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SHORT COMMUNICATION

Isolation and Characterization of Chitinolytic Bacteria and Their Potential to Inhibit Plant Pathogenic Fungi

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A study on the isolation and characterization of chitinolytic bacteria and their potential to inhibit plant pathogenic fungi has been done. The bacteria were isolated from the soil of Karo, Langkat, and Bangka, Sumatra. *Ganoderma boninense*, *Fusarium oxysporum,* and *Penicillium citrinum* of the stock cultures in Laboratory of Microbiology Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara were used for growth inhibition assay by the isolated bacteria. KR05 and LK08 shared similar morphological and physiological characters; like wise, KR07 shared property similarities with BK08. All bacterial isolates inhibited the growth of *G. boninense*, *F. oxysporum*, and *P. citrinum* at a different extent. LK08 showed the highest inhibition rate followed by BK07 and BK09. However, *P. citrinum* was inhibited more by BK07 and BK09. The crude enzyme preparation of the latter isolate exhibited the highest chitinase activity. The result suggested that their swarming activity seemed to contributed to inhibition of fungal growth.

Key words: chitinolytic bacteria, growth inhibition, pathogenic fungi, chitinase activity.

Telah dilakukan isolasi dan karakterisasi bakteri kitinolitik dan kajian tentang potensi bakteri ini dalam menghambat pertumbuhan jamur pathogen tanaman. Bakteri diisolasi dari contoh tanah Karo, Langkat, dan Bangka, Sumatra. *Ganoderma boninense*, *Fusarium oxysporum* dan *Penicillium citrinum* merupakan koleksi Laboratorium Mikrobiologi, Fakultas Matematika dan Ilmu Pengetahuan Alam, Universitas Sumatera Utara digunakan dalam uji daya hambat oleh bakteri kitinolitik. KR05 dan LK08, serta KR07 dan BK08 memiliki sifat morfologi dan fisiologi yang sama. Semua isolat bakteri menghambat pertumbuhan *G. boninense*, *F. oxysporum*, dan *P. citrinum*. LK08 menunjukkan daya hambat terbesar diikuti oleh BK07 dan BK09. Namun demikian kelihatannya *P. citrinum* lebih terhambat oleh BK07 dan BK09. Enzim kasar dari isolat BK09 menunjukkan aktivitas kitinase tertinggi. Hasil menunjukkan bahwa aktivitas keriap memberikan sumbangan terhadap kemampuan menghambat pertumbuhan jamur patogen.

Kata kunci: bakteri kitinolitik, penghambatan pertumbuhan, jamur patogen, aktivitas kitinase.

The use of biological sources for plant disease control remains an important potential alternative to the use of pesticides. This method has been proposed for the replacement of chemical control of plant diseases. Biological control using microorganisms has been studied intensively since there is not many alternatives to control left (Kotan *et al*. 2009; Oskay 2009). Health problems, environmental concerns, development of resistance in target populations also contribute to developing biological control using natural enemies (Ningthoujam *et al*. 2009; Mejía *et al*. 2008).

Antagonistic microorganisms, by their interactions with various soil-borne plant pathogens, play a major role in microbial equilibrium and serve as powerful agents for the control of biological diseases (Alabouvette *et al*. 2006; Ozbay and Newman 2004). The antagonism may operate through antibiosis, competition, predation, or parasitism (Alabouvette *et al*. 2006; Ozbay and Newman 2004). Antibiosis is the antagonism resulting from the production of secondary metabolites by one microorganism that is toxic to other microorganisms. Antibiosis is a very common phenomenon responsible for the activity of many biological control agents, such as fluorescent *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp. and *Trichoderma* spp. (Alabouvette *et al*. 2006), and *S. halstedii* AJ-7 that suppressed the growth of *Phytophthora capsici*, the causal agent of phytophthora blight in red-peppers (Joo 2005). Parasitism involves the production of several hydrolytic enzymes that degrade cell walls of pathogenic fungi (Alabouvette *et al*. 2006; Ozbay and Newman 2004). The lytic activity of bacteria is one of many mechanisms that has been implicated in biocontrol (Anitha and Rabeeth 2010; Patel *et al.* 2007; Gohel *et al*. 2006). A number of fungi are particularly susceptible to degradation by microorganisms (Kim *et al.* 2008).

