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Isolation of Chitin-utilizing Bacterium and Production of Its Extracellular Chitinase

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A bacterial strain, designated as WY22, producing extracellular chitinase was isolated from the soil around the Youngduck area, after enrichment culture in a medium containing 1% (w/v) wet colloidal chitin as a sole carbon source. The isolate was identified as a strain of *Bacillus* sp. based on its morphological and physiological characteristics. It was observed that *Bacillus* sp. WY22 could inhibit the growth of *Fusarium oxysporum* with hyphal extention-inhibition assay on potato dextrose agar plate supplemented with 1% collidal chitin. Optimum culture conditions of *Bacillus* sp. WY22 were examined for chitinase production in a chitin medium. High level production of chitinase was observed not only in the chitin medium but in a medium supplemented with 1% *N*-glucosamine or lactose instead of chitin. The optimum concentrations of colloidal chitin and yeast extract were 3.0 and 0.5%, and the optimum culture conditions for initial pH of medium and temperature were 7.0 and 30°C, respectively, for the production of chitinase.

Chitin, a β-1.4-linked polymer of N-acetylglucosamine, is one of the most abundant biopolymers found in nature next to cellulose (10). It can be found in insect exoskeletons, outer shells of crustaceans, the cell walls of most groups of fungi, and nematodes etc. In recent years, there have been a good number of reports on the possible use of chitin and related materials in many fields including heavy metal recovery from water, drug delivery, wound dressing, and dietary fiber (10). Chemical derivatives of chitin may be complemented and extended by the use of chitin degrading and modifying enzymes (3, 12). In the enzymatic degradation of chitin by microorganisms, the first step is that the polymer is hydrolyzed into oligomers by chitinase (E.C. 3.2.1.14). The second step is the further hydrolysis of the produced oligomers, mainly dimers into N-acetylglucosamine with chitobiase (E.C. 3.2.1.30) (13). Chitinase is known to be produced by a wide range of bacteria (7, 11, 12, 15, 19, 20, 24, 26), actinomycetes (5, 16, 21), mold (1, 14, 18, 22), and yeast (2, 25). Some higher plants are also known to produce chitinases, which probably serve as fungicides or fungistants (4). In this study, a new bacterial strain producing extracellular chitinase was isolated from soil and the optimum culture conditions for the production of chitinase were investigated.

MATERIALS AND METHODS

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Key words: extracellular chitinase, *Bacillus* sp. antifungal activity

Media

The medium for the isolation of chitinase producing bacteria was composed of 1% (w/v) wet colloidal chitin (equivalent to 0.1% dry colloidal chitin), 0.07% KH_2PO_4, 0.03% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.4% NaCl. For the the production of chitinase, the chitin medium used was composed of 3% (w/v) wet colloidal chitin, 0.5% yeast extract, and various mineral salts containing 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.4% NaCl, 0.01% $FeSO_4 \cdot 7H_2O$, 0.001% $MnCl_2$, 0.001% $ZnCl_2$ and 0.001% $CaCl_2$ as described (11). For the growth of fungi in the antifungal activity assay of the isolate, potato dextrose agar medium was used which was composed of 2% infusion from potatoes 0.2% glucose, and 1.5% agar (19).

Isolation of Chitinase-producing Bacteria

One gram of each soil sample collected from around the Youngduck area in Korea was inoculated into 50 ml of the liquid isolation medium in a Erlenmeyer flask. The soil samples were incubated at 30°C with shaking (100 rpm) for one week for the enrichment culture of chitin utilizing bacteria. The cultures grown rapidly were diluted in sterilized 0.9% NaCl solution and spreaded on the isolation media plates. The plates were then incubated at 30°C for 72-96 h. The bacteria that formed clear zones around their colonies were isolated and used further for chitinase activity assay.

Identification

The isolate was identified according to the Bergey's

Mannual of Systematic Bacteriology (7) based on its morphological, physiological, and nutritional characteristics. The morphlogical aspects were studied using a scanning electron microscope.

Antifungal Activity Assay

Antifungal activities of the isolate against *Botryosphaeria dothidiea* and *Fusarium oxysporum* were assayed according to the method of Roberts and Selitrennikoff (17) as follows. A paper disc inoculated with fungal conidia suspension was placed at the center of the potato dextrose agar plate supplemented with 1% colloidal chitin on which the isolate and *E. coli* strain as a negative control were streaked on each side. Growth inhibition of fungal hyphae was examined after incubation of the plate at 30°C for 7 days.

