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Selection and Identification of Cellulase-Producing Bacteria Isolated from the Litter of Mountain and Swampy Forest

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The isolation and selection of cellulase-producing bacteria was conducted to identify the species of cellulolytic *Bacillus*. The bacteria were isolated from the litter of swampy forest in Pesisir Selatan and mountain forest in Lembah Anai Tanah Datar. These bacteria were cultivated in selective media to obtain bacteria from the genus *Bacillus*. Six *Bacillus* isolates were obtained from swampy forest and three *Bacillus* isolates from mountain forest. These isolates were cultivated in agar medium with carboxymethylcellulose as the carbon source. Colonies which produced clear zones were assumed to be cellulolytic *Bacillus*. Based on biochemical and morphological examinations the result indicated that these two isolates were *Bacillus coagulans* and *B. amyloliquefaciens*. The cellulase activity of *B. coagulans* and *B. amyloliquefaciens* were 0.812 and 1 200 unit ml^{-1} to C_1(β-exoglucanase) respectively, 0.368 and 0.488 unit ml^{-1} to C_x(β-endoglucanase) respectively.

Key words: identification, cellulolytic, *Bacillus*, litter

Agricultural and agro-industrial wastes, which are mostly in the form of lignocelluloses, basically have no economic value may be even be considered of negative value because they still need further treatment. Lignocelluloses consist of hemicelluloses, celluloses, and lignin. Celluloses are the biggest component and can be transformed into energy sources, paper, single-cell protein, glucose, and sorbitol (Putarau 1969; Coral *et al.* 2002).

One of the goals in biotechnological development is to open the way to utilize microbes in waste bioconversion. Microbe used to treat cellulose-containing wastes could produce extra-cellular enzymes that were able to degrade cellulose material into their smaller components (Bedford and Partridge 2001).

The potency of utilizing cellulase is varied. However, there are some constraints in producing it such as the unavailability of superior microbial strains and a lack of knowledge in enzyme production technology. On the other hand, experts from developed countries acknowledge that countries rich in biodiversity, including Indonesia, are a potential source of microbes for bioprocessing (Fox 1994). Cellulase is usually produced by bacteria and fungi. At present, fungi are usually needed in producing cellulase and for the bioconversion process to improve animal feed quality, but there is a constraint arising from the increase of crude fiber content due to the presence of hyphae which is counted as crude fiber (Coral *et al.* 2002) .

One of the alternatives is using forest litter decomposer bacteria i.e. *Bacillus* spp. *Bacillus* spp. have the biggest number of bacteria, can be found in almost every location, and when chemically tested, they were the most active out of seven genera of bacteria (Jusfah *et al.* 1995; Yusuf 2000). Litter is organic material residue from dead plants that can be found on the earth's surface or buried in its soil minerals. The speed of the litter decaying process by decomposer microorganisms (decomposition) depends on the enzymes produced (Spurr and Barnes 1980).

Bacillus spp. has been known to be the producer of various enzymes such as cellulase, hemicellulase, protease, α-amylase, urease, xylanase, and chitinase (Cowan 1974; Alexander 1977). These enzymes are expected to be able to transform and change complex molecules, especially lignocellulose which is a limiting factor in animal feed, into simpler molecular components. The objective of this research is to select and obtain a collection of cellulase-producing *Bacillus* sp. based on their characteristics.

MATERIALS AND METHODS

Isolation and Selection of Cellulolytic *Bacillus*. Sampling locations used were several places in the forest litter area of Lembah Anai Tanah Datar and the swampy forest litter in Pesisir Selatan, West Sumatera. One gram of soil sample from each litter was diluted to 10^{-5} cfu ml^{-1} using fisiologis NaCl solution. One ml of this dilution was spread on solid medium of *Bacillus* that contained 0.25% bacto agar, 0.6% peptone, 0.3% pancreatic digest of casein, 0.3% yeast extract, 0.3% beef extract, and 0.001% $MgSO_4 \cdot 7 H_2O$ for 24 h at 37 °C (Cowan 1974). Well-grown colonies were chosen to obtain a pure culture of the bacteria using two loops of an inoculating needle to transfer and inoculate *Bacillus* sp. onto a petri dish containing NA medium (Cappucino 1987).

The selection of cellulolytic *Bacillus* sp. was conducted based on the ratio of clear zone to colony diameter after 48 h on carboxy methyl cellulase (CMC) medium (Sigma) (Cowan 1974). To be able to identify the clear zone more distinctly, a qualitative test was conducted by pouring five ml of 0.1% congo red on CMC medium after 24 h incubation. A clear zone indicated that the isolate was a pure culture of cellulolytic *Bacillus* sp. After that, each of the grown colonies was recultured again on medium specific for *Bacillus* and incubated for 24 h at 37 °C. The colonies were observed

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under microscope. The colonies that looked uniform and grew well indicated that the isolates obtained were pure cultures of cellulolytic *Bacillus* sp.

