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Isolation and Characterization of *Acinetobacter* sp. WC-17 Producing Chitinase

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The bacterial strain WC-17 able to produce chitinase was isolated from soil using an enrichment technique. The isolated strain was identified as *Acinetobacter* sp. judging by their morphological and physiological characterisitics. The optimal culture conditions for the production of chitinase of *Acinetobacter* sp. WC-17 are 1.5% colloidal chitin and 1% tryptone at 30°C with pH 6.5. Since the enzyme was rapidly produced in a culture supplied with chitin, glucose, or N-acetylglucosamine but not with other polymers and monosaccharide, the enzyme was considered to be an inducible enzyme. Notably N-acetylglucosamine and glucose were found to be effective inducers at low concentrations but repressors at excessive concentrations. The cultural supernatant of *Acinetobacter* sp. WC-17 inhibited the growth of phytopathogenic fungi such as *P.oryzae*, *R.solani*, and *F.solani*. Among the phytopathogenic fungi tested, *P.oryzae* was the most sensitive. The conventional agar plate (PDA containing 1% colloidal chitin) method also produced the same result.

Chitin, a polymer of β-1.4-bonded N-acetyl-D-glucosamine (NAG) residues occured in fungal cell wall and exo-skeleton of arthropod, is the most abundant biopolymer found on earth after cellulose. The chitin is degraded by chitinase to the dimer, chitobiose; this is degraded to monomer, NAG, by chitobiase. The largest potential use of chitinase and chitobiase is the treatment of the chitin-containing wastes produced by the seafood packing industry. Chitin degradation products can be used as feedstock chemicals, for the production of single-cell protein and for animal or aquaculture feed. Other industrial uses currently under consideration include adhesive, wound dressing, heavy metal recovery from waters, delayed-release agrochemicals or drug, and dialysis membranes. Chitinases have been detected in a variety of organisms, not only in those containing chitin as their major structural component, such as fungi (4, 22), but also in ones which do not contain chitin, such as bacteria (19, 25, 6, 24), higher plants (5, 3). The physiological functions of chitinases depend on their sources. In plant, the enzymes are thought to be a defense system against fungal pathogens (5, 23). Plant chitinase degrade isolated cell walls containing chitin from some fungi, and some also degrade bacterial cell walls due to their lysozymal activity. In fungi, chitinases seem to play a physiological role in cell division and differentiation, as well as a nutritional role (2). In bacteria, the enzyme appears to have a nutritional role. In past decade, chitinase have also recieved immense attention because they might play a role in plant defense systems against chitin-containing pathogens (3) comparing with agrochemicals which have been criticized severely for causing environmental pollution and residual toxicities. In attempts to reinforce the endogenous mechanisms of plants defense, chitinase-encoded genes have been cloned from plants, bacteria and introduced into plants (15).

To research the various application of chitin and the use as biocontrol agents against phytopathogenic fungi, we have investigated: (i) the isolation, selection and identification of bacterial strain producing extracelluar chitinase, (ii) the optimum condition of chitinase production of selected strain, (iii) antifungal activity against various phytopathogenic fungi.

MATERIALS AND METHODS

Isolation of Chitinase Producing Bacterial Strain

The soil samples obtained from an area of Taegu were incubated in a basal salt medium containing colloidal chitin as a sole carbon and nitrogen source for enrichment. The bacterial strain WC-17 was selected from the enriched microorganisms by measuring the degradation of activity toward colloidal chitin. The composition

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of the basal salts medium was 0.8g/l K_2HPO_4, 0.8g/l KH_2PO_4, O.9g/l $MgSO_4$, 16g/l Agar and Cyclohexamide (50 g/ml) for removing fungi. The selected microorganism was identified by investigating its physiological, biochemical and morphological characteristics according to Bergey's Manual of Determinative Bacteriology (7). The morphorlogical aspects were studied by using a scanning electron microscope.

