Original Research Article

Microbial profile and detection of bio-catalytic enzymes from bacterial derivatives in selected agrowastes

ABSTRACT

The bio-catalytic enzyme potential of microbial consortium in selected agrowastes was evaluated in this study. The isolation, enumeration and biochemistry of bacteria and fungi from agrowastes were conducted via standard microbiological techniques. Screening of agrowaste substrates for enzyme synthesis was conducted. Potato peels had the highest bacterial count of 3.85×10^4 CFU/ml as maize husk had the highest fungal load of 6.55×10^4 SFU/ml, while wheat shaft had the lowest fungal load of 1.5×10^4 SFU/ml. *Acinetobacter baumanii, Staphylococcus aureus, S. epidermidis, Micrococcus luteus, Bacillus subtilis, B. cereus, B. licheniformis, B. megaterium* were among bacterial consortium significantly enumerated. *Saccharomyces cerevisiae, Rhodotorula glutins, Candida albicans* and *Geotrichum candidum* were yeasts characterized from the fermented agrowastes while *Aspergillus niger, A. terreus, A. flavus* constitute some of the mould consortium enumerated. Amylase and cellulase content of the agrowaste substrates increased after fermentation while protease and pectinase content of all the agrowastes remained unchanged after the fermentation process. *Acinetobacter baumanii, Bacillus subtilis, B. cereus, B. licheniformis, B. megaterium, B. subtilis* and *G. candidum* were positive for amylase, protease, cellulase and pectinase production. *Penicillium chrysogenum* and *Microsporum canis* were negative for the enzyme production. The findings of this study demonstrated the enzymatic-production potential of *Bacillus* species associated with agrowastes.

Keywords: Agrowastes; substrate; enzyme; fermentation; bacteria, fungi

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1. INTRODUCTION

Enzymes are complex protein molecules, often called biocatalysts, which are produced by living cells, highly specific both in reactions that they catalyze their closely relates reactants known as substrates [1]. Agricultural wastes are defined as the residues from the growing and processing of raw agricultural products such as fruits, vegetables, meat, poultry, dairy products, and crops [2]. They are the non-product outputs of production and processing of agricultural

products that may contain material that can benefit man but, whose economic values are less than the cost of collection, transportation, and processing for beneficial use [2]. The composition of agricultural wastes depends on the system and type of agricultural activities and can be in the form of liquids, slurries, or solids [2].

A broad study carried out by Chaturvedi and Verma (2016) has additionally demonstrated that perplexing substrates, for example, agrowastes are oxidized by various gathering of microorganisms bringing about the age of power. Agrowastes are useful materials emerging from different agrarian activities, for example, cultivating, poultry handling ventures, butcher houses, and agro enterprises [3].

A few examinations have been led in the course of recent many years inspecting the successful use of agro-industry squander as an expected crude material for the creation of significant worth added items. The majority of these examinations have been led in nations whose economies are vigorously dependent on agribusiness [4]. Agro-mechanical squanders comprise of variable structure that underpins the development of microorganisms.

These wastes are utilized as a crude material for delivering diverse significant compounds. Ravindran and Jaiswal, (2016) detailed that lignocellulosic deposits that emerge from agribusiness wastes are profoundly utilized in polysaccharides, for example, cellulose, hemicellulose, starch, gelatin, and inulin. Salim *et al.* (2017) conveyed another strain of *Bacillus* to contemplate the creation of four compounds (α-amylase, protease, cellulase and pectinase) utilizing diverse rural deposits as development substrates (sunflower feast, maize grain, maize pericarp, olive oil cake and wheat). Hence, it is appropriate to survey the bio-enzyme production potential of microbial derivatives of agrowastes. Consequently, the bio-enzyme production potential of microbial consortia from agrowastes is assessed in this study.

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2. MATERIALS AND METHODS

2.1. Collection and preparation of agrowaste samples ~~types~~

Maize husk, sweet potato peels, wheat shaft and sugar cane shaft were all aseptically collected from different agricultural waste sites within Akure South Local government area of Ondo State, Nigeria. They were kept separately in sterile air tight polythene bags and transported to the Microbiology Laboratory, Federal University of Technology, Akure, Nigeria, for further analysis within 24 hours. The substrates were dried in the drying cabinet (Gallenkamp, UK) for a period of 14 days after which they were milled individually into powder using an electric blender (Binatone blender/grinder- BLG 450).

