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Isolation and Characterization of a Chitinolytic Enzyme Producing Marine Bacterium, *Aeromonas* sp. J-5003

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A chitinolytic enzyme-producing bacterium was isolated from sea water on the coast of Busan. The bacterium was identified as *Aeromonas* sp. based on its morphological, cultural and biochemical characteristics and designated *Aeromonas* sp. J-5003. The strain produced two chitinolytic enzymes: chitinase and chitobiase. The optimum culture conditions of the strain for production of chitinolytic enzymes were investigated. For the production of chitinase, the major components of medium were colloidal chitin 0.5%, glucose 0.2%, yeast extract 0.25% and peptone 0.25%, while for the production of chitobiase, they were colloidal chitin 0.5%, galactose and tryptone 0.2%. The optimum cultural temperature and initial pH for the production of chitinase and chitobiase were 30℃ and pH 7.0, respectively.

Key words: Chitinase, Chitobiase, *Aeromonas* sp. J-5003

Introduction

Chitin is an insoluble polysaccharide consisting of β-(1,4)-linked N-acetyl-glucosamine (GlcNAc) units. This material is abundant in the marine crustaceans such as shrimps or crabs. Researches have been performed in various aspects including the drug delivery, wound healing, dietary supplementation and anticancer therapy (Jeuniaux, 1961; Kim, 1997).

Chitin oligomer or monomer has been conventionally acquired by chemical treatment which cause several problems such as environmental pollution and harmfulness, thus at present hydrolysis by microbial enzymes are common method. Various enzymes are related in the degradation process of chitin. Of these, two major enzymes play an important role in that process. First, chitinase degrades chitin to chitobiose, and chitobiase degrades chitobiose to N-acetlyglucosamine (Somerville and Colwell, 1993). Chitobiose is known to be a major inducer of chitin degradative enzymes, but there are few papers on the microbial production of chitobiase.

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The main purpose of the present study is to describe isolation and characterization of a novel chitin-degrading bacterium, *Aeromonas* sp. J-5003, which secretes potent chitinase and chitobiase degrading colloidal chitin. We also report the optimum conditions for production of the enzymes.

Materials and Methods

Isolation and cultivation of chitinolytic microorganism

Sea water and sediments for detection of chtinolytic enzymes-producing bacteria were collected from Gwangalli, Haeundae, Gammandong, Youngdo and Chagalchi on the coast of Busan. First, the samples were inoculated into the selective medium containing colloidal chitin and incubated for 1 week at 37℃. Clear zone-producing colonies on the selective medium plate were selected as chitinase -producing bacteria. And chitobiase-producing bacteria were also isolated through the filter paper spot method using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (O'Brien and Colwell, 1987). Finally, we determined the chitinolytic activity of these colonies on the colloidal chitin-containing

medium and then selected one strain showing the most strong activity. Compositions of the selective medium were colloidal chitin 1.0%, yeast extract 0.25%, peptone 0.25%, NaCl 1.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.03%, K_2HPO_4 0.07%, $FeSO_4 \cdot 7H_2O$ 0.1%, $ZnSO_4$ 0.01% and $MnCl_2$ 0.01%, and pH 7.0. To preserve the isolated strain, 1.5% NaCl-containing nutrient agar slant was used.

Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Hsu and Lockwood (1975). Commercial chitin (Sigma Co.) 100 g was hydrolyzed with 2 ℓ of conc. HCl for 12 hrs at 4℃ and the HCl-dissolved chitin was removed by filtering. The cold distilled water was added to filtrate and stirred until white suspension was formed. This suspension was centrifuged at 4,000×g for 20 min and distilled water was added to the pellet. Then, the pellet was neutralized with 5 N NaOH, centrifuged at 4,000×g for 20 min, washed with distilled water several times, and dried at 40℃ for 48 hrs.

