Malaysian Journal of Microbiology, Vol 11(2) Special Issue 2015, pp. 185-194

Malaysian Journal of Microbiology
Published by Malaysian Society of Microbiology
(In SCOPUS since 2011)

Isolation and characterization of cellulose degrading ability in *Paenibacillus* isolates from landfill leachate

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ABSTRACT

Aims: Cellulases are enzymes that convert cellulose into glucose molecules, and are produced by various microorganisms in the environment. Due to their importance to the biofuel industry, there is a need to screen for more efficient varieties of cellulases. In this study, leachate samples from a landfill site were screened for cellulolytic bacteria.
Methodology and results: Leachate samples obtained from a landfill collection pond were cultured in an enriched cellulose medium. Two cellulolytic isolates, designated MAEPY1 and MAEPY2, were isolated and further characterized. Phenotypic profiles and phylogenetic analyses using sequences of 16S rRNA, *gyr*B and whole genome suggested that these isolates are new strains of the *Paenibacillus* genera. The crude enzyme extracts from both isolates have cellulose degradation activity at approximately 0.1-0.2 IU/mg under working conditions of pH 6 and 55 °C. Assays using other lignocellulosic substrates showed that the crude enzyme extracts also have high xylan degradation activity.
Conclusion, significance and impact of study: *Paenibacillus* sp. are known to produce multiple enzymes for lignocellulolytic degradation and the present results suggest that isolates described in this study, MAEPY1 and MAEPY2, are excellent candidates deserving further study as potential producers of efficient cellulases for use in industries associated with cellulosic biomass.

Keywords: Bioprospecting, celluloytic bacteria, landfill leachate, microbial cellulase, *Paenibacillus*

INTRODUCTION

In Malaysia, municipal solid wastes are generally composed of more organic materials as compared to their non-organic counterparts. Such materials are commonly derived from food/organic wastes, papers and horticultural wastes, and often weigh more than half of the total municipal solid wastes, even in urban areas such as Kuala Lumpur city (Kathirvale *et al.*, 2004; Ismail and Manaf, 2013). These plant-derived wastes are slow to degrade due to the presence of lignocellulose and are usually discarded into landfills where decomposition can be accelerated by the microbial community (Reinhart and Al-Yousfi, 1996; Vavilin *et al.*, 2006). This process occurs primarily due to the action of a variety of microbial cellulases and hemicellulases that shear the lignocellulosic biopolymers into simple sugars. For this reason landfill microorganisms are of interest for application in many industries and represent excellent candidates for enzymatic studies (Westlake *et al.*, 1995; Huang *et al.*, 2004).

Cellulolytic microorganisms play a pivotal role in the degradation processes in landfills and are mostly bacteria. This is due to the aquatic and anaerobic nature of landfills to which bacteria are more adapted than fungi (Lynd *et al.*, 2002). Several known bacterial species with cellulolytic potential include the anaerobic fermenters of the genera *Clostridium*, *Fibrobacter*, and *Ruminococcus*, as well as the aerobic *Cellulomonas* and *Thermobifida.* Generally, it is believed that enzymes employed in aerobic degradation of lignocelluloses are secreted extracellularly in large quantities and have relatively simple structure. Conversely, anaerobic fermentative characteristically involves a few membrane-anchored enzyme complexes with multiple catalytic sites catering to various substrates (Lynd *et al.*, 2002). The latter strategy, which involves the multi-enzyme complex termed polycellulosomes, is probably best exemplified by the anaerobe *Clostridium thermocellum* (Schwarz, 2001). The complex nature of such a macromolecule, coupled with the possibility of excellent applicative potential, has spurred research initiatives into screening for new cellulolytic strains.

It has been suggested that the landfill environment is highly heterogeneous in terms of waste materials, but does not mix well due to the high solid content and poor substrate transfer (Westlake *et al.*, 1995). These factors present unique settings within landfill wastes in which multiple micro-environments exist, subject to influences from waste composition, stages of degradation and leachate migration, and in turn affect the associated microbial.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

In this paper, we report work on the enrichment of microbial communities obtained from landfill leachate samples and the subsequent isolation of cellulose degrading bacteria. The cellulolytic potential of these isolates was then assessed via several preliminary characterization studies. The study aims to prospect for new cellulases that may be useful for future enzymatic applications.

MATERIALS AND METHODS

Sample collection

Sample wastewater was acquired from the leachate collection pond of Jeram Sanitary Landfill, Selangor. Some of the leachate was kept anaerobically using AnaeroGen™ (Oxoid) in sealed containers. The leachate was then transported to the laboratory for immediate use.

