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Isolation and characterization of potential compost degrading bacteria isolated from domestic waste

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Domestic wastes accumulation becomes a great challenge in many parts the world, especially in rapid growing cities. Over the past decade, generation of municipal solid wastes in Malaysia has increased more than 90%. Waste materials may contain many potential bacteria with the ability to degrade waste by producing various endo- and exoenzymes. Isolation of the potential compost degrading bacteria were carried out from a disposal site at Kampung Paya Rawa, Besut, Terengganu, Malaysia using serial dilution and spread plate technique. Primary screening for the enzyme production was carried out using selective media (Congo Red, xylan, starch, skim milk and Vincent's agar medium). A total of 40 potential bacteria were isolated on Tryptic Soy Agar (TSA). Only 10 bacteria isolates exhibited xylanase, amylase and protease activity by producing halo zone on the selective media tested. Identification of the bacteria strains were performed using phenotypic (colony morphology and Gram staining) and genotypic approach (16S rRNA gene sequence). Based on NCBI BLAST analysis, 10 bacteria strains have been identified according to their percentage of similarity. They were *Pseudomonas stutzeri* strain SGAir0442 (Strain A2 and B2), *Providencia* sp. (Strain C2), *Pseudomonas stutzeri* strain KG-2 (Strain G2), *Pseudomonas aeruginosa* (Strain H2), *Micrococcus* sp. (Strain I2), *Pseudomonas putida* (Strain D2), *Lelliottia nimipressuralis* (Strain E2), *Enterobacter* sp. (Strain F2) and *Bacillus thuringiensis* (Strain J2). It can be concluded that these potential waste degrading bacteria can be further applied towards waste management system as an approach to breakdown wastes in eco-friendly way.

Keywords: waste degrading bacteria, domestic waste, 16S rRNA sequence, biochemical test.

INTRODUCTION

Rapid industrialisation and urbanisation in many countries including Malaysia have brought adverse effects to the environment due to increase waste generated. Domestic waste can be categorized into two types of waste known as organic waste consist of food and kitchen waste, yard trimmings or other garden waste and inorganic waste consists of paper, plastic, glass, wood, and metal products such as aluminium drink cans (Maczulak and Elizabeth, 2010). In Malaysia, approximately 30,000 tons of municipal solid wastes are produced daily (Fauziah and Agamuthu, 2009). Previous study reported that food debris was the major waste generated with 93.1% of the total domestic waste discarded mainly due to household activities. Following by plastics (64.3%), bottles/cans (47.3%), paper (36.0%), and old clothes (21.2%) (Yoada et al. 2014). Accumulation of wastes will degrade the environment quality and ability to sustain life, thus threaten human ability to remain healthy. Amount

of toxic, heavy metals and pathogens in raw biowaste cannot be measure accurately in environment will eventually get back into our food chain and effecting human health (Fourti, 2008, Bartkowiak et al., 2016).

Compost degrading bacteria can be defined as the synergy action of various types of bacteria which potentially produce a series of enzymes that contribute to the biodegradation of waste materials (Pérez et al., 2002). In general, bacterial enzymes can be grouped into two categories known as intracellular enzymes (endoenzymes) and extracellular enzymes (exoenzymes) (Maire et al., 2013). Exoenzymes are the enzymes secreted by the bacteria cells to the surrounding to hydrolyse complex substances into smaller subunits (Hussain et al., 2013). The end product usually composed from the stable by-products of this decomposition, the biomass of both dead and living microorganisms as well as the non-degradable parts of the raw material is called compost (Saranraj, 2014).

Earlier studies reported that cellulolytic and hemicellulolytic bacteria have been isolated from a wide range of environments such as soils, composts, decaying plant wastes and the faeces of ruminant animals (Maki et al., 2009). Generally, decomposition of domestic waste consist of different types of aerobic bacteria such as a novel bacterium known as *Ideonella sakaiensis* 201-F6 PET had been successfully isolated (Yoshida et al., 2016) as well as some of the major lipase producing bacterial genera were isolated commonly known as *Bacillus, Pseudomonas* and *Burkholderia* (Vakhlu et al., 2006). Previous studies reported that soil receiving kitchen wastes is one of the rich sources of starch degrading microorganism as it contains mostly starchy substrate. The two bacterial strains found known as *Bacillus amyloliquefaciens* and *B. licheniformis* have been exploited for the industrial scale (Kathiresan and Manivannan, 2006; Alariya et al., 2013).

