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Isolation and Screening of Mannanase Producing Bacteria from Agricultural Wastes

O. O. Olaniyi[1*] and D. J. Arotupin[1]

[1]Department of Microbiology, Federal University of Technology, P.M.B 704, Akure, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author DJA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author OOO managed the analyses of the study and the literature searches. The authors read and approved the first manuscript.

Research Article

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ABSTRACT

Aim: The work focused on the isolation and screening of mannanase-producing bacteria associated with selected agricultural wastes.
Study Design: The first experiment, mannanase-producing bacteria were screened for mannanase production on Locust Bean Gum (LBG) agar medium and total bacterial count was determined. In the second experiment, the isolated bacteria were further screened for mannanase production in submerged state fermentation.
Place and Duration of Study: Microbiology Research Laboratory Federal University of Technology, Akure and Postgraduate Research Laboratory, Obafemi Awolowo University Ile-Ife, Nigeria between September 2011 and March 2012.
Methodology: The associated bacterial isolates were isolated on agar medium containing LBG and counted by standard microbiological methods**.** Quantitatively, mannanase production was conducted in mineral salt medium into which copra meal had been incorporated as the sole carbon source and enzyme activity was determined by dinitrosalicylic acid method.
Results: The highest bacteria counts were recorded in compost from wood dust with 5.5×10^{11} cfu/g, while cassava peels had the least of 1.02×10^{6} cfu/g. In this study, 23 bacterial isolates showed positive results with clear zone around the cultures. Bacterial isolate 1A showed the highest ratio of clear zone to colony, while the lowest was observed in isolate 4B. In liquid broth, all the 23 isolates displayed mannanase activity between 0.28 to13.89 U/ml for static and 0.56 to13.43 U/ml for shaken condition, with the highest

**Corresponding author: Email: microladit@gmail.com;*

mannanase activity observed with isolate IA for both culture conditions. In the comparative study between static and shaken conditions, it was revealed that shaken cultures exhibited better yield than static cultures. According to the morphological and biochemical studies, the isolate 1A was primarily identified as the *Klebsiella edwardsii.*

Conclusion: In this investigation, bacterial isolates evaluated for mannanase production from agricultural wastes elaborated considerable mannanase activity and this could be applied in feed and prebiotic.

Keywords: Agricultural wastes; bacterial counts; mannanase; shaken and static conditions.

1. INTRODUCTION

The Mannan endo-1,4-β-mannosidase or 1,4-β-D-mannanase (EC 3.2.1.78), commonly named β-mannanase, is an enzyme that can catalyze random hydrolysis of β-1,4-mannosidic linkages in the main chain of β-1,4-mannans, glucomannans and galactomannans. It transforms the abundant heteromannans to manno-oligosaccharides [1] and a small amount of mannose, glucose and galactose [2]. Mannan endo-1,4-β-mannosidases are produced by a number of plants, bacteria, fungi, and by various invertebrates. The enzyme has found a number of applications in different sectors [2], including food, feed, pharmaceutical, and pulp/paper industries, gas well stimulation [3], as well as pre-treatment of lignocellulosic biomass for the production of second generation biofuel [4].

The application of mannan endo-1,4-β-mannosidase for the production of prebiotic manno-oligosaccharides from cheap agricultural by-products such as copra has recently gained significant interests [3,5]. It has been reported that mannooligosaccharides is a special nutrient or growth promoter for probiotics, such as *Bifidobacterium* sp. and *Lactobacillus* sp also have potential application for mannooligosaccharide preparation to be used as prebiotic, which is expected to improve the growth performance of animal.

Lignocellulose is the major structural component of plant cell walls and is mainly composed of lignin, cellulose and hemicellulose, and represents a major source of renewable organic matter. The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value [6]. Large amounts of lignocellulosic “waste” are generated through forestry and agricultural practices, paperpulp industries, timber industries and many agro-industries and they pose an environmental pollution problem. However, the huge amounts of residual plant biomass considered as “waste” can potentially be converted into various different value-added products including biofuels, chemicals, and cheap energy sources for fermentation, improved animal feeds and human nutrients [7].

Biotechnology research into microbial enzymes has been driven by the need to isolate and identify organisms which are either hyper-producer and/or sufficiently robust to withstand conditions of the intended application and/or are producers of novel extracellular enzymes. Hence, importance is placed on industrial enzymes of microbial origin with catalytic efficiency that is relatively stable under wide pH range, high temperature, and salts concentration with a view of producing industrial enzymes of desirable physicochemical properties [7]. The demands for microbial enzymes for industrial application have increased in the search of microorganisms with potential and adoption of biotechnological methods of enhancing enzyme production.This work focused on the isolation and screening of mannanase-producing bacteria that may be present in agricultural wastes.

