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RESEARCH ARTICLE

Identification and characterization of potential compost degrading bacteria from agro-waste

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Abstract

Agricultural industry plays a significant role in the global economic growth. It is estimated more than 15% of total waste in Asia are contributed by agro waste. Bacteria is known as one of the useful organisms actively found to surround the waste industry. They are considered as chemical decomposers and act as driving agent of composting that changes the chemistry of organic waste to simple compounds. Thus, this study was conducted to isolate and identify potential compost-degrading bacteria from agriculture waste at several sampling areas in Besut district, Terengganu, Malaysia. A total of 49 bacteria strains were isolated using Tryptic Soya Agar (TSA) from seven groups of raw agro wastes (paddy husk, paddy straw, paddy soil, rock melon waste, rock melon soil, corn waste, and corn soil). Primary screening for potential enzyme production was carried out using selective media containing different substrates (sucrose, xylan, starch, skim milk, and pectin). Only 13 bacterial strains were found positive for protease, nine bacteria strains positive for xylanase, and three bacteria strains were found positive for amylase. Identification of bacteria strains were performed using phenotypic, biochemical tests, and genotypic approaches by 16S rRNA gene sequence. Based on NCBI BLAST analysis, we have identified several bacteria strains: *Bacillus cereus* (strain B), *Alcaligenes faecalis* (strain C), *Micrococcus* sp. (strain D), *Pseudomonas stutzeri* (strain E), *Enterobacter cloacae* (strain G), and *Serratia marcescens* (strain J). Strain F and strain H were identified under distinct family of Enterobacteriaceae, while strain I was identified from Pseudomonadeles order which might represent a new type of proteobacteria strain. These potential waste degrading bacteria could be further analyzed and studies for their true potential in many areas including agriculture and industrial waste management as an approach to reduce waste accumulation in eco-friendly way.

Keywords: Agriculture waste, compost-degrading bacteria, biochemical test, 16S rRNA, characterization

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INTRODUCTION

Agriculture waste or agro waste is defined as waste produced by any agricultural activities, including manures, plant stalks, leaves, and vegetables matter. Agro-waste has been generated in large amounts throughout the year and apparently, it is one of the most abundant renewable resources on the earth [1]. In Malaysia, overall waste composition is dominated by municipal solid waste (MSW) (64 %) followed by industrial waste (25 %) commercial waste (8 %) and construction waste (3 %) [2]. Statistics have shown that these organic wastes contribute to major sources of environmental problems throughout the world. Secretion of phenolic and other toxic compounds from the waste degradation has caused deterioration in the environment [3].

Recent studies have found that agro-waste contains insoluble chemical constituents such as cellulose and lignin, as well as soluble constituents, for instance amino acid, sugars, and organic acids which are the major carbon sources for microbial community [4]. Therefore, discovery of potential compost-degrading bacteria has been actively studied for the potential application in compost, bio-fertilizer, or bio-degradation processes [5]. Based on Mehta *et al*. [6], decomposition of organic matter which occurs mostly in aerobic decomposition and anaerobic decomposition were usually occurred to transform raw organic materials into biologically stable components especially in soil amendments. Organic wastes are usually biodegradable and thus would facilitate composting bacteria to carry out biodegradation activity on a mixture of substrates, in which to help in managing agro waste accumulation [7].

Several bacterial species have been found as composting bacteria in previous studies such as *Enterobacter aerogenes* and *Pseudomonas brenneri* [8]. Another study also reported the presence of composting bacteria in vegetable compost such as *Serratia marcescens*, *Klebsiella* sp., and *Pseudomonas aeruginosa* [9]. Therefore, discovery of new potential compost-degrading bacteria from agro waste was carried out in order to isolate and identify potential compost-degrading bacteria from different agriculture wastes. Discovery of various types of

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compost-degrading bacteria could be applied in many fields especially in the agriculture and industrial waste management as an approached to a green technology and eco-friendly ways to reduce wastes in our country and to help eliminate their negative impacts to our environment.

EXPERIMENTAL

Sample Preparation

Samples were collected at several areas near Besut district, Terengganu, Malaysia. The types of samples and coordinates of sampling location are recorded in Table 1. All samples were sun-dried for a few days until their weights were constant. After completely dry, each sample was weighed to 10 g and homogenized in 90 ml of 0.9 % phosphate buffer saline. Sample was shaken for 30 minutes before centrifuged at 3000 rpm for 15 minutes to obtain the supernatant.

