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Isolation of Chitinolytic Bacteria from the Viscera of Korean Bony Fishes and Optimization of the Enzyme Production

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In order to produce functional chitin oligosaccharides, a chitinolytic bacterium was newly screened from the viscera of Korean bony fishes, and identified as *Bacillus* sp. LJ-25. For the production of chitinolytic enzymes, 1.0% nutrient broth and 0.3% colloidal chitin were used as nitrogen and carbon source, respectively. The optimal temperature, initial pH and concentration of NaCl for the enzyme production by *Bacillus* sp. LJ-25 were 30℃, 6.5~7.0 and 1.0%, respectively. The enzyme activity of *Bacillus* sp. LJ-25 increased until the incubation time of 168 hr, followed by a decrease in activity.

Key words: chitinolytic bacterium, viscera of Korean bony fishes, *Bacillus* sp. LJ-25, optimization of the enzyme production

Introduction

Chitin, poly-β-(1→4)-N-acetylglucosamine, is a cellulose-like biopolymer distributed throughout nature, especially in marine invertebrates, insects, fungi, and yeasts (Austin et al., 1981). Chitin has been well known as a functional material, but had drawbacks such as a low solubility and high viscosity in respect to the rheological behaviour. Thus, for a wide application in industrial fields, chitin was hydrolyzed by the chemical or the enzymatic method. The chemical method has a lot of problems leading to marine environmental pollution resulting from high concentrations of acid/alkali, the accumulation of salts by neutralization and chemical reactions during hydrolysis like deamino reaction and pigmentation (Yamaguchi et al., 1997). On the other hand, the enzymatic hydrolysis has merits in this aspect of being nonpoisonous and capable for a selective mass production of functional chitin oligosaccharides (Lee et al., 1998).

The enzymatic hydrolysis of chitin is carried out by various enzymes such as chitinase (EC 3.2.1.14), β-N-acetylhexosaminidase (EC 3.2.1.52), lysozyme (EC 3.2.1.17) and chitobiase (EC 3.2.130) (Hamaguchi and Funatsu, 1959; Loshi et al., 1989). These chitinolytic enzymes are known to be widely distributed in the natural world. The chitinolytic enzymes in some insects, bacteria, and plants have been well investigated for their important roles in molting and digestion of chitinous foods, and defense systems against parasites, respectively (Kono et al., 1990). But there has been little research on chitinolytic enzymes from aquatic species (Danulat and Kausch, 1984; Lindsay, 1984; Grisley and Boyle, 1990; Matsumiya and Mochizuki, 1997), moreover, there are no reports about them in Korea.

In this study, a new bacterial strain producing chitinolytic enzymes was isolated from the viscera of 12 species of Korean bony fishes and the optimum culture conditions for the production of chitinolytic enzymes were investigated.

Materials and Methods

Materials

Korean bony fish used in the experiments - Sting fish (*Sebastes inermis*), Armorclad rockfish

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(*Sebastea hubbsi*), Hairtail (*Trichiurus lepturs*), Mackerel (*Scomber japonicus*), Eel (*Anguilla japonica*), Black seabream (*Acanthopagrus schlegeli*), Amberjacks (*Seriola quinqueradiata*), Squid (*Todarodes pacificus*), Common mullet (*Mugil cephalus*), Bastard halibut (*Paralichthys olivaceus*), Seabass (*Lateolabrax japonicus*), Yellowfin sole (*Limanda aspera*) - were purchased from fish markets in Pusan and Kijang. The viscera of all bony fishes were immediately removed and used as materials for the isolation of chitinolytic bacteria.

Media

The solid medium for the isolation of chitinolytic bacteria was composed of 0.3% colloidal chitin, 0.1% NH_4Cl, 0.85% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $ZnSO_4 \cdot 7H_2O$, 0.0001% $CuSO_4 \cdot 5H_2O$, 0.0001% $MnSO_4 \cdot 7H_2O$, 0.0001% $CaCl_2 \cdot 2H_2O$ and 1.5% Bacto-agar, pH 7.0. For the production of the chitinolytic enzymes, a liquid medium used was composed of 0.3% colloidal chitin, 0.5% nutrient broth and 1.0% NaCl, pH 7.0. Colloidal chitin used in the chitinase assay was prepared by the method of Jenuiaux (1966).