Preparation of Enzyme Solution

After the bacteria were grown at 30°C for 72 h in 500 ml of the chitin medium, the supernatant was collected by centrifugation at 20,000 g for 20 min. Chitinase in the supernatant was collected by precipitation with ammonium sulfate saturated to 80% followed by centrifugation at 20,000 g for 20 min. The precipitate was dissolved in 15 ml of 20 mM sodium phosphate buffer (pH 6.0) and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) for 12 h.

Chitinase Assay

Chitinase assay was done based on the estimation of reducing sugars released during the hydrolysis of colloidal chitin. The reaction mixture containing 0.5 ml of 0.2% colloidal chitin, 0.5 ml of 0.2 M McIlvaine buffer (pH 5.0) and 0.5 ml of enzyme solution was incubated at 37°C for 2 h with gentle shaking, and then centrifuged at 12,000 g for 10 min. The amount of reducing sugar in the supernatant was measured according to the method of Imoto and Yagishita (6). Enzyme unit was defined as the amount of enzyme which released 1 μmol of *N*-acetyl-D-glucosamine per minute under the conditions of this study.

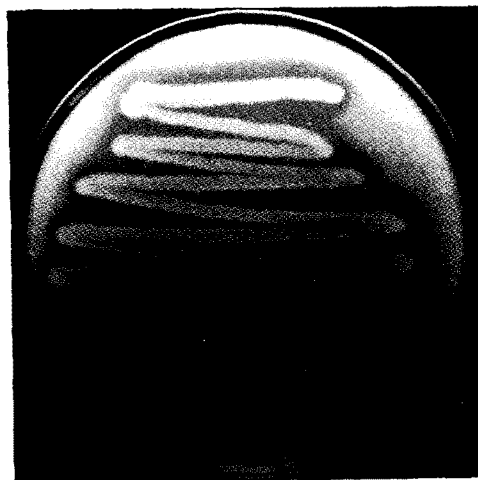

Fig. 1. Halo formation of the chitinase producing bacteria WY22 around their colonies.

The bacteria were grown at 30°C for 3 days on the isolation medium plate containing 1% (w/v) wet colloidal chitin as a sole carbon source.

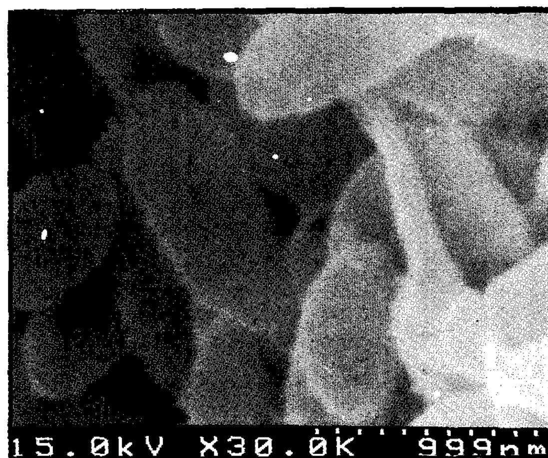

Fig. 2. Scanning eletron micrograph of the strain WY22.

RESULTS AND DISCUSSION

Isolation and Identification of Chitinase-producing Bacteria

About 300 strains of chitinolytic enzyme-producing bacteria that formed large clear zones around their colonies on the isolation media plates were isolated from soil samples. Among them, strain WY22 showed the formation of the largest clear zone. Therefore, strain WY22 was selected for further characterization.

Fig. 1 shows the strong halo formation of the bacterial strain WY22 around its colony because of the hydrolysis and utilization of colloidal chitin added in the medium as a sole carbon source. The shape of strain WY22 was noted to be a rod type on a scanning electron microphotograph (Fig. 2). Morphological and physiological characteristics of strain WY22 are summarized in Table 1. It was found to be Gram-positive, motile and rod-shaped. These characteristics as well as the oxidase, catalase, methyl red test, and indole formation were consistent with the description of *Bacillus* sp. in Bergey's Manual of Systematic Bacteriology (7). Strain WY22 was found to be similar to B. circulans, but to be different in some respects such as V-P test below pH 7.0 and acid production from carbohydrates including sorbitol, melibiose and raffinose.

Antifungal Activity of *Bacillus* sp. WY22

The antifugal activity of *Bacillus* sp. WY22 was investigated on the potato dextrose agar plate supplemented with 1% colloidal chitin. As shown in Fig. 3, in-

Table 1. Morphological, cultural and physiological characteristics of the chitinase-producing bacterium strain WY22.