Preparation of Chitinase Enzyme

The isolated strain was grown in 200ml chitin-trypton medium and culture supernatant was collected by centrifugation at 12,000 rpm for 15 min. Protein in the culture supernatant was precipitated with 60~70% ammonium sulfate saturation and collected by centrifugation at 15,000 rpm for 30 min. The precipitate was dissolved and dialysed against a 10mM Sodium phosphate buffer (pH 6.0). Concentrated enzyme solution was obtained by dissolving protein precipitate in minimum volume.

Chitinase Assay

Colloidal chitin used for the preparation of chitin plates and chitinase assay was prepared following the method of Lingappa (13). The enzyme assay mixture contained 500 µl of 0.5% (w/v) colloidal chitin in 100mM sodium phosphate (pH6.0) and 500 µl of enzyme solution. The enzyme reactions were conducted in a shaking water bath at 37°C, at 120 rpm for 3 h. Chitinase activity was determined by measuring the amount of N-acetyl-D-glucosamine released from colloidal chitin by the Somogyi method (19) using a standard curve for NAG. One unit of chitinase activity was defined as the amount of the enzyme required to produce 1 mol of NAG per min under described condition.

Detection of Chitinase Activity after *SDS-PAGE*

For the identification of existing isozymes and molecular weight in the gel, Trudel and Asselin method was used (20). SDS-PAGE was performed with 12% of polyacrylamide containing 0.01% glycol chitin and 1% SDS. After SDS-PAGE, the gel containing glycol chitin was incubated for 2 h at 37°C with reciprocal shaking in 0.1M sodium acetate buffer (pH5.0) containing 1% (v/v) Triton X-100, washed with distilled water, and incubated further for 6 h at 37°C to renature chitinases. Gel was then stained with 0.01% (w/v) calcoflour white M2R in 0.5M Tris-HCl (pH 8.9) for 2 h and destain in distilled water for 2 h at room temperature. The lytic zone produced by the hydrolysis of glycol chitin by chitinase appeared as a dark band under the UV transilluminator at 254nm.

Antifungal Activity of Selected Strain

To confirm the antifungal activity of selected strain against phytopathogenic fungi such as *Fusarium solani, Rhyzoctonia solani, Pyricularia oryzae,* bacterial culture was grown at 30°C for 72 h. Culture broth was centrifuged at 12,000 rpm for 15 min. The supernatant was filtered through 0.45 µm pore size membrane filter and then the filtrate was mixed with precultured phytopathogenic fungi in 1l flask containing 2.7% potato dextrose broth (PDB) and incubated at 28°C for 6 days. Antifungal activity was determined by measuring dry cell weight of fungal mycelium comparing with a control (without filtrate). And conventional agar plate method was also used. The phytopathogenic fungi was spotted on the center of PDA plate containing 1% colloidal chitin and isolated strain and *E.coli* JM109 were inoculated at its periphery. The antifungal activity was examined after the incubation at 30°C for 7 days.

RESULT AND DISCUSSION

Isolation and Selection of Microorganism

About 300 strains of bacteria, which produce chitinolytic enzyme, judging by the halo formation around their colonies, were isolated from soil samples. The chitinase activities of isolated strains were tested by the method of Somogyi (19). As shown in Fig. 1, strain WC-17 was chosen for showing the best chitinolytic activity.

Identification of the Isolated Bacterial Strain

The morphological, biochemical and cultural properties of the isolated bacterial strain WC-17 which produced chitinolytic activity is shown in Table 1. and an electron micrograph in Fig. 2. The strain is rods which become spherical in the stationary phase of growth. The strain commonly occurs in pairs and also chains of variable length. Cells stain Gram negative and do not form spores. They are oxidase negative, catalase positive, nonmotile and require oxygen as the terminal electron acceptor.

