

Research Paper

Screening and identification of mannanase producing fungi isolated from selected agricultural wastes

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Author's contributions

This work was carried out in collaboration between both authors.

Author OOO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author TCE managed the analyses of the study and the literature searches. The authors read and approved the first manuscript.

ABSTRACT

Aim: The study evaluated potential performance of different fungal isolates from agricultural by-products for mannanase production.

Methodology: The associated fungal isolates were isolated on locust bean gum containing agar medium by plate assay techniques and counted by standard microbiological methods. Mannanase production was conducted under submerged state fermentation (shaken & static) into which copra meal had been incorporated as the sole carbon source and enzyme activity determined by dinitrosalicylic acid method.

Results: In this study, 11 fungal isolates showed positive results with clear zone around their cultures. Fungal isolate 5A showed the highest activity ratio of 1.8, while the least was observed in isolate 9A12 with activity ratio of 0.64. The highest fungal counts were recorded in fermented coconut with 7.4×10^2 sfu/g, while cocoa pod and groundnut shell had no fungal growth. In terms of percentage occurrence of fungal isolates from selected agro-wastes, it was revealed that *Rhizopus japonicus* had the highest occurrence of 66.67%, while the same value of 8.33% was observed for *Aspergillus fumigatus*, *A. glaucus*, *R. stolonifer* and *Trichosporonoides oedocephalis*. In fermentation broth, all the 11 isolates displayed mannanase activity which was between 0.370 to 21.667 U/ml for static and 0.278 to 3.982 U/ml for shaken condition, with the highest mannanase activity observed with isolate 5A for both culture conditions. According to the cultural characters and microscopic morphology, the isolate 5A was identified as the *Aspergillus fumigatus*.

Conclusion: In this investigation, fungal isolates evaluated for mannanase production from agro-wastes elaborated considerable mannanase activity and this could be exploited for economic uses.

Key words: *Agricultural wastes, fungal counts, mannanase, shaken and static conditions*

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34 1. INTRODUCTION

35 Mannans function as carbohydrates storage in the bulbs and endosperm of some plants.
 36 Galactoglucomannans and glucomannans in softwoods and hardwoods are both branched
 37 heteropolysaccharides requiring several enzymes for their complete degradation [1]. β -
 38 Mannanase (1,4- β -D-mannan mannanohydrolase; mannan endo 1,4- β -mannosidase; EC
 39 32.1.78) is the enzyme that cleaves the β -1,4-mannosidic linkages of mannans,
 40 galactomannans, glucomannans, and galactoglucomannans [1,2]. The β -mannanases have
 41 been grouped into two families, glycosyl hydrolase 5 (GH5) and glycosyl hydrolase 26
 42 (GH26). The protein folding, catalytic mechanism and mechanism of glycosidic bond
 43 cleavage are conserved in both enzyme families [2]. β -Mannanase, required for the
 44 utilization of various β -mannans, occurs in certain endosperms such as copra and ivory palm
 45 nuts, in the beans of guar, locust, and coffee, and in the roots of konjak [1,3]. This enzyme
 46 has found several industrial applications such as the liquefaction and extraction of fruit and
 47 coffee beans [1]. An important industrial application of mannanases is likely to be seen in
 48 pulp and paper industry. Treating the pulp with β -mannanase alone [2] or in combination
 49 with cellulase-free xylanase improves lignin extraction [4]. In doing so, one can save on
 50 bleaching chemicals and at the same time reduce wastes which may harm the environment
 51 [5]. The bleaching process requires a high temperature and alkaline conditions, but in
 52 industrial applications β -mannanases treatment is done under milder conditions [6].
 53 Many microorganisms have been reported as mannanase producers. Mannanases from fungi
 54 such as *Aspergillus* species [7,3], *Trichoderma reesei* [8], *Sclerotium rolfsii* [9], and
 55 *Penicillium occitanis* [5] have been well described. Fungal mannanases are generally
 56 produced in the presence of different mannan-rich substrates. These included locust bean
 57 gum (LBG) [1], guar gum [10], konjac flour [5], and copra meal [3,11].
 58 Lignocellulose is the major structural component of plant cell walls and is mainly composed
 59 of lignin, cellulose and hemicellulose, and represents a major source of renewable organic
 60 matter. The chemical properties of the components of lignocellulosics make them a substrate
 61 of enormous biotechnological value [12]. Large amounts of lignocellulosic “waste” are
 62 generated through forestry and agricultural practices, paperpulp industries, timber industries
 63 and many agro-industries and they pose an environmental pollution problem. However, the
 64 huge amounts of residual plant biomass considered as “waste” can potentially be converted
 65 into various different value-added products including biofuels, chemicals, and cheap energy

