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The Production and Enzymatic Properties of Extracellular Chitinase from *Pseudomonas stutzeri* YPL-1, as a Biocontrol Agent

LIM, HO-SEONG AND SANG-DAL KIM*

Department of Applied Microbiology, Yeungnam University, Kyongsan 712-749, Korea

An antagonistic bacterium *Pseudomonas stutzeri* YPL-1 liberated extracellular chitinase and β-1,3-glucanase which are key enzymes in the decomposition of fungal hyphal walls. The lytic enzymes caused abnormal swelling and retreating at the hyphal tips of plant pathogenic fungus *Fusarium solani* in a dual culture. Scanning electron microscopy revealed the hyphal degradation of *F. solani* in the regions interacting with *P. stutzeri* YPL-1. The production of chitinase and properties of a crude preparation of the enzyme from *P. stutzeri* YPL-1 were investigated. Peak of the chitinase activity was detected after 4 hr of cultivation. The enzyme had optimum temperature and pH of 50°C and pH 5.3, respectively. The enzyme was stable in the pH range of 3.5 to 6.0 up to 50°C. The enzyme was significantly inhibited by metal compounds such as $HgCl_2$, but was stimulated by $CoCl_2$. *P. stutzeri* YPL-1 produced high levels of the enzyme after 84 hr of incubation. Among the tested carbon sources, chitin was the most effective for the enzyme production, at the concentration level of 3%. As a source of nitrogen, peptone was the best for the enzyme production, at the concentration level of 4%. The maximum amount of enzyme was produced by cultivating the bacterium at a medium of initial pH 6.8.

Chitinases, which hydrolyze β-1,4 glucosidic bonds of chitin as a major structural component of many agronomically important pests including phytopathogenic fungi, the exoskeleton and digestive tract of some insects, some nematodes, and arthropod integuments, are commonly found in a wide variety of organisms including bacteria (11, 17, 27, 28), fungi (5, 15, 20, 26), higher plants (25, 29), insects (9, 10), crustaceans (13), and some vertebrates (7). The roles of these chitinases could be devided into several catagories. In fungi, chitinase activity has a physiological role in apical growth and morphogenesis of fungal hyphae (1, 8, 18). Bacterial chitinase appears to have a nutritional or scavenging role in the decomposition of insoluble chitin and also in the utilization of chitin as a carbon and energy source. The production of chitinases by plants is considered to be a part of their defense mechanism against fungal infections (3, 19). These observations imply the significance of chitinase in the ecological interactions between organisms.

Recently, chitinases have received considerable attention because they may play a role in the plant defense systems against chitin-containing pathogens (4). The enzymatic digestion or deformation of the chitin component of these organisms by chitinase could present an effective method for their control. Thus, chitinolytic enzymes have been purified from many microorganisms and their enzymatic properties have been investigated.

A *Pseudomonas stutzeri* YPL-1 which actively lysed *Fusarium solani* causing root-rots leading to considerable losses in many important crops was found to liberate extracellular chitinase and β-1,3-glucanase which are enzymes essential for the decomposition of fungal hyphal walls as a factor of biocontrol (11). In this paper, we describe the culture conditions for the production of chitinase and properties of a crude preparation of the enzyme from *P. stutzeri* YPL-1.

*Corresponding Author
Key words:extracellular chitinase, *Pseudomonas stuctzeri*, biocontrol agent.

MATERIALS AND METHODS

Chemicals

Chitin powder extracted from crab shell was purchased from Sigma Chemical Co. Colloidal chitin used in the chitinase assay was prepared by the method of Bemiller (2). All other chemicals were special grade pro-

ducts.

Microorganisms and Cultivation

Pseudomonas stutzeri YPL-1, originally isolated from rhizosphere in ginseng root-rot-suppressive soil, was used in this study (11). The bacterium was grown and maintained on a nutrient agar (NA). *Fusarium solani*, which causes plant root rot, was provided by the Korean Ginseng and Tobacco Research Institute and grown on a potato dextrose agar (PDA). The microorganisms were lyophilized for long-term storage.