2.2. Isolation of bacteria and fungi from samples

Serial dilution was carried out to reduce the microbial population within the substrates. One gram (1 g) of each of the sample~~s~~ was separately transferred into 9 ml of sterile distilled

water in test tubes. A stepwise serial dilution was carried out until the required dilution was obtained. A 1 ml aliquot (10^{-5} and 10^{-3} dilution factors for bacteria and fungi isolation respectively) of the samples was introduced into the Petri dishes using sterile pipette. Nutrient agar (Hi-Media, India), McConkey agar (Hi-Media, India), Eosine methylene blue agar (Hi-Media, India), Potato dextrose agar (Hi-Media), and Sarbrouid dextrose agar (Hi-Media, India) were aseptically prepared ~~in petri plates~~ under laminar flow hood using pour plate technique and allowed to solidify. The plates were later incubated at 37°C for 24 hours (bacteria) and at 25°C for 48 to 72 hours (fungi). After 24 hours of incubation, the bacterial plates were examined for growth. Distinct bacterial colonies were sub-cultured on freshly prepared media to obtain pure culture. After 72 hours of incubation, fungal plates were observed for fungal colonies and were sub-cultured on freshly prepared media to obtain pure culture. Thereafter, the plates were incubated at 37°C for 24 hours and at 25°C for 48 to 72 hours respectively. The pure isolates were stored temporarily on slants and kept at 4°C for further use [7]; [8].

Calculation of colony forming unit (CFU) per ml and spore forming unit (SFU) per ml for bacteria and fungi respectively was based on the formula utilizing the technique of Olutiola *et al.* (2001)

$$\text{CFU/SFU} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Number of millilitre suspended}}$$

The frequency of occurrence of microbial isolates was calculated using the formula as demonstrated by Cheesbrough (2010). Frequency of occurrence of microbial isolate=

$$\text{Number of particulate isolate} \div \text{total microbial population} \times 100\ (\%)$$

2.3. ~~Cultural c~~Characterization and biochemical identification of ~~bacterial and fungal~~ isolates

Cultural characteristics of the discrete bacterial colonies such as colour, shape, pigmentation, elevation, margin, texture and opacity were observed ~~and noted~~ after 24 hours of incubation. Microscopic characterization was carried out using Gram staining procedure, biochemical tests were carried out according to the methods of Olutiola *et al*. (2001); Fawole and Oso (2007); Tiwari *et al*. (2009); Cheesbrough (2010) and identification of bacterial isolates was carried out using the method of Cowan *et al*. (1993).

The morphological, cultural and microscopy identification of fungal isolates were examined based on the colour, types and shapes of spores, conidia and hyphae. The isolates were further stained with two drops of lactophenol-cotton blue dye and viewed under the light microscope using medium and high power objective lens for nature of conidia shape, sporangiophore, anthrospores, spores head, rhizoid and hyphae (septate or non septate) [12]

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Isolated yeasts were biochemically characterized by testing for ability of the yeasts to ferment and assimilate sugars, utilize nitrate, form spores, mycelium, pseudo-mycelium and pellicle. Morphological characterization of all the isolated yeasts was carried out by adding two drops of lactophenol-cotton blue dye to smears of isolates on clean glass slides. These were examined under a light microscope using oil immersion. Comparison of morphological characteristics that were observed was carried out with the standard text described by Barnett *et al.* (2000). The pseudo-mycelium/mycelium production, ascospore formation, sugar fermentation and nitrate utilization techniques for the identification of yeasts were carried out according to the method of Barnett *et al.* (2000).

2.4. Screening of substrates for enzyme activity

The substrates; both raw and fermented were screened for enzyme activity. The enzymes assayed for included: cellulase, protease, pectinase and amylase.

2.4.1. Cellulase

Zero-point-two millilitres (0.2 ml) of 1% carboxy-methyl cellulose (CMC) was added to 0.2 ml of the substrate solution and incubated at 37^{o}C for 30 minutes. One millilitre of 3, 5- Di-nitrosalicyclic acid (DNSA) was added to the solution and heated for 5 minutes in a boiling water bath. The solution was allowed to cool and 10 ml of distilled water was added. The same procedure was carried out on the substrate without the addition of the enzyme solution instead distilled water was added. The absorbance was read at 540 nm. The absorbance at 540 nm obtain was extrapolated from the glucose standard curve to obtain the amount of glucose liberated [14]

2.4.2. Protease

One mililitre (1 ml) of 1 % casein was pipetted into test tubes. It was incubated at 37^{o}C for 15 minutes. Zero-point-two millilitres (0.2 ml) of the enzyme solution was added and allowed to cool for one hour inside the water bath. Three millilitres (3 ml) of 10% tri-carboxylic acid was added to terminate the reaction. The tubes were centrifuged at 3000 rpm and the supernatant was read at 280 nm for un-precipitated protein hydrolysate using UV spectrophotometer [15].

2.4.3. Pectinase

The reaction mixture contained 0.1 ml of 1% pectin (in 0.1 M citrate-0.2 M phosphate buffer, pH 7.2) and 0.2 ml of substrate, the mixture was incubated at 35 ^{o} C for 30 minutes. The reaction was terminated and colour developed by 3, 5-Dinitrosalicylic acid (DNSA) method. One unit of pectinase activity (U) was the amount of enzyme that will release 1µg of glucose per minute [16].