2. MATERIALS AND METHODS

2.1 Materials and Chemicals

The coconut residual cakes were collected from farm field in Akure, Ondo State, Nigeria and it was used as a carbon source for medium formulation. The residual were treated with petroleum ether and dried at 60ºC for 2 h, blended, milled and sieved to obtain uniform particle size of 0.5 mm. Locust Bean Gum was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2 Sample Sources

Thirteen agricultural wastes (pineapple peels, cotton seeds, cassava peels, cocoa pod/shell, yam peels, groundnut shell, banana peels, locust bean wastes, orange peels, potato peels and compost from wood dust, fermented coconut and rice bran) were collected from farm fields, domestic sources and local market. The samples were blended and milled to obtain uniform particle size of 0.5 mm using sieve and these were used as sources for the isolation of mannanase-producing bacteria.

2.3 Isolation and Enumeration of Associated Bacteria

The sample, 1 g of solid sample was suspended in 9 ml of sterilized 0.85% normal saline (NaCl). The solution was mixed by vortex for 60 seconds. One percent (v/v) of the solution was transferred into 20 ml of sterilized isolation medium [8] containing 1% copra meal for bacteria. The bacterial cells were grown under aerobic condition by shaking at 150 rpm for 24 h at 37ºC.

2.4 Primary Screening

The culture broth from enumeration step was serial diluted and spread on bacterial isolation medium (BIM) containing LBG instead of copra meal and cells were allowed to grow at 37ºC for 18-24 h. 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.

2.5 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. Enzyme production was performed in 250 ml Erlenmeyer flask containing 50ml of enzyme producing medium (PM) modified method of [8]. The composition was as followed: 1% Copra meal, 0.1% peptone, 0.1% yeast extract, 1.4% KH_2PO_4, 0.06 % $MgSO_4.7H_2O$, and 1% inoculums, pH 6.8. The flasks were incubated at 37^0C for 24 h in a static condition while shaken condition was performed on a rotary shaker (Gallenkamp) at 120rpm. Then, the culture broth was centrifuged at 6,000 rpm, 4ºC for 15 min. The supernatant was collected and kept at -20ºC for further study. Mannanase activity was assayed in the reaction mixture composing of 0.5 ml of 50mM potassium phosphate buffer pH 7.0 and 1% LBG with 0.5 ml of supernatant at 45ºC for 60 min [8]. Amount of reducing sugar released was determined by the dinitrosalicylic acid method (DNS method) [9]. One unit of mannanase activity was defined as

amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

2.6 Bacterial Identification

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations. The parameters investigated include colonial morphology, Gram reactions, endospore formation, catalase production, Voges-Proskauer (V-P) reaction, Indole production, starch hydrolysis, citrate utilization and gelatine hydrolysis. The results were compared with Bergey's Manual of Determinative Bacteriology [10].

3. RESULTS AND DISCUSSION

Thirteen agricultural wastes were used as sources of isolation for mannanase-producing bacteria. The primary screening was based on the clear zones formed on mannan-agar medium containing 1% LBG (Sigma). A total of 23 bacterial isolates showed clear zone of mannanase activity in BIM at 37ºC (Table 1). Bacterial isolate 1A showed the highest ratio of clear zone to colony of 9 on LBG medium and also isolates 2C, 6A, X3, X1, BS, BP, X4, 1D and X5 showed a ratio of clear zone to colony of 4 and above which was higher than other isolates. The production of mannanase on LBG medium had been reported for *Bacillus circulans* [8], *Chryseobacterium indologenes* [11], *Bacillus* sp. MG-33 [12] and *Bacillus amylolequifaciens* 10A1 [7]. The formation of clear zone by these isolates on LBG medium could be attributed to the ability of their genetic make up to secrete active mannanase with high diffusion rate [11].

Table 2 showed total bacterial counts from each of the agricultural wastes. Compost from wood dust (5.5×10^{11} cfu/g) recorded the highest number of bacterial population, while cassava peels (1.02×10^{6} cfu/g) recorded least bacterial counts. The high bacteria counts may be due to lack of efficient control measures in the discharge of agricultural wastes into the environment [13]. Howard et al. [14]; [7] reported that large amounts of lignocellulosic "waste" are generated through forestry and agricultural practices, paper pulp industries, timber industries and many agro-industries thus posing an environmental pollution problem. These singular activities tend to expose the agro-wastes to microbial contamination. The reports of [7] and [14] also revealed structural component of agricultural wastes to contain lignin, cellulose, hemicellulose, and presence of some components (activators or inhibitors). The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value [6]. The chemical composition of the wastes is linked to its ease of colonization, hence may account for the high bacteria counts. Apart from this, bacterial isolates may probably have originated from soil, water and material used during harvesting of agricultural produces, while the variations of the isolates may be due to the handling process and the prevailing environmental conditions. The low bacterial counts in cocoa pod may be due to the regular sanitation and fumigation of the environment where they were been deposited [13].