Chitinase Preparation

A cell wall-degrading chitinase was prepared from the culture supernatant of *P. stutzeri* YPL-1. The bacterium was grown at 30°C for 84 hr on a rotary shaker in a chitin-peptone medium containing 0.5% glucose, 0.5% peptone, 0.5% chitin, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% NaCl. The pH of the medium was adjusted to 6.8 prior to autoclaving. The culture was centrifuged aseptically at 12,000×g for 20 min at 4°C. The enzyme was partially purified by ammonium sulfate precipitation, column chromatographies on DEAE-cellulose and DEAE- Sephadex A-50, and gel filteration on Sephadex G-100.

Chitinase Assay

Chitinase activity was determined by measuring the amount of *N*-acetyl-D-glucosamine (GlcNAc) released from colloidal chitin by the method of Reissig *et al.* (16). The assay mixture consisted of 0.3 m*l* of 1M sodium acetate buffer (pH 5.3), 0.5 m*l* of 0.2% colloidal chitin, and 0.2 m*l* aliquot of appropriately diluted enzyme. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per min under these conditions.

Protein Estimation

Protein concentration was determined spectrophotometrically at 280 nm or by the method of Lowry *et al.* (12) with bovine serum albumin as a standard.

Scanning Electron Microscopy

Microscopic observations were made of the interacting regions of *F. solani* grown with *P. stutzeri* YPL-1 in a dual culture. The samples were fixed with 3% glutaraldehyde in a 0.2 M phosphate buffer (pH 6.5) for 3 hr, and washed with the same buffer for 15 min, fixed with 2% OsO_4 for 2 hr, and finally washed again with the buffer. The material was dehydrated with ethanol at 4°C by using a series of steps for 10 min each. The specimens were dried in a Hitachi HCP-2 critical point drier with CO_2 as the carrier gas. The dried specimens were mounted on stubs with Television Tube Koat to prevent charging. The specimens were sputter coated with gold palladium in an Ion Coater Giko IB-5 and observed with a scanning electron microscope (ISISS 103).

RESULTS AND DISCUSSION

Antagonism and Lytic Activity

P. stutzeri YPL-1 released extracellular chitinase and β-1,3-glucanase, which are key enzymes in the decomposition of fungal hyphal walls, when grown in a medium containing chitin, laminarin, or dried mycelium of *F. solani* as carbon sources (11). Scanning electron microscopy revealed the degradation of *F. solani* mycelium when its cell wall components served as the sole carbon source for *P. stutzeri* YPL-1 in a dual culture (Fig. 1). Abnormal hyphal swelling and retreating were caused by the excretion of lytic enzymes from the bacterium. Also, swift swelling and retreating at the hyphal tips of *F. solani* were observed. It showed that the fusarial hyphal walls of this region interacting with accumulation of the bacterial cells were rapidly lysed, causing a leakage of cytoplasm. The use of electron microscopy provided more detailed information about the mode of antagonism and helped to localize sites of interaction between hyphae of *F. solani* and *P. stutzeri* YPL-1. The results indicate that the β-1,3-glucanase and chitinase produced by antifungal agent attack these sites and completely degrade the hyphae.

Chitin Hydrolysis

Time course of the hydrolysis of colloidal chitin by the action of a partially purified chitinase from *P. stutzeri* YPL-1 is shown in Fig. 2. The chitinase hydrolyzed with similar rates in the initial stage of the reaction. However, the rate of hydrolysis reached maximum after 4 hr of incubation, and did not increase during the prolonged incubation.