2.4.4. Amylase

Zero-point-two millilitre (0.2 ml) of enzyme solution (1% soluble starch was prepared in 0.02 M sodium phosphate of pH 6.9 containing 0.006 M NaCl) was added to 0.2 ml of substrate and incubated at 25^{o}C for 3 minutes. One mililitre of 3, 5-DNSA was added. The mixture was then heated in water bath at 100^{o}C for 5 minutes. After heating, the mixture was cooled and 10 ml of distilled water was added, and then read in a colourimeter at 540 nm against a blank containing buffer without enzyme. A calibration curve was made with maltose [17].

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2.5. Primary screening of microbial isolates for enzyme production

Screening of microorganisms for enzyme production such as; cellulase, protease, pectinase and amylase were carried out using standard assay methods.

2.5.1. Cellulase production

The inoculum of bacterial and fungal isolates were inoculated into carboxyl methylcellulose (CMC) agar plates (containing (g/L), 10 g CMC, 0.2 g $MgSO_4.7H_2O$, 0.75 g KNO_3, 0.5 g K_2HPO_4, 0.02 g $FeSO_4.7H_2O$, 0.04 g $CaCl_2$, 2 g yeast extract and 1 g D-glucose) and autoclaved at 121℃ for 15 minutes. Zero point one percent (0.1%) chloramphenicol was used to restrict bacterial growth for fungal screening. The CMC agar plates were incubated for 2 days and 5 days for bacteria and fungi respectively. The plates were flooded with 1% Congo red for 20 minutes and washed with 1 M NaCl for 15 minutes. The clear zone formed by the isolates was indicated their cellulase activity [18]; [19].

2.5.2. Protease production

All the bacterial and fungal isolates were screened for their ability to produce protease on skim milk agar plate (casein 0.5 g, yeast extract 0.25 g, dextrose 0.1 g, skimmed milk powder 2.8 g and agar 1.5 g). The skim milk agar media was prepared in 100 ml of distilled of water before autoclaving at 121℃ for 15 minutes. Agar medium was amended with 0.1% chloramphenicol to restrict bacterial growth for fungal screening. Bacterial colonies and fungal spores were spot inoculated and plates were incubated at incubated at 37^{o}C for 24 hours and 25℃ for 72 hours for bacteria and fungi respectively. The formation of the clear zone around the bacterial colonies and fungal spores indicates the production of protease [20].

2.5.3. Pectinase production

The microbial isolates were cultivated on modified Czapek-Dox agar medium. The medium contained (g/L): $NaNO_3$ - 3.0 g, K_2HPO_4 - 1.0 g, $MgSO_4.H_2O$ - 0.50 g, KCl - 0.50 g, $FeSO_4$ - 0.01 g, Sucrose – 30 g, agar - 15.0 g and 1.5 g pectin). pH value was adjusted to 5.6 before autoclaving at 121℃ for 15 minutes. Agar medium was amended with 0.1% of

chloramphenicol to restrict bacterial growth for fungal screening. Bacterial colonies and fungal spores were spot inoculated and plates were incubated at 37^{o}C for 24 hours and 25℃ for 72 hours for bacteria and fungi respectively. Pectin utilization was detected by flooding the culture plates with freshly prepared iodine-potassium iodide solution (Iodine-1.0 g, Potassium iodide - 5.0 g in 330 ml distilled water). This solution gives colour to the medium containing pectin resulting in a translucent halo region where pectin was degraded, which indicated the pectinolytic activity [21].

2.5.4. Amylase production

A two-point-five gram~~me~~ (2.5 g) of starch agar (0.3 g meat extract, 0.5 g peptone, 0.2 g soluble starch, 1.5 g agar) was diluted in 100 ml of distilled water in a conical flask and autoclaved at 121^{o} C for 15 minutes. Chloramphenicol (0.1%) was used to prevent the growth of bacteria for fungal screening. Each isolated microorganism was spot inoculated on to starch agar plates and incubated for 24 hours at 37 °C and 72 hours at 25^{o}C for bacteria and fungi respectively. After incubation, the plates were flooded with iodine solution (0.3% iodine and 1% potassium iodide (KI). Amylase positive bacteria and fungi were identified and recorded based on the clear zone formation around the microbial growth [22].

2.6. Statistical analysis

Data obtained were analyzed by one-way analysis of variance (ANOVA) and means were compared by Duncan's New Multiple Range test (SPSS 23.0). Differences were considered significant at $p<0.05$.

