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Optimal Conditions for Chitinase Production by *Serratia marcescens*

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Abstract A chitinase-producing bacterium was isolated from seashore mud around Beobseongpo in Chunmam province through the use of a selective enrichment culture. The best chitinase producing strain was isolated and identified as *Serratia marcescens* KY from its characteristics. For effective production of chitinase, optimum pH, temperature, and agitation speed were investigated in flask cultures. The optimum pH using *Serratia marcescens* KY was between pH 6 and 7 and the chitinase produced was 37.9 unit/mL. On the other hand, the optimal pH of the *Serratia marcescens* ATCC 27117 was 7.5, and the produced amount of chitinase was 35.2 unit/mL. The optimal temperature for chitinase production for *Serratia marcescens* KY and *Serratia marcescens* ATCC 27117 was 30°C. The cell growth pattern at different temperature was almost identical to the chitinase production. To investigate the optimal shaking speed under optimal culture, speeds were varied in the range of 0~300 rpm. The maximum production of chitinase was carried at 200 rpm although the cell growth was the highest at 150 rpm. It indicates that oxygen adjustment is required for the maximum chitinase production. Using optimal conditions, batch cultures for comparing *Serratia marcescens* KY and *Serratia marcescens* ATCC 27117 were carried out in a 5 L fermentor. The oxygen consumption was increased with the increase of culture. Especially, at 120 h of culture *Serratia marcescens* KY and *Serratia marcescens* ATCC 27117 produced 38.3 unit/mL, and 33.5 unit/mL, respectively.

Keywords: chitinase, *Serratia marcescens* KY, pH, O_2, CO_2

INTRODUCTION

The main source of chitin originates from waste like shells of crabs, shrimps, or lobsters produced by food processing industries. Microorganisms that decompose chitin are used for the production of single cell protein (SCP), *N*-acetyl-D-glucosamine (NAG), glucosamine, and glucose. There have been many studies on enzymatic treatment of seafood waste that was related to chitinase production through decomposition by microorganisms [1,2]. It has been known that chitinase and chitobiase act together when NAG, a component of chitin, is decomposed by several kinds of microorganisms or bacteria [3]. Chitinolytic enzymes, most of which are inducible enzymes, are easily induced by the addition of substrates. Extensive researches have been carried out on the direct use of chitin as an inducible substrate to produce enzymes [4,5]. Microorganisms that produce chitinase are found in soil, and most are usually of the *Streptomyces* sp. The research on *Streptomyces* sp. focused specifically on *Streptomyces griseus* [6]. Using *Serratia marcescens*, chitinase was also refined from the strain to compare its characteristics with the chitinase produced from microorganisms, and the results demonstrated that has a significantly higher productivity [7,8].

A number of studies on chitin decomposition microorganisms and chitinase application exist in the literature. Carroad *et al.* [9] presented a four-step bioconversion model that use chitin decomposition to produce monosaccharides by treating chitin waste [8]. Research on decomposition of the nucleus has been performed using *Streptomyces species* chitinase [10,11]. Using protease as an origin of microorganisms, cell walls of *Rizopus* was decomposed to 95% [12]. In order to use chitin industrially, novel chitinase-producing microorganisms should be selected, and enhanced producing processes should be carried out for high productivity. Characteristics of bacterial strains, such as bacteri, *Penicillium islandicum* [13], a filamentous fungus, and yeast that are known as producing chitinase were studied to isolate *Serratia marcescens* with a high productivity from the tidal flat of the sea [14]. Then, their cultural characteristics were also examined for chitinase production.

This study describes the isolation and characteristics of the chitinase-producing bacterium and the optimal culture conditions required to increase the productivity of

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chitinase from *Serratia marcescens* using the variation of two key parameters (pH and temperature). The productivity of chitinase from *Serratia marcescens* KY, was compared with that from *Serratia marcescens* ATCC 27117. In addition, the effect of oxygen transfer to the reactive culture medium on chitinase production and the optimal chitinase culture medium in a flask were examined. The agitation speed for chitinase production was examined under optimal culture condition. To identify the optimal culture conditions for chitinase production in a fermenter, the concentrations of oxygen and carbon dioxide that were discharged were also examined.