Identification of Cellulolytic *Bacillus* sp. Further identification of these species was conducted by means of macroscopic and microscopic examinations, and biochemical characterization. Macroscopically, the observed characteristics were: color, shape, colony surface, and edge. Microscopically, the tests included: Gram reactions, shape, and cell size. Biochemical tests included: carbohydrate utilization, indole, H_2S, urease, citrate utilization, catalase activity, motility, and environmental tolerance test by growing the isolate on medium with pH 4.0 and 7.0 in the presence of NaCl at a final concern of 5.0 and 7.0%. The reactions observed in chemical tests were compared to the identification keys (Buchanan and Gibbons 1974).

Determination of Cellulase Activity of *Bacillus* sp. Cellulase activity can be measured from the fermentation results by two selected species of *Bacillus* and are compared with that of *Trichoderma harzianum* (as the control) in a mixed substrate from sago pulp and rumen content (7:3). Inoculum dosage, fermentation time, fermentation temperature, thickness of substrate, water content, and particle size for the determination of cellulase activity were 2%, 48 h, 40 °C, 2 cm, 60%, and 1 mm respectively.

Isolation of Cellulase Crude Extract. Sampling was conducted an a daily bases to observe the development of cellulase activity. Culture medium was homogenized by adding 75 ml citrate buffer 0.05M (pH 6) (5x medium weight) for each sample, followed by shaking and filtering. The filtrate was obtained by placsing the homogenate inside a 250 ml Erlenmeyer flask which was placed inside a 500 ml glass beaker filled with ice cubes. During filtration, the homogenate was stirred at low speed and the temperature was kept at 4 °C. The filtrate was centrifuged at 4 500 g for around 15 min (twice) under refrigeration and then filtered using Whatman no. 1 to obtain crude extract of the enzyme.

Determination of Cellulase Activity. The cellulase activity test was conducted employing the Somogy-Nelson method (Bergmeyer *et al*. 1981). A volume of 0.5 ml 1% (v/w) CMC substrate was pippeted into a reaction tube and was then preincubated for 5 min in 50 °C water bath. After adding 0.5 ml enzyme filtrate, the incubation was continued for 45 min (Chaabouni *et al*. 1994). Enzymatic reactions were stopped by heating the mixture in a boiling water bath for 15 min. Then 1 ml of Somogy-Nelson reagent was added, followed by 15 min heating. Finally, 1 ml of arsenomolibdic reagent was added into the solution. This mixture was shaken until no more gas was produced, and then diluted with 7 ml distilled water. After the filtrate was separated from its sediment, its absorbance was read at 620 nm wavelength. Control treatments were the same, except the enzyme was first inactivated. To determine the reducing sugar content from the above enzyme reactions we used a standard curve calibrated with glucose monohydrate. Cellulase activity was defined as the quantity of enzyme that can release 1 mmol glucose min^{-1} (Chaabouni *et al*. 1994).

RESULTS

Isolation and Selection of Cellulolytic *Bacillus*. From the isolation and selection results conducted in the laboratory of Research Institute for Animal Diseases (BPPH) Baso Bukittinggi, there were six isolates of *Bacillus* sp. (GT1, GT2, GT3, GT4, GT5, and GT6) from swampy forest litter and six isolates of *Bacillus* sp. (LA1, LA2, LA3, LA4, LA5, and

Table 1 Isolation and selection of *Baccillus* from the litter of swampy forest Pesisir Selatan (GT)

Evaluation	Isolate					
	GT_1	GT_2	GT_3	GT_4	GT_5	GT_6
Staining						
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Spore location	Center	Center	Center	Center	Center	Center
Gram test	Positive	Positive	Positive	Positive	Positive	Positive
Morphological observation						
Liquid medium	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Colony						
Shape	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular
Elevation	Flat	Flat	Flat	Flat	Flat	Flat
Edge	Serrated	Serrated	Serrated	Serrated	Serrated	Serrated
Motility	Motile	Motile	Motile	Motile	Motile	Motile
Biochemical test						
Catalase	+	+	+	+	+	+
Oxidase	+	-	-	-	-	-
Glucose fermentation	+	+	+	+	+	+
Starch hydrolysis	+	-	-	-	-	-
Indole	-	-	-	-	-	-
Urease	±	-	-	-	-	-
Citrate	-	-	-	-	-	-
Gas	-	-	-	-	-	-
H_2S	-	-	-	-	-	-
MR (Methyl Red)	-	-	-	-	-	-
VP (Voge Prokauer)	+	+	+	-	-	-
Nitrate reduction	+	+	+	-	-	-
Casein hydrolysis	-	-	-	-	-	-
Gelatin hydrolysis	+	-	-	-	-	-
TSIA	r/r	r/y	r/y	r/y	y/y	y/y

r = red, y = yellow.