Morphological characteristics	
Form	Rods
Size	0.3-0.6×1.4-2.1 μm
Gram stain	Positive
Cultural characteristics	
Nutrient broth	Moderate growth, Circular, Entire, Smooth
Physiological characteristics	
Catalase	Positive
Methyl red test	Negative
V-P test	Negative
V-P test(below pH 7.0)	Negative
Indole production	Negative
Starch hydrolysis	Positive
Gelatin liquefaction	Negative
Utilization of citrate	Positive
Hydrogen sulfide production	Positive
Gas from glucose	Negative
Urease test	Negative
Pigment production	Negative
Optimum growth temperature.	25-30°C
pH of growth	4.0-10.0
Acid production	Glucose, Arabinose, Xylose
Growth in 5% Nacl	Positive
Carbon Assimilation	
Glucose	Positive
Fructose	Positive
Maltose	Positive
Sorbitol	Weak positive
Lactose	Positive
Surcose	Positive
Sorbose	Weak positive
Galactose	Positive
Ribose	Positive
Xylose	Weak positive
Melibiose	Negative
Cellobiose	Positive
Arabinose	Positive
Raffinose	Positive
Mannitol	Positive
Inositol	Negative
Xylitol	Negative
Methanol	Negative
Ethanol	Negative
Glucosamine	Weak positive
Glycine	Positive
Succinate	Negative
Salicine	Weak positive
Arbutin	Positive
Gluconic acid	Positive
Dextrin	Weak positive

hibitions of hyphal growths of *Fusarium oxysporum* as well as Botryosphaeria dothidiea were observed around the colonies of *Bacillus* sp. WY22, but not around those

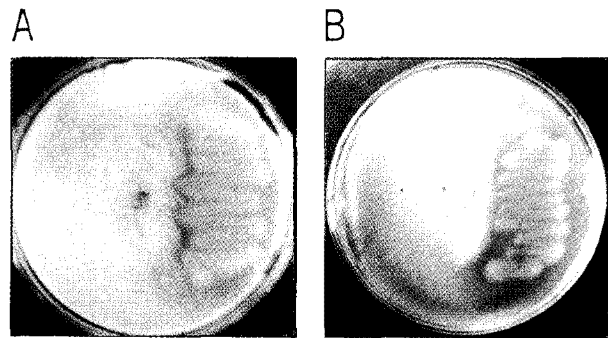

Fig. 3. Antifungal activities of *Bacillus* sp. WY22 against *Botryosphaeria dothidiea* (A) and *Fusarium oxysporum* (B) by the modified hyphal extension-inhibition assay.
A paper disc inoculated with each fungal conidia suspension was placed at the center of potato dextrose agar plate on which *Bacillus* sp WY 22 at left and *E. coli* (negative control) at right side were streaked. The growth inhibition of fungal hyphae was examined after incubation of the plate at 30°C for 7 days.

of *E. coli* used as a negative control. However, not very strong inhibition of fungal grówth was observed even around the colonies of *Bacillus* sp. WY22. The bacterial chitinases, being exochitinases, are known to be restricted to locate nonreducing termini of chitin as substrates which may be difficult in intact fungal cell walls. Inaccessibility of termini may also play a role in the inability of exochitinases, but not endochitinases, to hydrolyze fungal cell walls, although this difference in specificity could easily be seen due to other factors (23).

Effects of Carbon Sources on the Chitinase Production

The effects of various carbon sources on the production of chitinase from *Bacillus* sp. strain WY22 were investigated (Table 2). After the bacteria were grown at 30°C for 3 days in a medium containing 1% colloidal chitin or other carbon sources, the chitinase activity was assayed under the conditions described in Materials and Methods. As shown in Table 2, high levels of chitinase were produced in a medium containing 1% colloidal chitin, *N*-glucosamine or lactose as a carbon source. In a medium containing 1% glucose, durcitol or starch instead of collidal chitin, it was shown that the bacteria could produce 68 to 78% of chitinase compared with that in the chitin medium. However, a very low level of chitinase activity was detected in a medium containing other carbohydrates such as fructose, mannose, sucrose, ribose and pectin. It was surprising that only about 46% of chitinase was produced in a medium containing 1% *N*-acetyl glucosamine, the reason for which is yet unknown. Fructose and raffinose have been reported to be good inducers for chitinase production in *Acinetobacter* sp. (19), while lactose is reported to be a good inducer in *Streptomyces lividans* (16). However, none of them except lac-

Table 2. Effects of various carbon sources on the chitinase production from *Bacillus* sp. WY22.