Mycolytic enzyme-producing microorganisms have a great potential in solving such problems (Patel *et al.* 2007; Gohel *et al*. 2006). Investigations on lytic activity of biocontrol agents have focused mainly on the characterization of enzyme systems capable of

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degrading fungal cell wall components, of which chitinases are among the most intensively studied (Anitha and Rabeeth 2010; Patel *et al.* 2007; Gohel *et al*. 2006). Fungi and bacteria are important degraders of chitin in the soil ecosystem and contribute to the recycling of vital carbon and nitrogen resources (Metcalfe *et al*. 2002; Tsujibo *et al*. 2002). Combined chitinolytic bacteria of *Serratia plymuthica* strain C-1, *Chromobacterium* sp. strain C-61, and *Lysobacter enzymogenes* strain C-3 inhibited the growth of *P. capsici*, *Rhizoctonia solani*, and *Fusarium* spp. (Kim *et al*. 2008). *T. koningii* and *T. harzianum* suppressed the growth of *Ganoderma boninense* (Susanto *et al.* 2005). Crude chitinase enzyme extracted from *S. griseus* showed zone of inhibition on *F. oxysporum*, *Alternaria alternate*, *Rhizoctonia solani, F. solani* and 2 isolates of *Aspergillus flavus* (Anitha and Rabeeth 2010). For the purpose of employing such microorganisms for biological control of fungal diseases of plant, isolation of bacterial candidates, and assay on their ability to inhibit fungal growth and their chitinase activity are one of the important steps to be done prior other steps.

Screening of chitinolytic bacteria was conducted by inoculating soil samples of Langkat, Karo, and Bangka in modified salt medium (0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.001 g $ZnSO_4$, and 0.001 g $MnCl_2$ in 1000 mL) containing 2% (w/v) chitin colloidal (MSMC) agar aerobically. After 4-5 days of incubation, a clear zone around the bacterial colony was formed, indicating that bacteria have chitinolytic activity. The colony was then transferred into different dishes several times until a pure culture was obtained.

All cultures were cultivated at 30 °C. All media were adjusted to pH 6.8. Unless mentioned otherwise, the seed cultures for all bacterial inoculation were taken from a 2-day-old culture of either MSMC broth or agar. The cultures were grown with the initial cell concentration of $\approx 10^8$ cfu mL^{-1}. All fungal cultures, *G. boninense*, *F. oxysporum* and *Penicillium citrinum* were of the collection of the Laboratory of Microbiology, Department of Biology, Universitas Sumatera Utara, Medan. The fungal cultures were maintained in potato dextrose agar (PDA).

Cell shape and Gram staining were evaluated microscopically, while the colony was observed directly. Motility was observed using swarming activity. Physiological properties including catalase, oxidase, gelatinase, citrate-, starch-, and sugar-utilization tests were observed using 3% H_2O_2, *Bactident® Oxidase (Merck),* and gelatine nutrien medium, Simmons Citrate Agar (SCA), starch agar (SA), and Triple Sugar Indole Agar (TSIA), respectively.

Swarming activity was observed by inoculating 5 µl of bacterial culture at the center of nutrient agar (NA) or MSMC agar dish. Swarming activity was measured every day as colonial expansion.

Bioassays against different fungal species were conducted to determine the antifungal activity of chitinolytic bacterial isolates. An agar plug (Ø 5-mm) of *G. boninense*, *F. oxysporum* and *P. citrinum* from the margin of an actively growing culture was inoculated at the center of petri dishes containing 20 ml of MSMC agar. A well (Ø 5-mm) was made at the edge of petri dishes opposite to the fungal inoculation at a distance of 3.5 cm from the center. After 1 day incubation, 30 µl of bacterial seed solution was inoculated into the wells. The plates were incubated at 25-30 °C for 3 to 8 days until there was an inhibition of fungal growth. Antagonistic activity was measured as radius of uninhibited mycelia substracted by radius of inhibited mycelia by bacterial chitinolytic activity.

One ml of chitinolytic isolate grown in MSMC broth was reinoculated into 20 ml of MSMC broth and incubated for 96 hours. Crude enzyme was obtained by spinning bacterial culture at 6 000 g for 20 minutes. A supernatant containing crude enzyme *was* mixed with substrate of 1% (w/v) chitin colloidal and incubated for 60 minutes. Reaction was stopped by placing the reaction mixture in boiling water for 15 min. The mixture was spinned at 6.000 rpm for 20 minutes. The chitinase activity was measured by comparing free N-acetyl glucosamine (GlcNAc) that was treated with untreated chitin, with chitinase. GlcNAc was measured by the colorimetric method of Reissig (1955). One unit of chitinase activity was defined as the amount of enzyme which produces 1 µmol GlcNAc per hour.