Culture and enrichment procedure

Approximately 1% (v/v) leachate was inoculated into primary enrichment (PE) solution as formulated by Sizova *et al.,* (2011) and incubated for six days at 30 °C to mimic the temperature of the leachate as measured at the sampling site. The enrichment step serves to exclude the development of potential sulphate reducing and denitrifying bacteria found in the samples. The bacterial culture was then repeatedly transferred into fresh media every five days for a total of five repetitions after the initial enrichment. The culture media used in these subsequent transfers consists of minimal salts as described in Sizova *et al.,* (2011) with carboxymethyl-cellulose (CMC) as the sole carbon source. For samples kept under anaerobic conditions, both the PE solution and culture media in serum vials were boiled and gassed with nitrogen prior to use and then kept sealed in an air-tight container with AnaeroGen™.

Isolation of cellulose degrading bacteria

The enriched cultures were incubated for 5 days at 30 °C on cellulose agar plates which contain 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.02% peptone, and 1.7% agar with either 0.2% w/v CMC or microcrystalline cellulose (MCC) as sole carbon sources (Kasana *et al.,* 2008). Cellulolytic colonies were identified as those producing halo zones after staining with Gram's iodine solution (2 g KI and 1 g I_2 in 300 mL dH_2O). Pure colonies were isolated, grown in tryptic soy broth (TSB, Merck Millipore) and kept as glycerol stocks at −80 °C for future studies. Isolates were resuscitated on tryptic soy agar (TSA, Merck Millipore) prior to any subsequent characterization work.

Biochemical and physiological tests

Physiological characteristics of cellulolytic isolates were determined using Gram staining, malachite green endospore staining, and expression of catalase and oxidase. Biochemical profiles were constructed using the protocol design of bioMérieux API kits performed for carbohydrate metabolism (API 50 CH and API 20E), semi-quantification of enzymatic activities (API 20E and API ZYM), and amino acid decarboxylation (API 20E). Morphology was examined microscopically for cells, and observed on plates for colonies. Motility was verified by growing them on sulfide-indole motility (SIM) agar. Branching patterns were observed after 5 days of incubation on TSA, following instructions described by Cohen *et al.*, (2000).

Microscopy

Cells were observed under scanning electron microscopy (SEM) for determination of shape, size and presence of appendages. The protocol follows those described in Pason *et al.*, (2006) with some modifications. Overnight cultures grown on tryptic soy broth (TSB) were washed and placed onto glass slides and then fixed using 2.5% glutaraldehyde for 2 h, followed by a serial dehydration using 0-100% ethanol. Slides were then sputter-coated with gold before observation under Hitachi S-3400N Variable Pressure SEM.

16S rRNA sequencing, whole genome sequencing and phylogenetic analysis

DNA of isolates was extracted using Qiagen DNeasy® Blood & Tissue Kit following manufacturer's protocol for Gram-positive bacteria. 16S rRNA PCR was carried out using 63f (5'-CAGGCCTAACACATGCAAGTC–3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3') under the conditions as described by Marchesi *et al.*, (1998) with slight modifications: an initial denaturation step of 1 min at 95 °C; 30 cycles of denaturation (15 sec at 95 °C), annealing (45 sec at 60 °C) and extension (45 sec at 72 °C), followed by a final extension at 72°C for 5 min. The PCR products were then viewed in a 1% w/v agarose gel for verification. The amplified bands (estimated 1300bp) were subsequently purified using DNA purification kit and then sent for sequencing. Sequences were assembled in Bioedit sequence alignment editor v7.13 (Hall, 1999) before BLAST analysis on NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification. Genomic DNA for both strains was extracted and then purified using the Agencourt AMPure XP purification kit. Quality control i.e. concentration, purity, and sizing of gDNA were determined using Qubit® 2.0 Fluorometer and Agilent 2100 Bioanalyzer. The samples were subsequently fragmented and tagged with sequencing adapters using Nextera XT DNA Sample preparation kit, before loading into MiSeq Reagent Kits v2. Genome sequencing was performed using the Illumina MiSeq Benchtop Sequencer (150-bp paired-end reads). The raw

ISSN (print): 1823-8262, ISSN (online): 2231-7538

reads were trimmed and assembled *de novo* using CLC Genomics Workbench 6 (CLC Bio, Denmark).

Comparative analysis was performed using Gegenees software (Ågren *et al.*, 2012) and bacterial genomes (both draft and complete) for the genus *Paenibacillus* available in NCBI genome database. The results were then evaluated using a heat-plot based on fragmented alignment using BLASTN made with settings 200/100 and cutoff threshold for non-conserved material at 30%. Additionally, phylogenetic trees were also constructed using complete 16S rRNA and DNA gyrase B (*gyr*B) sequences where the closest matches to isolates MAEPY1 and MAEPY2 were determined via NCBI BLAST analysis and then aligned in MEGA4 (Tamura *et al.*, 2007) using CLUSTAL W (Larkin *et al.*, 2007). A bootstrap test with 1,000 replicates was performed to evaluate robustness.