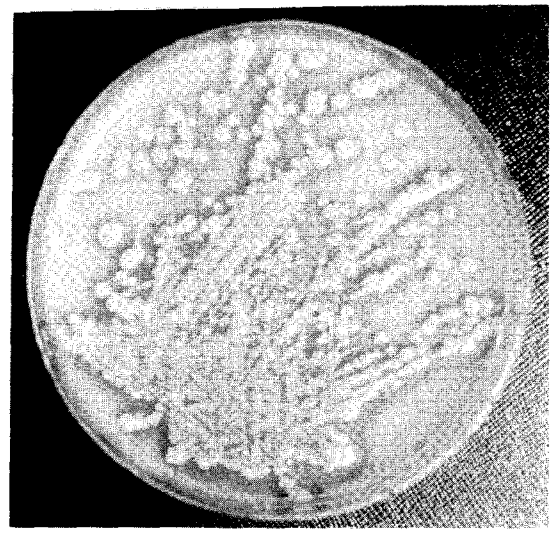

Fig. 1. The selelcted strain WC-17 shows halo formatiom around their colonies on 1% colloidal chitin at 30°C for 24 h.

Table 1. Characterization of the isolated chitinase-producing strain WC-17

Properties	Strain WC-17
Gram reaction	-
Cell shape	Rod or coccobacilli
Cell size	0.6×1.2 μm
Motility in liquid media	-
PHB accumulated	-
Grow at 44°C	-
41°C	+
Grow at pH 4	+
pH 8	+
Fix N_2 *invitro* aerobically	-
Requiring 0.1% NaCl or more	-
Grow with 3% NaCl	-
Cause lysis in soil of Gram(+) and G(-) bacteria	-
Oxidase	-
Catalase	+
Nitrate reduction	+
H_2S production	-
Acid from	
Glucose, Fructose, Sucrose	+
Maltose. Dextrose	+
Raffinose, Inositol, Sorbitol	-
Lactose, Manitol	-
β-Xylosidase	+
Indole from tryptophan	+
Hydrolysis of	
Gelatin, Pectin, Lipid	-
Starch, Patato Starch	+
Casein, Chitin, Xylan	+
Utilization of	
Glucose, Maltose, Sucrose, Fructose	++
Lactose, Arabinose, Raffinose, Xylose	+
Sorbitol, Xanthin	+
Galactose, Mannitol Rhamnose, Melibiose	-
Hydroxyproline, L-Hidtidine, Phenylacetate	-
β-Alanine, L-Leucine, L-Valine, L-Tyrosine	+
D-Malate, Malonate, 2,3-Butanediol, L-Valine	+
L-Arginine, L-Asparagine	++
L-hydroxyproline, L-Phenylalanine	+

++: Strong positive, +: Positive, -: Negative

The strain isolated, has temperature and pH of optima 28°C and pH 6.5 respectively. According to Bergey's Manual of Systematic Bacteriology (7), the isolated strain WC-17 was identified as a strain of *Acinetobacter* unmamed species "3". Thus, the isolated bacterial strain was named as *Acinetobacter* sp. WC-17

Time Course and Initial pH of Cell Growth and Chitinase Production

The growth curve and time course of chitinase production are shown in Fig. 3. The cells were grown at 30°C in a chitin-tryptone medium and the chitinase production was monitored at 12 h intervals. Chitinase activity appeared when bacterial growth was in the exponential phase, and its production increased rapidly after 36 h of growth. The chitinase production increased