sources for fermentation, improved animal feeds and human nutrients [5]. In this study, the isolation and screening of mannanase producing fungi that may be present in agricultural wastes was investigated.

69

70 **2. MATERIALS AND METHODS**

71

72 **2.1 Materials and chemicals**

73 The coconut residual cakes were collected from farm field in Akure, Ondo State, Nigeria and
74 it was used as a carbon source for medium formulation. The residual were treated with
75 petroleum ether and dried at 60⁰C for 2 hr. After that, the residual were blended, milled and
76 sieved to obtain uniform particle size of 0.5 mm. Locust bean gum was purchased from
77 Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

78

79 **2.2 Sample sources**

80 Twelve agricultural wastes were collected from farm fields, domestic sources and local
81 market. The samples were blended and milled to obtain uniform particle size of 0.5 mm using
82 sieve and these were used as sources for the isolation of mannanase producing fungi.

83

84 **2.3 Isolation and enumeration**

85 The sample, 1 g of solid sample was suspended in 9 ml of sterilized 0.85% normal saline
86 (NaCl). The solution was mixed by vortex for 60 second. One percent (v/v) of the solution
87 was transferred into 20 ml of sterilized isolation medium (IM) [13] with 1% copra meal for
88 fungi. The microbial cells were grown under aerobic condition by shaking at 150 rpm for 24
89 hr at 28±2⁰C.

90

91 **2.4 Fungal identification**

92 Identification of the positive isolates was conducted according to the method designed by
93 [14] on the bases of cultural characters (colour, shape of colony, surface and reverse
94 pigmentation and texture of the colony) as well as microscopic structure (septate or
95 nonseptate hyphae, structure of hyphae and conidia).

96

97 **2.5 Primary screening**

98 The culture broth from enumeration step was serial diluted and spread on isolation medium
99 (FIM) containing locust bean gum instead of copra meal and cells were allowed to grow at 28

$\pm 2^{\circ}\text{C}$ for 3-7 days. The colonies with a clear zone of mannanase activity were observed and the ratio of diameter of clear zone to colony was calculated. The positive isolates were selected and kept for further study.

2.6 Secondary Screening

The positive isolates from primary screening were further screened for their ability to produce mannanase under submerged (static and shaken condition) state fermentation. Mandels and Weber's medium modified by [1] was used for the production of mannan degrading activity. Enzyme production was performed in 250 ml Erlenmeyer flask containing 50ml of enzyme producing medium (PM). This medium contained the following ingredients (g/L): Copra meal 10, NaNO_3 2, KH_2PO_4 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, KCl 0.5, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ traces, pH 6.8. The medium was sterilized at 121°C for 15 mins. After sterilization, each flask was inoculated with 2 discs of 8 mm diameter of the organisms from malt extract agar culture plates using cup borer. The cultures were harvested after 7 days of incubation by centrifugation at 6000 rpm for 15 min at 4°C using refrigerated centrifuge. The supernatants were used as the crude extracellular enzyme source. Each treatment was carried out in triplicates and the results obtained throughout the work were the arithmetic mean of at least 3 experiments. Mannanase activity was assayed in the reaction mixture composing of 0.5 ml of 50mM potassium phosphate buffer pH 7.0 and 1% Locust Bean Gum (LBG) with 0.5 ml of supernatant at 45°C for 60 min (modified method of [1]). Amount of reducing sugar released was determined by the dinitrosalicylic acid reagent (DNS) [15]. One unit of mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

3.0 RESULTS AND DISCUSSION

Twelve agro-wastes were used as sources for the isolation of mannanase producing fungi. The primary screening was based on the clear zones formed on mannan-agar medium containing 0.5% locust bean gum (Sigma). A total of 11 fungal isolates showed clear zone of mannanase activity on solid medium at $28 \pm 2^{\circ}\text{C}$ (Table 1). Fungal isolate 5A showed the highest activity ratio (1.8) on locust bean gum medium followed by isolate 12C (1.42), while the least (0.64) was observed in isolate 2A. The production of mannanase activity on locust bean gum medium had been demonstrated by [13] and [4]. The formation of clear zone by these isolates on locust bean gum medium could be attributed to the ability of their genetic make up to secrete active mannanase with high diffusion rate as reported by [4].