Production of enzymes

Isolates were cultured in M9 minimal salts media containing a single source of carbon as described in Hazlewood *et al.,* (1992). Five different sources of carbon were offered individually including filter paper (strips of 1×6 cm) (Whatman, U.K.), carboxymethyl-cellulose (CMC) (Nacalai Tesque, Japan), microcrystalline-cellulose (MCC) (Acros Organics, U.S.), beechwood xylan (Sigma-Aldrich, Germany), and lignin. (Sigma-Aldrich, Germany). Approximately 0.5% w/v of the carbon source was used for each flask of M9 media, and inoculated with 1% bacteria culture (adjusted to McFarland standard 0.5). The bacteria cultures were prepared by growing the isolates in TSB overnight and then washed three times with phosphate buffer saline.

The cultures inoculated in M9 media were incubated at 30 °C and pH 8, until they achieved stationary phase. Subsequently, the media was harvested and centrifuged (5,000 × *g* for 30 min at 4 °C). Both pellet and supernatant were kept as crude extracts under refrigeration until further use. The pellets were re-suspended in 0.5 M phosphate buffer pH 7 and then subjected to sonication (ELMA Ultrasonics LC-130H, Germany) for cell lysis to release any intracellular and/or membrane-bound enzymes. Both fractions were then tested for enzyme activity.

Determination of enzyme activity potential

Protein concentrations of the extracts were quantified using Protein Kit (Bradford Method) reagent solution (Merck) and assessed spectrophotometrically at 595 nm. A standard IUPAC dinitrosalicylic acid (DNS) assay was performed to detect enzymatic activity in the crude extracts according to Ghose (1987) and Zhang *et al.*, (2009). Approximately 0.5 mL of the crude enzyme extracts were added to equal volumes of 0.5% carbon substrate in buffered solution and incubated for 2 h. DNS reagent was then added into the mixture and heated to 95 °C for 10 min. Presence of reducing sugar turns solution from yellow to red, in which the intensity was determined spectrophotometrically at 540 nm. Calculation of 1 enzymatic unit (IU) is defined as an average of 1 µmole of reducing sugar equivalents converted per min of assay reaction.

Characterization of enzymatic potential

Effects of pH and temperature on the activity of crude enzyme extracts were determined for temperatures ranging from 30-70 °C (intervals of 10 °C) and pH 3-8 (intervals of 1) using citrate-phosphate buffer. The specificity of the enzymes on substrates was tested on 0.5% (w/v) CMC, MCC, xylan and lignin. A comparison of enzymatic activity was made between crude enzyme extracts and 3 commercial enzymes (Celluclast®, Shearzyme®, Viscozyme® L from Novozymes, Denmark) at the controlled assay conditions using xylan and CMC as substrates. All 3 enzymes have activities corresponding to the lignocellulosic substrates used in this study, either acting on multiple substrate types i.e. Celluclast® (endoglucanase and xylanase) and Viscozyme® L (cellulolytic enzyme mixture) or in a specific manner i.e. Shearzyme® (endo-1,4-xylanase).

Statistical analysis

All assays were performed in triplicates and data obtained was analyzed using one-way analyses of variance (ANOVA) and pairwise comparisons of the means were conducted using Tukey's post hoc test at a 95% confidence level. All the statistical analysis was performed using SPSS 18 software.

RESULTS

Sampling and isolation of pure cellulolytic cultures

Plating of the enriched cultures on cellulose agar resulted in the growth of bacterial colonies with clear halo zones when stained with iodine solution. Only colonies which were able to grow on both the carboxymethyl-cellulose (CMC) and microcrystalline cellulose (MCC) agar plates were selected. In total six distinct colonies were selected and from these four were removed due to similar morphological characteristics. The remaining two isolates, designated MAEPY1 and MAEPY2, are facultative anaerobes that display a difference in colony morphology and branching patterns but are otherwise identical based on phenotypic characteristics and observation under the microscope (Figure 1).

Biochemical and physiological characteristics

The cells of both MAEPY1 and MAEPY2 stained both Gram positive (>1 day) and negative (<1 day) depending on culture age. Both isolates are rod shaped with an average width of 0.6±0.1 µm and length of 3-5 µm and are able to form endospores resulting in distinctive swelling (Figure 1). These characteristics matched the descriptions for the family Paenibacillaceae in Bergey's

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Manual of Systematic Bacteriology (Vos *et al.*, 2009). Isolates are mesophilic and are able to grow at low temperature (4 °C). Isolates are motile but no apparent appendages were observed on cells under SEM.

