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Isolation of Cellulose Degrading Microorganisms from Agro-Wastes for Industrial Utilisation.

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Abstract

Cellulose is a simple polymer, which forms insoluble crystalline micro fibrils that are highly resistant to enzymatic hydrolysis. Cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, the most abundant carbohydrate produced by plants. Twelve cellulose degrading microorganisms were isolated from different substrates: decomposing corn cob and corn husk, decomposing saw dust from timber shades and poultry beddings. The isolation of cellulose degrading bacteria (CDB) and fungi was done by inoculating 0.1ml aliquots of serially diluted samples on Cellulose agar medium, Nutrient agar, Potato dextrose agar and cellulose Congo-red agar. Cellulose activity of the organisms was determined by diameter of clear zones around the colonies on Cellulose Congo red agar medium. The highest zone of clearing for bacteria was seen with *Cellulomonas*, 4.10mm; followed by *Bacillus*, 3.7mm; *Pseudomonas* 3.1mm and *Staphylococcus* 2.8mm etc. The highest for fungal isolates was *Aspergillus*, 16.40mm; *Penicillum*, 14.70mm and *Mucor*, 12.4mm. Most of the biological conversions are aimed at changing cellulose into ethanol by fermentation, to replace fuel currently supplied by petroleum. These applications are based on the modification of cellulose and hemicelluloses by partial hydrolysis. Total hydrolysis of cellulose into glucose, which could be fermented into ethanol, isopropanol or butanol is not yet economically feasible. However, the need to reduce emissions of greenhouse gaseprovides an added incentive for the development of processes for generating biofuels from cellulose, a major renewable carbon source.

Key words: Cellulose, conversion, clear zones, renewable, biofuel.

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Introduction:

Lignocellulosic biomass offers a great potential as biomass sources for bioethanol production. They include cereal husks and hulls, corn cobs, wood dropping or residues (sawdust), and animal excreta. They are materials containing cellulose and lignin which are formed during photosynthesis. They are defined as natural, abundant, renewable and cheap sources of energy. It is one way to reduce both the consumption of crude oil and environmental pollution (Srivastava and Agrawal, 2012; Bhatia *et al*., 2014). Lignocellulosic biomass is composed primarily of carbohydrate polymers (≈ 45% of dry weight cellulose and ≈30% of dry weight hemicellulose) and phenolic polymers (≈25% of dry weight lignin). Cellulose $(C_6H_{10}O_5)$ n is a linear polysaccharide polymer of glucose made of cellobiose units that are packed by hydrogen bonds. The structure of this polymer is rigid and compact, so the biomass needs pretreatment that breaks its structure to facilitate the action of enzymes. The individual cellulose chains are packed and organized into crystalline microfibrils. Within these microfibrils, cellulose is found in two forms, namely amorphous and crystalline. The crystalline form of cellulose is very difficult to degrade (Bhatia, *et al.*, 2014).

Cellulose, the most abundant polymer on earth is composed of thousands of molecules of anhydroglucose linked by β(1, 4) - glycosidic bonds.Cellulose can be effectively hydrolyzed and depolymerized into fermentable sugars by the enzyme cellulase (Srivastava and Agrawal, 2012). A number of microorganisms are capable of producing extracellular cellulase enzyme and among which fungi are the widely used candidates for cellulase enzyme production. Currently, most commercial cellulases are produced from *Phanerochaete* species, *Aspergillus* species and *Trichoderma reesei*, usually used to describe a mixture of cellulolytic enzymes whose synergistic action is required for effective breakdown of substrate to its monomeric units (Srivastava and Agrawal, 2012). The action of cellulases involves the concerted action of (i) endoglucanase(s), which randomly attacks the internal, β(1,4)- linkages, (ii) Cellobiohydrolase, which cleaves off cellobiose units from the non reducing ends of the glucan,

and (iii) β 3- glucosidase, which hydrolyzes cellobiose to glucose (Srivastava and Agrawal, 2012).

Hemicellulose, such as xylan (C_5 H_8 0_4) n is a short polymer of pentose and hexose sugars.The dominant sugars in hemicelluloses are mannose (six-carbon sugar) in soft woods and xylose (five carbon sugars) in hardwoods and agricultural residues (Bhatia *et al.,* 2014). Hemicellulose also contains galactose, glucose and arabinose. This polymer is amorphous and easier to hydrolyse than cellulose. Lignin [(C_9 H_{10} O_3) (OCH_3)] n is a phenyl propane polymer that contains many functional groups such as hydroxyl, methoxyl and carbonyl. Unlike cellulose and hemicellulose, lignin cannot be utilized in the fermentation process. In fact, it has high resistance to chemical and enzymatic degradation (Bhatia, *et al.,* 2014).

The main technological barrier that impedes the widespread utilization of lignocellulosic biomass for production of fuels and other commodity product is the lack of low-cost technologies to overcome the recalcitrance of lignocellulose. The conversion of lignocellulosic biomass into ethanol requires pretreatment step to change the physical and chemical structure of biomass and to enhance the hydrolysis rate (Bhatia, *et al.,* 2014**;** Srivastava *et al*., 2012). Numerous pretreatment strategies have been developed to enhance the reactivity of cellulose and to increase the yield of fermentable sugars. The aim of this study was to isolate cellulose degrading microorganisms from agro wastes.