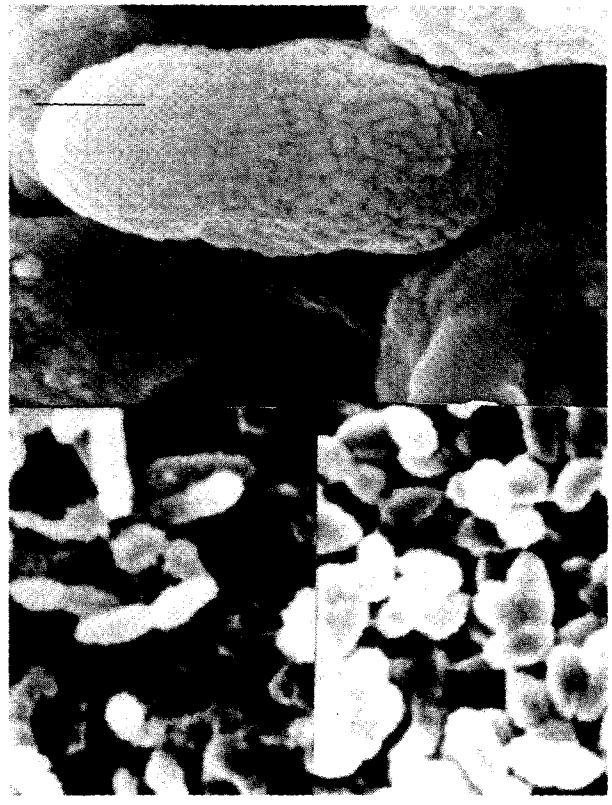

Fig. 2. Scanning electron microscoph of strain WC-17.
Left of bottom shows cells in exponential phase of growth and right of bottom in stationary phase. The bar represents 3 μm.

in proportion to the growth of the cell, and reached its maximum after 80 h of cultivation. Thereafter, the enzyme activity in the culture broth gradually decreased owing to cell's proteolytic activities. The effect of pH on the culture medium was examined to determine the optimum initial pH for chitinase production. *Acinetobacter* sp. WC-17 was grown at 30°C for 72 h and the enzyme activity was measured. The maximum yield of the enzyme was achieved when the initial pH of the medim was adjusted to pH 6.5.

Optimization of Chitinase Production by *Acinetobacter* sp. WC-17

The effect of various carbon sources on the production of chitinase is summarized in Table 2. *Acinetobacter* sp. WC-17 was grown at 30°C for 72 h in basal a medium containing 0.5%(w/v) of carbon sources and 0.5%(w/v) $(NH_4)_2HPO_4$ as a nitrogen source. The enzyme activity was then measured under the standard conditions. As shown in our results, high chitinase activity was found only in cultures supplied with chitin, but not with other polymers such as pectin, inulin and starch, which is indicative of induction on enzyme synthesis. The most effective inducers of chitinase among monomeric carbon sources were glucose and N-acetylglucosamine. Other

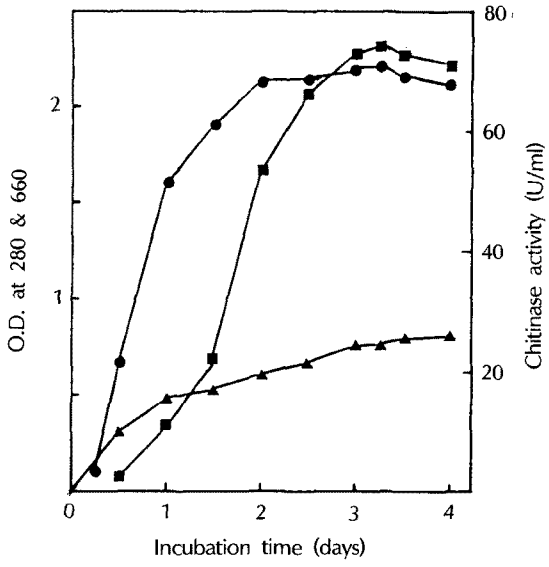

Fig. 3. Time course of growth and chitinase production of *Acinetobacter* sp. WC-17.
Chitinase activity(U/ml)(■), Cell growth(O.D.660nm)(●), and Extracelluar protein(O.D.280nm)(▲)

Table 2. Effect of various carbon sources on chitinase production

Carbon source (0.5%)	cell growth (660nm)	Extra-protein (280nm)	Chitinase Activity (U/ml)
Chitin	1.462	0.547	45.3
Inulin	2.318	0.492	11.4
Pectin	0.275	0.113	2.6
Starch	2.420	0.750	8.2
Galactose	0.127	0.502	2.2
meso-inositol	2.035	0.510	6.1
Sorbitol	1.515	0.505	4.3
Raffinose	1.893	0.472	10.1
Fructose	4.033	1.541	12.3
Lactose	2.115	0.847	4.9
Arabinose	3.980	1.651	5.2
Polygalacturonic acid	1.463	0.660	5.6
Rhamnose	0.545	0.266	0
Maltose	3.163	0.874	0
Mannitol	0.382	0.280	0
Xylose	0.488	0.420	2.4
Dextrose	4.682	1.592	5.8
Glucose	4.281	1.247	22.6
NAG*	3.563	1.048	24.7