Biochemical characteristics determined using the API kits are described in Table 1 together with comparisons to other closely related *Paenibacillus* sp.

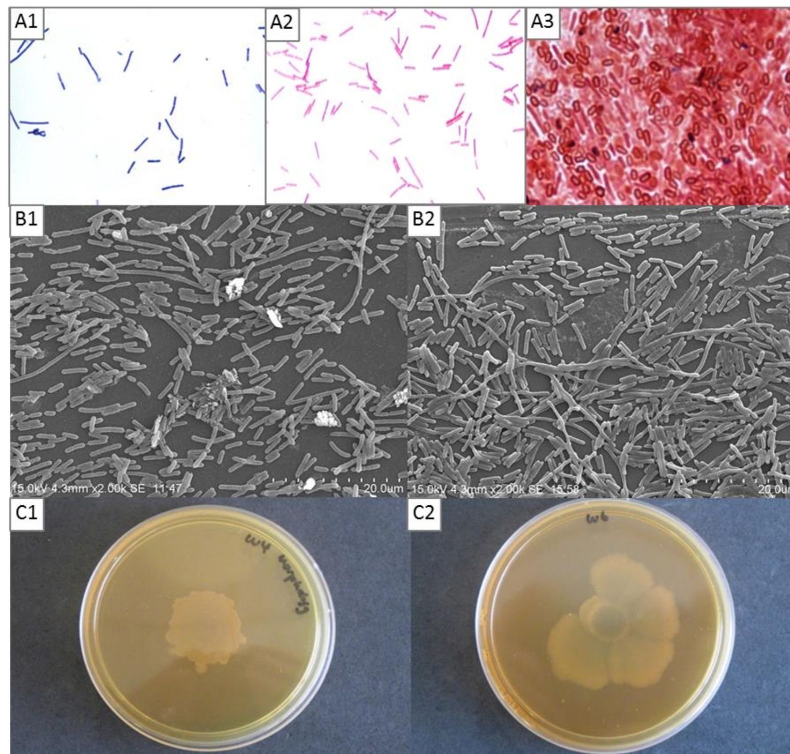

Figure 1: Images of *Paenibacillus* isolates. (A) Gram stains of landfill leachate *Paenibacillus* isolates using cultures incubated for (A1) 14-16 hours, (A2) > 24 hours, and (A3) > 3 days. (B) 18-hour old cells viewed under scanning electron microscopy for isolates (B1) MAEPY1 and (B2) MAEPY2. (C) Colony morphology of a single colony on TSA after 4 days for isolates (C1) MAEPY1 and (C2) MAEPY2.

Table 1: Biochemical characteristics that differentiate isolates MAEPY1 and MAEPY2 from other closely related Paenibacilli

Characteristics	MAEPY1	MAEPY2	1	2	3	4	5	6
API CH50								
L-Arabinose	+	+	+	+	−	+	+	−
D-Ribose	+	+	+	+	+	+	NT	−
D-Xylose	+	+	+	+	+	+	+	−
L-Rhamnose	−	−	−	−	−	−	+	−
Inulin	−	−	+	+	+	NT	NT	−
D-Melezitose	−	−	+	+	−	NT	NT	−
API 20E								
Voges-Proskauer	+	+	+	+	NT	−	−	NT
Gelatinase	−	−	−	−	NT	−	+	NT
API ZYM								
Alkaline phosphatase	−	−	NT	+	NT	NT	NT	NT
Leucine arylamidase	+	−	NT	+	NT	NT	NT	NT

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Valine arylamidase	+	−	NT	+	NT	NT	NT	NT
α-chymotrypsin	−	−	+*	+	NT	NT	NT	NT
Acid phosphatase	−	+	−*	+	NT	NT	NT	NT
N-acetyl-β-glucosaminidase	−	−	+*	+	NT	NT	NT	NT
Misc.								
Oxidase	+	+	−	+	−	NT	−	−
Catalase	+	+	+	+	+	+	+	+
DNA G+C (mol%)	45.9	45.9	49	46.7	50.3	44.3	50.5	46.4

Species: 1, *P. pabuli* NRRL NRS-924^T (=BCRC 15857^T =HSCC 492^T) (Nakamura, 1984; Shida *et al.*, 1997) *data obtained from Lee *et al.*, (2008); 2, *P. taichungensis* BCRC 17757^T (Lee *et al.*, 2008); 3, *P. tundrae* A10b^T (Nelson *et al.*, 2009); 4, *P. tylopili* MK2^T (Kuisiene *et al.*, 2008); 5, *P. xylanilyticus* XIL 14^T (Rivas *et al.*, 2005); 6, *P. xylanexedens* B22a^T (Nelson *et al.*, 2009). Tests were run using API CH50, 20E and ZYM kits from bioMerieux. All results from other strains were obtained from literature. Abbreviations: +, > 90% strains positive; −, < 10% strains positive; NT, not tested.