3.0. RESULTS AND DISCUSSION

3.1. Total microbial load of agrowastes

Potato peel recorded the highest bacterial count 3.85×10^{4} CFU/ml compared to other agrowastes examined. Maize husk had the highest fungal load with value of $6.55x10^{4}$sfu/ml while wheat shaft recorded the lowest fungal load with value of $1.5\ x10^{4}$sfu/ml as shown in Figure 1. The higher fungal load recorded in maize husk as compared to bacterial load may be due to the ability of fungi to produce cellulase, hence they were able to utilize the cellulose in the waste as source of energy for their growth [23]. Anusuya and Geetha, (2013) also reported the occurrence of *Aspergillus* and *Rhizomucor* species from mixed vegetable wastes, which is in alignment with the fungal load of agrowastes utilized in this study.

Morphological and biochemical characterization of the bacteria isolated from the agrowastes as shown in Tables 1a and 1b revealed the presence of *Acinetobacter baumanii, Staphylococcus aureus, S. epidermidis, Micrococcus luteus, Bacillus subtilis, B. cereus, B.*

licheniformis, B. megaterium, Paenibacillus dendritiformis, Lactobacillus plantarum, Proteus mirabilis, Corynebacterium spp, *Escherichia coli, Klebsiella* spp, *Pseudomonas aeruginosa, Xanthomonas campestris* and *Enterococcus faecalis*. Tables 2a and 2b showed the presence of moulds including; *Aspergillus flavus, R. stolonifer, A. niger, A. terreus, Trichophyton mantagrophytes, Microsporum canis, Fusarium oxysporum, Penicilluim chrysogenum, Alternaria alternate, Fusarium solani,* and *Neurospora crassa.* The yeasts isolated were; *Saccharomyces cerevisiae, Candida albicans, Rhodotorula glutins* and *Geotrichum candidum.* Microorganisms isolated from this study have similarly been reported to be responsible for the degradation of agrowastes. This is an indication that these microorganisms grow in close association with the agrowastes as supported by Rao *et al*. (2016); Njoku *et al*. (2019) who enumerated *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtilis,* from agrowastes substrates. Additionally, the enumeration of *K. pneumoniae*, *Ps. aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *B. subtilis, B. megaterium, B. cereus, B. licheniformis* was in consonance with Ndubuisi *et al.* (2018) who observed the occurrence of these bacterial consortia from feed stocks used in agrowastes.

Amylase and cellulase content of the agrowaste substrates increased after fermentation. Protease and pectinase content of all the agro wastes remained unchanged after fermentation process as shown in Table 3. All the agrowastes used as substrate in this study were reported to be good sources for enzyme production. This agreed with the findings of Salim *et al*. (2017), who studied the production of some extracellular enzymes using different agrowastes as the growth substrate. Increase in amylase and cellulase content after fermentation may be attributed to the proliferation of the enzyme-producing microorganisms in the fermenting medium. The increase recorded in the enzyme content after fermentation is in accordance with the report of Oliveira *et al*. (2017). This researcher reported an increase in lipase production after few days of fermentation. Similarly, Buenrostro *et al*. (2013) also reported an increase in the production of ellagitannase enzyme after fermentation of some selected agrowastes.

Acinetobacter baumanii, Bacillus subtilis, B. cereus, B. licheniformis, B. megaterium, B. subtilis, Pseudomonas aeruginosa, Enterococcus faecalis, and *Geotrichum candidum* were positive for amylase, protease, cellulase and pectinase production. The fungal isolates namely: *Penicillium chrysogenum* and *Microsporum canis* were negative to the enzymes production as illustrated in Table 4. Amylases are industrially produced via submerged or solid-state fermentation using *Bacillus* and *Aspergillus* species [30]. A large spectrum of bacterial and fungal species has been found with the potential to produce amylases of different characteristics such as thermos-stability, halo-tolerance, psycho-tolerance and alkali-stability [31]; [32]. Cellulases, which are enzymes of great commercial importance, especially because of the key role they play in bio-ethanol production similarly derived from *Bacillus* species and other

Fusarium and *Geotrichum* species in this study was also evident in a study conducted by Singhania *et al*. (2014). Ferreira *et al*. (2014) had also reported wide range of bacterial and fungal species that produce cellulases. As observed in this study, potato peel waste contains sufficient amount of starch, hemicellulose, cellulose, lignin and fermentable sugars in parallel with Israilides *et al*. (2008).

All the microorganisms isolated showed ability for enzyme production except *Penicillium chrysogenum* and *Microsporum canis.* Although, the enzyme synthesis potential for the microorganisms varies. This agree with the report of Adeleke *et al*. (2017), who documented that the production of enzymes by microorganisms in the fermentation media depend on the availability of suitable and utilizable substrate. Hasan *et al*. (2013) also stated that the capacity of microorganisms to produce extracellular enzymes was influenced by environmental conditions such as temperature, pH, aeration, inoculums age and the presence of inducer or repressor substrates. All the *Bacillus* species isolated from this study screened positive to amylase, protease, cellulase and pectinase production. This agrees with the findings of Arogunjo and Arotupin (2018), who reported that all the *Bacillus* species isolated during fermentation of millet cob were screened positive to pectinase, lipase, cellulase, amylase and protease. In the same way, this result conforms to the report of De Veras *et al*. (2018), who reported that *Bacillus subtilis* showed potential to secrete protease, cellulase, amylase and xylanase.