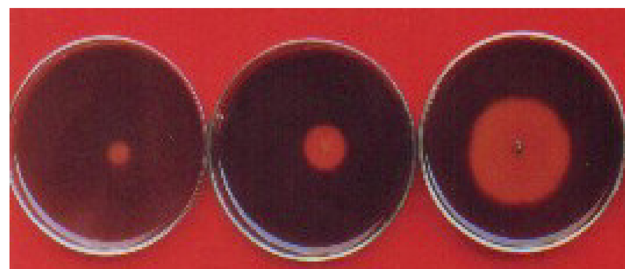
Table 2 Isolation and selection of *Bacillus* from the litter of Lembah Anai forest (LA)

Evaluation	Isolate					
	LA_1	LA_2	LA_3	LA_4	LA_5	LA_6
Staining						
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Spore location	Center	Center	Center	Center	Center	Center
Gram test	Positive	Positive	Positive	Positive	Positive	Positive
Morphological observation						
Liquid medium	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Colony						
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Elevation	Convex	Convex	Convex	Convex	Convex	Convex
Edge	Serrated	Serrated	Serrated	Serrated	Serrated	Serrated
Motility	Motile	Motile	Motile	Motile	Motile	Motile
Biochemical test						
Catalase	+	+	+	+	+	+
Oxidase	+	-	-	-	-	-
Glucose fermentation	+	+	+	+	+	+
Starch hydrolysis	+	-	+	-	-	+
Indole	-	-	-	-	-	-
Urease	±	-	±	-	-	±
Citrate	-	-	-	-	-	-
Gas	-	-	-	-	-	-
H_2S	-	-	-	-	-	-
MR (Methyl Red)	-	-	-	-	+	-
VP (Voge Prokauer)	-	+	-	-	+	-
Nitrate reduction	-	+	+	-	-	+
Casein hydrolysis	+	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-
TSIA	r/r	r/y	r/y	r/y	y/y	y/y

r = red, y = yellow.

LA6) from Lembah Anai mountain forest litter (Table 1 and 2). From the classification based on "Bergey's Manual of Determinative Bacteriology", there were seven different isolates out of 12 selected isolates, i.e. (GT1), (GT2/LA2), (GT3/LA4), (GT4/GT5 GT6), (LA1), (LA3/LA6), and (LA5).

After that, selection on the ability to degrade was conducted qualitatively by inoculating the above isolates on CMC medium. After 48 h, three out of seven isolates were found to produce clear zones. To examine further about the clear zones and their diameters as depicted in Fig 1 and Table 3. Sufficiently wide clear zones were obtained from *Bacillus* sp.1 (GT1) and *Bacillus* sp.2 (LA1) with diameters 37.4 mm and 12.40 mm respectively. These two isolates with wide clear zones were then examined to determine their species in the Microbiology laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung.



sp.3/GT2/LA2 sp.2/LA2 sp.1/GT1

Fig 1 Clear zones of *Bacillus* sp. on CMC medium.

Table 3 Clear zone diameters of isolates obtained from isolation and selection on CMC medium (48-hour inoculation)

Isolate	Species	Clear zone diameter (mm)
(GT1)	sp.1	37.40
(LA1)	sp.2	12.40
(GT2/LA2)	sp.3	09.32
(GT3/LA4)	sp.4	-
(GT4/GT5GT6)	sp.5	-
(LA3/LA6)	sp.6	-
(LA5)	sp.7	-

Identification of *Bacillus* sp. Species observation by means of macroscopic and microscopic evaluations as well as biochemical reactions on GT1 and LA1 showed some similarities and some differences. The similarities are: staining tests indicated Gram positive, rod shaped, produced elliptical endospores. From the morphological observation, growth on liquid medium formed a pellicle (aerobic), serrated colony edge, and motile. From biochemical tests, these microbes hydrolyzed starch and casein: sugar fermentation was positive, produced no H_2S, produced catalase, indicated as alkaline by litmus milk. The differences were: spore location of GT1 in the center of vegetative cell, irregular colony shape with flat elevation, positive hydrolysis of gelatin, and nitrate reduction. Whereas, spore location of LA1 were in the center to the end of vegetative cell, circular colony shapes with convex elevation, negative hydrolysis of gelatin, and negative nitrate reduction. The caracteristic of GT1 and LA1 as well as the defferences of *B. amyloliquefaciens* and *B. coagulans* at Table 4.