Phylogenetic analysis

BLAST analysis based on 16S rRNA PCR (~1,203 bp) revealed that the closest matches to both isolates MAEPY1 and MAEPY2 were *P. pabuli* (99.67%), *P. taichungensis* (99.58%), *P. tundrae* (98.42%), *P. tylopili* (98.25%), *P. xylanilyticus* (98.25%) and *P. xylanexedens* (98.00%). This placed both isolates in the genus *Paenibacillus* but was not enough to further identify the isolates to the species level. Reanalysis using the complete 16S rRNA sequences (~1540 bp for both isolates) obtained from the subsequent whole genome sequencing confirmed this result by placing both isolates MAEPY1 and MAEPY2 in close proximity to *P. taichungensis* and *P. pabuli* (Figure 2).

For further verification of the identity matches following the 16S rRNA sequences, a second highly conserved gene encoding DNA gyrase subunit B was used. Similarity of *gyr*B sequences has been correlated to levels of DNA-DNA relatedness in the *B. subtilis* group by Wang *et al.*, (2007) and has also been applied in the identification of *P. taichungensis* Lee *et al.*, (2008). In the *gyr*B phylogenetic tree (Figure 3) isolates were placed together with *P. amylolyticus, P. pabuli* and *P. barcinonensis.*

An alignment was made based on whole genome sequences of the isolates and all other listed *Paenibacillus* in NCBI Genbank database (total of 70 species and strains). The score generated between the two isolates by the heat plot is close to 100 (Figure 4), suggesting that both isolates may be strains of the same species. There were no scores above 70 which is the threshold for clustering under the same species level in the heat plot. However, it should be noted that the list did not include some of the more closely matched species such as *P. pabuli* and *P. taichungensis* due to the absence of whole genome sequences for these species. The draft genomes of strains MAEPY1 and MAEPY2 were deposited at DDBJ/EMBL/GenBank under accession no.AWUJ00000000 and AWUK00000000 (Chua *et al.*, 2014).

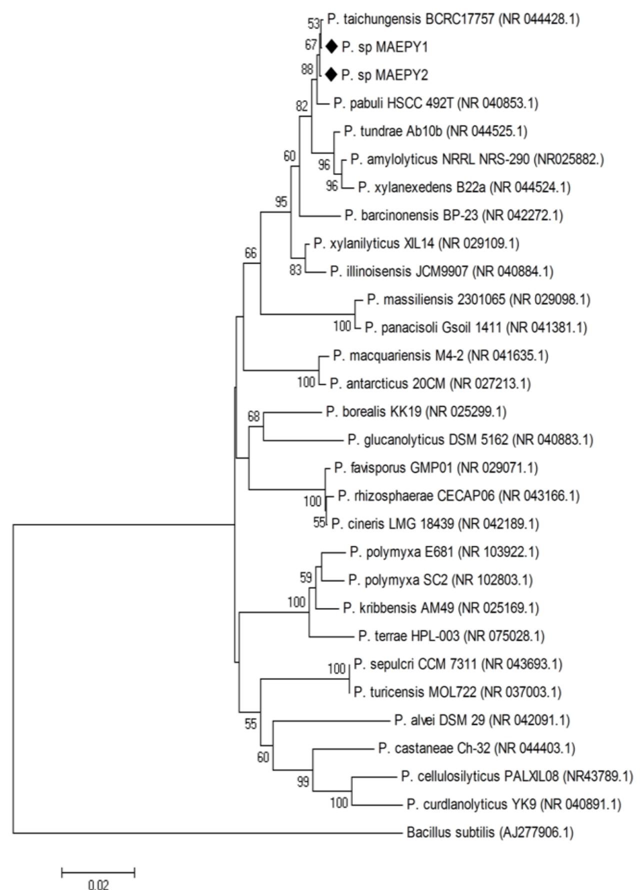

Figure 2: Neighbour-joining (NJ) tree based on 16S rRNA gene sequences. 27 *Paenibacillus* species were used in the comparison (accession numbers provided). Only bootstrap percentages above 50% are shown (based on 1000 replications). *Bacillus subtilis* strain Subtyl was used as outgroup. Bar, 1% nucleotide substitution rate.

