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Short Communication

Screening and isolation of a cellulolytic and amylolytic *Bacillus* from sago pith waste

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The final waste product in the extraction of starch from the sago palm is the starchy fibrous pith residue. This residue is abundantly and cheaply available especially in the state of Sarawak, Malaysia. It is usually washed off into drains or nearby streams together with wastewater, thus contributing to pollution load, or deposited in the factory's compound, which can lead to serious environmental problems. It has no significant industrial or commercial use, except to a small extent as animal feed mainly for pigs and poultry (Yeong and Ali, 1982) or as a soil conditioner (Bintoro and Sianapar, 1993). Studies have shown that sago pith waste is composed mainly of starch and fiber, including a fair amount of minerals. The crude starch and crude fiber contents range from 41.7% to 65.0 and 14.8%, respectively (Wina et al., 1986). When the fibrous and starch component of the pith waste are considered, it is logical to find ways to utilize this waste. The cellulose and starch components both have a good potential for bioconversion into value-added products. An attractive and efficient means is through a biotechnological approach in which microbial strains are employed to degrade the sago waste. Microorganisms such as fungi and bacteria are known to play a major role in the degradation of cellulose and starch components (Coughlan, 1985). The breakdown of these components produce simple sugars that find many uses in the feed and fermentation industries.

The first important step in this approach is to isolate and identify the microorganisms with the necessary characteristics as active cellulolytic and amylolytic microorganisms. Habitats that contain these substrates are the best sources in which to find these microorganisms. This paper described the screening and isolation of an indigenous *Bacillus* that is capable of degrading the substrates. The isolate was identified as *Bacillus amyloliquefaciens.* To our knowledge, this is the first report of a *B. amyloliquefaciens* that can utilize the waste of sago palm.

Three types of sago waste samples were collected for this study. First, pith residue, the final waste product from starch extraction, was obtained from a sago processing factory in the district of Pusa, Sarawak. Samples were collected around the discharge point at a distance of about 3 m from the factory. Second, partially decomposed fibrous waste comprising pith residue and bark was collected from around the same factory's compound. Samples were collected by using a clean spade and placed in separate sterile plastic bags. Third, waste effluent samples were collected from a sago processing plant in Mukah, Sarawak. The wastewater containing a large amount of pith residue and some unseparated starch was collected from sev-

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eral points along an open drain. The effluent samples were collected in sterile bottles and kept at 4°C until analyzed.

A 10% suspension (w/v) of waste samples was prepared in sterile distilled water, and 0.1 ml aliquots were plated on nutrient agar (NA) for the isolation of bacteria. Four replicates were prepared for each sample, and the plates were incubated at 30±2°C. The bacterial colonies recovered were subcultured on NA until pure cultures were obtained.

The bacterial isolates were initially identified by means of morphological examination and some biochemical characterization. The parameters investigated included colonial morphology, Gram reactions, endospore formation, catalase production, anaerobic growth, Voges-Proskauer (V-P) reactions, starch hydrolysis, citrate utilization, lecithinase, and growth in the presence of 7.5% NaCl and at 50°C. Further identification was conducted by using two commercial identification systems, the API 50 CHB test kit (BioMerieux, Marcyl'Etoile, France) and the Biolog system (Hayward, U.S.A.). All procedures were conducted as suggested by the manufacturers. To screen for cellulolytic organisms, the isolates were grown in a minimal agar plate consisting of yeast extract (0.2%), KH_2PO_4 (0.1%), $MgSO_4$ (0.5%), and a soluble form of cellulose, carboxymethylcellulose (0.5%). Negative control plates inoculated with laboratory *E. coli* strain (HB101) were included in all tests. The test plates were incubated at 30±2°C for 2 days. To visualize the hydrolysis zones, the plates were flooded with an aqueous solution of Congo-red (1 mg/ml) for 15 min and washed with 1 M NaCl (Teather and Wood, 1982). Cellulolytic organisms produced a clear zone around the colonies because of the digestion of carboxymethylcellulose (CMC). To screen for amylolytic organisms, the organism was inoculated on minimal agar plates (as above) containing soluble starch (0.5%, w/v) in place of CMC. Degradation of the starch would result in the formation of a clear zone around the colonies after flooding the plates with Lugol's iodine (Hyun and Zeikus, 1985). The diameters of the clear zone produced on CMC and starch plates were measured and used as an indication of the cellulolytic and amylolytic activities of the organisms. Besides the clearing zones on plates, a more quantitative assay method was used to determine the cellulase and amylase activity of the selected bacterial isolate in liquid medium. The reducing sugar content from the hydrolysis of sago pith waste was determined by using the dinitrosalicylic acid (DNS) method (Miller, 1959). Cellulase activity as measured by carboxymethylcellulase activity was determined as described by Mandels et al. (1974), using CMC as the substrate, and amylase activity was determined by using starch as substrate. The amount of reducing sugars released from the assay was detected by the DNS method. To detect the sugar component released into the culture medium from the hydrolysis of the sago pith waste, the method of high-performance liquid chromatography (HPLC) was used. The components were separated by using a Hitachi HPLC system (Model L-6000) equipped with a refractive index (RI) detector (ERC-7515A, ERC Inc., Tokyo, Japan), autosampler (Model AS-2000), and chromato-integrator (Model D-2500). The unit was fitted with a fermentation monitoring column (150 mm×7.8 mm, Bio-Rad, U.S.A.) and a guard column (Cation H, Bio-Rad). Before injection, the eluent was degassed and filtered through a 0.45 μm filter membrane. Exactly 20 μl of the sample were injected into the column with the autosampler. The analysis conditions were performed at 60°C and at a flow rate of 0.6 ml/min. The mobile phase was 1.0 mM H_2SO_4, and the refractive index was set at attenuation 3.

