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Effect of Culture Conditions on Cathepsin B Inhibitor Production by a Marine Bacterium, *Pseudomonas* sp. Strain PB01

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A novel cathepsin B inhibitor-producing bacterium was isolated from marine sediments and identified based on its 16S rDNA sequence as *Pseudomonas* sp. strain PB01 (Accession No. EU126129). The growth and enzyme inhibitor production were investigated under various culture conditions. A mixture of organic nitrogen source was required for the optimal production, whereas both glucose and maltose proved to be the effective carbon sources for cathepsin B inhibitor production. Other optimal culture conditions included temperature range between 25 and 28°C, initial medium pH of 6.6, and shaking speed of 200 rpm. Under these optimal conditions, the maximum inhibitory activity from culture broth was approximately 50% after 30 h of cultivation. Additionally, kinetic study revealed that inhibitor production paralleled with cell growth, which suggested that the inhibitor may be a primary metabolite of that bacterium.

Keywords: *Pseudomonas* sp., cathepsin B inhibitor, culture conditions

Cathepsin B (E.C. 3.4.22.1) belongs to the lysosomal cysteine proteases of the papain family [14]. Cathepsin B is a unique cysteine member showing dual roles as both an endopeptidase and exopeptidase, owing to the presence of the occluding loop in the structure [15]. Hence, it has been described as a promising candidate for protein processing in the lysosome [10, 16]. Overexpression and mislocation in the cell membrane of cathepsin B, however, have contributed to the invasive and metastatic processes of cancer. Cathepsin B is implicated in the proteolytic process as directly degrading extracellular matrix components or activating other proenzymes such as plasminogen, matrix metallosis proteases [21]. Therefore, cathepsin B inhibitors may serve as therapeutic drugs for cathepsin B disorders that are related to many diseases.

Marine organisms, including microorganisms, represent as treasure sources for therapeutic natural products. As these compounds generated from natural evolution procedures, their biological receptors play key roles in a certain cellular circuit and signal pathway [9]. Protease inhibitors have been reported to be produced by *Actinomyces* [7, 8], some fungi [19], and animals [23]. Moreover, for industrial purpose, microorganisms are preferable to other natural sources, such as sea animals, algae, *etc.*, owing to some geographic advantages, seasonal viability, and especially in large-scale production [11]. Many pure cultures of microorganisms capable of producing cathepsin B inhibitors have been documented from fungi such as *Aspergillus japonicus* [6], *Aspergillus oryzae* O-1018 (FERM P-15834) [24], and *Streptomyces* spp. [13], and some of them have become commercial products [6]. Continuing this current trend, we were screening for isolating a marine bacterium that showed potential in producing a cathepsin B inhibitor. Culture broths from more than 300 marine bacteria were investigated by enzyme inhibitory assay. The most interesting bacterium was chosen and identified by 16S rDNA.

The aim of this study was to investigate the capability of the isolated bacterium as a novel cathepsin B inhibitor-producer and to optimize the culture conditions to prepare for large culture processes.

Materials and Methods

Materials

Glucose, peptone, yeast extract, and beef extract were all obtained from BD (U.S.A.). Cathepsin B and REDTaq DNA Polymerase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Carbobenzoxy-L-arginyl-L-arginine 4-methyl-coumaryl-7-amide

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(Z-Arg-Arg-MCA) was purchased from Bachem AG (Switzerland). The DNA recovery kit was purchased from Takara Bio Inc. (Japan). Other chemicals and reagents used were of analytical grade.

Bacteria and Culture Conditions

All marine bacteria were provided by the Korea Research Institute of Bioscience and Biotechnology, Korea, and kept in liquid medium during transport.

Initially, a basal medium (10 g/l glucose, 1 g/l beef extract, 1 g/l peptone, and 20 g/l agar in the case of solid medium) was used and prepared with 60% of sea water. Before the experiment, strain PB01 was activated by two successive transfers in liquid medium and one to slant culture. The bacterium was stored at -80°C in the same medium with 20% glycerol stock.

The inocula were prepared by transferring a loopful of the activated bacteria to 50 ml of medium in 250-ml Erlenmeyer flasks, shaking at 200 rpm, 28°C for 24 h. Four ml of culture broth was transferred and inoculated under the same conditions and used for the study of the culture condition.

The growth of strain PB01 was determined by measuring the optical density at 620 nm with a GENios microplate reader (Tecan Austria GmbH, Austria).