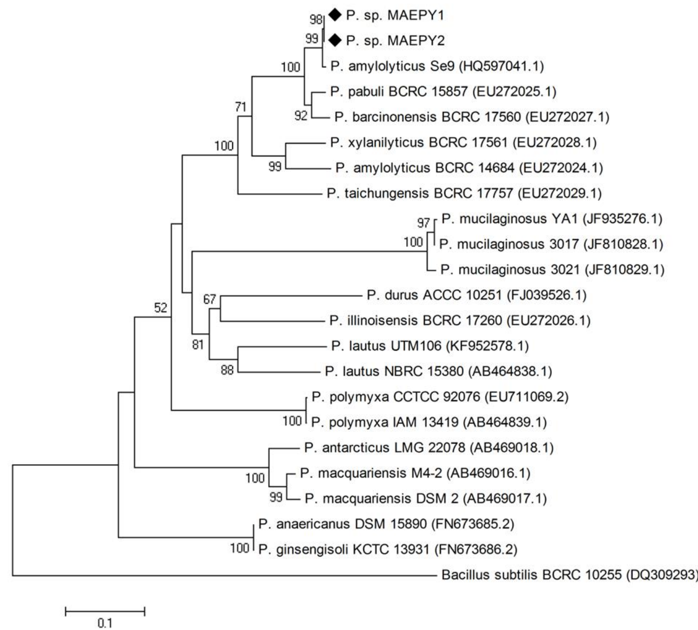

Figure 3: Neighbour-joining (NJ) tree based on *gyrB* gene sequences. Database accession numbers are provided beside the *Paenibacillus* species used. Only bootstrap percentages above 50% are shown. *Bacillus subtilis* BCRC 10255 was used as outgroup. Bar, 10% nucleotide substitution rate.

Organism	1	2
1: Paenibacillus sp. MAEPY1	100	100
2: Paenibacillus sp. MAEPY2	100	100
3: Paenibacillus alginolyticus DSM 5050	60	60
4: Paenibacillus alvei A6-6i	57	57
5: Paenibacillus alvei DSM 29	65	66
6: Paenibacillus alvei TS-15	57	58
7: Paenibacillus assamensis DSM 18201	54	54
8: Paenibacillus azotofixans ATCC 35681	51	51
9: Paenibacillus barengoltzii G22	58	58
10: Paenibacillus curdlanolyticus YK9	67	67
11: Paenibacillus daejeonensis DSM 15491	51	51
12: Paenibacillus dendritiformis C454	55	55
13: Paenibacillus ehimensis A2	54	54
14: Paenibacillus elgii B69	53	53
15: Paenibacillus fonticola DSM 21315	51	51
16: Paenibacillus forsythiae T98	51	51
17: Paenibacillus ginsengihumi DSM 21568	43	43
18: Paenibacillus graminis RSA19	54	54
19: Paenibacillus harenae DSM 16969	50	50
20: Paenibacillus lactis 154	57	56
21: Paenibacillus larvae 04-309	66	67
22: Paenibacillus larvae BRL 230010	54	54
23: Paenibacillus larvae subsp. larvae B-3650	66	66
24: Paenibacillus lentimorbus NRRL B-30488	52	52
25: Paenibacillus massiliensis DSM 16942	52	52
26: Paenibacillus mucilaginosus 3016	67	67
27: Paenibacillus mucilaginosus K02	67	67
28: Paenibacillus mucilaginosus KNP414	68	68
29: Paenibacillus panacisoli DSM 21345	52	53
30: Paenibacillus pasadenensis DSM 19293	48	48
31: Paenibacillus peoriae KCTC 3763	56	56
32: Paenibacillus pinihumi DSM 23905	56	56
33: Paenibacillus polymyxa ATCC 12321	53	52
34: Paenibacillus polymyxa ATCC 842	56	56
35: Paenibacillus polymyxa CR1	67	67
36: Paenibacillus polymyxa E681	68	68
37: Paenibacillus polymyxa M1	68	68
38: Paenibacillus polymyxa OSY DF	56	56
39: Paenibacillus polymyxa SC2	68	68
40: Paenibacillus popilliae ATCC 14706	54	55
41: Paenibacillus riograndensis SBR5	55	55
42: Paenibacillus sanguinis DSM 16941	50	50
43: Paenibacillus sonchi X19-5	53	53
44: Paenibacillus sp. 1-18	55	55
45: Paenibacillus sp. 1-49	57	57
46: Paenibacillus sp. A9	53	53
47: Paenibacillus sp. Aloe-11	56	56
48: Paenibacillus sp. HGF5	56	56
49: Paenibacillus sp. HGF7	57	57
50: Paenibacillus sp. HGH0039	61	61
51: Paenibacillus sp. HW567	57	57
52: Paenibacillus sp. ICGEB2008	62	62
53: Paenibacillus sp. J14	51	51
54: Paenibacillus sp. JC66	57	57
55: Paenibacillus sp. JCM 10914	56	56
56: Paenibacillus sp. JDR_2	67	67
57: Paenibacillus sp. oral taxon 786 str. D14	52	52
58: Paenibacillus sp. OSY-SE	54	54
59: Paenibacillus sp. PAMC 26794	66	66
60: Paenibacillus sp. UNC217MF	51	51
61: Paenibacillus sp. UNC451MF	58	58
62: Paenibacillus sp. UNCCL52	57	57
63: Paenibacillus sp. URHA0014	50	50
64: Paenibacillus sp. WLY78	56	56
65: Paenibacillus taiwanensis DSM 18679	51	51
66: Paenibacillus terrae HPL 003	65	65
67: Paenibacillus terrigena DSM 21567	53	53
68: Paenibacillus vortex V453	61	61
69: Paenibacillus Y412MC10	64	64
70: Paenibacillus zanthoxyli JH29	55	56