Isolation of the chitinolytic bacteria

Samples were diluted in sterilized 0.9% NaCl solution and spreaded on the solid medium, then incubated at 30 ± 2℃ for 5 days. Strains that formed large clear zones around the colonies were picked up and then incubated in the liquid medium under the same culture conditions. One of the chitinolytic bacteria was isolated by measurements of reducing sugar released during the hydrolysis of colloidal chitin in the liquid medium. The amount of reducing sugar was measured according to the method of Ressing et al. (1955).

Taxonomical studies

The morphological, physiological and nutritional characteristics of the isolated bacterium were analyzed according to Bergey's Manual Systematic Bacteriology (Sneath et al., 1984) and the method of Gibbs and Skinner (1966). The morphological characteristics were examined using a scanning electron microscope.

Preparation of the enzyme solution

After the chitinolytic bacterium was cultured at 30 ± 2℃ for 5 days in a liquid medium, the supernatent was collected by centrifugation at 12,000 ×g for 10 min. The enzymes in the supernatent were collected by precipitation with ammonium sulfate followed by centrifugation at 12,000×g for 10 min. The precipitate was dissolved in 30mM Tris-HCl buffer (pH 7.0) and dialyzed at 4℃ for 20hr.

Enzyme activity assay

The enzyme activity was assayed with colloidal chitin as a substrate according to the following method. The reaction mixture containing 3.0 ml of 0.3% colloidal chitin and 1.0 ml of enzyme solution was incubated at 37℃ for 60 min, and then the enzyme activity was measured. The enzyme activity was determined by measuring the amount of reducing sugars.

Results and Discussion

Isolation and identification of the chitinolytic bacterium

The chitinolytic bacteria of marine species were mainly isolated from bony fishes such as red sea bream (Kono et al., 1987), eel (Kono et al., 1990), mackerel (Matsumiya and Mochizuki, 1995), and squid (Matsumiya and Mochizuki, 1997), so that 12 species of Korean bony fishes were selected as isolation source for chitinase producing bacteria. Sixty three strains forming significantly large clear zones around the colonies were isolated. Among them, 12 strains produced reducing sugars (Table 1). Especially, the strain No. 25 producing the highest reducing sugar amount was used for further characterization. The formation of the clear zone and the scanning electron micrograph of strain No. 25 are shown in Fig. 1 and Fig. 2, respectively. The morphological and physiological chracteristics are summarized in Table 2. This strain was a gram-positive, mobile by flagella, and rod-shaped bacterium. These characteristics as well as the

Table 1. Ability of reducing sugar formation of the isolated strain

Strain No.	Reducing sugar (μg/ml)	Strain No.	Reducing sugar (μg/ml)
7	4.7	15	18.4
8	13.9	16	16.9
11	6.6	17	54.7
12	17.8	18	47.1
13	12.2	25	60.8
14	6.8	39	48.9

The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium. Liquid medium composition and growth condition : 0.3% colloidal chitin, 0.5% nutrient broth, 1.0% NaCl, initial pH 7.0, temp. 30 ± 2℃, time 120 hr.

ability to utilize ribose, maltose, glucose, lactate and proline were consistent with the description of the geneus *Bacillus* in Bergey's Manual of Systematic Bacteriology. Accordingly strain No. 25 was named *Bacillus* sp. LJ-25. Among the chitinolytic bacteria, there has already been reported about *Bacillus* sp. in Korea and abroad (Watanabe et al., 1990; Takayanagi et al., 1991; Woo et al., 1996; Hong et al., 1996).