Carbon sources	Relative activities (%)
Colloidal chitin	100.0
Arabinose	25.6
Fructose	13.8
Galactose	24.7
Glucose	78.2
Mannose	ND[a]
Ribose	14.1
Xylose	29.3
N-Acetylglucosamine	45.8
N-Glucosamine	99.0
Dulcitol	68.7
Inositol	44.4
Sorbitol	38.3
Lactose	96.0
Maltose	46.1
Sucrose	9.8
Raffinose	52.9
Pectin	ND
Starch	68.7

The chitinase activity was determined, after the bacteria were cultured at 30°C for 3 days in the chitin medium supplemented with each carbon source instead of colloidal chitin. [a]Not detected.

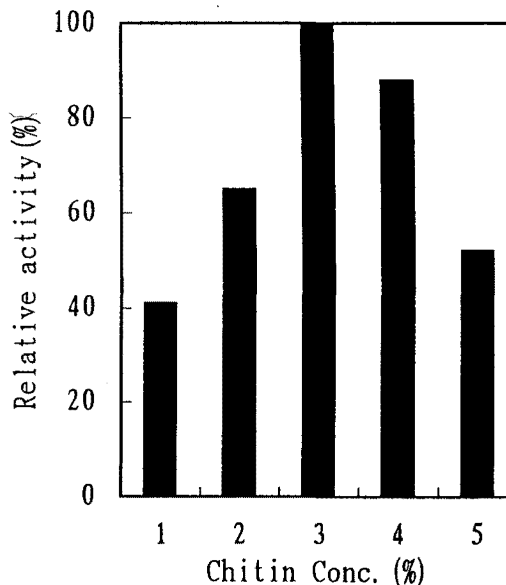

Fig. 4. Effect of the concentration of collidal chitin on the chitinase production from *Bacillus* sp. WY22.
The chitinase activity was measured after the bacteria were cultured at 30°C for 3 days in the chitin medium containing each concentration of collidal chitin.

tose could induce a high level of chitinase in *Bacillus* sp. WY22 isolated in this study.

The effect of chitin concentration on the chitinase production of *Bacillus* sp. WY22 is shown in Fig. 4. After the bacteria were grown at 30°C for 72 h in the chitin medium containing various amounts of colloidal chitin, chitinase activity was assayed. According to the increase of chitin concentration in the media, higher levels of chitinase were produced but activity gradually decreased at over 3% colloidal chitin. It has been reported that *Aeromonas salmonicida* YA7-625 (11), *Pseudomonas stutzeri* (12), *Serratia marcescens* (15) and *Acinetobacter* sp. (19) could produce maximum levels of chitinase in a media containing 1.26%, 3%, 1.5% and 1.5% chitin, respectively.

Effects of Nitrogen Sources on Chitinase Production

The effects of various nitrogen sources on the production of chitinase with *Bacillus* sp. WY22 are shown in Table 3. Yeast extract was found to increase chitinase production twice as much as that in a control medium (the chitin medium without 0.5% yeast extract). Some inorganic nitrogen sources such as $(NH_4)_2SO_4$ and NH_4Cl were shown to repress the production of chitinase. Little effect on chitinase production was observed compared with the control when bactopeptone and tryptone were used as nitrogen sources. These results appeared to be completely different from the patterns of chitinase production with other microorganisms. A tryptone was reported to be the most effective nitrogen source for chitinase production in *Aeromonas salmonicida* YA7-625 (11), *Acinetobacter* sp. (18) and peptone was the most effective nitrogen source for *Rhizopus* (21). The effect of a yeast extract concentration on chitinase production is shown in Fig. 5. The maximum amount of chitinase was produced at 0.5% yeast extract in the chitin medium.

Table 3. Effects of various nitrogen sources on the chitinase production by *Bacillus* sp. WY22.

Nitrogen sources	Relative activity (%)
None[a]	100.0
Yeast extract	234.3
Peptone	134.3
Tryptone	97.1
$(NH_4)_2SO_4$	13.7
NH_4Cl	3.9

The chitinase activity was determined, after the bacteria were cultured at 30°C for 3 days in the chitin medium supplemented with each nitrogen source instead of yeast extract. [a]The chitin medium devoid of 0.5% yeast extract was used as a control medium.