Acinetobacter sp. WC-17 was grown at 30°C for 72 h in a basal medium consisting of 0.5% of a carbon source and 0.5% $(NH_4)_2HPO_4$ as a nitrogen source and the enzyme activity was assayed under the standard conditions.
*NAG; N-acetylglucosamine

monomeric carbon sources only showed a rather inhibitory effect. In the case of *Streptomyces lividans*, glycerol and lactose were found to be effective. Glucose had a negative effect on chitinase production (17). The effect of various nitrogen sources, including both inorganic and

Table 3. Effect of various nitrogen sources chitinase production

Nitrgen sources (0.5% w/v)	Cell growth (660 nm)	Extra protein (280 nm)	Chitinase Activity (U/ml)
Peptone	3.042	0.964	20.2
Tryptone	2.246	0.742	28.7
L-Asparagine	1.542	0.521	12.7
$(NH_4)_2HPO_4$	0.862	0.246	1.1
KNO_3	0.556	0.122	2.4
NH_4Cl	0.424	0.125	1.5
$(NH_4)_2SO_4$	0.292	0.144	2.8
$NaNO_3$	0.386	0.221	3.2
$(NH_4)_2NO_3$	1.024	0.601	9.1

Acinetobacter sp. WC-17 was grown at 30°C for 72 h in a basal medium consisting of 0.5% of a nitrogen source and 0.1% glucose as a carbon source and the enzyme activity was assayed under the standard condition.

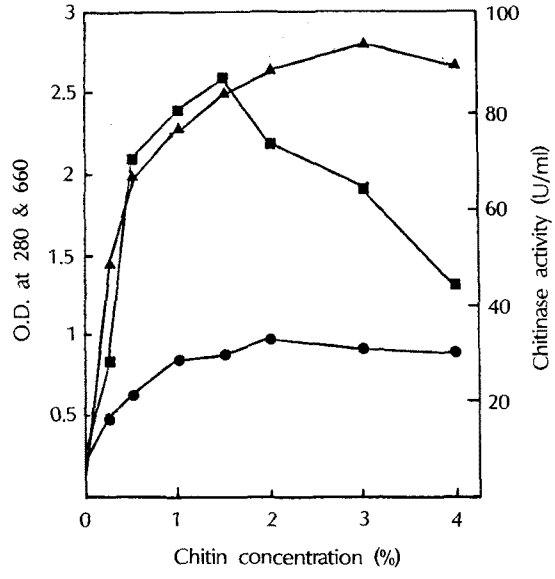

Fig. 4. Effect of chitin concentration on chitinase production
Acinetobacter sp. WC-17 was cultivated at 30°C for 72 h in chitin-tryptone medium with various amounts of chitin. Chitinase activity(U/ml)(■), Cell growth(O.D.660nm)(▲), and Extracelluar protein(O.D.280nm)(●)

organic compounds, was examined. Table 3 shows the influences of the different nitrogen sources on the production of chitinase. None of the inorganic nitrogen sources, except for $(NH_4)_2NO_3$, tested, appeared to affect chitinase activity. Among the organic nitrogen sources, tryptone was found superior to the others examined. In other cases, however peptone was the most effective on chitinase production (21). The effect of a chitin concentration on the chitinase production is shown in Fig. 4. *Acinetobacter* sp. WC-17 was cultivated at 30°C for 72 h in a chitin-tryptone medium with various amounts of chitin, and enzyme activity was measured using standard assay conditions. Chitinase activity increased rapidly when the chitin concentration reached 1.5% (w/v).