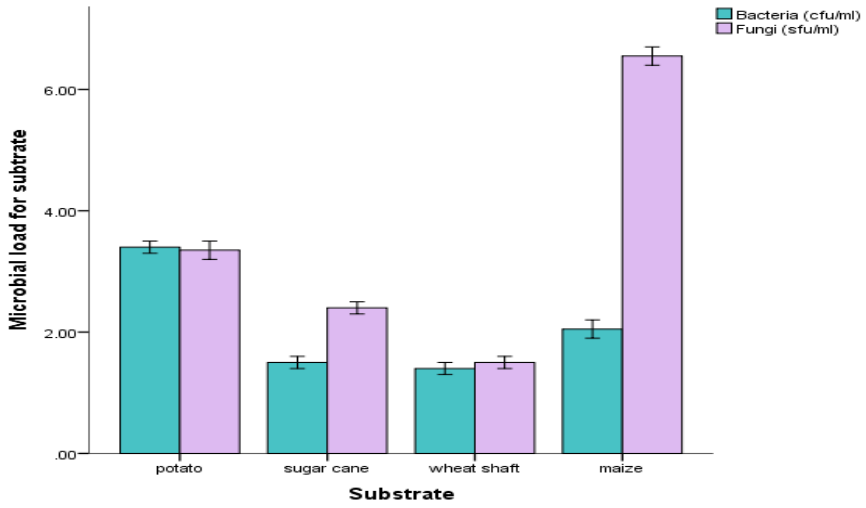

Fig. 1. Microbial load ($\times 10^4$) of substrates

Table 1a. Morphological and biochemical characteristics of isolated bacteria from the substrates

Isolate code	Colony colour	Gram rxn/ shape	Catalase	Coagulase	Motility	Spore	Indole	Starch	Citrate	Urease	Oxidase	H_2S test	MR/VP	Glucose	Fructose	Galactose	Lactose	Sucrose	Mannitol	Probable organisms
1	Creamy	-ve/ cocci	+	-	-	+	+	+	+	-	-	-	-/-	A	A	A	A	A	A	*Acinetobacter baumanii*
2	Creamy	+ve/cocci	-	+	-	-	-	-	-	-	-	-	-/-	A	A	A	A	A	A	*Staphylococcus aureus*
3	Creamy	+ve/cocci	+	-	-	-	-	-	+	-	-	+	-/-	AG	-	-	AG	AG	AG	*S. epidermidis*
4	Yellow	+ve/cocci	+	-	-	-	-	-	+	-	-	-	-/+	AG	A	AG	AG	AG	A	*Micrococcus leteus*
5	Creamy	+ve/rod	+	-	+	+	-	+	-	-	-	-	-/+	A	A	A	-	A	A	*Bacillus subtilis*
6	Creamy	+ve/rod	+	-	+	+	-	+	-	-	-	-	-/-	A	A	-	-	-	-	*B. cereus*
7	Creamy	+ve/rod	+	-	+	+	-	+	-	-	-	-	+/+	A	A	A	-	A	A	*B. licheniformis*
8	Creamy	+ve/rod	+	-	+	+	-	+	-	-	-	-	+/+	A	A	A	A	A	A	*B. megaterium*
9	Creamy	+ve/rod	+	-	+	+	-	+	+	-	-	-	+/+	A	A	A	A	A	A	*Paenibacillus dendritiformis*
10	Opaque	+ve/rod	+	-	+	+	-	-	-	-	-	-	-/-	A	A	A	A	A	A	*Lactobacillus plantarum*

Key: += positive, -=negative, A/G= Acid and Gas present, A= acid only

Table 1b. Morphological and biochemical characteristics of isolated bacteria

Isolate	Colony colour	Gram rxn/ shape	Catalase	Coagulase	Motility	Spore	Indole	Starch	Citrate	Urease	Oxidase	H_2S test	MR/VP	Glucose	Fructose	Galactose	Lactose	Sucrose	Mannitol	Probable organisms
1	Cream	-	-	-	+	-	-	-	-	+	-	+	+/	A	A	A	A	A	A	*Proteus*
2	y	ve/rod											+							*mirabilis*
1	Cream	-	-	-	-	-	+	-	-	-	-	+	-/-	A	A	A	A	A	A	*Corynebacter*
3	y	ve/rod													G	G	G	G	G	*ium diphtheriae*
1	Shiny	-	-	-	-	-	+	-	+	-	-	-	-	A	A	A	A	A	A	*Escherichia*
4		ve/rod											/+							*coli*
1		-	-	-	-	-	-	-	+	-	-	-	-/-	A	A	A	A	A	A	*Klebsiella*
5	Mucoid	ve/rod																		*pneumoniae*
1	Greeni	-	-	-	-	-	-	-	-	-	+	-	-/-	A	A	A	A	A	A	*Pseudomona*
6	sh	ve/rod																		*s aeruginosa*
1	Cream	-	-	-	-	-	-	-	-	-	-	+	-/-	A	A	A	A	A	A	*Xanthomona*
7	y	ve/rod																		*s campestris*
1	Cream	-	-	-	-	-	-	-	-	-	-	-	-/-	A	A	A	A	A	A	*Enterococcus*
8	y	ve/co cci																		*faecalis*