Effects of nitrogen sources on the chitinase production

The effects of nitrogen compounds on the production of chitinase from *Bacillus* sp. LJ-25 was examined in a liquid medium containing 0.3% colloidal chitin and other nitrogen sources. The

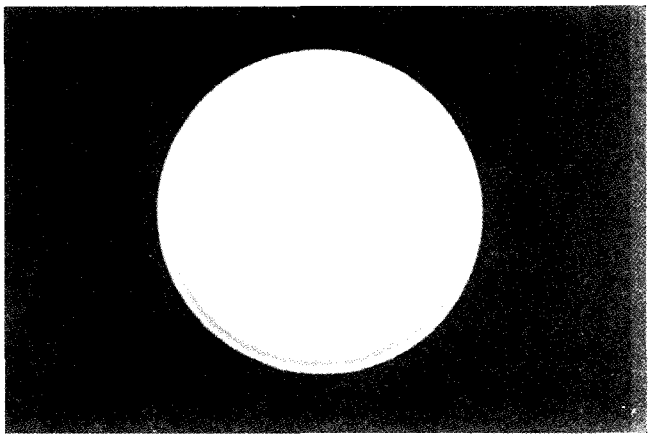

Fig. 1. Fomation of clear zone by chitin degrading bacterium.

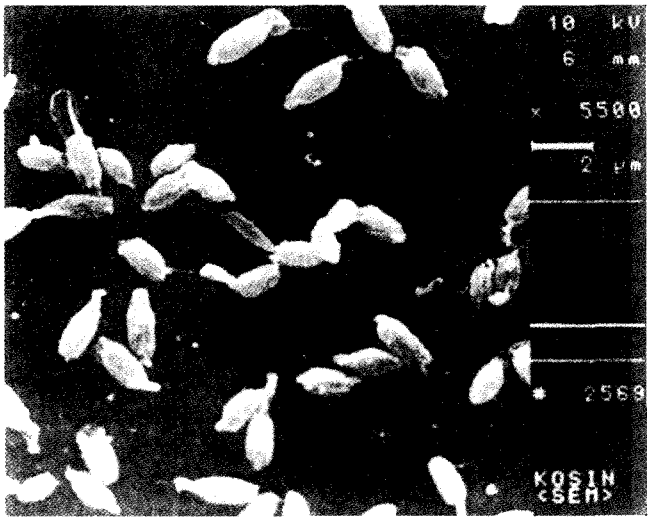

Fig. 2. Scanning electron micrography of strain No. 25 cultured in the liquid medium at 30 ± 2℃ for 5 days.

Table 2. Physiological and biochemical characteristics of the isolated strain No. 25

Gram staining	+	Salicine	−
Cell shape	Rod	Melibiose	−
Motile	+	Fucose	−
Catalase	+	Sorbitol	−
Citrate	+	Arabinose	−
Indole production	−	Propionate	−
Rhamnose	−	Caprate	−
N-Acetyl-D-glucosamine	+	Valonate	−
Ribose	+	Citrate	+
Inositol	−	Histidine	−
Sucrose	−	5-Ketogluconate	−
Maltose	+	Glycogen	+
Malonate	−	3-Hydroxy-butylrate	−
Acetate	−	2-Ketogluconate	−
Lactate	+	4-Hydroxy-butylrate	−
L-Alanine	−	Serine	−
Mannitol	−	Itaconate	−
Glucose	+	L-Proline	+

chitinase activity was assayed under the conditions described in Materials and Methods. As shown in Table 3, nutrient broth was the most effective among the nitrogen sources tested for the chitinase production. On the other hand, the other nitrogen sources such as yeast extract, peptone, tryptone, and marine broth were ineffective for the chitinase production from *Bacillus* sp. LJ-25. Also, the amount of reducing sugar in complex nitrogen sources containing yeast extract and tryptone or containing yeast extract, peptone and tryptone was very low. It is known that the most effective nitrogen sources on the chitinase production are completely different from the patterns of chitinase production with other microoganisms. In the case of *Bacillus licheniformis* KFB-C14 (Hong et al., 1996) and *Bacillus* sp. WY22 (Woo et al., 1996), Yeast extract was reported to be the most effective nitrogen source for chitinase production, and the most effective nitrogen source for *Pseudomonas stutzeri* YPL-1 (Lim and Kim, 1994) and *Aeromonas salmonicida* YA7-625 (Lee et al., 1990) were peptone and tryptone, respectively.

