Rice bran is one of the most abundant and locally available agricultural wastes which contain 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 (Oshoma and Ikenebomeh 2005). In addition, Nigeria is reported to have produced 4.8 million metric tonnes of rice in 2012 (FAOSTAT, 2012) therefore the rice bran waste is extensively available.

The most extensively studied cellulase producer is *Trichoderma* spp. (Jahangafeer *et al.,* 2005). However, this fungus was not isolated in this study. Its absence may be due to its suppression by other rapidly growing fungi such as *Aspergillus, Rhizopus, Fusarium* and *Penicillium* isolated in this study (Table 1). *Aspergillus* spp. was the dominant fungi isolated in this study and represents 40% of the total fungi isolated (Table 2). *Aspergillus* spp. have been reported to possess all components of the cellulase enzyme system (Vries and Visser, 2001) which may account for their dominance as recorded in this study. Other common highly efficient cellulase producers are *Fusarium, Rhizopus,* and *Penicillium* (Vries and Visser, 2001). Results of this study showed that *Penicillium citrinum* recorded the highest cellulase activity followed by *P. chrysogenum, Fusarium oxysporium* with *Aspergillus* spp recording the lowest cellulase activity (Figs 1 and 2). Other workers using different substrates have recorded high cellulase activity species of *Aspergillus, Trichoderma* and *Penicillium* (Khokhar *et al.,* 2012).

Cellulase activity in SSF culture by the organism was higher than that obtained by SmF (Figs 1 and 2). This may be due to the process of SSF in which filamentous fungi produce hyphae which have the natural ability to cover the nutritive surface of the substrate and even enter its pores, thus become strongly attached to the substrate. The easy growth of filamentous fungi on solid media relies on the high capacity of hydrolytic enzymes synthesis in the media along with a high content of polymerized sugars which are the inducers of gene expression of these enzymes (Sachslehner *et al.,* 1998, Kilikian *et al.,* 2014). In addition, substrates of SSF

besides being strong inducers of cellulase also induce hemicellulases such as xylanases and ligninases, if the substrate is composed of hemicellulose and lignin (Sachslehner *et al.,* 1998; Acharya *et al.,* 2008). Also, several workers proposed the use of SSF for cellulase production, using different solid agricultural and agro-industrial residues as substrates such as wheat bran and soy bran (Philippidis, 1994).

4. Conclusion

In this study, out of the ten cellulolytic fungi isolated from rice bran, *P. citrinum* has the highest total cellulase activity. Higher level of cellulase activity was obtained by SmF compared to SSF. The use of rice bran for cellulase production will not only reduce the production costs of the enzyme but also help decrease pollution-load due to the agro-industrial waste.

References

Acharya, P. B., Acharya, K. and Modi, H. A. (2008): Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate. *Afr. J. Biotechnol.* **7** (22), 4147-4152.

Chandel, K., Savita, J., Vandna, K., Shilpa, S., Ankita, S., Deep, K. and Naresh, K. (2013): Isolation, Purification and Screening of Cellulolytic Fungi from Mushroom Compost for Production of Enzyme (Cellulase). *Int. J. Curr. Res.* **5**(1), 222-229.

Cherry, J.R. and Fidants, A.I. (2003): Directed Evolution of Industrial Enzymes: An Update. *Curr. Opin. Biotechnol.* **14**(4), 438-443.

Delalibera, I., Handelsman, J. and Raffa, K. F. (2005): Contrasts in Cellulolytic Activities of Gut Microorganisms between the Wood Borer, *Saperda vestita*, the Bark Beetles, *Ipi pini* and *Dendrocttonus frontalis. Environ. Ent.* **34**(3), 541-547.

FAOSTAT, (2012): Final 2012 Data and Preliminary 2013 Data for 5 Major Commodity Aggregates. Http://faostat.fao.org/site/339/default.aspx. Retrieved April 30 2015.

Ghose, T.K. (1987): Measurement of Cellulase Activities. *P. Appl. Chem.* **59**(2), 257-268.

Jahangeer, S., Khan, N., Jahangeer, S., Soail, M., Shahzab, S., Ahmad, A. and. Khan, S.A. (2005): Screening and characterization of fungal Cellulases isolated from the native environmental source. *Pakist. J. Bot*., **37**(3), 739-748.

Jeffries, T.W. (1996): Production and Applications of Cellulase Laboratory Procedures Handbook, US Army Materials Laboratories. 10pp.

Kaurr, P.P., Arneja, J.S. and Singh, J. (2011): Enzyme hydrolysis of rice straw by crude cellulose from *Trichoderma reesei*. *Bioresour Technol* **66**, 267-269.

Khokhar, I., Haider, M.S., Mushtaq, S. and Muhhtar, I. (2012): Isolation and Screening of Highly Cellulolytic Filamentous Fungi *J. Appl. Sci. Environ. Mgt* **16**(3), 223-226.

Kilikian, B.V., Afonso, L.C., Souza, T.F.C., Ferreira, R.G. and Pinheiro, I.R. (2014): Filamentous Fungi and Media for Cellulase Production in Solid State Cultures. *Braz. J. Microbiol.* **45**(1), 279-286.

Lynd, L.R., Weimer, P.J., van Zyl, W.H. and Pretorius, I.S. (2002): Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiol. Mol. Biol. Rev.* **66**, 506-577.

.

Maki, L.M., Michael, B., Leung, K. T., Qin, W. (2011): Characterization of some efficient cellulase producing bacteria isolated from paper mill sludge and organic fertilizers. *Int J. Biochem. Mol. Biol*. **2**(2), 146-154.

Omojasola, P. F., Jilani, O.P. and Ibiyemi, S.A. (2008): Cellulase Production by some Fungi Cultured on Pineapple wastes. *Nat. Sci.* **6**(2), 64-79.

Onions, A. H. S., Allsopp, D. and Eggins, H. O. W. (1981): Smiths Introduction to Industrial Mycology. Edward Arnold, London, 398.

Oshoma, C.E. and. Ikenebomeh, M.J. (2005): Production of *Aspergillus niger* Biomass from Rice Bran. *Pakist. J. Nutr.* **4** (1), 32-36.

Philippidis, G.P. (1994): Cellulase Production Technology In: Enzymatic Conversion of Biomass for Fuel Production. (Eds.): M.E. Himmel *et al.*, *ACS Symposium Series* **566**.

Raji, A.I. and Balogun, M.A. (2003): Waste paper as a potential substrate for microbial cellulase production. *Nig. J. Pure Appl. Sci.* **18**, 1334-1339.

Sachslehner, A. Nidetzky, B. Kulbr, K.D. and Haltrich, D. (1998): Induction of Mannanase, Xylanase and Endoglucanase Activities in *Sclerotium rolfsii Appl. Environ. Microbiol.* **64**, 594-600.

Saliu, B.K. and Sani, A. (2012): Bioethanol potentials of corn cobs hydrolysed using cellulases of *Aspergillus niger* and *Penicillium decumbens*. *EXCLI J.* **11**, 468-479.

Sethi, S., Datta, A., Gupta, B. L. and Gupta, S. (2013): Optimization of Cellulase Production from Bacteria Isolated from Soil. *ISRN Biotechnology* vol. **2013**, Article ID 985685, 7 pages, doi: 10.5402/2013/985685.

Vries, R.P. and Visser, J. (2001): *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharide. *Micro. Mol. Bio. Rev*. **65**, 497-522.

Walsh, G. (2002): Industrial enzymes: proteases and carbohydrases. In: *Proteins; Biochemistry and Biotechnology*. John Wiley and Sons, UK.