Table 1 Types of agro wastes collected and their coordinate locations.

	Sample	Area	Latitude	Longitude
A	Paddy husk	Rendang, Jabi	5°41'50.9	102°34'25.7'
B	Paddy straw	Sg. Kotak, Jertih	5.6615°	102.5824°
C	Paddy soil	Rendang, Jabi	5°41'50.9	102°34'25.7'
D	Rockmelon waste	KETARA, Alur Lintang	5.7654335°	102.5570615°
E	Rockmelon soil	KETARA, Alur Lintang	5.7654335°	102.5570615°
F	Corn waste	UNISZA, Besut	5°45'7.89"	102°37'55.58
G	Corn soil	UNISZA, Besut	5°45'7.89"	102°37'55.58

Bacteria isolation and enumeration

Each sample had undergone serial dilution before plating on Tryptic Soy Agar (TSA) by spread plate technique. Plates were incubated at 37 °C for 2 to 3 days to allow for bacterial growth. Individual colony was picked and re-streaked on TSA medium until pure colonies were obtained.

Screening of potential secretion enzyme

All the isolated bacterial strains were screened using five different selective media as stated in Table 2. All media were sterilized by autoclaving at 121 °C, 15 psi for 20 minutes. Bacteria colonies were streaked onto each of the selective media and incubated at 37 °C for 2 to 3 days. Colonies with the ability to form halo zone on the selective media were observed. Diameters of halo zone around colony and colony diameter were measured as Solubilization Index (SI) to indicate bacteria degradation activity [10]. The Solubilization Index (SI) formula is stated below:

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

Table 2 Selective media used with its targeted substrate.

Selective medium	Substrate	Reference
Congo Red Agar (CRA)	Cellulose	Gupta *et al.* [11]
Xylan agar medium	Xylanase	Nagar *et al.* [12]
Starch agar medium	Amylase	Shanmugasundaram *et al.* [13]
Skim Milk Agar (SMA)	Protease	Dutta *et al.* [14]

Phenotypic bacteria identification

Phenotypic bacteria identification is an extensive phenotypic investigation which includes colony morphology and Gram staining. Colony morphology for each bacteria strain was observed under stereomicroscope (Leica) and colony characteristics were described based on Bergey's manual of Systematic Bacteriology [15]. For Gram staining procedure, method described by Coico [16] was used as the standard protocol in this study. All bacteria isolated also undergo 7 types of biochemical test (oxidase test, catalase test, sulphide test, indole test, motility test, triple sugar iron test (TSI), and urease test) in order to identify their biological enzymatic activities.

Genotypic bacteria identification

DNA samples were extracted from overnight bacteria culture using DNA Isolation and Purification Kit from Promega. PCR amplification was carried out using a set of 16S rRNA primers as listed in Table 3. Each PCR reaction mixture contained 1X PCR buffer, 0.2 mM dNTPs mix, 3 mM $MgCl_2$, 1 µM forward and reverse primers, genomic DNA, 1U Taq Polymerase, and sterile distilled water. PCR reaction was set for 30 cycles using PCR machine from Applied Biosystems (Verify 96 Well Thermal Cycler). The following PCR profile was used with initial denaturation at 95 °C for 5 minutes, denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and extension at 72 °C for 2 minutes. The final cycle included extension for 5 minutes at 72 °C to ensure full extension of the PCR products. 1 % agarose gel electrophoresis was performed at 80 V, 400 A for 1 hour.

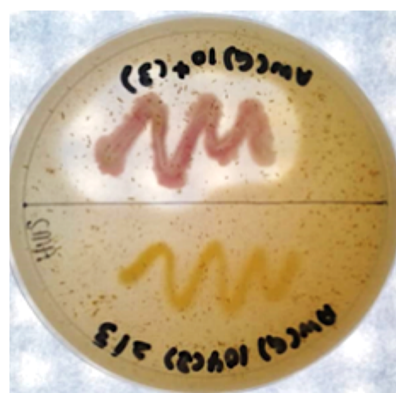
Finally, the PCR products were purified before being sent out for sequencing service at 1st Base Laboratory (M) Sdn. Bhd. Raw sequencing data obtained were then analyzed using BLAST-N software available at National Center Biotechnology Information (NCBI). While, for sequence alignment and comparison, Clustal Omega software was used (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Table 3 List of PCR Primers for 16S rRNA amplification.