Table 1. Mannanase activity of 23 bacterial isolates expressed as ratio of Clear zone/ Colony

Source	Isolate	Colony (mm)	Clear zone (mm)	Ratio of clear zone/ Colony
PAP	1A	1.0	9.0	9.0
	1B	1.0	2.0	2.0
	1D	2.0	8.0	4.0
CS	2B	1.4	2.7	1.7
	2C	0.2	0.9	4.5
CP	3A	0.5	1.2	2.4
CPS	4A	0.6	0.9	1.5
	4B	0.6	0.5	0.8
YB	5A	0.3	0.6	2.0
	5D	0.9	1.9	2.1
GNS	6A	0.3	1.2	4.0
BP	7A	1.1	2.5	2.3
LBW	8B	0.9	1.7	1.9
ORP	9B	0.9	1.7	1.9
	9E	0.7	1.8	2.6
PP	10B	0.8	0.9	1.1
CWD	11B	1.5	2.6	1.7
FCN	X3	0.3	1.2	4.0
	X1	0.2	0.8	4.0
	BS	0.2	0.9	4.5
RB	BP	0.3	1.2	4.0
	X4	0.2	0.8	4.0
	X5	0.3	1.2	4.0

***1A**=Klebsiella edwardsii 1A, **1B**=K. edwardsii 1B, **1D**=K. edwardsii 1D, **2B**=K. edwardsii 2B, **2C**=K. edwardsii 2C, **3A**=K. edwardsii 3A, **4A**=K. edwardsii 4A, **4B**=K. edwardsii 4B, **5A**=K. edwardsii 5A, **5D**=Escherichia intermedium 5D, **6A**=K. edwardsii 6A, **7A**=K. edwardsii 7A, **8B**=K. edwardsii 8B, **9B**=K. edwardsii 9B, **9E**=K. edwardsii 9E, **10B**=K. edwardsii 10B, **11B**=K. edwardsii 11B, **X3**=K. edwardsii X3, **X1**=K. edwardsii X1, **BS**=Bacillus subtilis BS, **BP**=Bacillus polymyxa BP, **X4**=Serratia marcescens X4, **X5**=Proteus rettgeri X5*

***RB**= Rice bran; **CP**= Cassava peels; **YP**= Yam peels; **FCN**= Fermented coconut; **PP**= Potato peels **CWD**= Compost from wood dust; **BP**= Banana peels; **GNS**=Groundnut shell; **RB**= Rice bran **CP**= Cassava peels; **YP**= Yam peels; **FCN**= Fermented coconut; **PP**= Potato peels **CWD**= Compost from wood dust; **BP**= Banana peels; **GNS**=Groundnut shell **PAP**=Pineapple peels; **LBW**=Locust bean wastes; **CS**=Cotton seeds; **ORP**=Orange peels; **CPS**=Cocoa pod/Shell; **cfu/g=** colony forming unit per gram*

Table 2. Total bacterial counts of different agricultural wastes (cfu/g)

Sources	Bacterial count (cfu/g)
PAP	1.5×10^{7}
CS	1.7×10^{7}
CP	1.02×10^{6}
CPS	1.31×10^{8}
YP	1.25×10^{8}
GNS	1.12×10^{8}
BP	1.61×10^{8}
LBW	1.37×10^{8}
ORP	1.55×10^{8}
PP	1.7×10^{8}
CWD	5.5×10^{11}
FCN	5.9×10^{8}
RB	1.06×10^{6}

***RB**= Rice bran; **CP**= Cassava peels; **YP**= Yam peels; **FCN**= Fermented coconut; **PP**= Potato peels; **CWD**= Compost from wood dust; **BP**= Banana peels; **GNS**=Groundnut shell; **PAP**=Pineapple peels; **LBW**=Locust bean wastes; **CS**=Cotton seeds; **ORP**=Orange peels; **CPS**=Cocoa pod/Shell; **cfu/g=** colony forming unit per gram*

