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Comparison of forest soils and waste-contaminated soils as sources of polysaccharide-degrading microbes

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Abstract
Objective: This study was conducted to determine the suitable soil types that can be used as sources of polysaccharide-degrading microbes. A comparison was done of the potential of isolating such microbes from non-polluted areas (forest soils) and waste-contaminated soils.
Methodology and Results: Waste-contaminated soils have more isolates producing polysaccharide-degrading enzymes (polysaccharases) than forest soils. *In vitro* plate screening using dye-labeled substrate detected 78 isolates producing polysaccharases from waste-contaminated soils, compared to 59 isolates from forest soils. Xylanase was the most common polysaccharase produced by isolates from both soil samples, followed by amylase and cellulase. Enzyme assay using dinitrosalicylic acid further established that bacteria from waste-contaminated soils produced the most xylanase (2.355 U ml^{-1}), amylase (1.420 U ml^{-1}) and cellulase (0.910 U ml^{-1}) activities, while bacteria isolated from forest soil samples have comparatively less xylanase (0.776 U ml^{-1}), amylase (0.223 U ml^{-1}) and cellulase (0.024 U ml^{-1}) activities. Similarly, polysaccharase activities in fungal isolates from waste-contaminated soils were higher than that of isolates from forest soils, except for cellulase activity.
Application of findings: Waste-contaminated soils are better sources for selection of candidates for bioremediation of solid wastes and for extraction of polysaccharases for industrial applications.

Key words: amylase, cellulase, industrial applications, polysaccharases, xylanase

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Introduction
Microbial derived enzymes are highly valued in many industrial applications (Akhtar *et al.*, 1993; Bhat, 2000; Strauss *et al*., 2001). Of the many types of microbial enzymes, polysaccharases are the most commonly used biocatalysts in various biotechnological activities. They are usually required in small amounts and can be easily obtained by manipulation of the microbes (Gupta *et al*., 2003). Polysaccharases make up the second largest group of industrial enzymes (Bhat, 2000), and are often used in the processing of food and beverage (Hebeda *et al*., 1991; Bhosale *et al*, 1996; Strauss *et al*., 2001), paper and pulp (Akhtar *et al*., 1993; Gupta *et al*., 2000), textile (Bhat, 2000), animal feed

(Walsh *et al*., 1993), detergent, cosmetic and chemical-synthesis processes (Ten *et al*., 2004). Other factors contributing to favorable use of microbial polysaccharases include the highly efficient and selective nature of the reactions they catalyze, hence causing low levels of pollution, and the low expensive and effort required compared to chemical-based methods (Cherry & Fidantsef, 2003). With the increasing demand for microbial polysaccharases projected to grow rapidly from the current market value of USD1.6 billion (Demain, 2000), novel enzyme-producing strains are continuously being sought to support industrial needs and to provide alternatives to the current strains.

To achieve this purpose isolation from soil is one of the oldest and most beneficial methods since many isolates can be recovered and screened to detect their beneficial properties. In soils, a higher diversity of microorganisms can be recovered, especially from nutrient rich environments, enhanced primarily by the presence of plant residues, organic matter, and organic substances from the plant roots (Coyne, 1999; Sylvia *et al*., 2005). Plant residues consist mainly of cellulose, starch and xylan (Smith & Wood, 1991; Mellerowicz *et al.,* 2001; Mazeau *et al.,* 2005). As such, an abundance of plant residues in soils would influence the predominance of a microbial community that is able to degrade and utilize these substrates.

Many types of soil-borne bacteria and fungi have been proven to produce amylase, cellulase and xylanase as their main polysaccharases. These isolates often produce more than one specific enzyme to achieve complete degradation of the substrates. *Bacillus* can produce both α-amylase and β–amylase, to degrade starch to form amylose and maltose, respectively (Walsh, 2002). Starch is also degraded by the extra-cellular enzyme amyloglucosidase, produced by the fungi *Aspergillus* and *Rhizopus* (Walsh, 2002). A variety of fungal species including *Trichoderma, Chaetomium, Penicillium, Fusarium,* and *Agaricus* efficiently degrade cellulose in plant debris (Walsh 2002; Zhang *et al.,* 2006). Other multiple-enzyme producing isolates include; *Bacillus* isolates for amylase and xylanase production (Rapp & Wagner, 1986); *Streptomyces* isolates that produce β–amylases (Walsh, 2002) and xylanases (Rapp and Wagner, 1986); *Cellulomonas* and *Trichoderma* that produce cellulase and xylanase (Rapp & Wagner, 1986; Walsh, 2002), and *Aspergillus* that degrade starch and xylan (Walsh, 2002). These isolates help to decompose remnants of living matter in the natural environment, and therefore have unlimited potential for management of wastes in the environment.