Effect of pH on Activity and Stability

The effects of pH on the activity of a partially purified *P. stutzeri* YPL-1 chitinase were determined by varying the pH of the reaction mixtures using a 1 M sodium acetate buffer (pH 3.0~5.5) and a 50 mM sodium phosphate buffer (pH 5.5~7.0). The chitinase

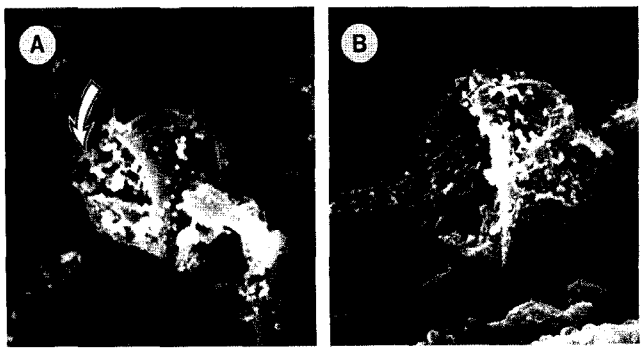

Fig. 1. Scanning electron micrographs of *F. solani* hyphae interacting with *P. stutzeri* YPL-1 in dual culture.
(A) Abnormal hyphal swelling and outflow of cytoplasm, (B) Swift swelling and retreating at the hyphal tip.

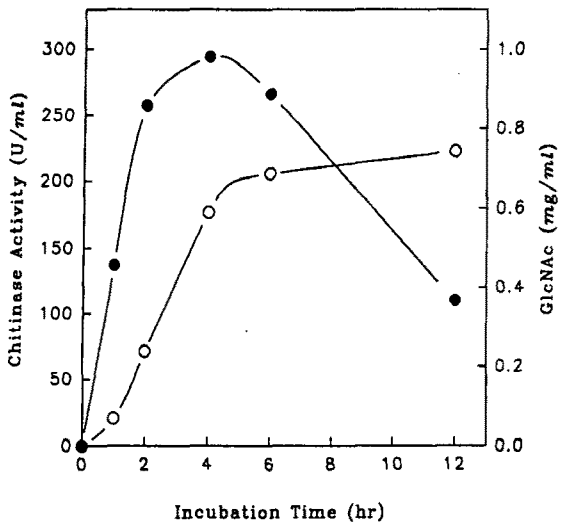

Fig. 2. Time course of reaction hydrolyzed by chitinase.
●: Chitinase activity, ○: Amount of N-acethyl-D-glucosamine (GlcNAc) released by chitinase.

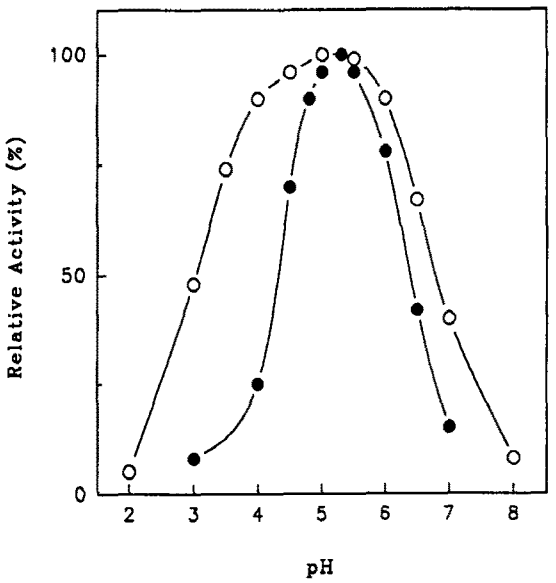

Fig. 3. Effect of pH on activity and stability of chitinase.
The chitinase activity (●) was assayed in a 1 M sodium acetate buffer (pH 3.0~5.5) and a 50 mM sodium phosphate buffer (pH 5.5~7.0) at the various pHs. A chitinase solution preincubated for 30 min in a 1 M sodium acetate buffer (pH 2.0~5.5) and a 50 mM sodium phosphate buffer (pH 5.5~8.0) of various pHs and the remaining activity (○) was assayed under the standard conditions.

activity was measured using colloidal chitin as a substrate at 50°C for 4 hr. The optimal pH range of the enzyme activity was 4.5~6.0 with a maximum activity at pH 5.3 (Fig. 3). This suggests that the enzyme is composed with a single protein whereas some chitinases are isoenzymes composed with several enzymes. The pH optimum for chitinases is usually in the range of pH 4.0 to 7.0, but exceptions to this are enzymes from *Streptomyces thermoviolaceus* (pH 8.0~10.0) (22) and *Alteromonas* sp. (pH 8.0) (23) with alkalic optimum pHs and *Saccharomyces cerevisiae* (pH 1.5~2.5)