134

135 **Table 1: Mannanase activity of fungal isolates expressed as ratio of clear zone/ colony**

	Isolate	Colony (mm)	Clear zone (mm)	Ratio of clear zone/ colony
136	1C	36	39	1.08
137	2A	19	21	1.1
138	5A	16	28	1.8
139	5B	44	49	1.1
140	7A	28	34	1.2
141	7B	26	32	1.2
142	8A	38	31	0.82
143	9A11	0.9	1.1	1.2
144	9A12	28	18	0.64
145	10A	2.3	2.0	0.9
146	12C	26	37	1.42

147 **1C**=*Rhizopus japonicus* PAP-1C, **2A**= *Aspergillus flavus* LAD-2A, **5A**=*A. fumigatus* LAD-
148 **A5**, **5B**=*Rhizopus japonicus* YP-5B, **7A**= *R. japonicus* BN-7A, **7B**= *R. japonicus* LBW-7B,
149 **8A**= *R. japonicus* LBW-8A, **9A11**= *A. glaucus* LAD-9A11, **9A12**= *R. stolonifer* LAD-9A12,
150 **10A**= *A. flavus* LAD-10A, **12C**= *Trichosporonoides oedocephalis* FCN-12C

151

152 Table 2 showed total fungal counts from each of the agro-wastes. Fermented coconut
153 recorded the highest number of fungal population with 7.4×10^2 sfu/g, while cocoa pod and
154 groundnut shell had no fungal growth. The high fungal counts may be due to lack of efficient
155 control measures in the discharge of agro-wastes into the environment [16]. [17] and [5]
156 reported that large amounts of lignocellulosic “waste” are generated through forestry and
157 agricultural practices, paper pulp industries, timber industries and many agro-industries and
158 they pose an environmental pollution problem. These singular activities tend to expose the
159 agro-wastes to microbial contamination. The reports of [17] also revealed structural
160 component of agro-wastes to contain lignin, cellulose, hemicellulose, and presence of some
161 components (activators or inhibitors). The chemical properties of the components of
162 lignocellulosics make them a substrate of enormous biotechnological value [12]. The
163 chemical composition of the wastes is linked to its ease of colonization, hence may account
164 for the high fungal counts. Apart from this, fungal isolates may probably have originated
165 from soil, water and material used during harvesting of agricultural produces, while the
166 variations of the isolates may be due to the handling process and the prevailing environmental
167 conditions [16]. The absence of fungal growth in cocoa pod and groundnut shell may be due
168 to the regular sanitation and fumigation of the environment where they were been deposited
169 [16].

The percentage occurrence of isolates from selected agro-wastes is reported in Table 3. The isolates consisted of *Aspergillus fumigatus*, *A. flavus*, *A. glaucus*, *Rhizopus japonicus*, *R. stolonifer* and *Trichosporonoides oedocephalis*. The highest percentage occurrence was obtained for *Rhizopus japonicus*, while *Aspergillus fumigatus*, *A. glaucus*, *R. stolonifer* and *Trichosporonoides oedocephalis* had the same value of 8.33%. The result clearly showed that certain fungal isolates are associated with the agro-wastes and that the growth and survival of these fungal isolates may not be unconnected with the fact that the wastes contained substances that can be utilized by the isolates. Therefore, the isolates can be said to be transient microorganisms surviving only in the absence of inhibitory substances [18,16].