Enzyme asay

One milliliter of chitinase solution was mixed with 1.0 mℓ substrate solution (1.0% colloidal chitin solubilized in 20 mM sodium phosphate buffer, pH 7.0) and allowed to react at 37℃ for 45 min. The reaction was terminated by boiling at 100℃ for 5−10 min and then the reaction mixture was centrifuged. The supernatant was used to determine chitinase activity by dinitrosalicylic acid (DNS) assay (Miller, 1959). One unit of chitinase was defined as the amount of enzyme required to produce the reducing equivalent of 1 μmol of N-acetylglucosamine per min under the assay conditions.

Chitobiase activity was determined with the following modification of Borooah et al. (1961). The solution contained 0.1 mℓ of the enzyme solution, 0.2 mℓ of 5 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate and 0.7 mℓ of 0.05 M sodium phosphate buffer. The assays were performed for 20 min at 37℃, and 2.0 mℓ of 0.25 M Na_2CO_3 was added to stop the reaction, and then A_{405} was determined. One unit of enzyme activity catalyzes the formation of 1 μmole *p*-nitrophenol per min.

Effect of pH and temperature on the produc-tion of chitinolytic enzymes

Two percents (v/v) preculture was inoculated into the colloidal chitin medium of pH 3.0−10.0, and incubated at 30℃ for 88−96 hrs with shaking. And then, the activity of chitinase and chitobiase was determined. Optimum temperature was also determined by incubating *Aeromonas* sp. J-5003 at 10−60℃.

Effect of colloidal chitin concentration on the production of chitinolytic enzymes

With change of the concentration of colloidal chitin in medium from 0% to 3%, the production of enzymes was investigated. For this reaction, incubation pH and temperature were 7.0 and 30℃, respectively.

Effect of carbon sources on the prodcution of chitinolytic enzymes

Carbon sources used for the enzyme production were xylose, sucrose, galactose, fructose, mannose, raffinose, rhamnose, sorbitol, arabinose, maltose and lactose. Concentration of carbon sources was adjusted to 0.2%.

Effect of nitrogen sources on the production of chitinolytic enzymes

Casein, peptone, urea, $(NH_4)_2HPO_4$, NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, $NH_4H_2PO_4$, tryptone and yeast extract were added into colloidal chitin medium. Concentration of nitrogen sources was adjusted to 0.5%.

Results and Discussion

Isolation and identification of chitinolytic micro-organism

Among the 140 isolates from Gwangalli, Haeundae, Gammandong, Youngdo and Chagalchi (Fig. 1), 65 isolates were found to produce chitinase by clear zone assay (Fig. 2) and 4-MUFGlcNAc method (Fig. 3). Of these isolates, one strain showing the strongest activity was selected and characterized. The selected strain was a Gram negative rod with motility. According to the results of physiological and biological test by API 21NE

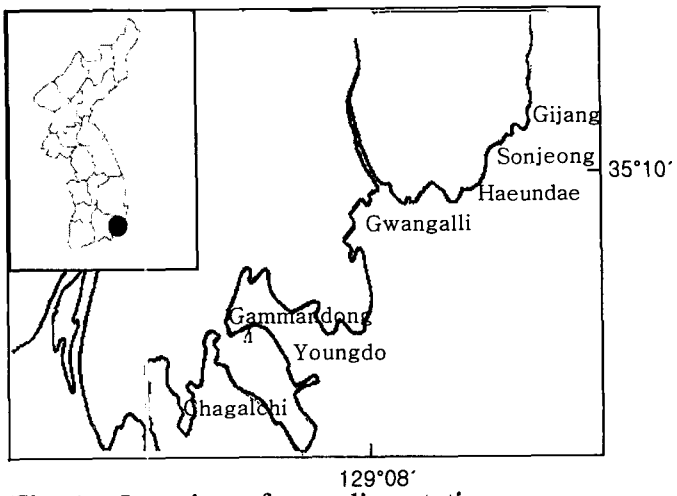

Fig. 1. Location of sampling stations.