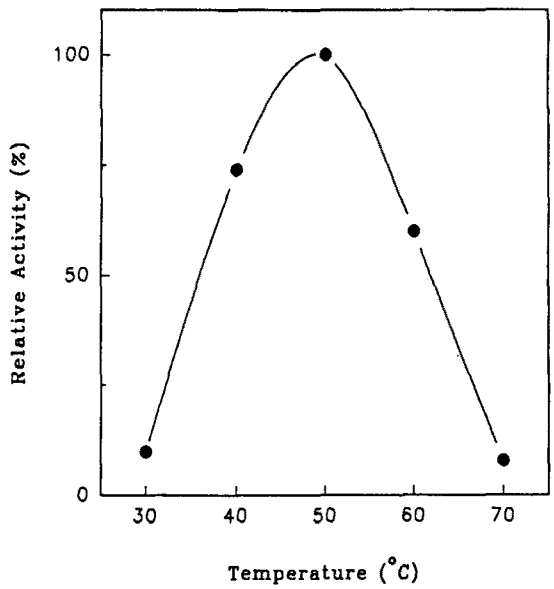

Fig. 4. Effect of temperature on chitinase activity.
The chitinase activity was assayed in a 1 M sodium acetate buffer of pH 5.3 at various temperatures under the standard conditions.

(6) with an extremely acidic optimum pH.

The denaturating effects of pH on the enzyme protein of *P. stutzeri* YPL-1 chitinase were investigated by incubating the enzyme solution for 30 min at various pH values. The chitinase was preincubated at 50°C for 30 min at various pHs from 2.0 to 8.0 and the remaining activity was measured using colloidal chitin at pH 5.3. More than 70% of the initial activity of the enzyme was retained at the pH range of 3.5 to 6.0, but was lost dramatically under pH 2.0 and over pH 8.0 (Fig. 3). This result suggests that the chitinase of *P. stutzeri* YPL-1 is more stable in the acidic pH range than in the alkalic pH.

Effect of Temperature on Activity and Stability

The effects of temperature on the activity of a partially purified *P. stutzeri* YPL-1 chitinase were determined by varying the temperatures of the reaction mixtures using a 1 M sodium acetate buffer of pH 5.3. The chitinase activity was measured using colloidal chitin at pH 5.3 for 4 hr in the temperature range of 30 to 70°C. The enzyme was most active at 50°C (Fig. 4). The optimum temperature for chitinases is usually in the temperature range of 40 to 50°C, but exceptions to this are enzymes from *Streptomyces thermoviolaceus* (22), with a high optimal temperature between 70 and 80°C and *Myrothecium verrucaria* (26), with a broad optimal temperature between 25 and 55°C.

Thermal denaturation of *P. stutzeri* YPL-1 chitinase was examined by maintaining the enzyme solution at different temperatures. The chitinase was preincubated at pH 5.3 at various temperatures from 50 to 70°C and the remaining activity was measured using colloidal chitin at pH 5.3. The enzyme was quite stable at tempera-

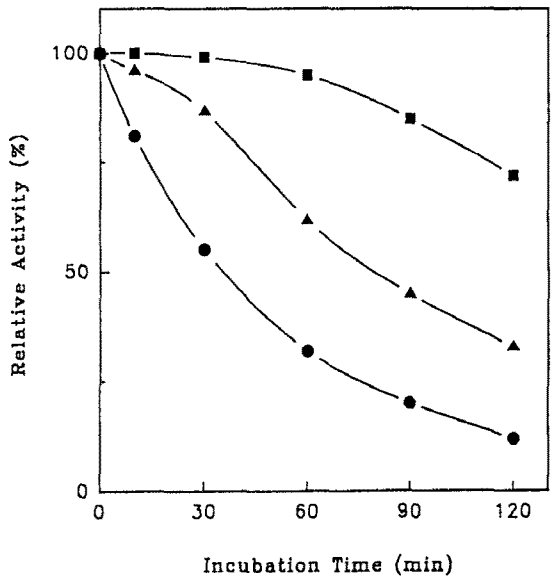

Fig. 5 Effect of temperature on chitinase stability.
The chitinase solution was preincubated in a 1 M sodium acetate buffer (pH 5.3) at various temperatures from 50°C (■), 60°C (▲) and 70°C (●) and the remaining activity was assayed under the standard conditions.