The effect of nutrient broth concentration on the chitinase production from *Bacillus* sp. LJ-25 is shown in Fig. 3. With increasing nutrient broth concentration, the amount of reducing sugar

increased continuously. However the amount of reducing sugar decreased above 1% nutrient broth.

Effects of colloidal chitin- and NaCl concentration on the chitinase production

Table 3. Effects of various nitrogen sources on the chitinase production from *Bacillus* sp. LJ-25

Nitrogen source (0.5%)	Reducing sugar** (μg/mℓ)
Yeast extract	12.1
Peptone	18.8
Tryptone	9.4
Marine broth	—
Nutrient broth	57.8
Casamino acid*	—
Yeast extract+Peptone	—
Yeast extract+Tryptone	6.6
Yeast extract+Peptone+Tryptone	0.1

*Casamino acid conc. : 1.0%.

**The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium. Liquid medium composition and growth condition : 0.3% colloidal chitin, 1.0% NaCl, initial pH 7.0, temp. 30 ± 2℃, time 120 hr.

Chitinase activity was not detected in a liquid medium containing other carbon sources such as glucose, galactose and maltose etc. except colloidal chitin (data not shown). So, the chitinase produced by *Bacillus* sp. LJ-25 was considered as a kind of induced enzyme which can be produced by adding colloidal chitin. Thus the effect of colloidal chitin concentration on the chitinase production was tested, and the results are summarized in Fig. 4. The highest level of chitinase activity was detected in a liquid medium containing 0.3% colloidal chitin as a carbon source, however the activity decreased at concentrations above and below 0.3%, respectively.

On the other hand, as it has been reported that NaCl plays an important role in the characteristics of the isolated marine microorganisms (Baxter, 19 59), the activity of the enzyme solution was also measured at different NaCl concentrations. As a result (Fig. 5), the chitinase activity was drastically increased up to a NaCl concentration of 1.0%. Above this concentration the activity gradually decreased. Thus it was concluded that the

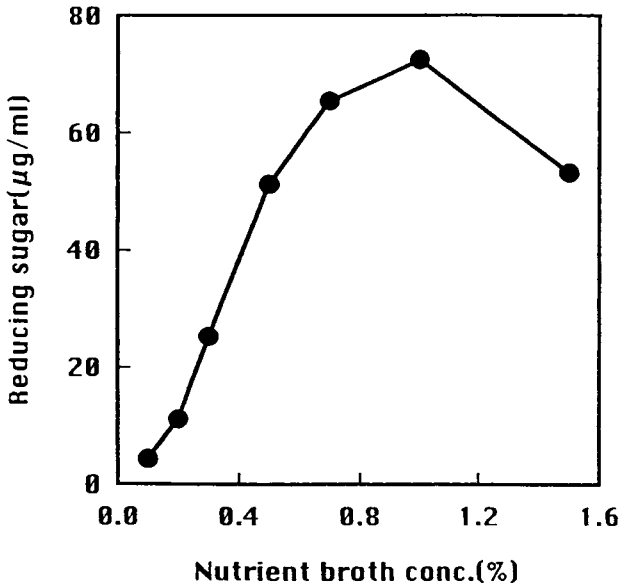

Fig. 3. Effect of nutrient broth concentration on the chitinase production from *Bacillus* sp. LJ-25
The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium containing each concentration of nutrient broth. Liquid medium composition and growth condition : 0.3% colloidal chitin, 1.0% NaCl, initial pH 7.0, temp. 30 ± 2℃, time 120 hr