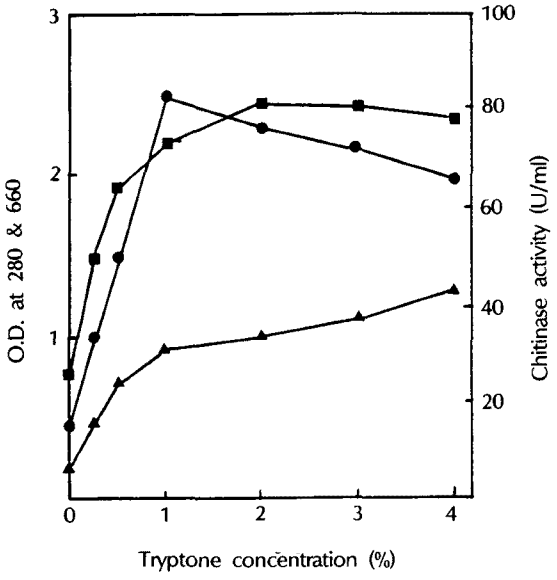

Fig. 5. Effect of Tryptone concentration on chitinase production

Acinetobacter sp. WC-17 was cultivated at 30°C for 72 h in chitin-trypton medium with various amounts of tryptone. Chitinase activity(U/ml)(●), Cell growth(O.D.660nm)(■), and Extracelluar protein(O.D.280nm)(▲)

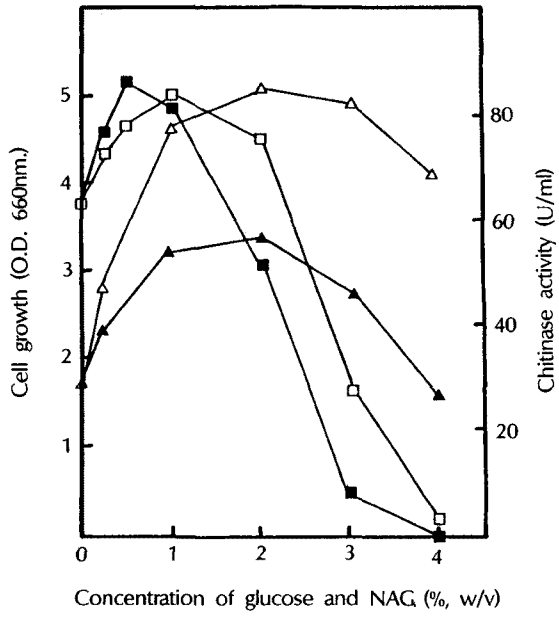

Fig. 6. Catabolic repression on chitinase synthesis

Acinetobacter sp. WC-17 was cultivated at 30°C for 72 h in chitin-trypton medium with various amounts of NAG and glucose. In glucose culture; Chitinase activity(U/ml)(□), Cell growth(O.D.660nm)(△), in NAG culture; Chitinase activity(U/ml)(■), Cell growth(O.D.660nm)(▲)

Thereafter, the enzyme activity in a chitin broth of high concentration gradually decreased. The decrease in the yield of the enzyme was probably due to the inhibition of enzyme action by excessive concentration of chitin.

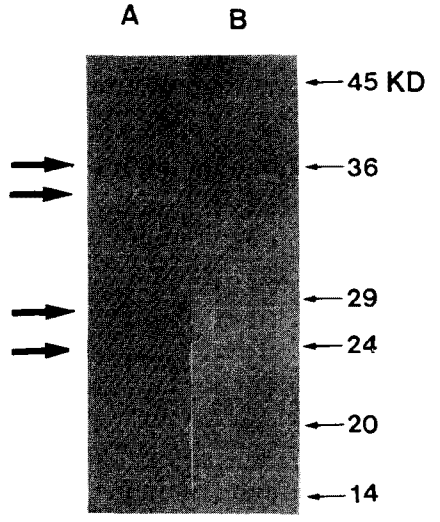

Fig. 7. Chitinase activity after SDS-PAGE in a gel containing 0.01% (w/v) glycol chitin as a substrate.

Arrows indicate chitinase isozymes. Chitinase isozymes of *Acinetobacter sp.* WC-17 can be renatured only if denatured in the presence of SDS alone (A) and not be renatured if denatured in the presence of SDS and reducing agent, such as β-mercaptoethanol (B).