Primer	Sequence (5'- 3')
PF3	CTAAGAGAAGCAAGTGTAATTGGCTAGCG
PR3	GTCATGGTCTTATAGTCCTTGGGAATG

RESULTS AND DISCUSSION

Altogether, 49 bacteria strains were successfully isolated from 7 different agro-waste samples. Pure bacteria colonies were screened in different selective mediums with specific substrates. A total of 25 bacterial strains were found to be positive on different screening media. From the screening, there were 13 bacteria strains positive for protease followed by 9 bacteria strains with xylanase activity, and another 3 bacteria strains were positive for amylase activity. From the total of 25 positive bacteria strains, only 9 bacteria strains were discussed in this report as shown in Fig. 1.



a) Positive bacteria strain growth on Skim Milk Agar (SMA).

b) Positive bacteria strain growth on starch agar.

c) Positive bacteria strain growth on xylan agar.

Fig. 1 Halo zone formation on different selective media for protease, amylase and xylanase enzyme activities.

Halo zone was observed around the growth colony which indicates enzyme production by the bacteria due to carbon source degradation [17]. However, no bacteria were found positive for pectinase and cellulase. Production of potential degrading enzymes usually affected by nature of solid, substrates, level of moisture content, temperature, and presence or absence of carbon and other minerals [18].

Areas of halo zone were measured quantitatively using Solubilization Index (SI) as presented in Table 4. SI formulation was used to observe the capability of bacteria colony to produce specific enzyme to degrade substrate presence in the media tested [19]. SI values for all the bacteria strains were listed in Table 5. Strain A was used as control for negative result. The highest SI value was detected for strain H at 4.1 for protease producer and the lowest SI was at 2.9 for strain D for protease production. The highest SI value for xylanase was at 3.8 for strain J and the lowest SI for xylanase was at 3.2 for strain E. However, amylase showed the highest SI value at 3.7 for strain B while strain E showed the lowest SI value which at 3.1. From the study, two bacteria strains (strain E and strain J) possess dual enzymes activities which secrete amylase and xylanase for strain E while strain J showed activity for protease and xylanase.

Based on Table 4, protease was found dominating the enzyme properties with 5 strains were found secreting this enzyme. Protease is the key enzyme involved in the aspects of plant growth, its development, and also plant defense, as well as in senescence and plant cell death [20]. Protease helps to catalyze hydrolysis of peptide bonds in proteins and are one of the most widely used industrial enzymes. Proteases also involved in simplifying downstream degradation process and most of the bacteria possessed this ability can easily obtained from plant and animal sources [21]. Microbial proteases have potential for application in different industries including detergent, leather, silver recovery, dairy, baking, beverages, and pharmaceutical industries. Moreover, proteases have demonstrated potentials in the development of high value-added products due to their characteristic nature of aided digestion [22].

Table 4 Solubility index (SI) value and potential degrading enzyme produced by the isolated bacteria strains.

Bacteria strain	Potential degrading enzyme	Solubility Index (cm)
A (CONTROL)	No enzyme	0
B	Amylase	3.7
C	Protease	2.9
D	Protease	2.9
E	Amylase	3.1
	Xylanase	3.2
F	Xylanase	3.7
G	Xylanase	3.7
H	Protease	4.1
I	Protease	4.0
J	Protease	3.9
	Xylanase	3.8

Besides, 4 bacteria strains were found positive for xylanase which were strain E, strain F, strain G, and strain J. Xylan has been reported as one of the major hemicellulosic polysaccharide found in the plant cell wall which represents about 30 % of dry weight hardwoods and 12 % in softwoods [23, 24]. Application of xylanase in waste treatment was not a new discovery as xylanases have been used for the conversion of xylan waste water released from agricultural and food industries into xylose [12]. Presence of xylan abundantly in plant biomass also can be found in solid agricultural and agro industrial wastes, which can be potentially used to produce various industrial based products such as biofuels, animal feeds and enzymes [25].

While, strain B and strain E were positive for amylase-producing-bacteria with the ability to hydrolyze starch in plants. Application of amylase enzyme commercially in industry are wide which include chemical production, bioconversion, and bioremediation [26]. In agro-based industry, amylase is widely used in production of sugar syrups from starch [27]. Interestingly, in agricultural waste, amylase has been actively found as one of the major enzymes secreted by microbial communities such as *Bacillus subtilis* [28], *Aspergillus oryzae* [29], and *Paenibacillus amylolyticus* [30]. Many potential amylase producers can be obtained from agriculture associated wastes with high application in industrial fields.