Waste or residual plant biomass have the ability to be converted into various value-added products that could be generated high profit such as biofuels, chemicals, improved animal feeds and human nutrients (Mabrouk and El Ahwany, 2008). Currently, the discovery of potential compost degrading bacteria has been actively study for the potential application in compost, bio-fertilizer or bio-degradation process in decaying waste material, soil fertility and crop productivity without resulting in harmful environmental effects. The aim of this study were to identify and characterize potential waste degrading bacteria isolated from local domestic dump site. Exploration in the search for potential bacteria with the ability to promote natural and eco-friendly waste degradation should be prioritized.

MATERIALS AND METHODS

Sample Preparation

Samples were collected at Kampung Paya Rawa, Besut, Terengganu at the latitude 5.77° E and longitude 102.58° W. The sampling site is the official disposal area under Besut district and the age of the wastes were around 5 years old as confirmed by Besut City Council. Waste samples labelled as A, B and C were consisted of mix of solid waste such as cloth, plastic, aluminium cans and food substrate whereas samples labelled as D and E were consisted of soil collected from the disposal site. Waste samples were dried under the sunlight for a couple of days until constant weight obtained. Each waste sample was weighed for 10 g and homogenized in 90 mL of 0.9% saline water respectively. All samples mixture were shaken on incubator shaker for 30 minutes before centrifuged at 3000 rpm for 15 minutes to obtain supernatant.

Isolation and enumeration of bacteria

Ten series of serial dilution for each samples were prepared before plating on Tryptic Soy Agar (TSA) by spread plate method. Colony forming unit (CFU) which indicates bacterial enumeration was performed using specific formula as stated by Fouzia and Amir (2011). Colonies obtained from the serial dilution were isolated and streaked individually onto TSA at 37°C for two days (Hayat et al., 2012). The colonies were re-streaked until pure colonies were obtained.

Screening of potential degrading bacteria

Five different selective media were used as shown in Table 1. All media were sterilized by autoclaving (15 psi, 121°C for 15 minutes) except for glucose was filter sterilized using 0.22 µm syringe filter before adding the component to the media. Plates were incubated at 37°C for two to three days. Colonies with the ability to form halo zone on the selective media were recorded carefully. The zone of clearing around the bacterial growth is an indicator of enzyme production due to the degradation of carbon source present in the media (Sethi et al., 2013;

Shanmugas et al., 2015; Rahman et al., 2018). Solubilization index (SI) was calculated to indicate bacterial degradation activity based on the formula stated by Kannapiran and Vijayan (2011):

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

Table 1: List of selective media and its respective substrate.

Selective Media	Substrate
Congo red agar medium (Eida et al., 2012)	Cellulose
Xylan agar (Kumar et. al, 2010)	Xylan
Skim milk agar (Rahman et al., 2018)	Skim Milk Powder
Vincent's agar medium (Kumari et al., 2013)	Pectin
Starch agar medium (Shanmugasundaram et al., 2015)	Starch

Phenotypic Identification

Colony for each bacterial isolates were observed under stereomicroscope (Leica) and identification of the strains predominantly were based on the phenotypic characteristics described in the Bergey's Manual of Systematic Bacteriology (Holt et al. 1994). Morphological properties such as colour, shape, margin, elevation and surface were identified. Bacteria Gram staining was performed according to Bartholomew and Finkelstein (1958). There were seven different biochemical tests carried out to analyse the enzymatic functions of the bacterial strains which were catalase, oxidase, sulphide, motility, indole, triple sugar iron (TSI) and urease test.

Genotypic Identification

The 16S rRNA gene of the bacteria strains were determined from genomic DNA isolated from bacteria culture. DNA extraction was carried out using DNA Isolation and Purification Kit from Promega. PCR amplification was performed in a final reaction of 25 µL and the reaction mixture contained 1X PCR buffer, 0.2 mM dNTPs mix, 3 mM MgCl2, 1 µM forward and reverse primers, genomic DNA, 1U Taq Polymerase and sterile distilled water.