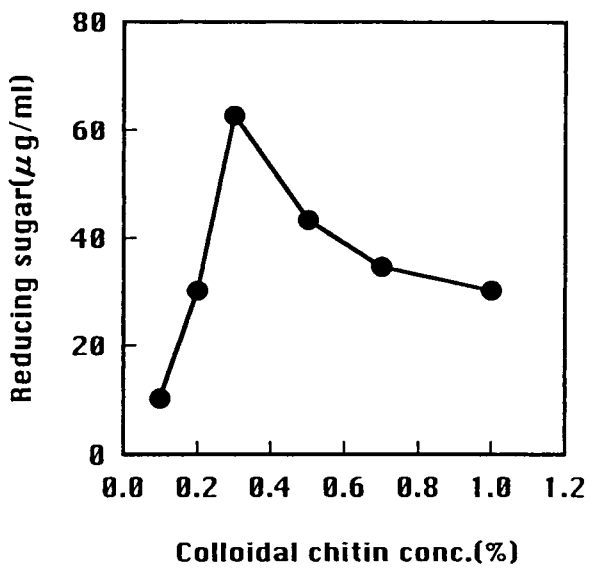

Fig. 4. Effect of colloidal chitin concentration on the chitinase production from *Bacillus* sp. LJ-25
The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium containing each concentration of nutrient broth. Liquid medium composition and growth condition : 1.0% nutrient broth, 1.0% NaCl, initial pH 7.0, temp. 30 ± 2℃, time 120 hr

chitinolytic enzyme produced by *Bacillus* sp. LJ-25 requires NaCl to reveal the maximum activity.

Effects of initial pH, temperature and incubation time on the chitinase production

The effects of initial pH, temperature and incubation time on the chitinase production with *Bacillus* sp. LJ-25 were examined by the help of the chitinase activity assay. The results are shown in Fig. 6, Fig. 7 and Fig. 8, respectively.

The highest activity of the enzyme solution was observed in 2 locations at pH 6.5 and 7.0, however the activity was suddenly decreased at a pH above 7.5. It is reported that the optimum pH for production of chitinase from isolated bacteria in Japanese sea bass and yellow fin tail were 5.5~7 and 7~7.5, respectively (Kono et al., 1987).

As the results of temperature variation, the highest level of activity was obtained at 30℃, but gradually decreasing above 30℃.

The effect of incubation time was as follows: The activity of chitinase produced by *Bacillus* sp. LJ-25 increased until the incubation time of 168hr, however, after 168hr the activity decreased.

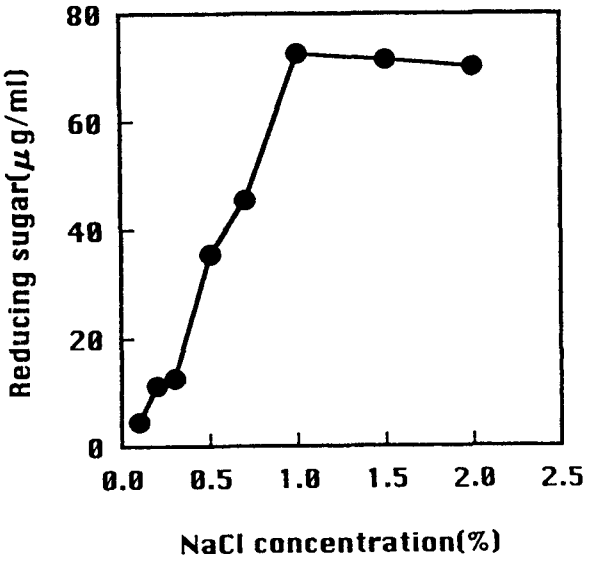

Fig. 5. Effect of NaCl concentration on the chitinase production from *Bacillus* sp. LJ-25
The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium containing each concentration of nutrient broth. Liquid medium composition and growth condition : 1.0% nutrient broth, initial pH 7.0, temp. 30 ± 2℃, time 120 hr