In this study, bacteria and fungi were isolated from samples of forest soils and waste-contaminated soils. Forest and waste-contaminated soils carry different residual composition, which may chart a different pattern of microbial community. Forest soils are covered with plant debris (Sylvia *et al*., 2005), while waste-contaminated soils mostly consist of organic waste, mixed paper, textiles and yard waste (MHLG, 2004). This study then aimed to determine which of the two soil types have better potential for isolation of microorganisms with polysaccharide-degrading activity, and to establish the polysaccharases produced by these isolates.

Materials and methods

Isolation of soil microbes*:* The waste-contaminated soils were collected from two different locations in Kuala Lumpur, Malaysia, while the forest soils were collected from Setapak and Johor, Malaysia. Four samples were randomly collected from each location. These soil samples were obtained from the layer between the residue litters to approximately 15 cm beneath the soil surface. The collected soil samples were first pooled and mixed together for a more

homogenous distribution, and air-dried at ambient temperature (28±2°C) for five days. Five grams of soil from each sample were then dissolved in 100 mL of sterile distilled water in a 250 mL conical flask. Serial dilutions to 10^{-9} were performed, and 0.1 mL aliquot pipetted from each dilution factor and spread onto NA (Nutrient Agar, Oxoid) plates and PDA (Potato Dextrose Agar, Conda) plates. Inoculated agar plates were incubated at room temperature (25±2°C) for 2 and 7 days for NA and PDA plates, respectively. Bacterial and fungal colonies formed on agar surface were carefully transferred onto fresh NA or PDA plates to establish pure cultures.

In vitro screening for polysaccharase production: The detection of polysaccharase production by the isolates was conducted using the dye-labeled substrate (plate-screening) method (modified from Ten *et al*., 2004). Xylan-red, starch-blue and cellulose-orange substrates were prepared and incorporated into the respective agar medium, to enable detection of xylanase, amylase and cellulose activities, respectively.

For xylan-red preparation, 2 g of beechwood xylan (Sigma®) was first dissolved in 30 mL of distilled water, followed by addition of 10 mL of 2M NaOH (Merck), 1.9 g of Cibacron Brilliant Red 3B-A (Sigma-Aldrich®) and 1.2 mL of 1,4-butanediol diglycidyl ether (Aldrich®). The mixture was allowed to solidify at room temperature (25±2°C) for 48 hours. The resultant gel-like xylan-red mixture was then mixed with 100 mL of distilled water and blended for 15 seconds. The ground particles were washed with boiling water (100±2°C) and filtered repeatedly with a vacuum pump until the filtrate was colorless to remove the unbound dye. The above procedure for xylan-red preparation was repeated for starch-blue and cellulose-orange preparation, with each producing 72 g, 63 g and 10 g of highly hydrated substrate, respectively.

The dye-labeled substrates were then supplemented to both NA and PDA medium, at a rate of 25.0 g L^{-1}, 50.0 g L^{-1} and 25.0 g L^{-1}, of xylan-red, starch-blue and α-cellulose-orange, respectively. Upon agar solidification, bacterial isolates were inoculated within a 0.5 cm diameter on the NA plates, while fungal mycelial discs measuring 0.5 cm in diameter were placed on PDA plates. The following isolates have been shown to be able to degrade a variety of polysaccharide-based substrates, and were inoculated as positive controls in the plate assay; *Bacillus subtilis* for xylanase activity (Isabel & Roncero, 1983), *Pseudomonas fluorescens* for cellulase activity (Yoshikawa, 1974) and *Aeromonas hydrophila* for amylase activity (Ten *et al*., 2004). These isolates were obtained from the existing stock cultures in Universiti Tunku Abdul Rahman. Media inoculated with sterilized distilled water was used as negative control. The NA and PDA plates were then incubated at room temperature (25±2°C). Formation of discolored zones was observed at day 5 and day 7 after inoculation, for bacterial and fungal isolates, respectively. Bacterial and fungal isolates that produced discoloration zones with the largest diameter discoloration were chosen for the DNS assay.