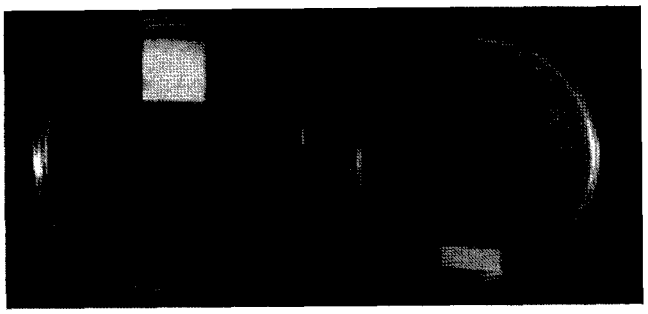

Fig. 2. Detection of chitinolytic enzyme-producing bacteria by colloidal chitin agar plate.

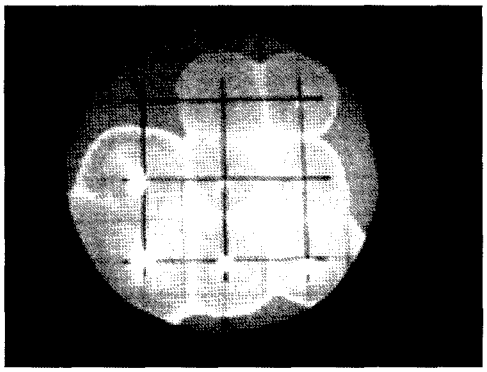

Fig. 3. Detection of chitinolytic enzyme producing-bacteria by 4-MUFGLcNAc assay.

kit, the strain was identified as a bacterium belonging to the genus *Aeromonas*. We designated this strain as *Aeromonas* sp. J-5003 (Table 1).

4-MUFGlcNAc assay was originally used for the detection of chitobiase which produce the N-acetyl-β-D-glucosamine (NAG) from chitin. However, O'Brien and Colwell (1987) have reported that chitinase-producing bacteria always possessed the chitobiase activity, so we also used this method to screen the chitobiase activity of chitinase-producing bacteria. The advan-tages of this method are rapid reaction rate and sensitivity for low activity.

Table 1. Biological and physiological characteristics of *Aeromonas* sp. J-5003

Tests	Results
Gram stain	−
Motility	+
Oxidase	+
Catalase	+
O/F	Fermentative
Growth at 0% NaCl	+
Growth at 5% NaCl	+
Growth at 10% NaCl	−
Growth at 4℃	−
Growth at 40℃	+
Gas from glucose	+
Urea	−
Arginine Dihydrolase	−
Esculin	+
Ornithine decarboxylase	−
Arabinose	+
Adonitol	−
Rhamnose	−
Mannitol	+
Sorbitol	−
Cellobiose	−
Raffinose	−
Melibiose	−
Indole	+
Malonate	−
Phenylalanine deaminase	−
Sucrose	+
5-ketogluconate	−
Palatinose	−
Galacuronate	−
Salicin	+
Colistin	−
Coumarate	+
Tetra thionate reductase	−
p-nitrophenyl-β-D-galactopyranoside	+
o-nitrophenyl-β-D-galactopyranoside	−
Glucuronidase	−
Mannose	+
Maltose	+
α-galactose	+
Indoxyl phosphate	−
Trehalose	+

Effect of initial pH and temperature on the production of chitinase and chitobiase

Effect of initial pH and temperature of the medium on the chitinase and chitobiase production were shown in Tables 2 and 3, respectively. *Aeromonas* sp. J-5003 could not grow at pH 3.0−

Table 2. Effect of initial pH on the chitinase and chitobiase production

pH	Chitinase		Chitobiase	
	Viable cell count (cfu/mℓ)	Relative activity (%)	Viable cell count (cfu/mℓ)	Relative activity (%)
3.0	NG	ND	NG	ND
4.0	NG	ND	NG	ND
5.0	NG	ND	NG	ND
6.0	2.2×10^{8}	34.7	4.0×10^{8}	86.5
7.0	2.4×10^{9}	100.0	5.6×10^{9}	100.0
8.0	2.3×10^{9}	87.5	3.0×10^{9}	93.3
9.0	1.6×10^{8}	61.4	1.6×10^{9}	86.2
10.0	8.1×10^{8}	40.7	8.1×10^{9}	81.1

NG, Not grow; ND, Not detected.