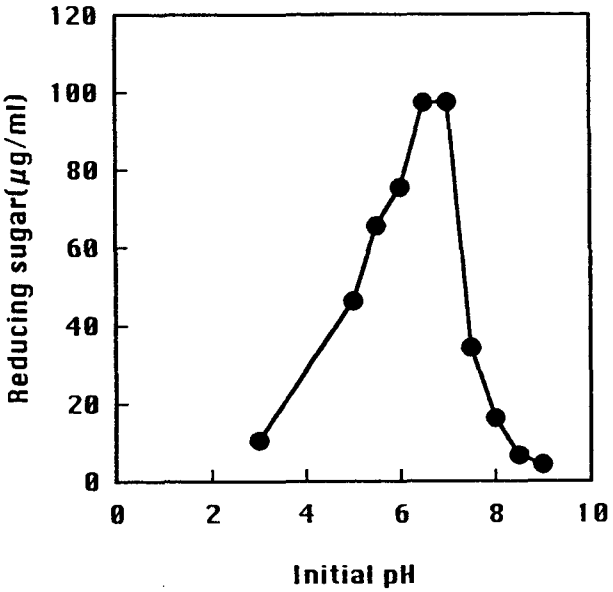

Fig. 6. Effect of initial pH of media on the chitinase production from *Bacillus* sp. LJ-25
The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium containing each concentration of nutrient broth. Liquid medium composition and growth condition : 1.0% nutrient broth, 1.0% NaCl, temp. 30 ± 2℃, time 120 hr

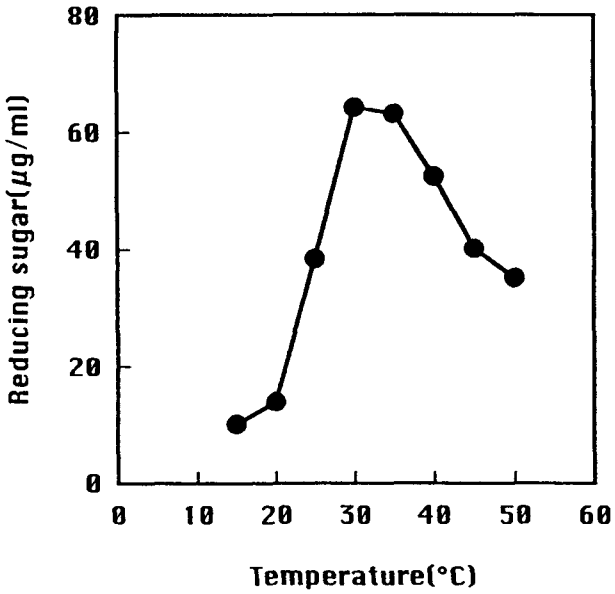

Fig. 7. Effect of temperature on the chitinase production from *Bacillus* sp. LJ-25
The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium containing each concentration of nutrient broth. Liquid medium composition and growth condition : 1.0% nutrient broth, 1.0% NaCl, initial pH 6.5, time 120 hr

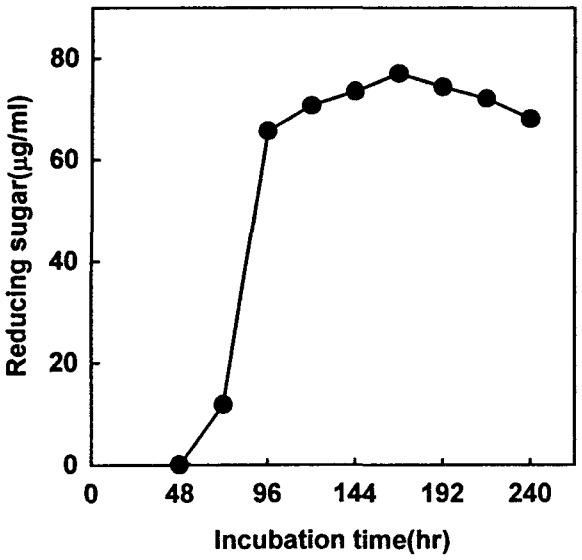

Fig. 8. Effect of incubation time on the chitinase production from *Bacillus* sp. LJ-25
The amount of reducing sugar was determined, after the bacteria were cultured in the liquid medium containing each concentration of nutrient broth. Liquid medium composition and growth condition : 1.0% nutrient broth, 1.0% NaCl, initial pH 6.5, temp 30 ± 20%.

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