Key: += positive, - =negative, A/G= Acid and Gas present, A= acid only

Table 1c. Morphological and biochemical characteristics of yeasts isolated from agrowastes

Isolate code	Morphology			Biochemical Properties								Yeast Identity
				Fermentation/Assimilation								
	Cell shape	Ascospore	Shape	Spore	Pseudomycelium	Glucose	Fructose	Sucrose	Lactose	Maltose	Nitrate	
$P_3$3	Oval	+	Oval	+	+	FA	FA	FA	-A	FA	-	*S. cerevisiae*
$P_3$1	Cylindrical	+	Oval	+	+	FA	FA	FA	--	FA	+	*C. albicans*
$P_3$5	Oval	-	Oval	-	+	-A	-A	-A	--	-+	-	*R. glutinis*
S.CA1	Cylindrical	+	Short cylindrical	+	-	FA	FA	-A	-A	FA	+	*Geotrichum candidum*

Key**:** + = Present, - = Absent, FA= Fermentation and Assimilation, -A= Assimilation

Table 2a. Cultural and morphological characteristics of moulds from the agrowastes

Isolate code	Cultural characteristics	Spores/conidia arrangement under the microscope	Identity of isolates
1	Spores are granulated	Globose to sub-globose colonies	*Aspergillus flavus*
2	Conidia grows rapidly and darkens	Mycelia are noticeable by copious stolons with sporangiophores	*Rhizopus stolonifer*
3	Compact white colonies	Conidial head with smooth-walled conidiophores	*Aspergillus niger*
4	Pinkish brown mycelia growth	Conidia are globose to ellipsoidal	*Aspergillus terreus*
5	Colonies are flat, white, powdery surface	Copious single-celled micronidia and multi-celled macronidia	*Trichophyton mentagrophytes*
6	Colonies are flat, spreading and whitish	Spindle-shaped and thick walled macroconidia	*Microsporum canis*

Table 2b. Cultural and morphological characteristics of fungal isolates from the agrowastes

Isolate code	Cultural characteristics	Spores/conidia arrangement under the microscope	Identity of isolates
7	Aerial mycelium with whitish purple tinge	Septate on phialides with branching conidiophores	*Fusarium oxysporum*
8	Greenish colonies with septate fruiting mycelium	Bluish-green filament metamorphosed to crumbly greenish brown.	*Penicillium chrysogenum*
9	Black to olivaceous black colonies	Branching chains of multi-celled conidia	*Alternria alternata*
10	Grey-whitish mycelium	Oval microconidia with cylindrical contour	*Fusarium solani*
11	Orange-coloured roughly scattering colonies with ascomata assembly	Ascospores roughly fusiform	*Neurospora crassa*

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Table 3. Enzyme content (mg/ml/min) of substrates (agrowastes) before and after fermentation

Samples	Amylase		Cellulase		Protease		Pectinase	
	Raw	Fermented	raw	fermented	raw	fermented	raw	fermented
A	0.08±0.00^{d}	0.24±0.02^{c}	0.11±0.00^{d}	7.15±0.03^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{b}	0.02±0.00^{b}
B	0.02±0.00^{c}	0.10±0.00^{b}	0.09±0.00^{c}	8.49±0.03^{b}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{b}	0.02±0.00^{b}
C	0.01±0.00^{b}	0.02±0.00^{a}	0.08±0.00^{b}	9.04±0.03^{c}	0.01±0.00^{a}	0.02±0.00b	0.00±0.00^{a}	0.01±0.00^{a}
D	0.00±0.00^{a}	0.01±0.00^{a}	0.03±0.00^{a}	9.71±0.0^{3}	0.01±0.00^{a}	0.02±0.00^{b}	0.00±0.00^{a}	0.02±0.00^{b}

Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (p<0.05). Key: A= potato peels; B= sugar cane shaft; C= wheat shaft; D= maize husk