PCR reaction was run for 30 cycles using PCR machine from Applied Biosystems (Verify 96 Well Thermal Cycler). The following thermal profile was used for the PCR: 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. Primers used were PF3 (Forward primer) and PR3 (Reverse primer) as listed in Table 2. The amplified PCR products were then purified and sent for sequencing service at 1st Base Laboratory (M) Sdn. Bhd.

Table 2: List of PCR primers for the amplification of bacteria 16S rRNA gene.

Primer	Sequence
PF3 (Forward)	5′-CTA AGA GAA GCA AGT GTA ATT GGC TAG CG-3'
PR3 (Reverse)	5′-GTC ATG GTC TTA TAG TCC TTG GGA ATG-3'

Source: Dev et al., (2016)

Sequencing and Statistical Data Analysis

All raw data obtained after sequencing service were analysed using online Bioinformatics software. DNA sequences were analysed using BLAST-N software available at National Center Biotechnology Information (NCBI). For sequence alignment, CLUSTAL Omega software was used for sequence alignment and comparison. Experimental data were analyzed using statistical data analysis for Microsoft Excel. The reported results in the study were represented as the mean values of triplicates ± standard deviation.

RESULTS AND DISCUSSIONS

Isolation and screening of potential compost degrading bacteria

Ten series of dilution were prepared for all the samples before plating on the Tryptic Soy Agar (TSA) by spread plate method. After 48 hours, the viable count method was carried out in TSA plate for bacteria enumeration. Separation and identification of pure colony were also carried out to obtain pure culture of the bacteria strains before proceed to identification and characterization phase. There were 40 bacterial colonies that have been successfully isolated as pure and single colony after sub culturing the samples. Out of all the bacteria strains, 10

bacteria strains were found positive with the presence of clear halo zone around the colonies when grown on different selective media as listed in Table 1.

Solubility Index (SI) formulation was used to measure quantitatively area of halo zone presence on the selective media tested. The purpose is to observe bacteria capability to produce specific enzyme in order to degrade substrate in the media (Kannapiran and Vijayan, 2011). Results of 10 bacterial strains with their Solubility Index (SI) and potential enzyme properties were shown in Table 3.

Table 3: List of ten potential compost degrading bacteria and its Solubilization Index (SI).

Samples Label	Strains	Degrading Enzyme	Solubilization Index (cm)
IW(C)-1a	A2	Amylase	4.1
IW(C)-1b	B2	Amylase	4.3
IW(C)-2	C2	Protease	3.8
IWS(B)-2	D2	Xylanase	3.9
IW(A)-2	E2	Xylanase	3.7
IWS(A)-2	F2	Xylanase	2.2
IW(B)-1	G2	Protease	3.6
IWS(A)-4a	H2	Protease	4.3
IWS(A)-4b	I2	Protease	4.2
IW(B)-1	J2	Xylanase	4.4

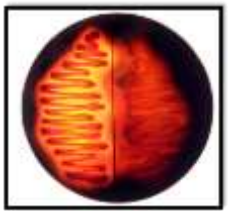
In this study, only starch agar, skimmed milk agar and xylan agar showed positive results of producing halo zones around the bacteria colonies (Figure 1). This indicates that the bacteria managed to produce certain enzyme to degrade carbon source present in the media (Maire et al., 2013). Some of the bacteria managed to produce xylanase, amylase and protease as shown in Table 3. However, no bacteria were found to produce pectinase and cellulase enzyme.

Ability of some bacteria to degrade waste are depends on the presence of degrading enzymes which are using substrate in the waste materials (Gaufam et al., 2012). It is involved a symbiotic interaction between various types of bacteria which potentially produce a series of enzymes that contribute to the biodegradation of waste materials (Pérez et al., 2002). In composting, different bacterial population plays an important role for substrate degradation and heat generation (Rebollido et al., 2008). A metabolic cooperation between different microbial communities and synergistic action of secreted enzymes help in waste degradation more efficiently (Taha et al., 2015; Jiménez et al., 2017).

Based on Table 3, the highest SI value was 4.4 produced by bacteria strain J2 for xylanase enzyme. The second highest SI value was 4.3 produced by bacteria strain B2 and H2 for amylase and protease enzyme respectively. The lowest SI value was produced by strain F2 for xylanase enzyme at 2.2 solubilization index.

Figure 1: Halo zone formation around the streaked area showed positive result for xylanase, amylase and protease producing bacteria.