PCR Amplification of the 16S rDNA Gene of Strain PB01

The 16S rDNA gene was amplified from a single isolated colony using forward primer FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer RC1492 (5'-TACGGCTACCTTGTTACGACTT-3'). The PCR reaction was carried out by a T1 Thermocycle (Biometra, Germany). Amplicons were obtained with a PCR cycling program of 80°C for 5 min, 30 cycles at 94°C for 30 s, a 55°C for 30 s and a 72°C for 60 s; and a final 7-min extension at 72°C. The amplified products were visualized by electrophoresis separation on 2% agarose gel stained with ethidium bromide.

DNA Sequencing and Data Analysis

PCR fragments were purified by using Easy trap-Glass power for DNA recovery. Total 1,400 nucleotide sequences of the 16S rDNA gene of strain PB01 were sequenced with the same primers by SolGent Co., Ltd. (Korea). The nearly complete 16S rDNA gene sequences were used to search on the GenBank database. A phylogenetic tree was constructed by generating a complete alignment of the 16S rDNA gene sequences of selected members in GenBank by using MEGA software bootstrap values (from 1,000 resamplings) [22].

Enzyme Inhibitory Assay

Cathepsin B inhibitory assay was performed in 96-well black plates according to the method of Barrett *et al.* [1] with some modifications. Because the cathepsin B inhibitor was secreted into the medium, the culture broth was centrifuged at 4,000 rpm for 5 min, and the supernatant was used for testing inhibitory activity. Each well contained 12.5 µl of a 0.4 M sodium potassium phosphate buffer (pH 6.0) containing 8 mM dithiothreitol (DTT) and 4 mM EDTA, 12.5 µl of 40 µunits/ml cathepsin B solution, and 12.5 µl of supernatant. After 10 min at 37°C, 12.5 µl of 20 µM Z-Arg-Arg-MCA in water was added to start the reaction. The reaction was stopped after 20 min by 50 µl of 100 mM sodium monochloroacetate in 100 mM sodium acetate (pH 4.3). The fluorescence of MCA release was measured at Ex 360 nm and Em 465 nm by a GENios microplate reader. The percentage of inhibition (I) was calculated by the following equation:

$$I(\%)=\frac{\text{Control}-\text{Test}}{\text{Control}-\text{Blank}}\times 100\%$$

Statistical Analysis

Data were presented as mean±standard error of the mean (SEM; n=3). Student's *t*-test was used to determine the level of significance. A p value <0.05 was considered significant.

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A   AGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGAGAGTAATGCCTAGGAATCTGCC
B   AGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAKGAATCTGCC

A   GGGGAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTAGGGGAG
B   TGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAG

A   AAAGGAGGGGACCTTCGGGCCTTGGGCTATCAGATGAGCCTAGGTCGGATTAGCTAGT
B   AAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTASCTAGT

A   TGGTGGGGTAATGGCTCACCAAGGCGACGGTCCGTAACTGGTCTGAGAGGATGATCAG
B   TGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAK

A   TCACACTGGAACTGAGACACGGTCCAAACTCATACGGGAGGCAGCAGTGGGGAATATT
B   TCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCTGCAGTGGGGAATATT

A   GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGAT
B   GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGAT

A   TG TAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGAC
B   TG TAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGAC

A   GTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGG
B   GTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGG

A   GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT
B   GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT

A   GGATGTGAAATCCCCGGGCTCAACCTGGGAAACTGCATTCAAAACTGACTGACTAGAG
B   GGATGTGAAATCCCCGGGCTCAACCTGGGAAACTGCATTCAAAACTGACTGACTAGAG

A   TATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAAATGCGTAGATATAGGAAG
B   TATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAAATGCGTAGATATAGGAAG

A   GAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCG
B   GAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCG

A   TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAG
B   TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAG

A   CCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
B   CCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG

A   GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
B   GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG

A   AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATG
B   AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATG

A   AACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTG
B   AACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTG

A   TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTC
B   TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTC

A   CTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGA
B   CTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGA

A   GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGC
B   GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGC

A   TACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACC
B   TACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACC

A   GATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAA
B   GATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAA

A   TCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
B   TCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA

A   CACCTTGGGAGTGGGTTGCACCAGAAGTAGCTAGTC
B   CACCATGGGAGTGGGTTGCACCAGAAGTAGCTATGT
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Fig. 1. The results obtained by BLASTN.
A. PB01; B. *Pseudomonas fluorescens*.