MATERIALS AND METHODS

Isolation of Chitinase-producing Bacterium

The strain was isolated from seashore mud around Beobseongpo in Chunnam province, Korea. After the samples were incubated at 30°C for 7 days on a shaking incubator (150 rpm), they were spread onto a chitinase detection medium containing 1.2% chitin. Among the 7 colonies, a single colony showing a chitinase-producing bacterium was selected and identified by examining its morphological, physiological, and biochemical characterization [15,16]. The ability to utilize organic materials was studied by adding small amounts of the organic materials into the basal medium containing chitin.

Strains, Culture Medium and Culture Conditions

Two chitinase-producing strains were used in this study [17]: *Serratia marcescens* KY and *Serratia marcescens* ATCC 27117. The bacteria were grown in 1.5% (w/v) colloidal chitin, 0.5 g/L of yeast extract, 1.0 g/L of tryptone, and 1.0 g/L of NaCl. The initial pH was adjusted to 7.0. To increase the chitinase concentration, the effects of temperature and pH were investigated in terms of chitinase production. To examine the cell growth in terms of the initial pH, the pH was adjusted between 3.0~10.0 using sterilized 0.5 N NaOH and 0.5 N HCl. For different culture conditions, the cell growth and chitinase production were examined. In order to examine the effect on cell growth and chitinase production, the culture temperature was changed from 20 to 37°C.

Chitinase Activity and Cell Concentration

Chitinase activity was measured by the colorimetric method of Jung *et al.* [18] with some modification to allow for multi-sample analysis. The absorbance was measured at 545 nm using ELISA plate reader. One unit of activity was defined as the amount of enzyme able to liberate 1 mg of NAG per hour. The cell concentration was measured by optical density (OD) at 600 nm. The dry weight cell concentration (g/L) was found to be 0.4 x $OD_{600\,nm}$ [19].

Optimization of Aeration

To identify the effect of oxygen transfer during the chitinase production with *Serratia marcescens*, chitinase production was investigated with changes in the agitation speed. Also, to discern the most appropriate culture medium for chitinase production, 50~300 mL culture media containing 1.5% colloidal chitin were prepared in an Erlenmeyer flask with a capacity of 500 mL. The samples were cultured at pH 7, 30°C, and 150 rpm, and the agitation speed was adjusted from 0 to 300 rpm.

Variation of O_2 and CO_2 Concentration Discharged from the Fermentation-tubculture

In order to investigate the optimal conditions of chitinase production with a fermenter, or tub culture, the concentration of O_2 and CO_2 discharged during the production was examined according to time passage [20]. To obtain the basic data for mass production of chitinase at the optimal temperature and pH, the strains were inoculated using the optimal medium, and the amounts of the discharge of O_2 and CO_2 were examined. The concentration of O_2 and CO_2 was measured by FOCA-1 (TOA Corporation, Japan).

The data was saved at intervals of 2 sec. First, *Serratia marcescens* grown to exponential growth phase at flask was inoculated into 5-L Jar Fermenter (MDL 300, Marubishi Bioeng. Co., Tokyo, Japan) containing 3 L of working volume containing 1.5% swollen chitin and the medium containing carbon and nitrogen sources. 2 L/min of sterilization-filtered oxygen was injected to it to investigate the chitinase production and the concentration changes of O_2 and CO_2 as time progressed.

RESULTS AND DISCUSSION

Screening of Chitinase-producing Bacterium

A chitinase-producing bacterium was isolated using selective enrichment culture, and in this culture, one isolate that was the best in producing chitinase was selected. The Nutrient or MacConkey medium were confirmed with the secretion of the prodigiosin pigment by the isolate or Serratia Marcescens ATCC 27117, and it was performed by the production of a clear zone around their colonies on medium containing chitin. Among them, the strain forming the largest clear zone was selected for further examination. The strain was subjected to a taxonomic analysis according to the Bergey's Manual of Systematic Bacteriology [16]. The morphological, physiological, and biochemical characteristics of this strain are summarized in Table 1. The isolate was almost the same compared to *Serrtia marcescens* ATCC 27117 in respect to its morphological, physiological, and biochemical characteristics with the exception of starch, citrate, catalase, and glucose. Based on these results, the isolated strain was designated as *Serratia marcescens* KY.