Table 3. Effect of temperature on the chitinase and chitobiase production

Temp. (℃)	Chitinase		Chitobiase	
	Viable cell count (cfu/mℓ)	Relative activity (%)	Viable cell count (cfu/mℓ)	Relative activity (%)
10	1.9×10^{4}	13.3	1.8×10^{4}	0.03
20	2.1×10^{8}	92.3	1.6×10^{8}	65.7
25	1.6×10^{8}	97.3	2.0×10^{8}	80.7
30	3.6×10^{9}	100.0	5.6×10^{9}	100.0
37	9.0×10^{8}	75.1	8.5×10^{8}	20.9
40	4.5×10^{4}	ND	4.1×10^{4}	4.8
50	NG	ND	NG	ND

NG, No grow; ND, Not detected.

5.0, and maximum production was obtained at pH 7.0. About 87.0% productivity remained at pH 8.0. Most of chitinase produced by general bacteria showed the best productivity in neutral range (Huang et al., 1996; Kang et al., 1998; Monreal and Reese, 1969). But, *Beauveria bassiena* and *Bacillus* sp. BG-11 had the best activity at pH 9.6 and pH 8.5 (Bhushan and Hoondai, 1998; Suresh and Chandrasekaran, 1999), and *Verticilluim lecanii* isolated in Antarctic Continent showed the maximum activity at pH 5.5 (Fenice et al., 1998). Optimum temperature for chitinase production of *Aeromonas* sp. J-5003 was 20−30℃ and chitinase activity was still remained with the culture broth at 37℃. But no activity was detected above 40℃. There has been reports that optimum temperature of chitinase from a thermostable *Bacillus* sp. BG-11 was 50℃ (Bhushan and Hoondai, 1998) and, *V. lecanii* had the best activity at 5−25℃ (Fenice et al., 1998).

Optimum pH and temperature for chitobiase production of *Aeromonas* sp. J-5003 were 7.0 and 25−30℃, respectively. In contrast to the chitinase produced by *Aeromonas* sp. J-5003, the chitobiase was produced in a wide pH range from 6.0−10.0. Chitobiase productivity was very low above 37℃.

Effect of colloidal chitin concentration on the production of chitinase and chitobiase

Productivity of chitinase by *Aeromonas* sp. J-5003 was highest at colloidal chitin concentration 0.5%. Above 0.5% concentration, the productivity was decreased (Table 4). In many of chitinase-producting bacteria, concentration of the added colloidal chitin was from 0.5−1.5% (Kang et al., 1998; Huang et al., 1996; Tsujibo et al., 1991; Yabuki et al., 1986). But, it has been also known that *A. hydrophila* H-2330 (Kazumi et al., 1997) and *B. thuringiensis* (Barboza-Corona et al., 1999) produced the greatest productivity at 5.0% and 13.0%, respectively.

Table 4. Effect of colloidal chitin concentration on the chitinase and chitobiase production

Colloidal chitin (%)	Chitinase		Chitobiase	
	Viable cell count (cfu/mℓ)	Relative activity (%)	Viable cell count (cfu/mℓ)	Relative activity (%)
0[a]	1.2×10^{9}	4.0	1.3×10^{9}	20.3
0.5	2.1×10^{9}	100.0	1.4×10^{9}	88.1
1.0	1.4×10^{9}	94.2	2.1×10^{9}	93.7
1.5	1.3×10^{9}	73.1	5.6×10^{9}	100.0
2.0	1.0×10^{9}	26.2	3.0×10^{8}	0.6
2.5	1.5×10^{9}	18.3	1.3×10^{8}	0.4
3.0	1.5×10^{9}	12.9	1.3×10^{8}	0.3

[a] The nutrient broth without colloidal chitin.

No chitnolytic activity was showed at colloidal chitin concentration 0%. This result suggests that this enzyme from *Aeromonas* sp. J-5003 is a colloidal chitin substrate-inducible enzyme.