The GenBank accession number of the sequence reported in this paper is EU126129.

RESULTS AND DISCUSSION

Identification of the PB01 Strain

The bacteria in sediments are rather abundant and play an important role in the mineralization of organic matter, resulting in many recent reports [20]. In our screening program, several marine bacteria isolated from marine sediments were chosen. Among them, strain PB01 isolated from Deagu stood out as a good candidate according to the enzyme inhibitory results. Strain PB01 was Gram-negative, aerobic. Colonies on solid media were circular, opaque, and low convex. Because of the result of the alignment of 16S rDNA on GenBank, there were 100 16S rDNA gene sequences having high similarity with that of the PB01 strain. The 16S rDNA sequence identity between strain PB01 and *P. fluorescens* (Accession No. AY622219) was approximately 99% (Fig. 1). These values fall within the range of 16S rDNA gene sequence similarities (93.7–99.9%) reported for species of the genus *Pseudomonas* [14]. Furthermore, strain PB01 conformed to the genus *Pseudomonas* with a high bootstrap value based on an analysis of the phylogenetic tree (Fig. 2). Hitherto relatively few species originating from marine environments are classified within *Pseudomonas* species including *Pseudomonas stutzeri* [2], *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes* [17], some strains of *Pseudomonas balearica* [3] from marine sediments, *Pseudomonas alcaliphila* [25] from seawaters, and *Pseudomonas pachastrellae* sp. nov. from sponge [18]. Strain PB01 is the first *Pseudomonas* species used for isolating cathepsin B inhibitor, and also is the only member that belongs to the marine bacteria.