Table 1. The morphological cultural and biochemical characteristics of the isolated strain (+: Positive, -: Negative)

Characteristics	*Serratia marcescens* KY	*Serratia marcescens* ATCC 27117
Morphological characteristics		
shape	short rod	rod
cell size(μm)	0.3~0.5	0.2~1.4
motility	+	+
gram stain	-	-
spore stain	-	-
Cultural characteristics		
colony color on nutrient agar	red	red
colony color on MacConkey agar	red	red
growth at 4°C	-	-
at 42°C	+	-
Biochemical characteristics		
aerobic growth	+	+
anaerobic growth	+	+
hydrolysis of gelatin	+	+
hydrolysis of starch	+	-
catalase test	-	+
oxidase test	-	-
citrate test	-	+
nitrate reduction test	+	+
indole test	-	-
voges-proskauer test	+	+
methyl red test	-	-
H_2S production	-	-
Glycolysis test		
glucose	+	+
maltose	-	+
sucrose	+	+
lactose	-	-

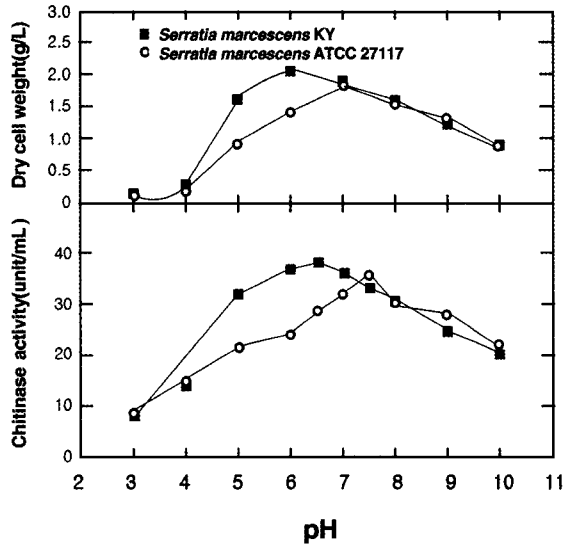

Fig. 1. Effect of pH on cell growth and chitinase production by *Serratia marcescens*.

Effect of pH and Temperature on the Chitinase Production

In order to investigate the optimal pH for chitinase production, the initial pH of the culture medium was adjusted between 3.0~10.0 using sterilized 0.5 N HCl and 0.5 N NaOH. The culture broth that was grown to exponential growth phases was inoculated on a separate culture medium containing 1.5% colloidal chitin and was cultured at 30°C for four days. The cell growth and chitinase production for different pHs are shown in Fig. 1. The maximum amount of chitinase production occurred between pH 6 and 7. This demonstrated that the optimal pH of chitinase production by the *Serratia marcescens* KY was 6.5, and the chitinase produced at this pH was 37.9 unit/mL. On the other hand, the optimal pH of the *Serratia marcescens* ATCC 27117 was 7.5, and the produced amount of chitinase was 35.2 unit/mL. The examination of chitinase production according to the cell growth in a separate culture medium containing colloidal chitin revealed that when there was a greater chitinase production, there was a higher level of cell growth. This indicates that cell growth and chitinase production follow the same trend. The maximum chitinase production under maximum cell growth of the two strains was compared for different pHs. The chitinase productions of the two strains were similar as the following results: the *Serratia marcescens* KY produced 18.1 unit/mg, and the *Serratia marcescens* ATCC 27117 produced 18.51 unit/mg. It indicates that. In general, *Serratia marcescens* had a tendency to be more acidic than pH 7.5, which is the value reported in the Bergey's Manual of Systematic Bacteriology [16] and by Monreal and Reese [20]. However, the values of pH corresponding to maximum cell growth were almost identical. Also, the maximum production pH of *Talaromyces emersonni* reported by McCormack *et al.* [21] tended to be more basic than the pH range of 5.0~5.5. The pH for optimal chitinase production reported by Monreal and Reese [20] or Roberts and Cubib [22] using *Serratia marcescens* was close to pH 7.7.