Quantification of polysaccharase activities: The DNS assay is a quantitative determination of reducing sugars that are released as a result of activities of polysaccharases. In this study, the DNS reagent used [10 gL^{-1} dinitrosalicylic acid (Sigma®), 0.5 gL^{-1} Na_2SO_3 (R&M Chemicals) and 15 gL^{-1} NaOH (Merck)] was prepared as described by Wang *et al*. (1997), with modification to the amount of NaOH used. Standard curves for glucose and xylose were established according to methods by Konsula & Liakopoulou-Kyriakides (2003).

The activities of the polysaccharases were determined by mixing 1% of the substrate (starch, beechwood xylan, or α-cellulose) with 1 mL of the inoculum suspension (10^7 cfu mL^{-1}) in a 15 mL centrifuge tube. Tubes were then incubated at 30±2°C for 3 hours in a water bath with slight agitation (140 rpm). After the incubation period, 2 mL of the DNS reagent was added to each of the centrifuge tubes. All tubes were heated at 90±2°C for 5 minutes, after which each tube was added with 1 mL of 40% potassium sodium tartrate solution and cooled on ice (0±2°C) to room temperature (25±2°C). The absorbance value for each tube was then recorded at A_{575nm}. Three parallel replicates were used for each of the tested substrate. The procedure was repeated for determination of DNS assay for fungal isolates, using a spore suspension of 5 x 10^4 cfu mL^{-1}.

Statistical Analysis: Data collected were analyzed using the Statistical Analysis System

(SAS) version 6.12 and the means compared using Tukey's Studentized Range Test ($HSD_{(0.05)}$).

Results

Isolation of microbes from soil samples: A higher number of bacterial and fungal isolates were obtained from forest soils compared to waste-contaminated soils. Of the 156 isolates recovered from forest soils, 100 isolates were bacteria and the remaining 56 fungi. Similarly, waste-contaminated soils also hosted more bacterial isolates, with only 24 fungal isolates out of a total of 108 isolates.

In vitro screening for polysaccharase production: Samples from waste-contaminated soils had more isolates producing polysaccharases compared to forest soils. Polysaccharase activities were detected in 78 isolates from waste-contaminated soils, with 52 isolates producing xylanase and 26 isolates showing amylase activities (Figure 1). In contrast, only 59 isolates from forest soils showed either amylase or xylanase activities. Thus 72% of microbes from waste-contaminated soils produce at least one polysaccharase, as compared to only 38% of microbes from forest soils.