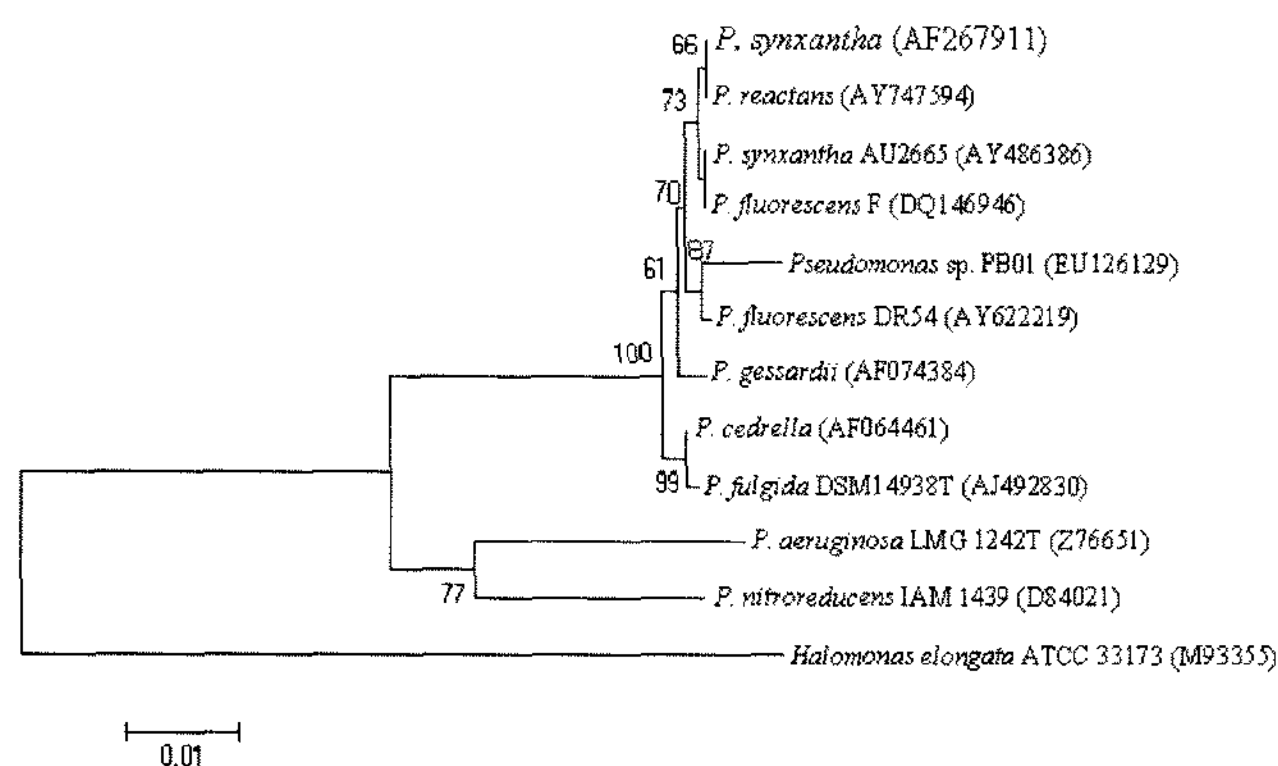

Fig. 2. Phylogenetic relationship of *Pseudomonas* sp. strain PB01 and some related *Pseudomonas* species on the basis of 16S rDNA gene sequence analysis.
The branching pattern was generated by the neighbor-joining method. Numbers indicate bootstrap values greater than 60%. Bar, 0.01 *Knuc* units.

Table 1. Effect of nitrogen sources on the growth and cathepsin B inhibitor production.

Nitrogen source (2 mg/ml)	Inhibitory activity (%)	Biomass (OD 620 nm)
Inorganic		
NH_4NO_3	40.78±1.06	1.10±0.02
$NaNO_3$	25.56±1.01	0.76±0.01
$Ca(NO_3)_2$	25.02±0.92	0.70±0.04
KNO_3	30.47±0.07	0.89±0.05
Organic		
Urea	36.28±1.10	1.02±0.02
Yeast extract	41.42±0.84	1.17±0.05
Peptone	42.32±0.06	1.13±0.02
Yeast and peptone	47.42±0.07	1.36±0.05

All experimental data are means±SEM of triplicate determinations.

Effects of Carbon and Nitrogen Sources on Biomass and Cathepsin B Inhibitor Production

Various carbon and nitrogen sources were tested in order to determine their influences on bacterium growth and cathepsin B inhibitor product. The effects of various nitrogen sources on the growth and cathepsin B inhibitor production are showed in Table 1. In comparison with inorganic nitrogen sources, organic nitrogen sources, gave rise to a higher biomass and inhibitory activity. Using the sole nitrogen source, however, did not give out noticeable differences. On the other hand, the mixture of yeast extract and peptone with added beef extract in the medium showed the highest cathepsin B inhibitory activity (47.42%). Therefore, a mixture of these was selected as the nitrogen source.

Table 2 summarizes the effect of various carbon sources on the growth and cathepsin B inhibitor production. Certain carbon sources were suitable for growth of strain PB01 including glucose, maltose, and mannose. Among the carbon sources tested, the maximum biomass (OD_{620}, 1.35±0.02) was obtained in the glucose medium. However, the difference in cathepsin B inhibitory activity between glucose (47.3%) and maltose (43.78%) was not so significant. From

Table 2. Effect of carbon sources on the growth and cathepsin B inhibitor production.

Carbon source (10 mg/ml)	Inhibitory activity (%)	Biomass (OD 620 nm)
Glucose	47.30±0.50	1.35±0.02
Maltose	43.78±0.90	1.26±0.01
Mannose	19.65±1.01	0.43±0.03
Starch	0	0.73±0.02
Lactose	0	0.61±0.01
Galactose	0	0.53±0.05
Sucrose	0	0.55±0.02

All experimental data are means±SEM of triplicate determinations.