Six isolates were characterized based on their morphological and physiological traits. The isolates showed different characters, except KR05 and LK08, and KR07 and BK08 (Table 1), indicating different species of bacteria, and all isolates were aerobe. Some isolates exhibited catalase activity, an important enzyme produced by aerobic bacteria. Five isolates were Gram-negative and one was Gram-positive. Chitinolytic bacteria spread among Gram-negative and Gram-positive (Anitha and Rabeeth 2010; Kotan *et al*. 2009; Kim *et al*. 2008). Bacterial genera *Achromobacter, Bacillus, Chromobacterium, Pseudomonas* and *Vibrio,* along with bacteria from the *Flavobacterium-Cytophaga* group and the *Enterobacterioceae* family (Donderski and Brzezińska 2001), and *Streptomyces* (Anitha and Rabeeth 2010; Prapagnee *et al*. 2009) are reported to produce chitinase.

All isolates were able to grow in chitin colloidal media. Chitin colloidal is one of the substrates

Table 1 Morphological and physiological traits of the chitinolytic bacterial isolates

Characters	Isolates					
	KR05	KR07	LK08	BK07	BK08	BK09
Colony shape	circular	circular	circular	circular	circular	circular
Colony color	transparent	cream	transparent	transparent	transparent	transparent
Cell shape	rod	rod	rod	rod	coccus	rod
Physiological traits						
Gram	-	-	-	-	-	+
Motility	+	+	+	+	+	+
Catalase	+	-	+	+	-	-
Oxydase	+	+	+	+	+	+
Starch	-	+	-	-	+	+
Citrate	+	+	+	+	+	-
Gelatin	-	+	-	+	+	+

Table 2 Swarming activity of the chitinolytic bacteria on chitin colloidal MSMC agar and NA

Isolates	Media	Colony expansion (mm) of days-			
		4	5	6	7
KR05	MSMC	6.63	7.85	8.90	9.60
	NA	9.18	12.15	13.07	14.13
KR07	MSMC	31.67	32.85	33.13	34.20
	NA	27.38	27.82	28.53	29.07
LK08	MSMC	8.60	9.60	10.65	11.58
	NA	4.00	5.00	5.00	6.00
BK07	MSMC	6.58	6.73	7.40	8.18
	NA	9.28	10.11	10.98	11.58
BK08	MSMC	16.75	18.75	19.38	20.07
	NA	8.07	8.78	9.25	10.10
BK09	MSMC	19.75	24.58	24.70	25.65
	NA	4.68	6.15	6.85	7.65

commonly used to induce hydrolytic enzymes such as chitinase (Nandakumar *et al*. 2007). Chitinolytic bacteria were often characterized by their ability to produce a clear zone around their colony in chitin containing media. The clear zone was formed because chitin was hydrolized into its soluble monomer or derivates, mainly GlcNAc by extracellular chitinase produced by the bacteria (Nandakumar *et al*. 2007). Finally, the degradation products are then taken up by the cells as carbon and nitrogen sources (Metcalfe *et al*. 2002; Tsujibo *et al*. 2002).

Instead of the SIM test, motility of isolates was examined through their swarming activity. The swarming activity was tested with chitin availability in the media. The isolates showed different responses of growth in NA and MSMC. The bacterial isolates swarmed on the agar surface, colonizing agar plate at different expansion rates (Table 2). The swarming activity test indicated that the isolates moved at different rates in different media. All isolates expanded rapidly in chitin containing media, except that of BK07. The isolate showed more swarming activity in complete media such as NA. KR07 showed optimum swarming activity both in NA and in MSMC agar with colony expansion of 34.20 and 29.07 mm respectively in 7-days incubation time, followed by BK09 (Table 1). This indicated that the movement of isolates was stimulated by chitin availability. An autoinduction phenomenon might be involved in this swarming activity (Eberl *et al.* 1999). Other factors affecting swarming activity were not examined in this study. However, Senes *et al*. (2002) showed that increasing mannitol concentration from 2 to 20 mM in swim tryptone agar (TrA) containing 0.25% agar inhibited the movement of *Bacillus cereus*. The swarming response of this strain, on the contrary, did not exhibit any changes when the mannitol concentration in swarm TrA (1% agar) plates was varied from 0.2 to 20 mM. These results suggested that chemotaxis itself, at least toward mannitol, is unlikely to play a role in *B. cereus* swarming motility.