Table 1. Effect of metal compounds on chitinase activity

Compounds	Relative activity (%)	
	10 mM	1.0 mM
$CoCl_2$	108.2	140.2
$BaCl_2$	96.9	98.5
$CaCl_2$	82.4	98.5
$MnSO_4$	80.4	90.9
$MgSO_4$	72.2	87.9
$Pb(CH_3COO)_2$	23.7	74.2
$ZnSO_4$	61.9	68.2
K_2CrO_2	30.9	50.8
$CuSO_4$	25.8	35.4
$HgCl_2$	18.6	24.2
None	100.0	100.0

Metal compounds were preincubated with chitinase solution in a 1 M sodium acetate buffer (pH 5.3) at 30°C for 1 hr and the remaining activities were assayed under the standard conditions.

tures below 50°C, lost 55% of the original activity at 60°C after incubation for 1 hr 30 min, and was almost inactivated at temperatures above 70°C for 2 hr (Fig. 5).

Effect of Metal Compounds

The variation of the activity of a partially purified *P. stutzeri* YPL-1 chitinase in presence of various metal compounds is summarized in Table 1. The enzyme solution was preincubated with metal ions of 1.0 mM and 10 mM concentrations at 30°C for 1 hr and then the residual activity was measured using the standard assay conditions. The enzyme was significantly inhibited by metal ions such as Hg^{2+}, which were markedly inhibitory to other chitinase enzymes (10, 21). The other metal

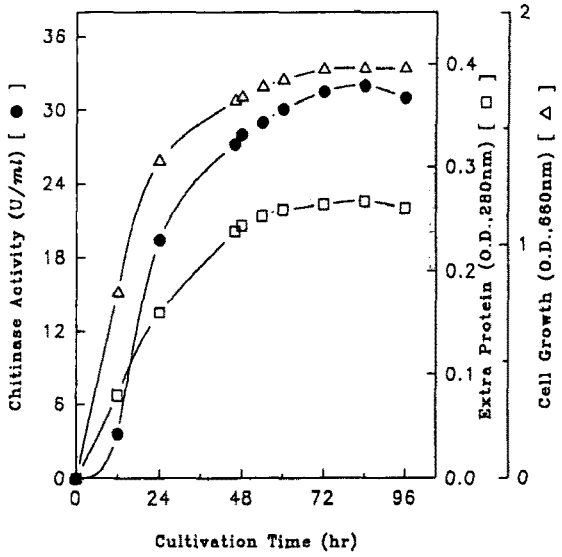

Fig. 6. Growth and chitinase production of *P. Stutzeri* YPL-1.
P. stutzeri YPL-1 was grown at 30°C in a chitin-peptone medium and the enzyme activity was assayed under the standard conditions.

compounds caused partial inhibition, whereas Co^{2+} increased the enzyme activity, by about 1.4 times at the concentration of 1.0 mM.

Growth Profile and Time Course of the Chitinase Production

Time course of cell growth and chitinase production of *P. stutzeri* YPL-1 are shown in Fig. 6. The cells were cultivated at 30°C in a chitin-peptone medium and the chitinase production was monitored while the bacterium was growing in the medium. Chitinase activity began when bacterial growth was in the log phase, and its production increased rapidly after 12 hr of growth. The chitinase production increased in parallel with the growth of the bacterium, and reached its maximum (32 U/ml) after 84 hr of cultivation. Thereafter, the enzyme activity in the culture broth gradually decreased. The decrease in the yield of the enzyme was probably due to the autolysis of cells and inactivation of chitinase during the death phase.