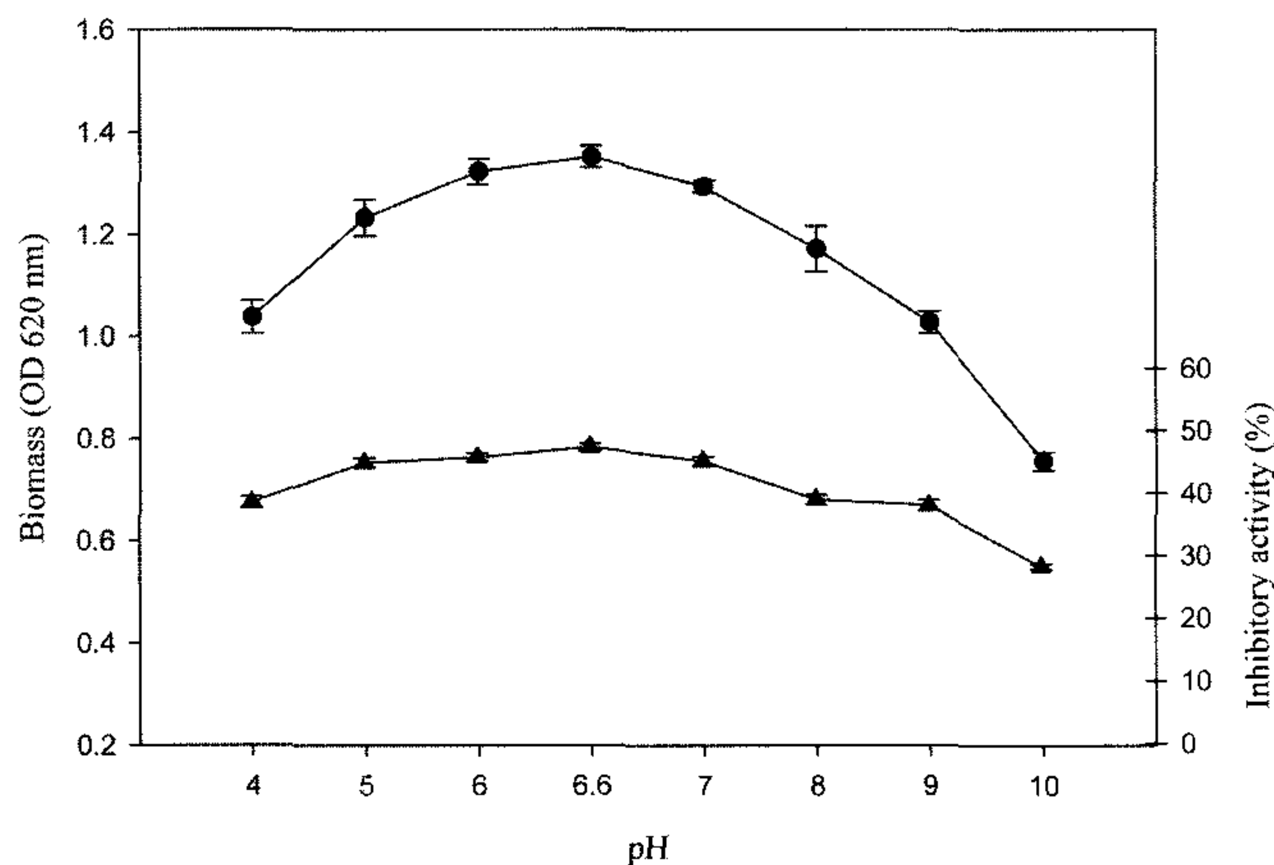

Fig. 3. Correlation between growth and inhibitory activity of strain PB01 at different initial pHs.
All experimental data are means±SEM of triplicate determinations. (●) Biomass, (▲) Inhibitory activity.