In this study, identification of bacteria strains was performed based on phenotypic and genotypic identification. Data on the phenotypic study which includes colony morphology was presented in Table 5, while Gram staining and biochemical test were presented in Table 6. Colony morphology was analyzed using three important features (form, elevation, and margin) according to the Bergey's Manual of Systemic Bacteriology [15]. These key features of bacteria colony serve as an important criterion for colony identification [31]. Most of the bacteria strains were recorded as rod and coccus when observed under light microscope as shown in Fig. 2. Gram staining was carried out to characterize different Gram of bacteria. Purple color stain on bacteria cell is referred as Gram-positive bacteria due to thick peptidoglycan cell wall while pink color stain is referred to Gram negative bacteria due to thinner layer of bacteria cell envelope [32]. From Gram staining analysis, there were 7 bacteria strains showed Gram-negative results which were strain C, E, F, G, H, I and J. Only strain B and D were observed as Gram-positive bacteria as stated in Table 6.

For biochemical test, most of the strains showed catalase positive as they were able to produce catalase enzyme which enables them to catalyze hydrogen peroxide into oxygen and water except for strain B bacteria which showed a negative result for catalase [33]. Therefore,

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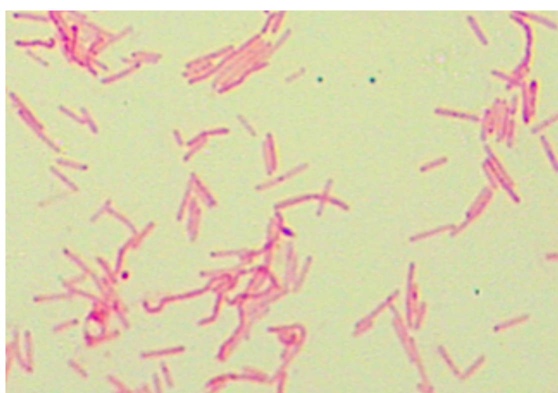
bubbles were produced during the catalase test for strain B which represented oxygen released by decomposition of hydrogen peroxide. While for oxidase, most of the bacteria showed positive results for oxidase test except for the strain D and E. Bacteria strain D and E were unable to produce cytochrome-c-oxidase which catalyzes the reaction of cytochrome and oxygen which resulted in no production of blue or deep purple color [34]. For the sulphide test, the production of sulphate showed positive results on bacterial strain B, D, F, and J. This result was also in agreement with Triple Sugar Iron (TSI) test which showed the production of hydrogen sulphide and fermentation of carbon source for strain B, D, F and J. From the data, bacteria strain C, G, H, and I showed acidic condition and only strain E showed alkaline conditions. Acidic condition is referred to the ability of bacterial strain to ferment glucose, lactose, and sucrose, while alkaline condition can be related to no carbon source is fermented but only peptone is catabolized [35].

Table 5 Colony morphology of nine isolated bacteria strains.

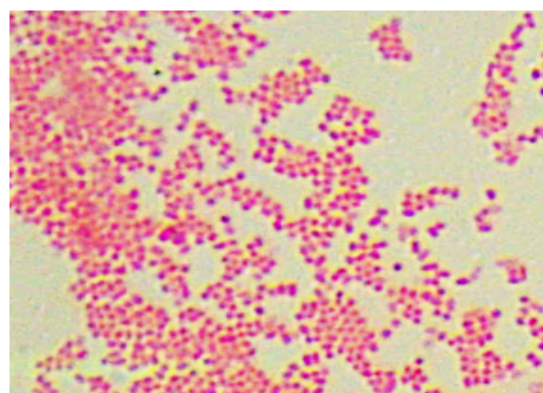
Sample	Form	Color	Elevation	Margin
B	Filamentous	Creamy	Flat	Undulate
C	Irregular	Creamy	Flat	Undulate
D	Circular	Greenish	Raised	Entire
E	Irregular	Transparent	Flat	Entire
F	Irregular	Yellow cream	Flat	Undulate
G	Irregular	Creamy	Raised	Undulate
H	Circular	Yellow	Flat	Entire
I	Filamentous	Yellow	Raised	Filiform
J	Circular	Creamy	Raised	Entire

Table 6 Biochemical tests for all 9 bacteria strains.