Table 2: Total fungal counts from different agro-wastes (sfu/g)

Agro-wastes	Fungi
PAP	2.0×10^2
CS	5.0×10^2
CP	1.0×10^2
CPS	ND
YP	4.0×10^2
GNS	ND
BP	1.0×10^2
LBW	2.0×10^2
ORP	1.0×10^2
PP	7.0×10^2
SDC	1.0×10^2
FCN	7.4×10^2

PAP= Pineapple peels

CS= Cotton seed

CP= Cassava peels

CPS= Cocoa pod/Shell

YP= Yam peels

GNS= Groundnut shell

ND= Not detectable

BP=Banana peels

LBW=Locust bean wastes

ORP=Orange peels

PP=Potato peels

SDC=Sawdust compost

FCN=Fermented coconut

sfu/g= Spore forming unit per gram

Table 3: Percentage occurrence of fungal isolates from selected agro-wastes

Fungal isolates	% occurrence of isolates
<i>Aspergillus fumigatus</i>	8.33
<i>A. flavus</i>	16.76
<i>A. glaucus</i>	8.33
<i>Rhizopus japonicus</i>	66.67
<i>R. stolonifer</i>	8.33
<i>Trichosporonoides oedocephalis</i>	8.33

Upon further quantitative determination of mannan degrading enzyme, all the 11 isolates displayed mannanase activity between 0.370 to 21.667 U/ml for static and 0.278 to 3.982 U/ml for shaken condition, with the highest mannanase activity lied on isolate 5A for both (Figure 1). However, the overall evaluation of all the isolates in static condition showed higher extracellular mannanase activity than what was displayed by isolates in shaken condition. The higher mannanase activity in static culture could be attributed to the shearing effect induced by the agitation speed on cells and enzyme activity which may contribute negatively towards cell growth and enzyme instability [19]. Apart from this, agitation speed might cause cell damage which in turn leads to enzyme inactivation [20,21]. [1] linked lower mannanase production to oxygen limitation induced by the highly non-Newtonian medium with the filamentous growth of the fungus.

In shaken culture, isolate 1C and 9A12 had highest protein content, while isolate 5A was recorded for static culture, although, there was no direct relationship between the protein content of the tested cultures and the production of mannanase (Figure 2). Protein concentration does not really indicate an increase in the production of mannanase. Different fungal isolates had been reported to produce variety of enzymes (amylases, cellulases, protease and xylanases) apart from the enzyme been examined for in this study. Besides that, the protein from fungal cells might also interfere with mannanase enzyme causing variation in protein content, since the protein assay could only identify accumulated protein in solutions. This view was supported by the findings of [22].

The final pH values obtained from the fermentation systems is revealed in Figure 3. It was observed that the final pH designed for fermentation system for isolates in static condition was at the acidic range (4.68- 6.97), while those of the shaken cultures ranged from acidic to alkaline (3.97- 7.45). The acidic pH obtained for the static culture might be due to organic

acid accumulation, while variation from acidic to alkaline states observed in shaken cultures may be attributed to an increase in soluble reducing sugars profiles during fermentation [4]. The colonies of isolate 5A, being the highest producer of mannanase on mannan-agar medium containing locust bean gum, morphological examination was therefore conducted. A morphological examination of the isolate revealed that the conidiophores were upright, simple, terminating in a globose bearing phialides at the apex or radiating from the entire surface. Furthermore, the conidia appeared in dry basipetal chains. From these cultural and morphological features, the isolate was identified as *Aspergillus fumigatus* [14].

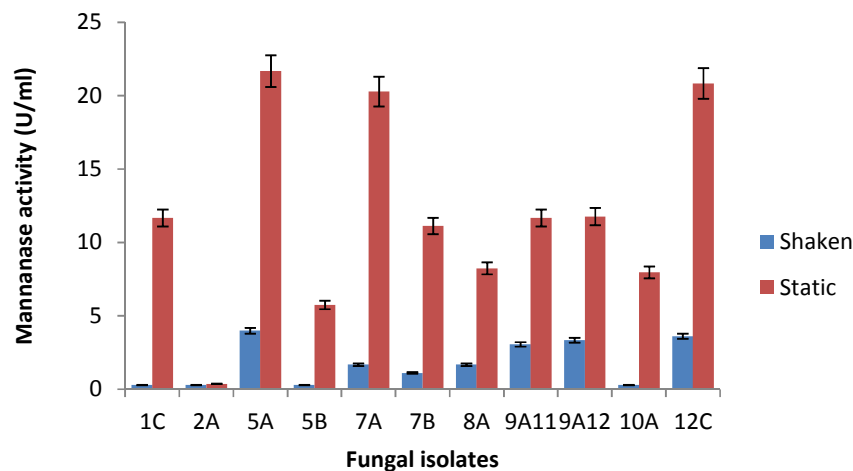


Figure 1: Mannanase production by fungal isolates at different fermentation conditions