Bacterial strains A2 and B2 which isolated from



(i) Cultured bacteria on xylan agar

(ii) Cultured bacteria on starch agar

(iii) Cultured bacteria on skimmed milk agar

waste sample were found positive for amylase. Basically, starch agar plate was stained with iodine after incubation to make the halo zone clearer. This indicated the starch (carbon source in the media had been consumed by the bacteria inoculated (Sethi et al., 2013). According to Burlacu et al., (2016), xylan normally can be found in plant as it made up the secondary cell walls of dicotiledon plants. From this study, four bacteria colonies (D2, E2, F2 and J2) exhibited positive acitivity towards xylanase which correlate to the majority of the waste were collected from soil and food substrates. Previous studies also found that major producers of xylanases were bacteria and actinomycetes (*Bacillus* spp*., Pseudomonas* spp*., Streptomyces* spp.) (Beg et al., 2001; Motta et al., 2013; Mandal, 2015).

Moreover, bacteria strains C2, G2, H2 and I2 exhibited positive for protease activity. Study done by Maheswari et al., (2018) reported, most of the protease degrading bacteria which have been isolated from soil are mainly originated from *Bacillus* sp*.* Other than that, *P. aeruginosa* and *B. cereus* which were isolated from soil also exhibited extracellular proteolytic activity as stated by Kalaiarasi and Sunitha (2009).

Phenotypic bacteria identification

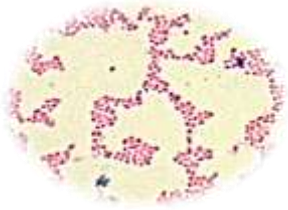
Phenotypic data were recorded based on colony morphology and biochemical test. In Gram staining, there were eight bacteria strain showed Gram-negative result which were bacteria strain A2, B2, C2, D2, E2, F2, G2, and H2 while bacteria strain I2 and J2 were reported as Gram-positive bacteria (Figure 2). Results of biochemical test were shown in Table 4. While results obtained for colony morphology identification were shown in Table 5.

Based on biochemical test, all ten bacteria strains show positive results on oxidase and catalase test. A positive oxidase test refer to strains that are able to produce cytochrome-c-oxidase which catalyzes the reaction of cytochrome and oxygen, thus lead to production of dark purple color (indophenols) as a result of tetramethyl-p-phenylenediamine reagent which has been oxidized (Shan, 2014). All bacteria that are oxidase positive are normally aerobic and use oxygen as a terminal electron acceptor in respiration (Wilber et al., 2018). Triple sugar iron (TSI) indicate test for the fermentation of lactose, glucose and sucrose. Results showed that strain C2, strain D2, strain E2, strain F2, strain G2 and strain J2 were acidic whereas strain A2, strain B2, strain H2 and I2 were alkali. Acidic bacteria strains can be referred to non-lactose fermented but there is a small amount of glucose fermented and the oxygen deficient slant turned into yellow (Karki, 2018). As for strain A2, strain B2, strain H2 and strain I2, result on TSI showed alkaline because neither lactose, glucose and sucrose were fermented and nor the slants turn into deep red or purple.

Colony morphology was analyzed using three important features according to Bergey's Manual of Systematic Bacteriology (Holt et al. 1994) which are form, elevation and margin. Key features of these bacterial colonies serve as an important criteria for their identification (Yang et al. 2016). The morphological characterization of all ten bacteria colonies were summarized in Table 5.

Table 4: Gram staining and biochemical test results for bacteria strains isolated.

Biochemical Test	**Bacteria Strains**									
	A2	B2	C2	D2	E2	F2	G2	H2	I2	J2
Gram Staining	- (rod)	- (rod)	- (rod)	- (rod)	- (rod)	- (rod)	- (rod)	- (rod)	+ (coccus)	+ (rod)
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Non motile	Motile
Indole	-	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-
Acid from TSI	Alkali	Alkali	Acid	Acid	Acid	Acid	Acid	Alkali	Alkali	Acid



i) Coccus shape of bacteria cells (Strain I2).

ii) Rod shape of bacteria cells (Strain E2).