Effect of Carbon Sources on the Chitinase Production

The effect of various carbon sources on the production of chitinase is summarized in Table 2. *P. stutzeri* YPL-1 was grown at 30°C for 84 hr in a basal medium consisting of 0.5%(w/v) of a carbon source. Then the enzyme activity was measured by the standard assay conditions. Among the carbon sources examined, chitin, maltose, and glucose were shown to be effective, while the other carbon sources only showed a rather inhibitory effect. Chitin was the most effective source on the chitinase production. In *Rhizopus* sp., dextrin, galactose, arabinose, and maltose were found to be effective on the chitinase production (24). The results indicate that

Table 2. Effect of various carbon sources on chitinase production

Carbon source (0.5%, w/v)	Cell growth (660 nm)	Extra protein (280 nm)	Chitinase activity (U/ml)
Chitin	2.400	0.268	31.2
Maltose	1.500	0.283	25.0
Glucose	1.990	0.350	24.2
N-acetylglucosamine	2.250	0.368	17.8
Starch	2.220	0.408	16.2
Ribose	1.910	0.475	16.0
Laminarin	4.000	0.369	15.8
Cellobiose	2.520	0.315	13.6
Mannitol	1.940	0.332	13.0
Chitosan	0.365	0.239	10.2
Polygalactronic acid	0.810	0.283	6.2
Pectin	7.475	0.475	5.8
Lactose	1.790	0.332	5.8
Arabinose	1.320	0.316	3.4
Fructose	1.740	0.368	1.8
Pulluran	1.010	0.189	0.0
Cellulose	5.200	0.177	0.0
Galactose	0.940	0.268	0.0
Xylose	2.820	0.388	0.0
Sucrose	2.140	0.368	0.0

P. stutzeri YPL-1 was grown at 30°C for 84 hr in a basal medium consisting of 0.5% of a carbon source and the enzyme activity was assayed under the standard conditions.

chitinase produced by *P. stutzeri* YPL-1 is inducible with chitin.

Effect of Nitrogen Sources on the Chitinase Production

The effect of various nitrogen compounds was examined in a basal medium by adding 0.5%(w/v) of a nitrogen source and measuring the resulting enzyme activity. Peptone was the most effective among the nitrogen sources tested for the chitinase production and also quite effective in promoting cell growth (Table 3). In the case of *Rhizopus* sp., peptone was also found to be the most effective on the chitinase production (24). The addition of $NaNO_3$ and KNO_3 was not effective in promoting the cell growth or chitinase production. The inorganic nitrogen sources were generally ineffective for the bacterium to produce the enzyme.

Effect of Chitin Concentration on the Chitinase Production

The effect of chitin concentration on the chitinase production is summarized in Fig. 7. *P. stutzeri* YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium with different amounts of chitin and the enzyme activity was measured. When chitin concentration increased to 3% from the initial 0.2%(w/v) in the original chitin-peptone medium, the chitinase production drastically increased from 8 to 63 (U/ml). *Serratia marcescens* showed a high enzyme activity when chitin at a concentration of 1.5% was added (14).

Table 3. Effect of various nitrogen sources chitinase production

Nitrogen source (0.5%, w/v)	Cell growth (660 nm)	Extra protein (280 nm)	Chitinase activity (U/ml)
Peptone	2.280	0.288	32.8
Tryptone	1.810	0.208	24.0
Asparagine	1.865	0.215	22.8
$(NH_2)HPO_4$	1.850	0.210	21.4
NH_4NO_3	1.610	0.168	13.4
$(NH_4)_2SO_4$	2.010	0.128	11.4
NH_4Cl	1.940	0.125	7.2
$NaNO_3$	0.460	0.042	1.4
KNO_3	0.305	0.031	0.8

P. stutzeri YPL-1 was grown at 30°C for 84 hr in a basal medium consisting of 0.5% of a nitrogen source and the enzyme activity was assayed under the standard conditions.