Furtherance to quantitative determination of mannan degrading enzyme, all the 23 isolates displayed mannanase activity between 0.2778 to13.889 U/ml for static and 0.556 to 13.426 U/ml for shaken condition, with the highest mannanase activity lied on isolate 5A for both (Table 3 and 4). However, the overall evaluation of all the isolates in static condition showed lesser extracellular mannanase activity than what was displayed by isolates in shaken condition except isolate 1A. The higher mannanase activity in shaken culture compared to static one could be attributed to the importance of agitation in fermentation to facilitate the maintenance of homogenous conditions, especially with respect to temperature and gaseous environment [15]. Agitation serves to replenish the inter-particle spaces with fresh air. This could not be achieved in static condition since only the upper most substrate was in contact with air, while others were not. Such condition could lead to reduction of oxygen in inter-particle spaces at limiting level and carbon dioxide can rise to inhibitory level [15,16]. Nevertheless, carbon dioxide that dissolved in the fermentation medium might also cause pH decrease that inhibits cell growth. In different cases, agitation plays important role in preventing and encouraging the agglomeration of solids [15]. It was observed that the final pH values designed for fermentation systems in all the isolates in shaken condition was at the alkaline range (7.11-7.91), while those of the static culture ranged from acidic to alkaline (4.93 to 7.70). In shaken culture, isolate 8B had highest protein content, while isolate 4B was recorded for static culture. It was observed that there was no direct relationship between the protein content of the tested cultures and the production of mannanase.

Table 3. Production of extracellular mannanase by bacterial isolates from agricultural wastes in shaken condition

Source	Isolate	Mannanase activity (U/ml)	Protein content (mg/ml)	Yield (U/g)	Final pH
PAP	1A	13.426	2.361	26.852	7.20
	1B	0.556	1.528	1.111	7.12
	1D	3.889	2.778	7.778	7.42
CS	2B	10.278	2.778	20.556	7.30
	2C	5.556	2.542	11.111	7.28
CP	3A	3.704	2.736	7.407	7.30
CPS	4A	3.889	2.500	7.778	7.26
	4B	2.500	2.639	5.000	7.45
YB	5A	2.500	2.500	5.000	7.39
	5D	3.611	3.153	7.222	7.47
GNS	6A	3.333	2.125	6.667	7.62
BP	7A	5.278	3.056	10.556	7.12
LBW	8B	3.704	3.472	7.407	7.18
ORP	9B	3.056	2.361	6.111	7.30
	9E	3.981	2.500	7.963	7.52
PP	10B	3.611	2.778	7.222	7.50
CWD	11B	2.583	2.958	5.167	7.91
FCN	X3	12.222	2.500	24.444	7.11
	X1	11.481	1.944	22.963	7.23
	BS	10.370	1.944	20.741	7.41
RB	BP	10.278	1.852	20.556	7.09
	X4	10.093	2.083	20.185	7.13
	X5	10.278	1.847	20.556	7.37

***1A**=Klebsiella edwardsii 1A, **1B**=K. edwardsii 1B, **1D**=K. edwardsii 1D, **2B**=K. edwardsii 2B, **2C**=K. edwardsii 2C, **3A**=K. edwardsii 3A, **4A**=K. edwardsii 4A, **4B**=K. edwardsii 4B, **5A**=K. edwardsii 5A, **5D**=Escherichia intermedium 5D, **6A**=K. edwardsii 6A, **7A**=K. edwardsii 7A, **8B**=K. edwardsii 8B, **9B**=K. edwardsii 9B, **9E**=K. edwardsii 9E, **10B**=K. edwardsii 10B, **11B**=K. edwardsii 11B, **X3**=K. edwardsii X3, **X1**=K. edwardsii X1, **BS**=Bacillus subtilis BS, **BP**=Bacillus polymyxa BP, **X4**=Serratia marcescens X4, **X5**=Proteus rettgeri X5*

***RB**= Rice bran; **CP**= Cassava peels;**YP**= Yam peels; **FCN**= Fermented coconut **PP**= Potato peels; **CWD**= Compost from wood dust; **BP**= Banana peels; **GNS**=Groundnut shell; **RB**= Rice bran; **CP**= Cassava peels;**YP**= Yam peels; **FCN**= Fermented coconut **PP**= Potato peels; **CWD**= Compost from wood dust; **BP**= Banana peels **GNS**=Groundnut shell; **PAP**=Pineapple peels; **LBW**=Locust bean wastes; **CS**=Cotton seeds*

***ORP**=Orange peels; **CPS**=Cocoa pod/Shell; **cfu/g=** colony forming unit per gram*

Table 4. Production of extracellular mannanase by bacterial isolates from agricultural wastes in static condition