The maximum chitobiase productivity was shown at 1.5% colloidal chitin concentration.

Effect of carbon sources and nitrogen sources on the production of chitinase and chitobiase

With the exception of glucose, enzyme production was inhibited by addition of carbon sources such as xylose, sucrose, galactose, fructose, mannose, raffinose, rhamnose, sorbitol, arabinose, maltose and

lactose (Table 5). Production of chitinase was significantly stimulated by organic nitrogen, particulary combination of peptone and yeast extract (Table 6).

Table 5. Effect of carbon sources on the chitinase and chitobiase prodcution

Carbon sources	Chitinase		Chitobiase	
	Viable cell count (cfu/mℓ)	Relative activity (%)	Viable cell count (cfu/mℓ)	Relative activity (%)
Colloidal Chitin 0.5%[a]	2.8×10^9	92.3	2.8×10^9	75.9
Glucose	4.5×10^9	100.0	4.5×10^9	90.8
Galactose	4.9×10^9	63.0	4.9×10^9	100.0
Maltose	9.1×10^9	53.4	9.5×10^8	77.4
Mannose	6.0×10^9	78.0	1.5×10^9	60.1
Fructose	7.0×10^9	52.2	3.0×10^9	97.4
Sucrose	4.5×10^9	81.0	1.6×10^9	86.7
Raffinose	4.7×10^8	53.4	1.0×10^8	76.4
Rhamnose	1.4×10^9	49.8	NE	NE
Lactose	9.7×10^8	46.2	5.0×10^8	86.7
Sorbitol	5.1×10^8	49.8	NE	NE
Arabinose	7.3×10^8	47.3	NE	NE
xylose	5.7×10^8	46.0	NE	NE

The concentration of carbon sources was 0.2%, and 0.5% colloidal chitin was added.
[a]No carbon source was added.
NE, Not examined.

Table 6. Effect of nitrogen sources on the chitinase and chitobiase production

Nitrogen sources	Chitinase		Chitobiase	
	Viable cell count (cfu/mℓ)	Relative activity (%)	Viable cell count (cfu/mℓ)	Relative activity (%)
None	1.5×10^8	18.4	3.0×10^8	13.9
Yeast extract	1.4×10^9	89.6	1.0×10^9	79.5
Peptone	2.8×10^9	87.5	9.0×10^8	97.3
Yeast extract +Peptone[a]	3.6×10^9	100.0	8.0×10^8	90.6
Casein	1.4×10^6	50.4	1.0×10^6	36.3
Tryptone	2.3×10^9	68.6	1.0×10^9	100.0
$NH_4H_2PO_4$	2.1×10^8	30.1	2.1×10^8	92.6
$(NH_4)_2HPO_4$	3.3×10^8	36.4	3.5×10^8	90.7
KNO_3	2.6×10^2	ND	NE	NE
$NaNO_3$	7.3×10^3	ND	1.0×10^4	16.8
NH_4Cl	2.7×10^5	19.6	2.5×10^4	15.6
$(NH_4)_2SO_4$	1.1×10^4	19.1	NE	NE
Urea	2.7×10^5	21.2	2.1×10^5	19.5

The concentration of nitrogen sources was 0.5%.
[a]Yeast extract (0.25%) and peptone (0.25%) were added.
ND, Not detected.
NE, Not examined.

Carbon source and nitrogen source for the maximum production of chitobiase were galactose and tryptone, respectively.

In *A. salmonicida* YA7-625 (Lee et al., 1990; Lee et al., 1992), addition of carbon sources inhibited enzyme production, and it was presumed catabolite repression. The enzyme activity of *Serratia marcescens* was also inhibited by addition of glucose, glucosamine and ribose, but stimulated by addition of sucrose and mannose (Monreal and Reese, 1969). These results suggest that the chitinolytic enzymes productivity from *Aeromonas* sp. J-5003 is likely to have the catabolite repression by several of carbon sources.

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