Genotypic bacteria identification

The presence of hyper variable regions in the 16S rRNA gene provides a species specific signature sequence which is useful for bacterial identification. This is due to the slow rates of evolution of this gene region (Clarridge, 2004). Generally, 16S rRNA gene sequence represent component in 30S small subunit of a prokaryotic ribosome and thus has been widely used in microbiology studies to identify the diversities mainly in prokaryotic organisms as well as phylogenetic relationships between the organisms (Xia et al. 2015; Dev et al., 2016). In this study, a pair of universal primer known as PF3 (forward primer) and PR3 (reverse primer) which targeting 16S rRNA region in the bacterial strains were used.

Figure 3 shows amplification of the targeted 16S rRNA gene from all the bacteria strains. The size of the amplified 16S rRNA gene was shown approximately 1500 bp on 1% agarose.

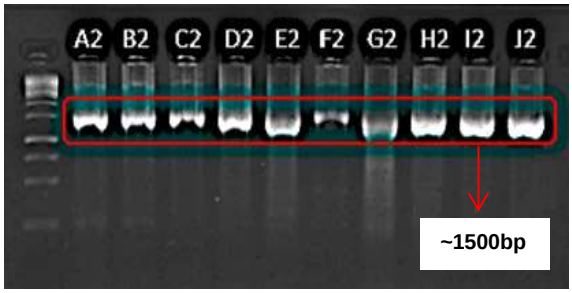


Figure 2: Observation of bacteria cells after Gram staining under light microscope (1000x magnification).

Table 5: Features of colony morphology of the isolated bacteria strains.

	Features		
Colony	Form	Elevation	Margin
A2	Filamentous	Filiform	Flat
B2	Filamentous	Filiform	Flat
C2	Irregular	Entire	Raised
D2	Round	Entire	Convex
E2	Irregular	Undulate	Flat
F2	Irregular	Entire	Flat
G2	Filamentous	Filiform	Flat
H2	Irregular	Entire	Raised
I2	Irregular	Entire	Raised
J2	Round	Entire	Raised

Basically, universal 16S rRNA primers are used as the represent part of 16S rRNA gene sequence in prokaryotic small subunit ribosome that binds to the Shine-Dalgarno sequence (Clarridge, 2004; Dev et al. 2016). Besides, the complete size of bacteria 16S rRNA gene is relatively short at 1.5 kb, which allows for rapid sequencing results in detecting unknown bacteria without utilizing the entire genomes (Langille et al., 2013).

The purified PCR products were sent to 1st Base Laboratory (M) Sdn. Bhd. for sequencing service. The raw sequences data were analysed using Basic Local Alignment Search Tool (BLAST) for nucleotide analysis using National Center of Biotechnology Information (NCBI) database. Result for nucleotide BLAST and its percentage similarity were shown in Table 6. Usually, nucleotide BLAST analysis results show percentage of similarity with the closest bacteria candidate found in the database. As a result, unknown bacteria can be identified according to the similarities of query sequence available in their information data (Pearson, 2013).

As a general rule-of-thumb for species-level bacteria determination, sequence similarity of 95% or above are considered to be a single species, thus it is not required to review the sequence once more in the future. However, if the top results fall below the range of 95% similarity, it is required to review the sequence for details confirmation and correct identification (Newell et al. 2013).

Table 6: Potential bacteria strains identified through NCBI Blast analysis and its percentage of similarity.

Label	Name of Bacteria	% Similarity
A2	*Pseudomonas stutzeri* strain SGAir0442	98.38%
B2	*Pseudomonas stutzeri* strain SGAir0442	98.26%
C2	*Providencia sp.* VibC-Oc-103	98.76%
D2	*Pseudomonas putida* strain MnS3200909	94.67%
E2	*Lelliottia nimipressuralis* strain SGAir0187	97.62%
F2	*Enterobacter sp.* DRSBII 7	99.66%
G2	*Pseudomonas stutzeri* strain KG-2	98.16%
H2	*Pseudomonas aeruginosa* strain H25883	96.97%
I2	*Micrococcus sp.* strain UCCB 141	92.19%
J2	*Bacillus thuringiensis* strain TERI SID4	95.79%

Based on the results, five out of ten bacteria strains were identified as *Pseudomonas* genus*.* known as *Pseudomonas stutzeri, P. putida* and *P. aeruginosa*. Another five bacteria strains were identified as *Providencia* sp*., Lelliottia nimipressuralis, Enterobacter* sp*., Micrococcus* sp*.* and *Bacillus thuringiensis.* In this study, multiple sequence alignment was carried out using Clustal Omega. It usually involves the input set of query sequences which are assumed to have an evolutionary relationship which share a linkage and are descended from a common ancestor (Pearson, 2013). From this analysis, bacteria strains A2 and B2 showed the closest percentage similarity to the same bacteria species identified as *Pseudomonas stutzeri* strain SGAir0442.