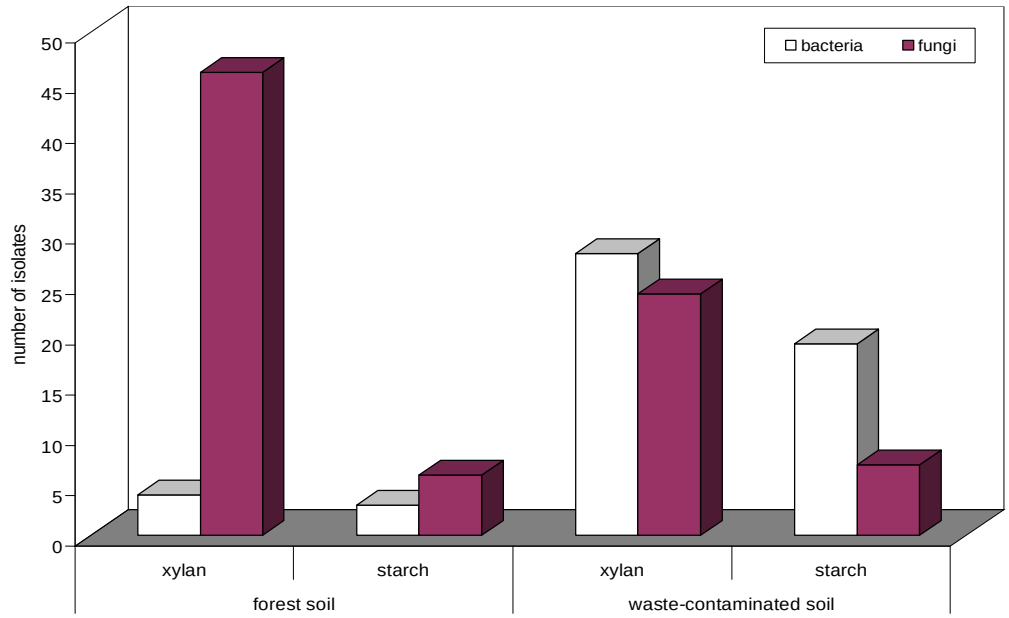

Figure 1: Total bacterial and fungal isolates from different soil samples, with positive xylanase and amylase activities.

In total, seventy fungal isolates were identified that produce xylanase, of which 46 were isolated from forest soils (Figure 1). Bacterial isolates that produce xylanase were mostly recovered from waste-contaminated soils. A total of 22 bacterial isolates were identified that produce amylase as compared to only 13 fungal isolates expressing amylase activity (Figure 1). Most of the amylase-producing bacteria were also recovered from the waste-contaminated soils.

By the plate-screening method, the type of polysaccharase produced was easily identifiable due to the distinct discoloration zones observed. A discoloration zone without colored particles denoted degradation of the corresponding substrate. Xylan-red particles were absent from the undersurface of 102 bacterial and fungal colonies tested. Thirty five isolates that were positive for amylase activity showed discoloration of the starch-blue substrate. Isolates that produced both

amylase and xylanase had no starch-blue or xylan-red particles beneath the colonies.

The diameters of the discolored zones were generally smaller for bacterial isolates than for fungi, ranging from 0.4 to 3.5 cm for bacteria, and discoloration of 1.9 to 7.2 cm for fungi. The discoloration zone for some isolates, mostly fungi, was observed to extend beyond the colony coverage, being as large as 6.3 cm diameter although the colony diameter was only 4.0 cm. All positive results were confirmed using known positive control isolates, except discoloration cellulase production, which was also not detected even with the positive control *Pseudomonas fluorescens* isolate. The cellulose-orange particles that were not degraded were found in the agar beneath the colonies.

From the many isolates tested, four bacterial and four fungal isolates were detected that produced relatively large discoloration zones (Table 1). Data shown includes only isolates that had discoloration zones more than 2.5 cm diameter, and more than one type of polysaccharase. All four isolates from waste-contaminated soils produced more than one type of polysaccharase (xylanase, amylase), with the fungal isolates F6103 and F6304 expressing larger discoloration zones compared to the bacterial isolates B2215 and B2402 (Table 1). The mean diameter of discoloration zones for isolates B2215 and B2402 was 4.2 and 2.7 cm for xylan-red and starch-blue substrates, respectively, while the mean diameter for isolates F6103 and F6304 was twice as large, measuring 6.75 and 4.1 cm for the same substrates, respectively.

Table 1: Diameters of discoloration zones (cm) observed for xylan-red, starch-blue and cellulose-orange substrate for the respective isolates tested

Source	Isolate	Diameter of discoloration zone (cm)		
		Xylan-red	Starch-blue	Cellulose-orange
Forest soils	B31201	3.8	-	-
	B61502	3.2	2.1	-
	F12204	3.4	-	-
	F42303	2.4	-	-
Waste-contaminated soils	B2215	5.6	3.5	-
	B2402	2.8	1.9	-
	F6103	7.2	4.0	-
	F6304	6.3	4.2	-

Note: - indicates no discoloration zone observed

Unlike isolates from waste-contaminated soils, bacterial isolates B31201 and B61502 from forest soils produced larger discoloration zones compared to the fungal isolates F12204 and F42303. However, only one isolate (B61502) from forest soils produced more than one type of polysaccharase, as compared to all four isolates from waste-contaminated samples. These eight isolates, with their relatively larger discoloration zones (Table 1) were selected for quantitative analysis to determine the mean activities of the polysaccharases produced.