Source	Isolate	Mannanase activity (U/ml)	Protein content (mg/ml)	Yield (U/g)	Final pH
PAP	1A	13.889	13.889	27.778	6.08
	1B	1.111	1.111	2.222	6.91
	1D	2.129	2.129	4.259	6.28
CS	2B	1.667	1.667	3.333	4.98
	2C	1.389	1.389	2.778	5.72
CP	3A	3.333	3.333	6.667	6.34
CPS	4A	2.037	2.037	4.074	7.13
	4B	1.667	1.667	3.333	7.47
YB	5A	2.500	2.500	5.000	6.47
	5D	2.222	2.222	4.444	6.17
GNS	6A	3.241	3.241	6.482	4.93
BP	7A	1.481	1.482	2.963	5.54
LBW	8B	0.278	0.278	0.556	6.96
ORP	9B	0.833	0.833	1.667	7.64
	9E	2.037	2.037	4.074	7.70
PP	10B	1.111	1.111	2.222	7.48
CWD	11B	0.556	0.556	1.111	7.64
FCN	X3	6.481	6.482	12.963	6.74
	X1	4.629	4.629	9.259	7.10
	BS	4.629	4.629	9.259	6.98
RB	BP	4.537	4.537	9.074	7.16
	X4	5.741	5.741	11.482	7.23
	X5	6.944	6.944	13.889	6.79

***1A**=Klebsiella edwardsii 1A, **1B**=K. Edwardsii 1B, **1D**=K. edwardsii 1D, **2B**=K. edwardsii 2B, **2C**=K. edwardsii 2C, **3A**=K. edwardsii 3A, **4A**=K. edwardsii 4A, **4B**=K. edwardsii 4B, **5A**=K. edwardsii 5A, **5D**= Escherichia intermedium 5D, **6A**=K. edwardsii 6A, **7A**=K. edwardsii 7A, **8B**=K. edwardsii 8B, **9B**=K. edwardsii 9B, **9E**=K. edwardsii 9E, **10B**=K. edwardsii 10B, **11B**=K. edwardsii 11B, **X3**=K. edwardsii X3, **X1**=K. edwardsii X1, **BS**=Bacillus subtilis BS, **BP**=Bacillus polymyxa BP, **X4**=Serratia marcescens X4, **X5**=Proteus rettgeri X5*

***RB**= Rice bran; **CP**= Cassava peels; **YP**= Yam peels; **FCN**= Fermented coconut; **PP**= Potato peels; **CWD**= Compost from wood dust; **BP**= Banana peels; **GNS**=Groundnut shell **RB**= Rice bran; **CP**= Cassava peels; **YP**= Yam peels; **FCN**= Fermented coconut; **PP**= Potato peels; **CWD**= Compost from wood dust; **BP**= Banana peels; **GNS**=Groundnut shell; **PAP**=Pineapple peels; **LBW**=Locust bean wastes; **CS**=Cotton seeds; **ORP**=Orange peels; **CPS**=Cocoa pod/Shell; **cfu/g=** colony forming unit per gram*

The colonies of isolate 1A appeared creamy on mannan-agar medium containing LBG. A microscopic examination of the isolate revealed that it was a Gram-negative bacteria with long rod and produced catalase enzyme. Furthermore, the isolate 1A displayed positive reaction on VP and nitrate reduction test, while negative reaction was displayed towards citrate utilization, MR and sulphide indole motility (Table 5). From these morphological and biochemical reactions, the isolate IA was presumptively identified as *Klebsiella edwardsii* [10].

Table 5. Biochemical reaction and characteristics of the isolate 1A

Characteristics/biochemical tests	Result
Cell shape	LR
Gram reaction	-
Spore formation	-
Methyl red test	+
Sulphide indole motility test	-
Oxidation-Fermentation test	F
Triple salt iron test	Y
Nitrate reduction test	+
Catalase	+
V-P reaction	+
Citrate utilization	-
Fermentation of maltose	NC
Glucose	Y
Mannitol	NC
Sucrose	NC
Lactose	NC

+= Positive, -=Negative reaction, F=Fermentative, Y=Acid production, LR=Long rod, NC=No change.

4. CONCLUSION

In this study, agro-wastes contained large array of bacteria with potential for the production of mannanase. The *Klebsiella edwardsii* with code number 1A of agro-waste origin showed a potential to convert substrates containing mannan into simple carbohydrates which could be readily used in many applications such as animal foods and a feed stock for production of prebiotics. Although, the most effective isolate for mannanase production is known to be pathogen but it could be detoxified to harmless form such that its potential can be fully harnessed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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