Both *Peudomonas stutzeri* (12) and *Serratia marcescens* (14) showed a high enzyme activity with 3% and 1. 5% (w/v) of chitin concentration, respectively. The effect of a tryptone concentration on the chitinase production is summarized in Fig. 5. *Acinetobacter* sp. WC-17 was grown at 30°C for 72 h in a chitin-tryptone medium with various concentration levels of tryptone and the enzyme activity was measured. The tryptone promoted cell growth and chitinase production. The maximum amount of chitinase was produced at a concentration at 1% (w/v) tryptone.

Catabolic Repression on the Chitinase Production

To test for sensitivity to catabolic repression, *Acinetobacter* sp. WC-17 was grown at 30°C for 72 h in a chitin-tryptone medium with various amounts of glucose and NAG and the enzyme activity was measured. As shown in Fig. 6, maximum enzyme activity appeared at 0.5% (w/v), 1.0% (w/v) and maximum growth were 1.5%, 2.0% of NAG and glucose concentrations, respectively. In both cases, after maximum chitinase activity, enzyme activity rapidly decreased. This result indicated that NAG and glucose were effective inducers of chitinase production in *Acinetobacter* sp. WC-17 at low concentration but repressors in exessive concentrations. Some species of *Streptomyces* were repressed by 0.5% (w/v) of glucose concentration (9).

Chitinase Activity after *SDS-PAGE*

To determine the number of isozyme and the molecular weight of chitinase in *Acinetobacter* sp. WC-17, a cultural supernatant was analysed using SDS-PAGE and

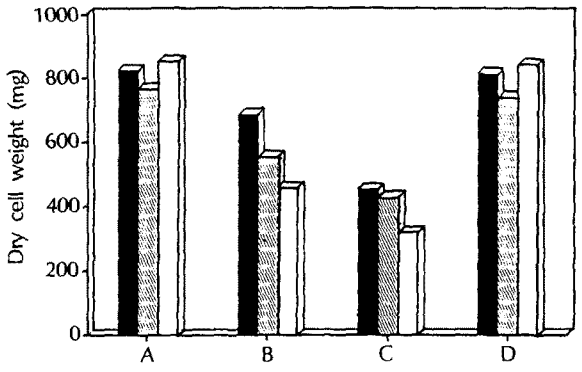

Fig. 8. Antifungal effect of *Acinetobacter* sp. WC-17 against phytopathogenic fungi.
The mixures of phytopathogenic fungi and cultural filtrate were cultured at 28°C for 6 days in PDB. *F.solani*(■), *R.solani*( ), *P.oryzae*( ), A; Control (containing water) B; 3% cultural filtrate, C; 7% cultural filtrate, D; 3% heat treated filtrate.

the active staining method. As shown in Fig. 7, *Acinetobacter* sp. WC-17 has four chitinase isozymes consisting of 37, 32, 26, and 23 KD in their molecular weight. The 37 KD chitinase showed the strongest activity among the isozymes. In some reports, *Serratia marcescens* has at least five chitinase isozymes consisting of 58, 52, 48, 36 and 21 KD (6, 8). In the case of *Streptomyces plicatus* and *Streptomyces lividans*, there are four chitinase isozymes consisting of 63, 61, 49, 47 and 65, 46, 41, 36 KD. respectively (16, 10). Chitinase isozymes of *Acinetobacter* sp. WC-17 can be renatured only if denatured in the presence of SDS alone (Fig. 7, A) and cannot be renatured if denatured in the presence of SDS and a reducing agent, such as β-mercaptoethanol (Fig. 7, B).