Quantification of polysaccharase activities: Isolates B2215, B2402, F6103 and F6304 from waste-contaminated soils produced more xylanase, amylase and cellulase, compared to isolates B31201, B61502, F12204 and F42303 from forest soils (Figure 2). Bacterial isolates B2215 and B2402 from waste-contaminated soils produced higher xylanase and amylase activities than the

fungal isolates F6103 and F6304, with means of 2.355, 1.42, 1.27, and 0.950 U mL^{-1}, respectively. Significantly higher cellulase activities were also detected among the bacterial isolates (0.910 U mL^{-1}) compared to the fungal isolate F6304, although all had less cellulase activity than isolate F6103's 1.180 U mL^{-1} (Figure 2).

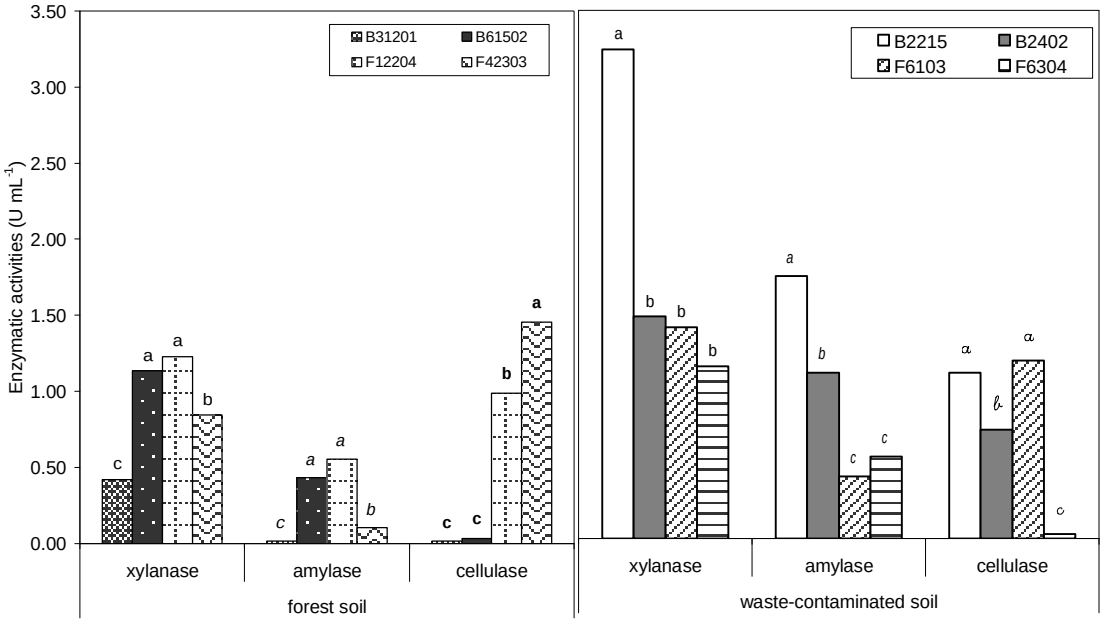

Figure 2: Xylanase, amylase and cellulase activities (U mL^{-1}) for eight selected bacterial and fungal isolates recovered from forest soils and waste-contaminated soils. Means of columns with the same letters and the same font type are not significantly different (HSD$_{(0.05)}$).

The fungal isolates F12204 and F42303 from forest soils produced more xylanase, amylase and cellulase compared to the bacterial isolates B31201 and B61502 from the same soils. Isolates F12204 and F42303 had mean xylanase, amylase and cellulase activities of 1.037, 0.328, and 1.220 U mL^{-1} respectively, compared to mean of the respective activities of 0.776, 0.223, and 0.024 U mL^{-1} for the bacterial isolates B31201 and B61502 (Figure 2). Compared all the eight isolates, xylanase and amylase activity was highest in isolate B2215 from waste-contaminated soils, and lowest in isolate B31201 from forest soils. Isolate F42303, from forest soil had the highest cellulase activity (1.454 U mL^{-1}) compared to the other isolates (Figure 2).