Figure 4: Heat plot of similarity matrices for Paenibacilli. The heat-plot is based on a fragmented alignment using BLASTN made with settings 200/100 for *Paenibacillus* sp. MAEPY1 and MAEPY2 against a list of all available *Paenibacillus* whole and draft genomes (total of 70 genomes). The cut-off threshold for non-conserved material was 30 %.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Characterization of enzyme activity

The enzymes produced by both isolates when grown in M9 minimal salt media were only detected in the extracellular fraction. This allowed for direct use of the cell-free supernatant from culture broths as the crude enzyme extract. Activity of crude enzymes peaked at approximately 55 °C, pH 6 (data not shown) and these parameters were therefore used in subsequent enzymatic assays. The mean activities of the crude enzyme extracts from both strains using CMC as the substrate were in a range of 0.1-0.2 IU/mg. When tested using other lignocellulosic substrates, the xylanase activity (≈ 1.1 IU/mg) was shown to be significantly higher, whereas activity using MCC was lower (≈ 0.05 IU/mg) (Figure 5a). Ligninase activity however, is negligible for both isolates.

A simple comparison of enzymatic activity was made with some commercially available enzymes for the assessment of the applicative potential of the isolates. In addition to the cellulolytic potential, xylanase activity was also included in the assessment using Celluclast® (endoglucanase and xylanase), Viscozyme® L (cellulolytic enzyme mixture) and Shearzyme® (endo-1,4-xylanase). Based on Figure 5b, the crude extracts from both strains had better xylanase activities than their commercial counterparts at the chosen assay conditions (55 °C, pH 6), although the cellulase activity is lower.

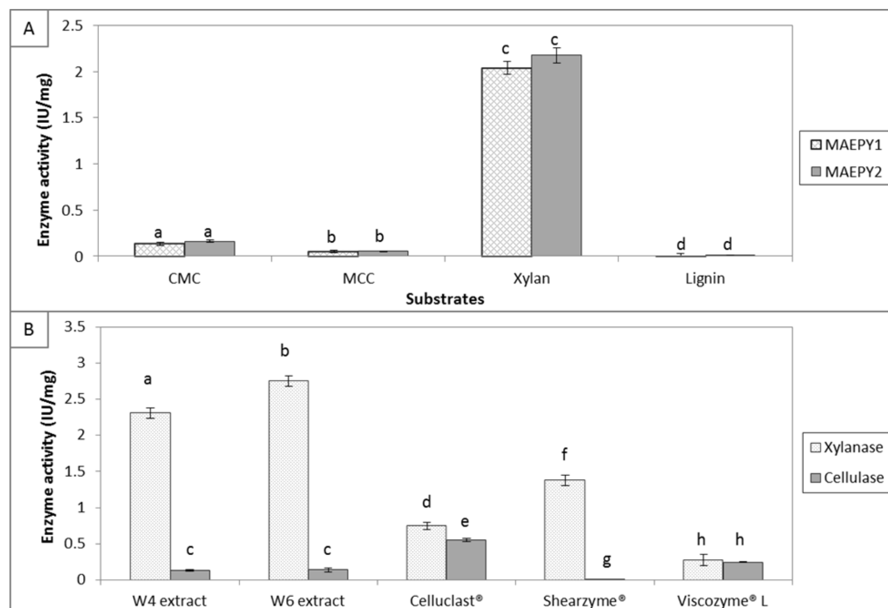

Figure 5: Catalytic activity of crude enzyme extracts from MAEPY1 and MAEPY2 assayed at 55 °C, pH 6 for 2 h. (A) Crude enzyme activity using CMC, MCC, xylan and lignin as substrates. (B) Comparison of xylanase and cellulase activity of enzyme extracts with commercial enzymes. Cellulase and xylanase activities were assayed using CMC and xylan as substrate, respectively. All results are presented as mean ± SD where n = 3; different letters within the graphs represent significant difference between the samples where $p < 0.05$.