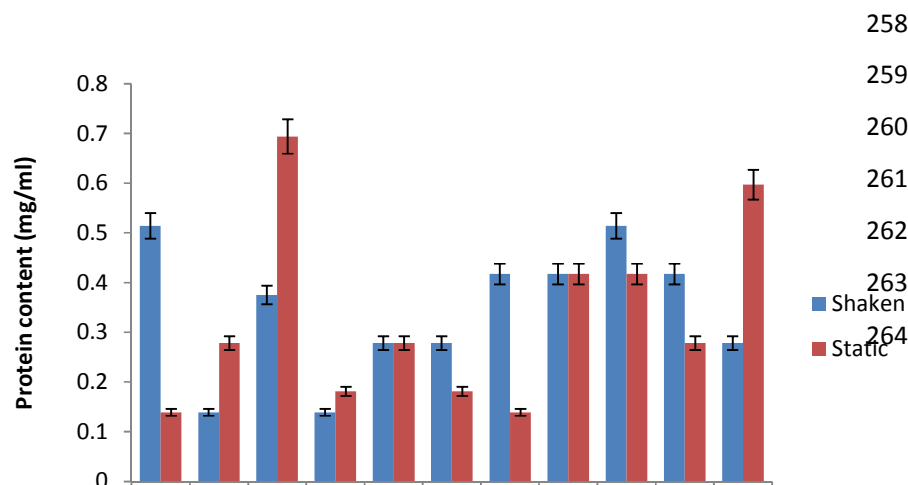


Figure 2: Protein content produced by fungal isolates at different fermentation conditions

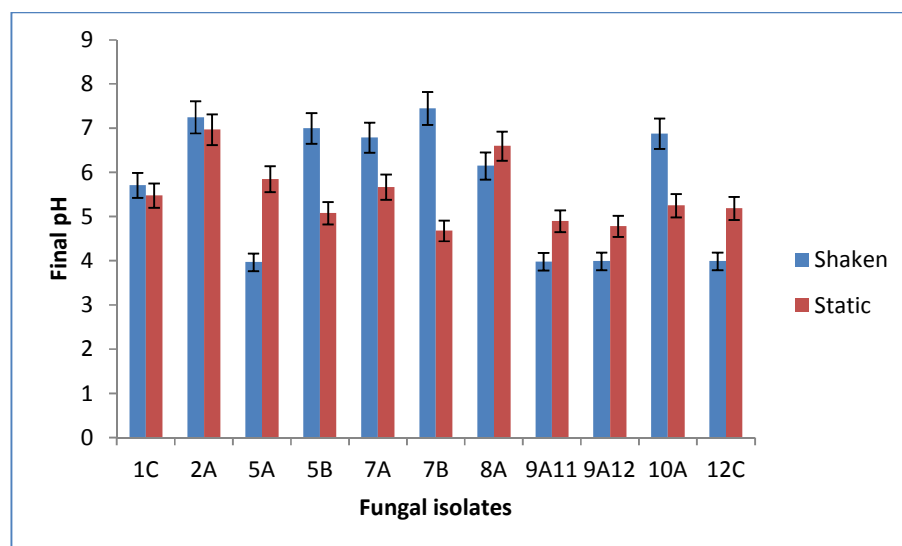


Figure 3: Final pH values obtained from the fermentation systems

4. CONCLUSION

Agro-wastes rich in β -mannan are abundant in nature and awaited to be converted into more valuable products used for mankind. Several microorganisms capable of converting mannan into simple carbohydrates had been discovered for decades. However, needs for newly isolated mannan-degrading microbes were still remained. The isolates showed a potential to convert mannan into manno-oligosaccharides which could be readily used in many applications such as animal foods and a feed stock for production of valuable organic compounds

COMPETING INTEREST

Authors have declared that no competing interests exist.

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