Antifungal Activity of *Acinetobacter* sp. WC-17 against Phytopathogenic Fungi

To test for antifungal activity by *Acinetobacter* sp. WC-17 against phytopathogenic fungi, a culture filtrate of *Acinetobacter* sp. WC-17 and precultivated fungal mycelium were mixed and incubated. As shown in Fig. 8, 3% culture filtrate reduced 17%~47% of dry cell weight of fungal mycelium compared with a control (without culture filtrate). Also, a 7% culture filtrate reduced fungal mycelium by 44%~62%. The more the percentage of culture filtrate increased, the more the percentage of reduction increased. As an alternative control, heated culture filtrate, was used. This control showed no differences with the water one. This result indicates that the antifungal substance was at least a heat labile one such as a protein. Among the tested fungi, *Pyricularia oryzae* was the most sensitive to chitinase of *Acinetobacter* sp. WC-17. Generally, cell walls of *Fusarium* sp. contain more protein than other fungi (1). The larger amounts of protein in the cell wall of *Fusarium* sp. may be responsible for their ability to resist lysis. The chitinase

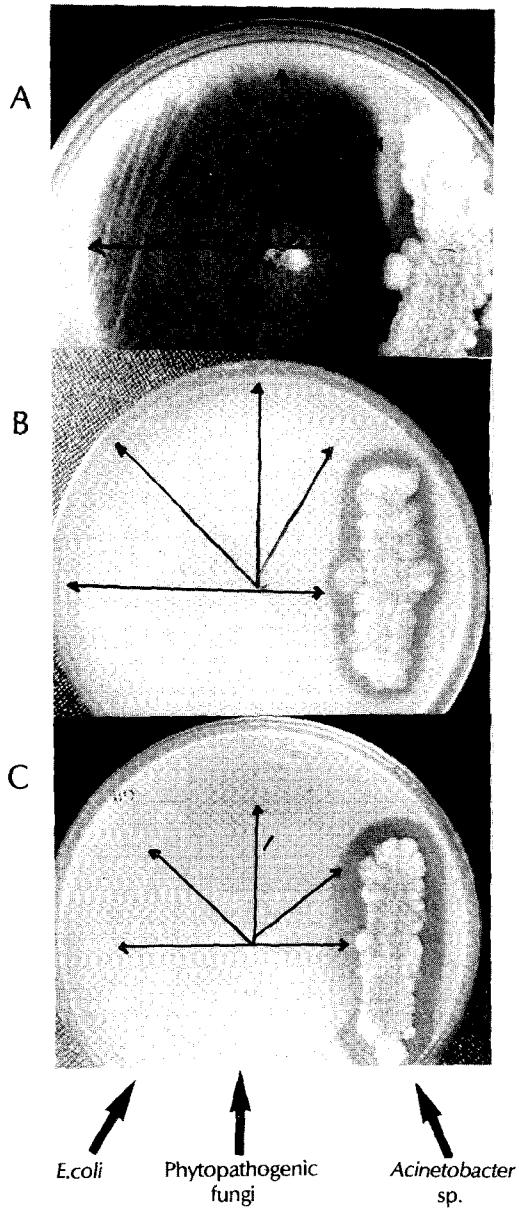

Fig. 9. Antifungal effect of *Acinetobacter* sp. WC-17 against phytopathogenic fungi on PDA plates.
Phytopathogenic fungi and *Acinetobacter* sp. WC-17 were cultivated on PDA containing 1% colloidal chitin at 30°C for 8 days. Arrow indicates the length of fungal mycelium. A; *P.oryzae*, B; *R.solani*, C; *F. solani*

of *Acinetobacter* sp. WC-17 was determined as an exotype enzyme by using a 4-MU oligosaccharide (unpublished data). The bacterial chitinases (exochitinases) as well as in our case, are restricted to locating nonreducing termini of chitin as substrates, which may be difficult in an intact fungal cell wall. Inaccessibility of termini may also play a role in the inability of exochitinases, but not endochitinase, to hydrolyse bacterial cell walls. However, both exochitinase and endochitinase

degrade chitin efficiently in standard chitinase assays, but these use purified, partially degraded chitin as substrate, and would contain numerous termini available for exo-chitinase attack. As shown in Fig. 9, Conventional agar plates (PDA containing 1% colloidal chitin) were also used for detecting inhibition of fungal growth. *P.oryzae* was more inhibited by chitinase of *Acinetobacter sp.* WC-17 than *F.solani* and *R.solani.*

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