Table 4. Microbial screening for enzymes production

Organisms	Enzymes			
	Amylase	Protease	Cellulase	Pectinase
Bacteria				
Acinetobacter baumanii	+	+	+	+
Bacillus subtilis	+	+	+	+
B. cereus	+	+	+	+
B.licheniformis	+	+	+	+
B. megaterium	+	+	+	+
B. subtilis	+	+	+	+
Corynebacterium diptheriae	+	-	+	+
Escherichia coli	+	+	-	+
Enterococcus faecalis	+	+	+	+
Proteus mirabilis	+	+	-	-
Paenibacillus dendritiformis	+	-	-	+
Pseudomonas aeruginosa	+	+	+	+
Staphylococcus aureus	+	-	-	-
S. epidermidis	+	-	-	-
Xanthomonas campestris	+	+	+	-
Moulds				
Microsporum canis	-	-	-	-
Fusarium solani	-	-	+	+
Fusarium oxysporum	-	-	+	+
Penicillium chrysogenum	-	-	-	-
Yeast				
Geotrichum candidum	+	+	+	+

Key: + = Positive, - =Negative

4. CONCLUSIONS

All the Bacillus species isolated from this study were screened positive to amylase, protease, cellulase and pectinase production. This study further accentuates the usefulness of these Bacillus species and other yeast (*Geotrichum candidum*), mould (*Fusarium solani* and *Fusarium oxysporium*) especially for cellulose and pectinase production. Enzyme produced can also serve as an enhancement for usage in industrial food production. The adoption of *Bacillus* species for enzyme production with beneficial economic importance is suggested to ease the burden of enhanced food production.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

References

1. Berj JM, Tymoczko JL and Stryer L. Biochemistry, 5th edn New York: WH Freeman., (2002).
2. Obi FO, Ugwuishiwu BO, Nwakaire JN. Agricultural waste concept, generation, utilization and management. Nigerian Journal of Technolnology. 2016; 35(4): 957-964.
3. Chaturvedi V and Verma P. Microbial fuel cell: a green approach for the utilization of waste for the generation of bioelectricity. Bioresources and Bioprocessing. 2016; 3(38): 1-14).
4. Ravindran R, Hassan SS, Williams GA, Jaiswal AK. A review of bioconversion of agro-industrial wastes to industrially important enzymes. Bioengineering. 2018; 5: 93-113.
5. Ravindran R. and Jaiswal A. Microbial enzyme production using lignocellulosic food industry wastes as feedstock: A review. Bioengineering. 2016; 3: 30.
6. Salim AA, Grbavcic S, Sekuljica N, Stefanovic A, Jakovetic TS, Lukovic N. and Knezevic-Jugovic Z. Production of enzymes by a newly isolated *Bacillus* sp. TMF-1 in solid state fermentation on agricultural by-products: The evaluation of substrate pretreatment methods. Bioresource Technology. 2017; 228: 193–200.
7. Fawole MO and Oso BA. Laboratory manual of Microbiology. 5th Edition. Spectrum Books Limited, Ibadan. 2007; pp. 22-23.
8. Cheesbrough M. District Laboratory Practice in Tropical Countries. Cambridge: Cambridge University Press. (2010).
9. Olutiola PO, Famurewa O and Sonntag HG. Introduction to general microbiology: A practical approach. 2nd Edition, Bolabay Publications, Ikeja, Nigeria. (2001).
10. Tiwari RP, Hoondal GS and Tewari B. Laboratory techniques in microbiology and biotechnology. Study of biochemical characteristics of bacteria.1st Edn Mehra, Offset Press, New Delhi pg.14, 75-77,140. (2009).
11. Cowan ST, Steel KJ, Barrow GI and Feltham RKA. Cowan and Steel's manual for the identification of medical bacteria. Cambridge, Cambridge University Press. (1993).

12. Jankiewicz U, Brzezinska MS and Saks E. Identification and characterization of a chitinase of *Stenotrophomonas maltophilia*, a bacterium that is antagonistic towards fungal phytopathogens. Journal of Bioscience and Bioengineering. 2012; 113(1): 30-35.

13. Barnett JA, Payne RW and Yarrow D. Yeasts: Characteristics and identification. Cambridge, UK: Cambridge University Press. (2000).

14. Miller GL. Use of dinitrosalycilic acid (DNSA) reagent for the determination of reducing sugar. Analytical chemistry. 1959; 31: 426-42.

15. Ladd JN and Butler JHA. Short-term assay on soil proteolytic enzyme activity using proteins and dipeptide substrate. Soil biology and biochemistry. 1972; 4:19-39.

16. Mandels M, Andreotti R. and Roche C. Measurements of saccharifying cellulase. Biotechnology Bioengineering Symposium. 1976; 6: 21-33.

17. Bernfeld P. Amylase α and β in Methods in Enzymes. Academic Press, New York. pp. 1951; 144-147.

18. Lisdiyanti P, Suyanto E, Gusmawati NF and Rahayu W. Isolation and characterization of cellulose produced by cellulolytic bacteria from peat soil of Ogan Komering Ilir, South Sumatera. International Journal of Environmental Bioenergy, 2012; 3: 145-153.

19. Saowapar K, Yupa P, Taweesak T and Somboon T. Screening and identification of cellulase producing bacteria isolated from oil palm meal. Journal of Applied Pharmaceutical Science. 2014; 4(4): 90-96.