Sample	B	C	D	E	F	G	H	I	J
Gram staining	+	-	+	+	-	-	-	-	-
Catalase	-	+	+	+	+	+	+	+	+
Oxidase	+	+	-	-	+	+	+	+	+
Sulphide	+	-	+	-	+	-	-	-	+
Indole	-	-	-	-	-	-	-	-	+
Motility	+	-	+	-	+	-	+	+	+
Acid in TSI	H_2S	+	H_2S	-	H_2S	+	+	+	H_2S
Urease	-	-	-	-	-	-	-	-	-



a) Bacteria cells in rod shape (Strain H)



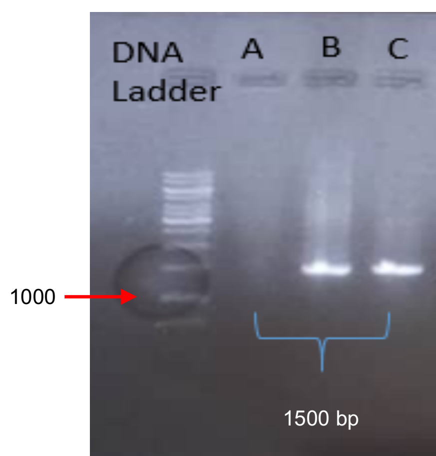
b) Bacteria cells in cocci shape (Strain C)

Fig. 2 Observation of bacteria cells after Gram staining under light microscope (1000x oil immersion).

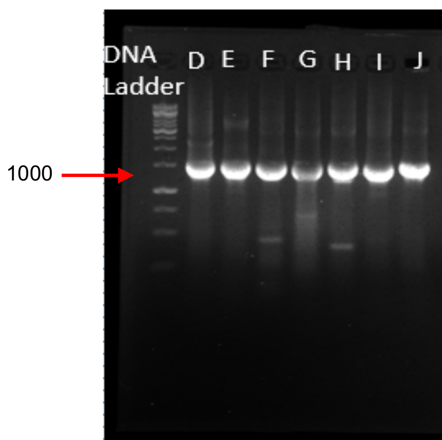
Moreover, for indole test, only bacteria strain I and J showed positive result while other bacteria strains were showed negative for indole test. Negative results in indole test are related to inability of the bacteria strain to produce tryptophanase which is responsible to degrade tryptophan into indole, ammonia and pyruvic acid [36]. While for motility test, most of the bacteria strains showed motile and only three bacteria strain (strains C, E, and G) showed negative results. For urease test, all the bacteria strains showed negative results which unable to produce urease enzyme to break down urea into ammonia and carbon dioxide via hydrolysis where the media remained yellow [37].

While, for genotypic bacteria identification, amplification of 16S rRNA gene was performed as this region gene sequences are commonly presences and universal for bacteria domain with multi gene family [38, 39]. The size of 16S ribosomal gene is 1500 base pair which is large enough for the verification purposes in the genotypic study [40]. Fig. 3 shows PCR amplification result of all 9 bacteria strains isolated. The size of PCR amplified products was approximately 1500 bp as compared to the 1 kb DNA Marker gene (Promega) by using PF3 and PR3 primers (Table 3). These universal primers were used because they could amplify a specific region present on the 16S rRNA gene sequences which considered to be universal for bacteria domain [39]. Sequences analysis were carried out using Basic Local Alignment Search Tool (BLAST) and the data were compared with the available sequences in National Center of Biotechnology Information (NCBI) database. Result for the BLAST nucleotide analysis and their percentage similarity were as shown in Table 7. From the findings, most

of the isolated bacterial strains showed higher percentage similarity with more than 95 % similarity. The accuracy recognition of unknown bacteria was identified based on percentage of similarity with respected to 95 % and 98.7 % for genus and species taxonomy as threshold value [41]. As stated by Yarza et al. [42], sequence identity of 94.5 % or lower for 16S rRNA genes is strong evidence for distinct genera, 86.5 % or lower is strong evidence for distinct families, 82.0 % or lower is strong evidence for distinct orders, 78.5 % or lower is strong evidence for distinct classes and 75.0 % or lower is strong evidence for distinct phyla. Moreover, these genetic criteria should always be accompanied by a discriminant phenotypic property and biochemical characteristics. We strongly agree that some of the bacteria strains isolated might represent a new type strain of the bacteria isolate (Table 7).



(A)



(B)

Fig. 3 PCR amplification of the targeted 16S rRNA gene from all nine bacteria strains; (A) for strains B and C; (B) for strains D till J.