Results from the quantitative assessment showed that the DNS assay effectively detected production of cellulase by the eight isolates (Figure 2) although no cellulose-degradation was observed in the plate assay (Table 1). Amylase production by isolates B31201, F12204, and F42303, which was also not observed by the plate-screening method, was clearly detected by the DNS assay. These results indicate that the DNS assay provides a better assessment method than the plate screening assay. The DNS assay was also more accurate in quantifying the enzymatic activities expressed, which is better compared to qualitative estimation based on the diameter of discoloration zones. For example, isolate B2402 which initially had

a discoloration zone measuring 1.9 cm diameter on the starch-blue substrate, was determined to have a much higher amylase activity of 1.100 U mL^{-1}, compared to isolate F6103 which had a 4.0 cm wide discoloration zone, but only 0.410 U mL^{-1} of amylase activity (Table 1, Table 2). Nevertheless, the plate assay was useful as a rapid detection method for initial screening of large numbers of isolates.

Discussion

Forest soils yielded more isolates than the waste-contaminated soils with bacteria isolates dominating the microbial community. This finding was as expected since the distribution of bacteria in soils is typically to a factor of 10^8 compared to a factor of 10^5 for fungi (Metting, 1993).

Although the initial plate-screening exercise using dye-labeled substrates was able to detect xylanase- and amylase-producing isolates, this method is noted to have limitations such as the false-negatives observed for cellulose degradation. We suspect this false outcome could be due to the substitution of hydroxylethyl-cellulose (HEC) used by Ten *et al.* (2004) with α-cellulose. In previous studies, cellulose-orange detection has always been established with HEC, and not yet with α-cellulose. The weakness of the plate-screening method was further proved after detection of cellulase-positive isolates by the DNS assay, confirming the possibility that some isolates that produce cellulases might have been screened out by the plate-screening test.

Generally, isolates from the different soils produced the same type of polysaccharases (xylanase, amylase, cellulase), but to varying degrees of activities. The plate-assay confirmed that these polysaccharases are extra-cellular in nature, as the enzymes were able to diffuse into the agar to degrade the dye-labeled particles. The highest number of isolates produced xylanase, followed by amylase and cellulase. Xylanase production was found to be fairly equal in bacterial and fungal isolates but bacterial isolates produce more amylase, while some fungal isolates, e.g. F42303, F12204 and F6103 had relatively higher cellulase activities.

Xylanase, the most produced polysaccharase, degrades xylan which is a major constituent of plant litter in forest soils, as well as inorganic, paper or yard waste-contaminated soils (MHLG, 2004). The rate of xylan decomposition is rapid and exceeds that of cellulose degradation (Sylvia *et al*., 2005), which might explain the clear xylan-red discoloration zones that were observed as early as the third day after inoculation. Amylases are produced mainly by the bacterial isolates from soils that are contaminated with food, organic or paper wastes (MHLG, 2004), but also from mixed plastic wastes consisting of plant-derived polymers, such as starch, xylan and cellulose derivatives (Kawai, 1995). Forest soils have less amylase-producing isolates since in the natural ecosystem, starch is mainly found in storage organs of plants, e.g. rhizomes and tubers (Sylvia *et al*., 2005), which may be utilized by other microfauna or larger organisms, rather than being degraded naturally by microorganisms.

Results here showed that cellulases are produced mostly by fungal isolates, especially those from forest soils. Cellulose is found in plant cell walls (Smith & Wood, 1991), and the incorporation of plant residues into the soils encourages the growth of cellulase-producing microbes. The few bacteria (B2215 and B2402) from waste-contaminated soils that produced substantial amounts of cellulase may have adapted to utilizing carbon sources from cellulose, possibly those degraded from cellulose-based wastes such as paper, textiles and yard wastes (MHLG, 2004).

In conclusion, this study has found that microorganisms from unpolluted forest soil or from waste-contaminated soils are able to produce the main microbial polysaccharases, i.e. xylanase, amylase and cellulase. The results show a higher probability of bacterial isolates from waste-contaminated soils producing more amylase, while fungal isolates from forest soils are likely to produce more cellulases. On the overall bacterial and fungal isolates from waste-contaminated soils appear to be better candidates for production of xylanase, amylase and cellulase activities, possibly due to better adaptation to degrade and utilize organic food, paper, and yard waste. Such isolates can be explored for bioremediation, industrial and pharmaceutical applications.

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