20. Hnin E, Zaw KO and Kyaw NA. Screening on proteolytic activity of lactic acid bacteria from various yogurts and fermented milk. International Journal of Advanced Science and Engineering Technology. 2015; 15: 34-37.

21. Hankin L, Zucker M and Sands DC. Improved solid medium for the detection and enumeration of pectolytic bacteria. Applied Microbiology. 1971; 22: 205-209.

22. Akpomie OO, Akponah E and Okorawhe P. Amylase production potentials of bacterial isolates obtained from cassava root peels. Agricultural Science Research Journal. 2012; 2(2): 95-99.

23. Bamigboye OO. Screening of some fungi associated with maize cob degradation for cellulase activity. Greener Journal Biological Science. 2013; 3(6): 208-212.

24. Anusuya D and Geetha M. Isolation and identification of fungal communities from vegetable wastes composts. International Journal of Scientific Invention. 2013; 3(3): 203-207.

25. Rao RKM, Sivagnanam KS and Syed FB. Isolation, characterization and identification of predominant microorganisms from agro-waste. Der Pharmacia Lettre, 2016; 8(5): 79-86.

26. Njoku NO, Mbachu IAC and Uba BO. Influence of physicochemical and microbiological properties on the composting of agro wastes using cow dung as a booster. Animal Research International. 2019; 16(1): 3238 – 3246.

27. Ndubuisi-Nnaji UU, Ofon UA and George QS. Bacterial survivors of agrowastes anaerobiosis: Their hemolytic activity and antibiotic susceptibility patterns. World Journal of Applied Science and Technology. 2018; 10(1): 86 - 90

28. Oliveira F, Carlos ES, Peclat VR, Salgado JM, Bernardo DR, Maria AZC, Armando V and Isabel B. Optimization of lipase production by *Aspergillus ibericus* from oil cakes and its application in esterification reactions. Food Bio-production and Processing. 2017; 17: 01-07.

29. Buenrostro J, Ascacio A, Sepulveda L, De la Cruz R, Prado-Barragan A, Aguilar-Gonzalez MA, Rodriguez R and Aguilar CN. Potential use of different agro-industrial by products as supports for fungal ellagitannase production under solid state fermentation. Food Bio-production and Processing. 2013; 1: 8-10.

30. Sundarram A and Murthy TPK. Amylase production and applications: A review. Journal of Applied Environmental Microbiology. 2014; 2: 166–175.

31. Roohi T and Kuddus M. Bio-statistical approach for optimization of cold-active _ amylase production by novel psychrotolerant *M. foliorum* GA2 in solid state fermentation. Biocatalysis and Agricultural Biotechnology. 2014; 3: 175–181.

32. Sen SK, Dora TK, Bandyopadhyay B, Das Mohapatra PK, Raut S, Sen SK, Bandyopadhyay B, Mohapatra PKD and Raut S. Thermostable alpha-amylase enzyme production from hot spring isolates *Alcaligenes faecalis* SSB17—Statistical optimization. Biocatalysis and Agricultural Biotechnology. 2014; 3: 218–226.

33. Singhania RR, Saini JK, Saini R, Adsul M, Mathur A, Gupta R and Tuli DK. Bioethanol production from wheat straw via enzymatic route employing *Penicillium janthinellum* cellulases. Bioresource Technology. 2014; 169: 490–495.

34. Ferreira NL, Margeot A, Blanquet S and Berrin JG. Use of cellulases from *Trichoderma reesei* in the twenty first century—Part I: Current industrial uses and future applications in the production of second ethanol generation. Journal of Advance Biology and Biotechnology. 2014; 245–261.

35. Israilides C, Vlyssides AG, Arapoglou D, Varzakas T, Marchant R and Vlysides AA. Integrated management of potato starch wastes. In: Proceedings of the Waste and Resource Management – A Shared Responsibility (Waste 2008), September 16–17, Stratford-upon-Avon, UK, No. 36 (2008).

36. Adeleke BS, Olaniyi OO and Akinyele BJ. Isolation and screening of bacteria associated with fermented cassava peels for linamarase production. International Journal of Biotechnology Research. 2017; 5: 20-26.

37. Hasan S, Ahmad A, Purwar A, Khan N and Kundan R. Production of extracellular enzymes in the entomopathogenic fungus *Verticillium lecanii*. Bioinformatics. 2013; 9: 238-242

38. Arogunjo AO and Arotupin DJ. Millet cobs: A source of microbial enzymes. *Journal of* Microbial Biochemistry andTechnology. 2018; 10: 124-133.
39. De Veras BO, Dos Santos YQ and Diniz KM. Screening of protease, cellulase, amylase and xylanase from the salt-tolerant and thermostable marine *Bacillus subtilis* strain SR60. F1000 Research. 2018; 7(1704): 1-8.