From the findings as listed in Table 7, two bacteria strains (strain E and strain I) were identified under the same genus of *Pseudomonas* sp., meanwhile strain G and strain H were similar to genus *Enterobacter* sp. Others bacteria strains (strain B, C, D, F and J) were as listed in Table 8. Through analysis of 16S rRNA gene identification, several bacteria genera have been identified such as *Bacillus cereus* (strain B), *Alcaligenes faecalis* (strain C), *Micrococcus* sp. (strain D), *Pseudomonas stutzeri* (strain E), *Enterobacter cloacae* (strain G), and *Serratia marcescens* (strain J). Strain F and strain H were identified under distinct family of Enterobacteriaceae, while strain I was identified from Pseudomonadeles order which might represent a new type of proteobacteria strain.

As stated in the previous report, *Pseudomonas* species was isolated from soil sediments and has been recorded as one of the useful nitrifying bacteria in degradation process [43]. Nitrifying bacteria is important to remove nitrogen for wastewater treatment in the biological nutrient removal process [44]. A study by Salam [45] showed extensive degradation abilities of *Pseudomonas aeruginosa* RMI and SKI strains on different hydrocarbon fractions of SAE40 engine oil. This *Pseudomonas* genus is known for their diverse group of bacteria with different physiological and genetic adaptability which possess broad substrate affinity towards different classes of hydrocarbons (heterocyclics, aromatic, and alicyclics).

Enterobacter sp. are well known bacteria in agriculture as natural biofertilizer. One of the strains, *E. cloaceae* has the ability to produce Indole Acetic Acid (IAA) hormone which help in stimulating root initiation and cell differentiation including plant external stress mechanism [46, 47]. Another species known as *Enterobacter asburiae* also was commonly used in sewage treatment and biodegradation process to reduce organic matter, toxic substances, and pathogen [48, 49].

Table 7 Potential bacteria identification through 16S rRNA gene sequences analysis.

Code	Description	Percentage Identity (%)
B	*Bacillus cereus* strain WJH	98.26%
C	*Alcaligenes faecalis* strain VIT-RAS	97.66%
D	*Micrococcus* sp. strain TM-S63	97.80%
E	*Pseudomonas stutzeri* strain SGAir0442	96.74%
F	*Providencia rettgeri* strain OK-62_NRB-DRDO/MP	85.24%
G	*Enterobacter cloacae* strain A1137	98.45%
H	*Enterobacter asburiae* strain CAV1043	89.35%
I	*Pseudomonas aeruginosa* strain BFM2	79.41%
J	*Serratia marcescens*	98.11%

Study carried out recently by Mehandia *et al.* [50] reported that *Alcaligenes faecalis* isolated from soil samples composed of plant waste materials has been applied in decolorization of industrial dyes. Another study by Zhang *et al.* [51], has found out that this bacterium which was isolated from soil also has ability to biodegrade mycotoxins from contaminated feed and food. While a previous study has reported that *Micrococcus* sp. has the ability to biodegrade vegetable oil wastes. This genus showed a good ability to grow in different concentrations of vegetable oil wastes as the carbon source [52]. Proteolytic bacteria isolated from municipal solid wastes (MSW) have been identified as *Serratia marcescens* A3 and *Pseudomonas putida* A2 which demonstrated the ability to use MSW as raw material for carbon and nitrogen source for the production of industrial protease. This protease enzyme was produced through the fermentation of organic MSW materials under optimized physicochemical parameters as described by Iqbal *et al.* [53].

CONCLUSION

Nine bacteria strains have been successfully identified from agro-waste materials. All of the bacteria strains showed the ability to produce different types of degrading enzymes such as protease (5 strains), xylanase (4 strains), and amylase (2 strains). Through analysis of 16S rRNA gene identification, a few bacteria genera have been identified such as *Bacillus cereus* (strain B), *Alcaligenes faecalis* (strain C), *Micrococcus* sp. (strain D), *Pseudomonas stutzeri* (strain E), *Enterobacter cloacae* (strain G), and *Serratia marcescens* (strain G). Strain F and strain H were identified under distinct family of Enterobacteriaceae, while strain I was identified from Pseudomonadeles order which might represent a new type of proteobacteria strain. With the discovery of these bacteria species, their ability to secrete important industrial enzymes has been determined in this study. Ability of these bacteria to play important roles in degrading different type of waste materials has leads to many researches on this matter. Furthermore, application of these bacteria strains towards green

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technology to accelerate the variety of wastes decomposition may need to be further explored.

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