Swarming activity in solid media was observed as an indication of the colonization ability of the bacteria in the environment. Interestingly, the ability to inhibit fungal growth seemed in line with the swarming activity, except for that of KR07 and BK08, hence the swarming activity test might be one useful step in selecting biological control agent. Swarming activity may indicate the colonization rate of microorganism in the environment. Swarming, therefore, is thought to be a successful strategy developed by flagellated microorganisms to ensure their rapid expansion in the natural environment, where microbial activities are often associated with solid surfaces (Senes *et al*. 2002).

Efficacy of all isolates in inhibiting fungal growth was examined by growing the isolate next to the fungi

Table 3 Diameters of the inhibition zone as a result of antagonism assays of the chitinolytic bacteria against fungi

Isolates	Fungi	Inhibition zone (mm) of days-			
		4	5	6	7
KR05	*Ganoderma boninense*	5.13	15.15	17.22	12.61
	Fusarium oxysparum	0	0	1.98	1.93
	Penicillium citrinum	0	0	0	1.34
KR07	*Ganoderma*	0	3.00	3.00	5.00
	Fusarium oxysparum	0	3.67	3.67	4.84
	Penicillium citrinum	0.19	0.19	0.85	0.85
LK08	*Ganoderma boninense*	0	13.60	18.87	25.34
	Fusarium oxysparum	0	6.96	14.98	21.96
	Penicillium citrinum	0.30	2.43	2.45	0.70
BK07	*Ganoderma boninense*	15.14	15.14	17.22	12.61
	Fusarium oxysparum	0	0	4.83	4.83
	Penicillium citrinum	2.25	2.25	3.30	8.12
BK08	*Ganoderma boninense*	0.94	0.94	0.63	0.31
	Fusarium oxysparum	0	0	1.37	2.37
	Penicillium citrinum	0.25	0.25	0.95	0.32
BK09	*Ganoderma boninense*	5.52	5.52	7.84	18.13
	Fusarium oxysparum	0	0	2.31	2.49
	Penicillium citrinum	2.52	2.52	3.49	7.92

in chitin-containing media to induce extracellular chitinase. The isolate ability in controlling fungal growth varied during cultivation (Table 3). Although LK08 showed optimum inhibition rate to all fungi, BK07 exhibited more inhibition to the growth of *P. citrinum*. Different ability of chitinolytic bacterial isolates to inhibit fungal growth was previously observed (Matroudi *et al*. 2009). In general, all isolates were more capable of inhibiting *G. boninense* growth rather than that of *F. oxysporum* and *P. citrinum*. This variation might be caused by species specific, different bacterial chitinase activity, chitin composition of the fungal mycelium, the growth rate of the bacterial and the fungi, and other antifungal metabolites. The fungal cell walls are usually composed not only of chitin but also of other sugars such as β-1,3 glucan, which is bound to chitin in an amorf structure. Since fungal cell wall is made up of mainly of glucan and chitin, the β-1,3-glucanase and chitinase are key enzymes responsible for fungal cell wall lytic and degradation (Anand and Reddy 2009; Gohel *et al*. 2006). The presence of other metabolites in addition to chitinase is also responsible for inhibiting fungal growth (Prapagdee *et al*. 2008; Getha and Vikineswary 2002).

All six isolates were grown in MSMC broth to find out their ability to utilize chitin. The result showed that their chitinase activity varied (Table 4). Although crude chitinase activity of BK09 was relatively high, this isolate was not as capable as LK08 to inhibit fungal growth. The high chitinase activity seemed not to correlate with the ability of an isolate to inhibit fungal growth. This is probably caused by the structural difference of the substrates. The structure of chitin in fungal cell walls, however, was more complex compared to that of chitin colloidal used as carbon source in chitinase activity assay. The mycelia of *Fusarium* and *Penicillium* is rather hard to be lysed since the cells contain protein and lipid, which act as a barrier to hydrolytic enzymes (Sivan and Chet 1989).

Table 4 Crude chitinase activities of the chitinolytic bacteria

Isolates	Activity (Unit mL^{-1})
KR05	0.088
KR07	0.036
LK08	0.060
BK07	0.092
BK08	0.363
BK09	0.726

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