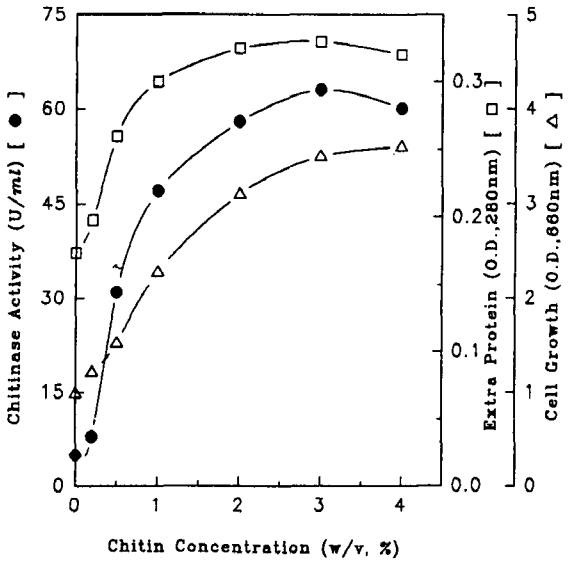

Fig. 7. Effect of chitin concentration on chitinase production.

P. stutzeri YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium with different amounts of chitin and the enzyme activity was assayed under the standard conditions.

Effect of Peptone Concentration on the Chitinase Production

The effect of peptone concentration on the chitinase production is summarized in Fig. 8. *P. stutzeri* YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium and the enzyme activity was measured at various concentration levels of the peptone. The peptone concentration was identified to be the most influential nutritional factor for the chitinase production. The maximum amount of chitinase was produced at a concentration of 4%(w/v). However, *Rhizopus* sp. showed the maximum enzyme activity at a concentration of 7% (24).

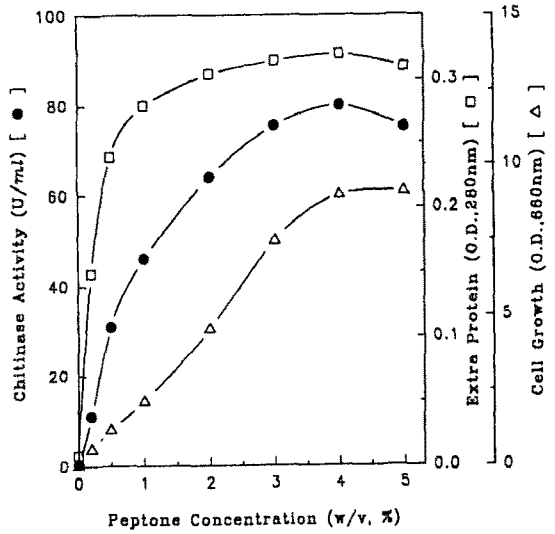

Fig. 8. Effect of peptone concentration on chitinase production.
P. stutzeri YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium with different amounts of peptone and the enzyme activity was assayed under the standard conditions.

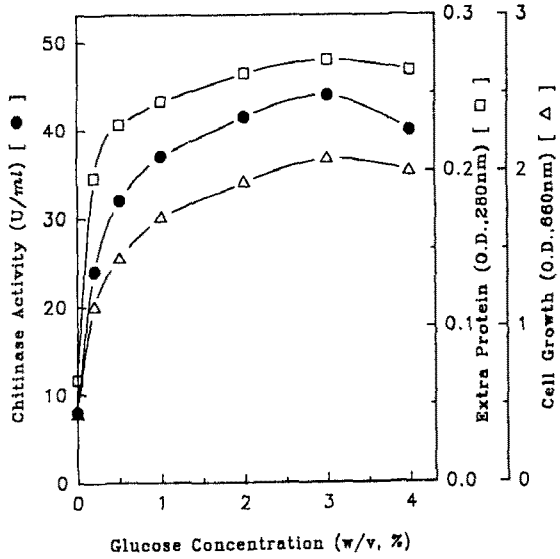

Fig. 10. Effect of initial pH on chitinase production.
P. stutzeri YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium with various initial pHs and the enzyme activity was assayed under the standard conditions.