Materials and Methods:

Samples of corn cob and corn husk were collected from dumped areas at Naze Owerri, Imo and Umuahia, Abia States respectively. Decomposing Sawdust from timber wood was collected in a sterile container from Timber Market at Weathral road,Owerri. Decomposing Sawdust from Poultry farm was collected in a sterile container from Emma-Rich Commercial Poultry Distributors at Naze Town, Owerri.

Isolation of Microorganisms:

Basal salt medium was used for the isolation of cellulolytic bacteria. It serves as buffer to sustain the cellulose degrading microorganisms.

Composition of Basal salt medium: $NaNO_3$ 2.5g, KH_2PO_4 2.0g, $MgSO_4$ 0.2g, NaCl 0.2g, $CaCl.6H_2O$ 0.1g, Distilled water 1000ml/1 Litre and Filter paper(Whatman no.1).

The mixture was sterilized by autoclaving at 121^0C for 15 minutes. The medium was allowed to cool before aseptically dispensed into bijoux bottle and allowed to set before labeling.

One gram each of the ground corn cobs, corn husk and saw dust was inoculated into sample bottles containing the basal salt medium. After which the culture was incubated for 7 days in a shaker incubator at 37^0C at 100rpm.The ones that turned cloudy were sub cultured on Cellulose agar medium for 48 hours at 37^0C.Growth on Cellulose medium was confirmed with zones of clearing on Congo red cellulose agar medium(Pratima *et al*.,2012).

Cellulose agar medium composition: KH_2PO_4 0.5g, MgSO4 0.25g, Cellulose 2.0g, Agar 15g, Gelatin 2g, Distilled water 1000ml/ 1 Litre.

Composition of Cellulose Congo –red agar medium: KH_2PO_4 0.5g, $MgSO_4$ 0.25g, Cellulose 2.0g, Agar 15g, Congo-red 2.0g, Gelatin 2.0g, Distilled water 1000ml/1Litre.

The use of Congo-red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria (Pratima,*et al*.,2012).

Colonies showing discolorations of Congo-red were taken as positive cellulose degrading colonies and only these were taken for further study.

Further clarification was done by inoculation of samples on Nutrient agar and Potato Dextrose agar. One gram each of samples was weighed and dissolved in 9mls of distilled water in different test tubes and labeled. Ten- fold serial dilutions were carried out. This was done up to 10^{-7} dilution. Then 0.1ml aliquot each from 10^{-5} to 10^{-7} dilutions were plated out on nutrient and potato dextrose agar plates using pour plate method. The colonies were incubated at 37^OC for 24hrs for nutrient agar and at 25^0C for 72 hours for potato dextrose agar (PDA). The purified colonies were stored in nutrient agar slants prior to screening for cellulase production and further identification.

Colonies that developed on Nutrient agar plates were sub-cultured using the streak plate method to obtain pure cultures and the zones of clearing of isolates on the Congo red cellulose medium (Confirmation test) were measured.

Identification of Isolates:

Colonial, morphological, microscopic and biochemical identification were used to identify the cellulolytic microorganisms present in the samples. Isolates were subjected to the following tests:

Gram staining, Catalase test, Indole, Methyl red and Voges Proskauer tests, Citrate test, Motility and Sugar fermentation tests (Cheesbrough, 2005).

Sugar Fermentation Tests:

Each test organism was tested for its ability to produce acid with or without gas from different sugars. The test was carried out using the following sugars: - glucose, maltose, lactose, mannitol and sucrose. Nutrient broth medium was prepared by incorporating 0.5% of each of the test sugars separately and a few drops of 0.01% phenol red indicator. The tubes containing the mixtures were labeled and the corresponding test organisms were inoculated and incubated at 30^0C for 24 hours. The tubes were examined daily for color change from red to yellow which shows the presence of acid (produced from the sugar utilized). Also the Durham tubes were examined for the presence of air spaces which also shows gas production for the test organisms (Cheesbrough, 2005; Obire, 2005).

Results and Discussion:

Isolation and Screening of Cellulose Degrading Microorganisms:

A total of twelve isolates was found to be positive on screening for cellulose degrading activity in this study. Eight bacterial species were identified, while four were fungi. The number of isolates and their percentage occurrence is shown in Table 1.0. Amonsgt the bacteria include: *Bacillus* species, *Streptococcus* spp, *Staphylococcus* spp, *Micrococcus* spp, *Cellulomonas* spp, *Pseudomonas* spp, *Escherichia coli*, and *Klebsiella* spp. Fungi had *Penicillum* spp, *Aspergillus*, *Mucor* and *Rhizopus* species isolated. This result corroborates previous reports from Akpomie *et al*., (2013) and Jones and Lee, (2008). *Escherichia coli* had the highest percentage occurrence of the isolates with 13.47%, followed by *Bacillus* spp, 11.22%, and *Streptococcus* spp 10.97% .Amongst the fungal isolates, Mucor spp had the highest percentage of occurrence of 6.98%, followed by *Aspergillus* spp with 5.49%.