Cellulase Activity. Average values of cellulase activities of *Bacillus* and *T. harzianum* in mixed substrate containing sago pulp and rumen content were as follows. Cellulase activity of C_x and C_1 of *B. amyloliquefaciens* were 0.368 and 0.812 unit ml^{-1} respectively, *B. coagulans* were 0.488 and 1 200 unit ml^{-1} respectively, and *T. harzianum* 0.6550 and 0.3070 unit ml^{-1} respectively (Table 5).

Table 4 Characteristics of *Bacillus* sp.1 (GT1), *Bacillus* sp.2 (LA1), *B. amyloliquefaciens* and *B. coagulans*

Evaluation	Isolate			
	Bacillus sp.1 (GT1)	*Bacillus* sp.2 (LA1)	*Bacillus amyloliquefaciens*	*Bacillus coagulans*
Staining				
Cell shape	Rod	Rod	Rod	Rod
Spore location	Center	Center-end	Center	Center-end
Gram test	Positive	Positive	Positive	Positive
Morphological observation				
Liquid medium	Aerobic	Aerobic	Aerobic	Aerobic
Colony				
Shape	Irregular	Circular	Irregular	Circular
Elevation	Flat	Convex	Flat	Convex
Edge	Serrated	Serrated	Serrated	Serrated
Motility	Motile	Motile	Motile	Motile
Biochemical test				
Catalase	+	+	+	+
Glucose fermentation	+	+	+	+
Lactose fermentation	-	-		
Sucrose fermantation	+	+		
Starch hydrolysis	+	+	+	+
Indole	-	-		-
Urease	-	-		
Citrate	-	-		
Triple sugar-iron	+	+		
H_2S	-	-	-	-
Methyl red	+	+		
Voges-Proskauer	-	-		
Nitrate reduction	+	-	+	-
Casein hydrolysis	+	+	+	+
Gelatin hydrolysis	+	-	+	
Litmus milk	alkaline	alkaline	alkaline	

Table 5 Average value of cellulase activities of *Bacillus amyloliquefaciens*, *B. coagulans* and *Trichoderma harzianum*

Isolates	Average value of cellulase activity (unit/ml)*	
	C_x (β-endoglucanase)	C_1 (β-exoglucanase)
B. amyloliquefaciens	0.4880 ± 0.046	1.2000 ± 0.150
B. coagulans	0.3680 ± 0.043	0.8120 ± 0.145
T. harzianum	0.6550 ± 0.045	0.3070 ± 0.013

*Cellulase activity is based on cellulase crude extract.

DISCUSSION

The results of this research are in line with the characteristics determined by Buchanan and Gibbons (1974) and Holt *et al.* (1994) based on qualifications from "Bergey's Manual of Determinative Bacteriology", that a *Bacillus* is classified into the kingdom of *Prokaryotes*, division *Bacteria*, class *Schyzomycetes*, order *Eubacteriales*, family *Bacillaceae*, and genera *Bacillus*. The characteristic of *Bacillus* sp. cultivated on NA medium was that the clear zone of *Bacillus* sp.1 (GT1) was wider than that of *Penicillium* on CMC medium as reported by Dharma (1998) (37.4 mm vs 15.60 mm).

Identification results of these two species were in line with Buchanan and Gibbons (1974) and Holt *et al.* (1994), that *B. amyloliquefaciens* was Gram positive, rod shaped, produced elliptical endospores, and located in the center of vegetative cell, growth on liquid medium formed pellicle (aerobic), irregular colony shape with flat elevation, motile, positive hydrolysis of gelatin and nitrate reduction, and *B. coagulans* was Gram positive, rod shaped, produced elliptical endospores, and located in the center to the end of vegetative cell, growth on liquid medium formed pellicle(aerobic), circular colony shapes with convex elevation, motile, negative hydrolysis of gelatin, and negative nitrate reduction. From the results of biochemical reactions, GT1 is *B. amyloliquefaciens* and LA1 is *B. coagulans*.

The value of the cellulase activity of both *Bacillus* species were lower compared to those of enzymes produced by *Bacillus subtilis* strain-CBTK 106 cultivated for 72 h on banana peel medium completed with C and N sources and several minerals, such as carboxy methyl cellulase (CM Case) 9.6 IU gds^{-1} (gram dry matter substrate). Filter paperase (FPase) 2.8 IU gds^{-1} and cellobiase 4.5 IU gds^{-1} (Chundakkadu 1999). Likewise, the cellulase activity in the fermentation of oil palm empty fruit bunch using *Penicillium* sp. was higher for C_x, it was 1 457 unit ml^{-1} in an 8-day fermentation and for C_1 0.01 unit ml^{-1} in a 14-day fermentation (Dharma 1998).

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