Generally, *Pseudomonas* sp*.* was isolated from wide range of natural environment mainly in soil, sediments and water as they used wide range organic materials for food. Soil and marine waters are two environments where *P. stutzeri* could be found (Kee et al. 2018). *P. stutzeri* has also been found in wastewater, where its unique ability to degrade some anthropogenic compounds may related to bioremediation (Chapla et al. 2012). Another study reported that *P. putida* has the ability to degrade common hexoses, such as glucose and gluconate which usually found in food wastes, to yield ATP for its energy consumption (Papadopoulou et al. 2018). While study carried out by Kämpfer et al. (2018) has successfully isolated and identified an environmental gamma-proteobacteria known as *Lelliottia nimipressuralis* from left food products and water sources.

Many have reported that a wide range of bacteria species have been isolated from agriculture environments, soils, water, agricultural wastes and natural reserves. Most common bacteria genera are *Clostridium, Cellulomonas, Cellulosimicrobium, Bacillus Thermomospora, Ruminococcus, Streptomyces, Staphylococcus, Fibroacter, Paenibacillus, Erwinia, Acetovibrio, Micrococcus,* Arthrobacter and *Pseudomonas* (Sanghi et al. 2008; Chapla et al. 2012; Liang et al. 2014; Gupta et al. 2015).

Other than that, recent study reported that variety of thermophilic cellulose-degrading bacteria such as *Bacillus* spp*., Thermus* spp*.,* and *Streptomycetes* spp*.* have been isolated from compost as stated by Zaccardelli et al. (2013). Another recent research also reported that genus of *Bacillus* especially *B. licheniformis, B. horikoshii, B. sphaericus, B. furmis, B. alcalophilus, B. subtilis* have been isolated from marine sediment and were protease producers (Maheswari et al. 2018). Other than that, extracellular proteolytic activity was also produced by *Pseudomonas aeruginosa* and *Bacillus cereus* isolated from soil (Kalaiarasi and Sunitha, 2009).

According to Rebollido et al. (2008), during composting, bacterial population act as the main role for substrate degradation and heat generation. Most of the studies reported that metabolic cooperation between different type of microorganisms and synergistic action of the secreted enzymes may lead to an efficient degradation process (Taha et al. 2015; Jiménez et al. 2017). This information were also supported by our findings which successfully isolated different types of bacteria genera from local domestic waste. This variety of bacteria would work in cooperative and synergestic manner to efficiently degrade waste materials. Therefore, by isolating and characterizing these bacteria, would provide more insight and understanding towards their potential values in industrial and waste management applications.

CONCLUSION

As conclusion, out of 40 bacteria strains isolated, 10 potential compost degrading bacteria have been successfully identified, consist of four protease producing bacteria, four xylanase producing bacteria and two amylase producing bacteria. All bacteria strains have been identified based on 16S rRNA gene sequence and biochemical test analysis. They were *Pseudomonas stutzeri* strain SGAir0442 (Strain

A2 and B2), *Providencia* sp. (Strain C2), *Pseudomonas stutzeri* strain KG-2 (Strain G2), *Pseudomonas aeruginosa* (Strain H2), *Micrococcus* sp. (Strain I2), *Pseudomonas putida* (Strain D2), *Lelliottia nimipressuralis* (Strain E2), *Enterobacter* sp. (Strain F2) and *Bacillus thuringiensis* (Strain J2). The ability of these bacteria strains to secrete important industrial enzymes have been determined. While, other potentials may need to be explored in order to shed more light on their application towards green technology to accelerate variety of wastes degradation.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NA designed the experiments, performed data analysis and also wrote the manuscript. NZN and SAMK performed sample collection, isolation of bacteria, biochemical tests, 16S rRNA analysis and data analysis. NZN and SAMK also wrote the manuscript. RW performed final revision and reviewed the manuscript. All authors read and approved the final version.

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