A total of 84 bacterial isolates was isolated from the pith residue, effluent samples, and partially decomposed fibrous waste. Of these isolates, 52 were Gram positive and 32 Gram negative. Among the Gram-positive isolates, 43% were rod shaped and spore formers, and the rest were cocci. Screening for the isolates with cellulolytic and amylolytic activity revealed that the spore formers were more prolific producers of both enzymes compared with the cocci-shaped isolates. However, screening for both cellulolytic and amylolytic activities on agar plates revealed only one isolate (UMAS 1002) having both activities. It is interesting that this isolate was also found to be the most active as revealed by the size of clearing zones on both types of agar plates. The diameters of clear zones on CMC and starch plates were 1.7 and 1.45 cm, respectively. On NA plates, UMAS 1002 produced creamy colonies with circular motions. A microscopic examination of the isolate showed that it is a spore former with an oval-shaped spore in the center. Further identification using standard biochemical tests showed positive reactions in the catalase test and in the V-P, ONPG, starch hydrolysis, gelatin, oxidase, and nitrate tests. From these morphological and biochemical reactions, the isolate

was tentatively identified as *B. amyloliquefaciens* (Table 1). Further identification, using the API 50 CHB kit, also revealed the isolate to be *B. amyloliquefaciens.* Another commercial identification system, the Biolog automated system, which is based on biochemical characterization for 95 different compounds, also confirmed the isolate to be *B. amyloliquefaciens.*

When the isolate was grown on sago pith waste, it hydrolyzed the pith substrate into reducing sugars. After 24 h, reducing sugars were detected in the supernatant at an estimated amount of 1.03 mg/ml (Table 2). Detection of the components of the reducing sugars by HPLC revealed the presence of two sugars, maltose and glucose. The isolate also produced the enzymes cellulase and amylase, based on the activities detected in the culture filtrate. The cellulase and amylase activities measured in the filtrate after 24 h were 0.63 and 0.38 I.U., respectively (Table 2). To determine the pattern of production of reducing sugars and the cellulase and amylase enzymes by the isolate on sago pith waste, growth of the isolate in sago pith waste medium was extended up to 72 h. Reducing sugars and enzymatic activity in the crude filtrate were assayed at an interval of 6 h for the first 24 h, followed by every 24 h until 72 h of incubation. Table 2 demonstrates the level of reducing sugars and the cellulolytic and amylolytic activities over a period of 72 h as measured by the ability of the culture filtrate to degrade CMC and starch, respectively. The maximum content of reducing sugars was 1.03 mg/ml at 24 h, which represents the highest level of production. After 24 h, the reducing sugars declined and the lowest level (0.07 mg/ml) was detected at 72 h. The cellulolytic ac-

Table 1. Biochemical and growth characteristics of the bacterial isolate (UMAS 1002).

Characteristics/biochemical tests	Observation
Gram reaction	Gram positive
Endospore forming/spore location	Oval central endospores
Motility	+
Catalase	+
Starch hydrolysis	+
Lecithinase	−
Growth at 50°C	+
Growth in 7% NaCl	+
Voges-Proskauer (V-P) reactions	+
Citrate utilization	+
NO_3 reduction into NO_2	+

+, positive reaction; −, negative reaction.

Table 2. Production of reducing sugars and the cellulolytic and amylolytic activities of *B. amyloliquefaciens* (UMAS 1002) in liquid medium.

Time (h)	Reducing sugars (mg/ml)[a]	Cellulolytic (CMCase) activity (I.U./ml)[a]	Amylolytic activity (I.U./ml)[a]
0	0.007	0.0	0.0
6	0.06	0.13	0.21
12	0.53	0.52	1.07
18	0.55	0.45	0.85
24	1.03	0.63	0.38
48	0.18	0.27	1.72
72	0.07	0.17	2.30

[a] Each value is mean of triplicates.