In order to investigate the effect of temperature on the chitinase production, the culture broth grown to the exponential growth phase was inoculated on a culture medium containing 1.5% colloidal chitin, and culturing was carried out at pH 7 and 20~37°C for four days. Fig. 2 shows the cell growth and chitinase production for various temperatures. According to the results, the optimal temperature was 30°C. The cell growth pattern was almost identical to the chitinase production. This agrees with the results obtained by Monreal and Reese [20] or Roberts and Cubib [22] that maximum production is obtained at 30°C. The comparison of maximum production of chitinaseby the two strains leads to the following results: the *Serratia marcescens* KY produced 15.41 unit/mg and the *Serratia marcescens* ATCC 27117 produced 17.47 unit/mg. These results indicate that chitinase production by the two strains are similar to each other.

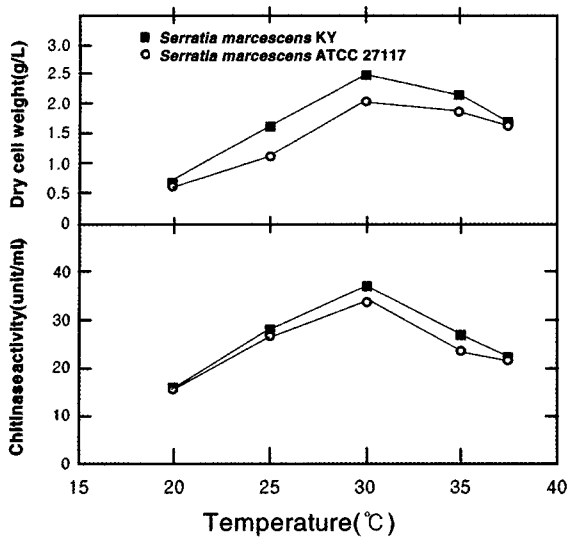

Fig. 2. Effect of temperature on cell growth and chitinase production by *Serratia marcescens*.

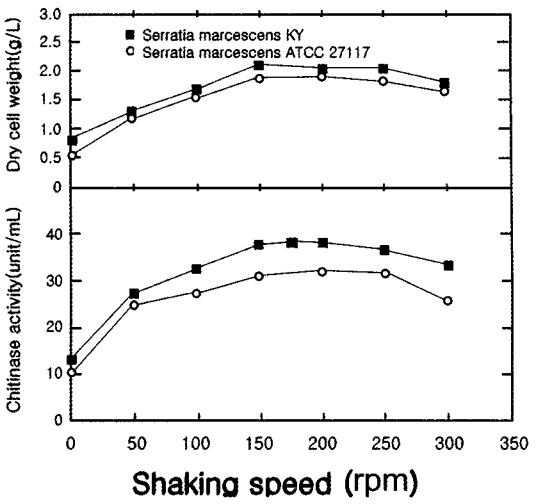

Fig. 3. Effect of shaking speed on cell growth and chitinase production by *Serratia marcescens*.

Table 2. Effect of working volume on the chitinase production by *Serratia marcescens*

Working volume (mL)	Chitinase activity (unit/mL)	
	Serratia marcescens KY	*Serratia marcescens* ATCC 27117
50	29.9	25.6
100	35.2	31.1
150	33.4	32.8
200	33.3	31.5
250	33.0	30.2
300	28.2	26.6

Effect of Aeration on Chitinase Production

For the mass production of chitinase using microorganisms, it is important to know the effect of oxygen transfer, there are several important impeding factors in culturing microorganisms in large quantities. There is a study, which indicates that the shear stress caused by the oxygen transfer represses the growth of microorganisms [23]. Therefore, this study was conducted to investigate the factors that influence optimal chitinase production in an Erlenmeyer flask.

In this study, the amount of separated culture medium containing 1.5% colloidal chitin in a 500 mL Erlenmeyer flask was adjusted from 50 mL to 300 mL of working volume. The pH and temperature were kept at 7 and 30°C, respectively, and the agitation speed was 150 rpm. As shown in Table 2, chitinase that was produced by *Serratia marcescens* KY at 100 mL of optimal culture medium was 35.2 unit/mL. Chitinase produced by the *Serratia marcescens* ATCC 27117 at 150 mL of optimal culture medium was 32.8 unit/mL. This experiment demonstrated that the most chitinase was produced with the optimal culture medium. To investigate optimal shaking speed under optimal culture medium of 100 mL of the *Serratia marcescens* KY and 150 mL of the *Serratia marcescens* ATCC 27117, the speed was varied in the range of 0~300 rpm. As shown in Fig. 3, chitinase production of both strains was higher at the shaking speed of 150~250 rpm. For both strains, the maximum production occurred at 200 rpm of maximum mixing speed although the cell growth was the highest at 150 rpm. This indicates that oxygen adjustment is required for the maximum production of chitinase.