Effects of Initial pH of Media and Temperature on Chitinase Production

The effects of initial pH of media and temperature on chitinase production with *Bacillus* sp. WY22 were examined by chitinase activity assay after the strain was cultured for 72 h in the chitin medium (Fig. 6). The maximum yield of chitinase was obtained when initial pH of media and temperature were 7.0 and 30°C, respectively.

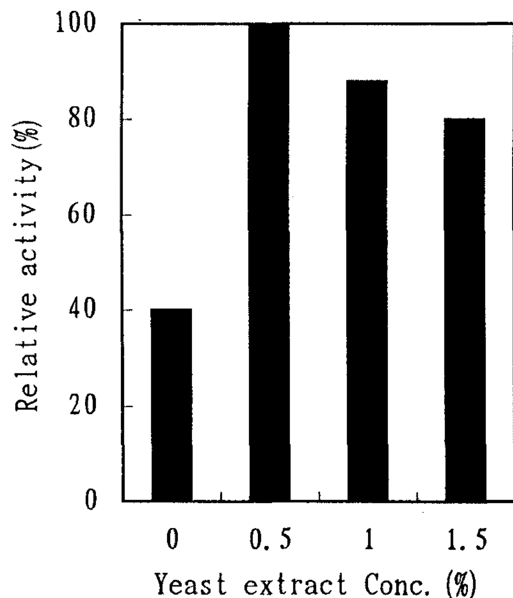

Fig. 5. Effect of the concentration of yeast extract on the chitinase production from *Bacillus* sp. WY22.
The chitinase activity was measured after the bacteria were cultured at 30°C for 3 days in the chitin medium containing each concentration of yeast extract.

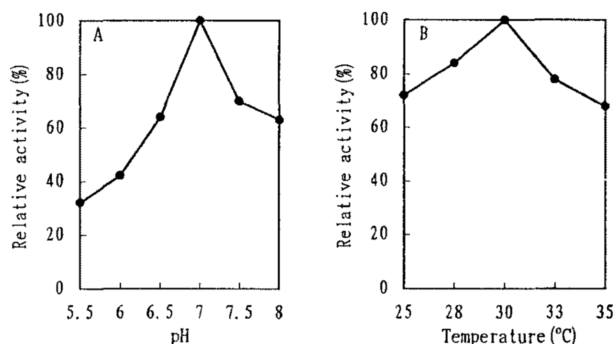

Fig. 6. Effects of initial pH of media (A) and temperature (B) on the chitinase production from *Bacillus* sp. WY22.
The chitinase activity was assayed after the bacteria were cultured at 30°C for 3 days in the chitin medium under the conditions of initial pH of media and temperature shown below the figures.

Time Course of Chitinase Production

During culture of *Bacillus* sp. WY22 at 30°C in the chitin medium, chitinase production was monitored at every 24 h (Fig. 7). The chitinase production was observed to increase rapidly according to the culture time, and to reach maximum level after 3 days. It then gradually decreased, and this is thought to be due to the increase of the proteolytic enzyme activities in cells. It was shown that the patterns of extracellular protein production were similar to those of chitinase production.

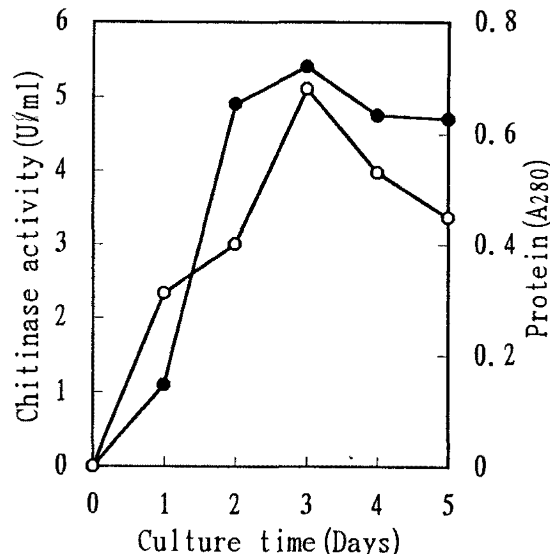

Fig. 7. Time course of the chitinase production from *Bacillus* sp. WY22.
The chitinase activity was monitored at every 24 h during the culture of *Bacillus* sp. WY22 at 30°C for 5 days in the chitin medium. ●—●, chitinase activity (U/ml); ○—○, Protein (A_{280}).

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