Effect of Glucose Concentration on the Chitinase Production

The effect of glucose concentration on the chitinase production is summarized in Fig. 9. *P. stutzeri* YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium with different amounts of glucose and the enzyme activity was measured. The result glucose showed to promote the chitinase production best at the concentration level of 3%.

Effect of Initial pH on the Chitinase Production

The initial pH of the chitin-peptone medium was adjusted to various pH values from 5.0 to 8.0 to determine the optimum initial pH for the chitinase production.

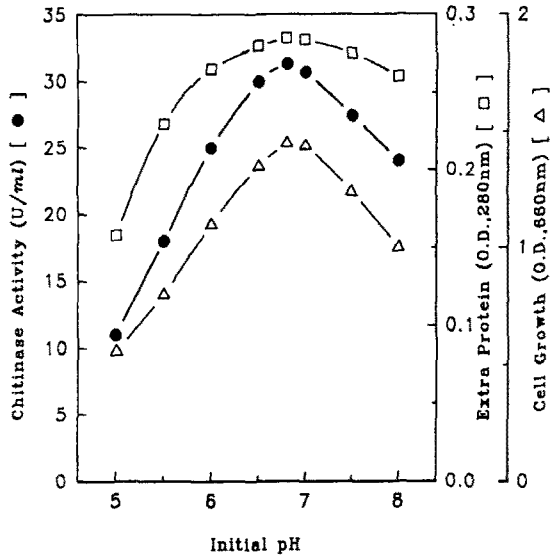

Fig. 9. Effect of glucose concentration on chitinase production.
P. stutzeri YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium with different amounts of glucose and the enzyme activity was assayed under the standard conditions.

Table 4. Suppression of chitinase production by addition of metabolites

Additive, %	Cell growth (660 nm)	Extra protein (280 nm)	Chitinase activity (U/ml)
Dextrin, 0.5%	3.250	0.290	56.2
Glucose, 0.5%	2.540	0.290	42.4
Glucose, 1.5%	3.250	0.329	46.8
N-AGA* 0.5%	1.850	0.256	32.8
N-AGA, 1.5%	2.290	0.283	33.4
Maltose, 0.5%	2.240	0.255	31.4
Arabinose, 0.5%	1.710	0.255	24.4
Fructose, 0.5%	2.800	0.329	25.4
Cellobiose, 0.5%	2.600	0.243	12.4
Xylose, 0.5%	2.770	0.308	10.8
Sucrose, 0.5%	3.000	0.318	8.8
Lactose, 0.5%	2.060	0.249	6.2
Ribose, 0.5%	2.875	0.310	6.2
Galactose, 0.5%	2.230	0.250	1.6
None	1.645	0.278	31.4

P. stutzeri YPL-1 was grown at 30°C for 84 hr in a chitin-peptone medium consisting of 1% chitin with various metabolites and the enzyme activity was assayed under the standard conditions.
*: *N*-acetylglucosamine.

P. stutzeri YPL-1 was grown at 30°C for 84 hr and the enzyme activity was measured. As shown in Fig. 10, the maximum yield of the enzyme was achieved when the initial pH of the medium was adjusted to pH 6.8. In the case of *Rhizopus* sp., the maximum enzyme production was achieved when initial pH used was adjusted to 8.5 (24).

Suppression of Chitinase Production by Addition of Metabolites

The production of chitinase by *P. stutzeri* YPL-1 grown

in a chitin-peptone medium consisting of 1% chitin at 30°C for 84 hr was repressed by the addition of various metabolites indicated in Table 4. Galactose, ribose, lactose, sucrose, xylose, cellobiose, fructose, and arabinose did not promote the enzyme production. The addition of 0.5% maltose did not influence on the chitinase production. However, 0.5% dextrin was the most effective in promoting both the cell growth and the enzyme production. The production of chitinase by *Serratia marcescens* grown on 1% chitin was repressed by the addition of N-acethylglucosamine, glucose, and ribose (14).

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