Table 1.0: Microorganisms isolated from the samples and their Percentage Occurrence

Microoganism	No. of Isolates	Percentage Frequency (%)
Bacillus spp	45	11.22
Streptococcus spp	44	**10.97**
Staphylococcus spp	**42**	**10.47**
Micrococcus spp	36	8.98
Cellulomonas spp	**39**	**9.73**
Pseudomonas spp	**39**	**9.73**
Escherichia coli	**54**	**13.47**
Klebsiella spp	**32**	**7.98**
Penicillum spp	**10**	**2.49**
Aspergillus spp	**22**	**5.49**
Mucor	28	6.98
Rhizopus	**10**	**2.49**
Total	**401**	**100**

The following bacteria: *Bacillus* spp, *Streptococcus* spp,*Micrococcus* spp, *Staphylococcus spp, Aspergillus spp, Rhizopus spp and Mucor spp* have been implicated in the degradation of cellulose (Schwarz, 2001). *Streptoccocus* spp are the cellulolytic microorganisms that are associated with the possession of complex cellulase enzyme system (Schwarz,2001), while *Aspergillus* spp is an important soil fungus having a great potential of producing a range of primary and secondary metabolites, including cellulase enzymes as well as an aromatic water – soluble product which can repress the cellulolytic action of the enzyme(Howard, 2003). Since it is known that these microorganisms can degrade or utilize cellulose, they can be used to turn cellulosic waste in the

environment to beneficial products such as fertilizer and as a soil conditioner so as to reduce environmental hazards caused by some cellulose wastes (Anbuselvi *et al*., 2009). In addition, these organisms can be explored for bio-fuel production, and can also be used to reduce environmental nuisance caused by cellulose wastes.

Confirmation of Cellulase activity:

Isolates were confirmed of cellulolytic activity by growing them on Cellulose Congo-red agar medium and measuring the zones of clear growth as shown in Table 2.0. *Cellulomonas* spp had the highest diameter of zone of clearing of 4.1mm, followed by *Bacillus* spp with 3.7mm, and *Pseudomonas* spp 3.1mm among others. Fungal species tend to have greater cellulolytic abilities than bacteria (Akpomie *et al*., 2013). Isolates from this study presented *Aspergillus* spp with the highest diameter of clearing of 16.4mm, followed by *Penicillum* spp 14.7mm, *Mucor* spp 12.4mm and *Rhizopus* spp 11.0mm.This result establishes the fact that these microorganisms can decompose the substrates used in this study from which they were isolated: namely corn cobs and husks from yellow and white maize eaten in Imo and Abia States, respectively; and decomposing saw dust from timber shades and poultry beddings. This conforms to the study of Lennox *et al.*, (2010) and Srivastava and Agrawal,(2012).Most of the isolates were able to utilize glucose, namely: *Cellulomonas* spp, *Streptococcus* spp, *Staphylococcus* spp, *Pseudomonas* spp, *Micrococcus* spp, *Escherichia coli* and *Klebsiella* spp. This is evidence that these isolates are potential agents in the biotransformation of cellulosic wastes to industrial products such as biofuel and biofertilizers, since glucose is the major monomer unit of cellulose (Bhatia *et al*., 2014).

Table 2.0 Diameter of Zones of Clearing of the Celluloytic Organisms (mm)

Cellulolytic Organism	Diameter of Zone of Clearing(mm)
Staphylococcus spp	2.8
Micrococcus spp	2.6
Pseudomonas spp	3.1
Bacillus spp	3.7
Cellulomonas spp	4.1
Escherichia coli	2.2
Klebsiella spp	1.8
Streptococcus spp	2.5
Aspergillus spp	16.4
Rhizopus spp	11.0
Mucor spp	12.4
Penicillum spp	14.7

The production of multi-enzyme cellulase by cellulolytic microorganisms has helped in the biogeochemical cycling of carbon in the ecosphere by degrading cellulosic wastes, humus and materials into simple sugar, utilizable by other organisms into water and carbon-dioxide which is the basis for carbon cycling. Cellulolytic microbes have been found to be important in the provision of simple sugars by degrading cellulosic biomass in nature (Boisset *et al*., 1999).Cellulolytic micro-organisms play an important role in the biosphere by recycling cellulose.

Conclusion:

Cellulolytic microorganisms are found to occur in decomposing sawdust from timber shades and poultry beddings, as well as in decomposing corn cobs and husks where they help to recycle cellulosic biomass into renewable source of energy and as an important component of carbon

cycle. The different sizes of the diameter of zones of clearing on cellulose Congo-red agar medium, with cellulose as the only carbon source indicated differences in the ability of the isolates to produce cellulase enzymes. This assay has proven these micro-organisms to have potential of being utilized in industrial processes. These organisms could also convert voluminous cellulose wastes to useful products like bio-fuel and bio-fertilizers that could benefit the society. These organisms are important as they could play a vital role in the reduction or even in the elimination of cellulosic wastes that constitute a nuisance to our environment if properly harnessed in industrial processes

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