Overnight culture (1.0% at $O.D._{600}=1.5$) was inoculated into 100 ml of sago pith waste medium in a 250 ml flask, which was then incubated with shaking (200 rpm) for 24 h at 30°C. The sago waste was dried at 65°C and milled into 40-mesh size before being added into the medium at a concentration of 0.5% w/v. The culture was then centrifuged at 2,500 rpm, and the supernatant was used as crude enzyme for the enzyme assays and also analyzed for its content of reducing sugars. The reducing sugars content from the hydrolysis of sago pith waste was determined by using the dinitrosalicyclic acid (DNS) method (Miller, 1959). Cellulase activity was determined as described by Mandels et al. (1974), using CMC as the substrate, and amylase activity was determined by using starch as a substrate. The reaction mixture consisting of 2 ml of 1.0% substrate solution (soluble starch or CMC dissolved in 0.1 M citrate-phosphate buffer, pH 5.8) and 1.0 ml of crude enzyme filtrate was incubated for 1 h at 50°C. The reducing sugar released was measured by the (DNS) reagent method (Miller, 1959). A standard calibration curve was constructed by using D-glucose. One International Unit (I.U.) of each enzyme was defined as the activity that produced 1 μmol of glucose equivalents per minute under the above assay conditions.

tivity continued to increase and reached the maximum (0.63 I.U./ml) at 24 h, although a slight decline (0.45 mg/ml) was observed at 18 h. After 24 h, cellulolytic activities declined, and at 72 h very little activity (0.17 I.U./ml) remained. The pattern of cellulolytic activities was found to follow the trend of reducing the production of sugar. In contrast, amylolytic activity was very low (0.38 I.U./ml) at 24 h, but it sharply increased at 48 h and continued until 72 h (2.30 I.U./ml). On the basis of the highest production of reducing sugars detected at 24, it shows that a high amount can be achieved in just 24 h. This suggests that a high production of reducing sugars can be achieved within 24 h, thus eliminating the need for extended incubation.

From this study it is clear that the indigenous isolate identified as *B. amyloliquefaciens* has the ability to hydrolyze sago pith waste into reducing sugars. Based on the maximum value of 1.03 mg/ml production of reducing sugars, it is calculated that approximately 315 mg of reducing sugars can be obtained from every one gram of sago pith waste used. On this basis, if 1 ton of sago pith waste is used in a batch fermentation using the bacterial isolate UMAS 1002, about 0.315 ton of reducing sugars can be obtained after 24 h. In the state of Sarawak, it has been estimated that each sago factory produced approximately 7 tons of sago pith waste every day (Bujang et al., 1996). This is a huge amount, and if the bacterial isolate UMAS 1002 can be used to convert this waste into reducing sugars, as shown here, a sizable contribution will have been made to its utilization. The reducing sugars produced can be readily used for human and animal food or as feedstock in the production of ethanol, fuel alcohol, and other related industries that are well established (Govindasamy et al., 1992; Mandels, 1985).

B. amyloliquefaciens has widely been reported to produce amylase enzymes (Abante et al., 1999; Priest, 1977; Yoo et al., 1988), but so far it has not been reported to also produce cellulase enzyme, though other *Bacillus* species have been widely shown to produce several components of the cellulase enzymes (Pajni et al., 1989). Our finding here has shown the ability of *B. amyloliquefaciens* to produce amylolytic and cellulolytic enzymes. The existence of these enzymes in one organism would be a definite advantage in the degradation of sago pith waste as it eliminates the need of adding another source of organisms or enzymes to improve the hydrolysis. The cellulolytic enzyme can act on the cellulose component while the amylolytic activity acts on the starch component of the sago pith waste. In this way the cooperative action of both the cellulolytic and amylolytic enzymes will lead to better or even complete hydrolysis of the sago pith waste. This kind of synergistic effect has been reported by Haska and Ohta (1993), who observed that the hydrolysis of sago starch by raw starch digesting amylase was greatly improved with the addition of cellulase. It was reported that cellulase promoted the activity of the amylase leading to increased hydrolysis of the starch granule into the final product, glucose. On the basis of the results of this work, it is highly probable that *B. amyloliquefaciens* UMAS 1002 could be effectively used in the bioconversion of sago pith waste. Further studies on optimizing the conditions for enzyme production will undoubtedly lead to an improvement in the activity of both enzymes. There is also a great potential for genetic manipulation of the isolate to increase the enzyme activity which can lead to improved hydrolysis of the sago waste substrate.

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