DISCUSSION

Over the last decade there have also been numerous descriptions of novel species from the genus *Paenibacillus* with cellulolytic or hemicellulolytic capabilities (Velázquez *et al*., 2004; Sánchez *et al*., 2005; Rivas *et al*., 2006; Khianngam *et al*., 2011). In the present study, two facultative anaerobic bacilli were isolated from the leachate of Jeram Sanitary Landfill. These were later suggested to be potentially new strains of the genus *Paenibacillus* using biochemical (Table 1), physiological characteristics (Figure 1) and phylogenetic analyses (Figures 2, 3 and 4). As the attempt made to isolate the positive colonies on the cellulose agars were not exhaustive, this may have caused the low variation in bacterial taxa observed in the successfully grown cultures. We suggest that employing different culture techniques, such as the procedures described for enrichment in Pourcher *et al*., (2001) or using molecular-based screening (Chen *et al*., 2003; Krishnamurthi and Chakrabarti, 2013) may yield different results. The methodologies used in this study can however be regarded as selective for viable strains that are adaptive and efficient cellulose degraders. Studies on bacterial demographics in landfills have described members of the family Bacillaceae to be the predominant inhabitants of landfills (Pourcher *et al*., 2001; Krishnamurthi and Chakrabarti, 2013). The high number of aerobes/facultative anaerobes described in these studies suggests a strong possibility that these bacteria have a more definitive role in cellulosic waste degradation compared to anaerobic fermenters. It is known that the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

degradation of municipal solid wastes in landfills occurs under anaerobic conditions but there is lack of literature that specifies if the decomposers are strictly or facultative anaerobic. However, such studies may be hindered by the difficulty in obtaining and culturing samples. Even so, the heterogeneity in the nature of landfill sites seems to favour the hypothesis that the microbial community generally retain characteristics that improve adaptability (Westlake *et al.*, 1995). The physiological traits of both MAEPY1 and MAEPY2, particularly motility and oxygen tolerance, may also be indicative of their adaptability in the landfill environment. The abilities to propel themselves using flagella, form spores and switch oxygen metabolism may have competitive advantage in the migration of such strains throughout the landfill and proliferation in the various pockets of diverse microenvironments under the buried waste.

The catalytic potential of the cell-free culture broth as well as absence of intracellular cellulases suggests that the enzymes were secreted extracellularly. This means that the enzymes might not be large polycellulosomes molecules anchored to cell membranes typical of cellulose degrading anaerobic fermenters but instead have the simpler structural characteristics of those from other cellulolytic aerobic bacteria/fungi (Lynd *et al.*, 2002). The presence of xylanase activity despite the absence of xylan substrate in the enzyme production medium suggests that: (i) expression of xylanase gene might be regulated by the same pathway as cellulase expression or (ii) the *Paenibacillus* isolates secreted enzyme complexes with multiple active sites capable of digesting both celluloses and hemicelluloses (e.g. xylan). Further work will be conducted to verify this observation but it suggests that MAEPY1 and MAEPY2 do not survive on cellulose alone. This is further borne out by the fact that the xylanase activity was shown to be higher than its commercial counterparts (Figure 5b) under selected reaction conditions. A preliminary investigation into the annotated gene sequences revealed presence of carbohydrate-binding domains in some of the proposed cellulase/hemicellulase genes (data not shown) which may contribute to the high activity observed. The roles of carbohydrate-binding domains are proposed to be involved in maintaining the close proximity of enzymes towards specific substrate sites to facilitate catalytic activity (Boraston *et al.*, 2004). Further investigation into the structural domains of the enzyme molecules secreted by MAEPY1 and MAEPY2 are currently being undertaken.

CONCLUSION

In the present study, two new cellulolytic strains of the genera *Paenibacillus* were obtained from leachate samples from a sanitary landfill. Preliminary assessment of the crude enzyme extracts secreted by the isolates revealed good catalytic potential. Specifically, the xylanase activities of the isolates were comparable to some of the commercial enzyme products. Our findings serve to help isolate and develop good cellulases/hemicellulases as well as provide insights to the microbial enzyme systems in landfill sites.

ACKNOWLEDGEMENTS

The research in this paper was financially supported by Monash University. The authors are also grateful to Mr How Lim Sek (KUB-Berjaya Enviro Sdn. Bhd.) and Mr Zamri Abd Rahman (Worldwide Landfills Sdn. Bhd.) for their assistance in collecting leachate samples, as well as Ms Lim Liew Gin (Novozymes Malaysia Sdn. Bhd.) for providing the commercial enzyme products.

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