Influence of O_2 and CO_2 on Chitinase Production in a Fermenter

In this study, the discharged concentration of oxygen and carbon dioxide during chitinase production in a fermenter was examined [23]. The change in concentrations of O_2 and CO_2 due to chitinase production was measured under conditions of optimal temperature and pH. For *Serratia marcescens* KY, 2.0 g/L of tryptone was added into 3 L of separated culture medium containing 1.5% swollen chitin in a 5-L fermentation tub. For *Serratia marcescens* ATCC 27117, the culture medium containing 2.5 g/L of tryptone was combined with the culture medium containing 0.2 g/L of $MgSO_4$ and K_2HPO_4. Then, 2.0% (v/v) of sub-culture broth was inoculated, and at pH 7 and 30°C, 2 L/min of sterilized air that passed through the filter (0.2 vvm) was injected. As time progressed, chitinase production and the concentration change of O_2 and CO_2 were examined, and the results were presented in Fig. 4.

At initial culture, the oxygen consumption of two strains was remarkably increased and then decreased from 18 to 48 h of culture.

The amount of produced chitinase of the two strains

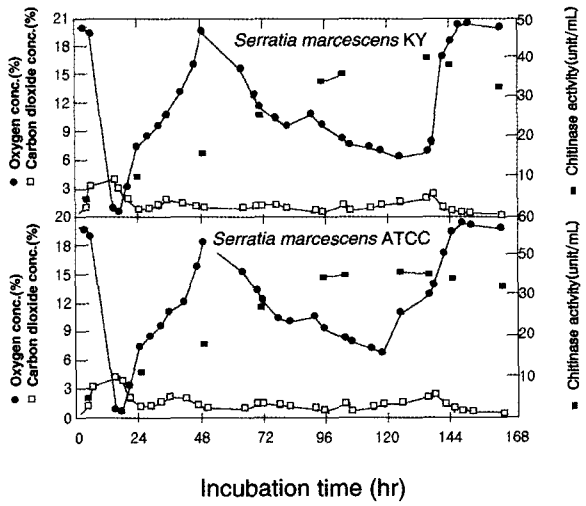

Fig. 4. Effect of O_2 and CO_2 on chitinase production by *Serratia marcescens*.

was only 5 unit/mL. But the oxygen consumption was again increased from 48 to 120 h of culture. During this period, *Serratia marcescens* KY produced 38.3 unit/mL, and *Serratia marcescens* ATCC 27117 produced 33.5 unit/mL. As shown in Fig. 4, the chitinase production increased with fermentation time. At 140 h of culture, maximum production was achieved, and after this period, the production was repressed. During this period, the concentration of O_2 increased while that of CO_2 decreased, and maximum production was. These results illustrated that where cell growth was active, as cell growth increased, oxygen consumption also increased. It led to a maximum discharge of carbon dioxide. During this period, maximum production was achieved.

CONCLUSION

This study describes the identification of chitinase-producing bacterium and the characterization of several of its properties. As a result, the isolated strain was designated as *Serratia marcescens* KY. Using *Serratia marcescens*, a chitinase-producing strain, a study on chitinase production was performed to investigate the effect of temperature, pH, and shaking speed. The optimum temperature and pH were 30°C and 6.5 for the *Serratia marcescens* KY and 30°C and 7.5 for the *Serratia marcescens* ATCC 27117, respectively. This study examined the chitinase production as a function of the culture medium and agitation speed. For *Serratia marcescens* KY, chitinase produced with 100 mL of optimal cultural medium was 35.2 unit/mL. For *Serratia marcescens* ATCC 27117, chitinase produced with 150 mL of optimal culture medium was 32.8 unit/mL. For both strains, maximum production was achieved at 200 rpm of shaking speed. This study also examined the chitinase production and the concentration of O_2 and CO_2 in a fermenter as a function of time. After 140 h, when the consumption of O_2 increased and that of CO_2 decreased, both strains produced a maximum amount of chitinase.

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