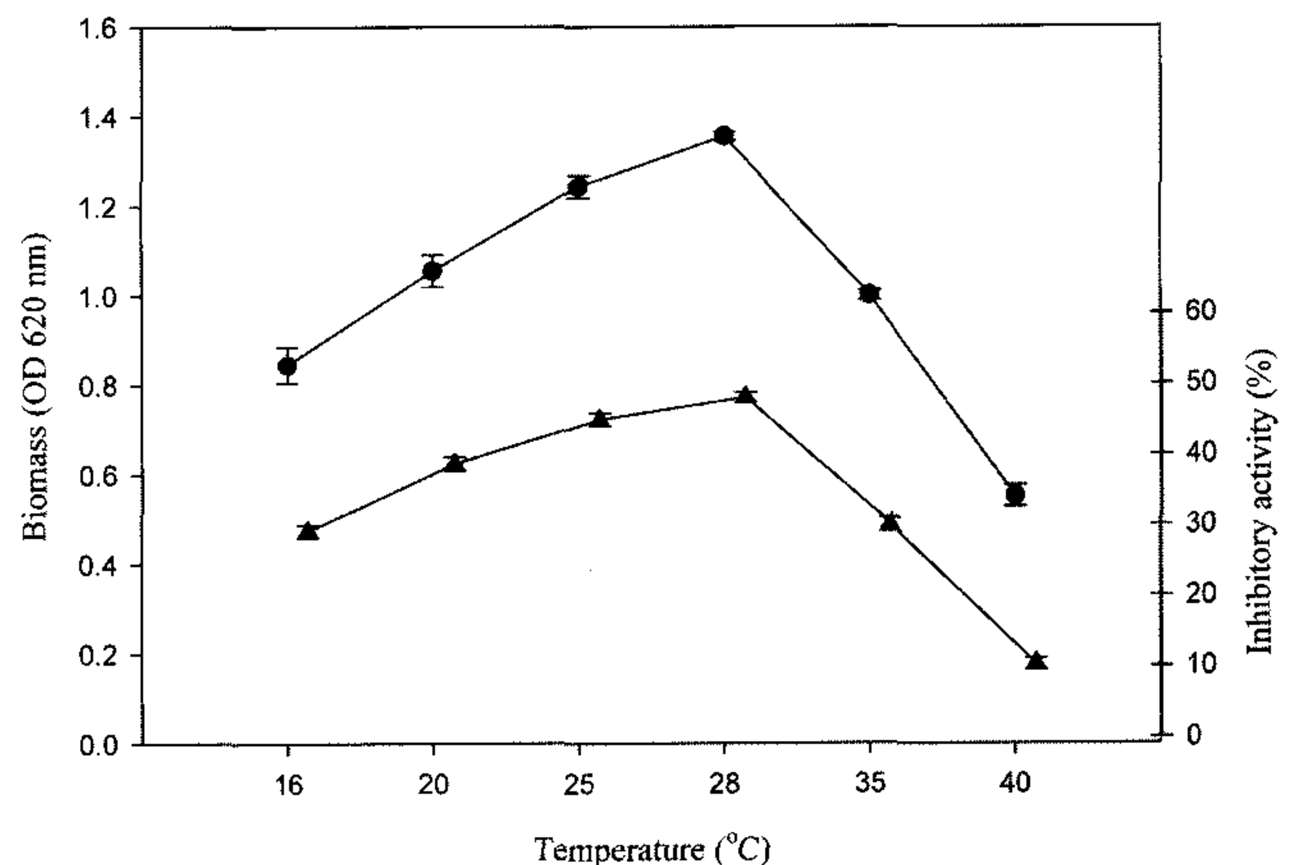

Fig. 4. Correlation between growth and inhibitory activity of strain PB01 at different cultivation temperatures.
All experimental data are means±SEM of triplicate determinations. (●) Biomass, (▲) Inhibitory activity.

the practical point of view, glucose is a good candidate for the carbon source because of its ease-of-use and low cost, compared with other carbon sources. Therefore, glucose was selected as the carbon source in the following experiments.

Effect of Initial Medium pH on Biomass and Cathepsin B Inhibitor Production

The initial pH of the medium was adjusted to various values (4.0–10.0) by sterile 1.0 N NaOH or 1.0 N HCl solution. Cultivation was carried out at 28°C and with shaking speed at 200 rpm for 24 h. As shown in Fig. 3, strain PB01 could grow well in a broad pH range and the best was at pH from 6.0 to 7.0; however, the highest inhibitory activity was attained at pH 6.6. From the initial pH 4.0, the inhibitory activity was 38.52% after 24 h of cultivation and it increased together with the increase of biomass of the bacterium. Further increase of the initial pH over 7.0 resulted in a reduced biomass and activity. In addition to growth, the inhibitor production was also affected by the initial pH of the medium.

Effect of Temperature on Biomass and Cathepsin B Inhibitor Production

Growth temperature is another critical parameter that needs to be controlled. Cultivation was conducted at pH 6.6 and with shaking speed at 200 rpm for 24 h. The productivity of strain PB01 changed together with different cultivation temperatures. As shown in Fig. 4, strain PB01 grew well at moderate temperatures from 25°C to 28°C, which is comparable to many kinds of *Pseudomonas* sp. that can grow at relatively higher temperatures. It may be because strain PB01 was originally isolated from a normal marine source. The difference of biomass between 28°C and 40°C was 2.5 times, and of inhibitory activity was 5 times. At 28°C, the inhibitory activity reached the highest (48.02%) among the various tests.

Effect of Shaking Speed on Biomass and Cathepsin B Inhibitor Production

As an aerobic bacterium, the suitable shaking speed during cultivation of strain BP01 was also tested at 0–200 rpm at initial pH 6.6 and 28°C. Even though strain PB01 is not so strictly aerobic, the increasing oxygen supply during culture favored for the growth and inhibitor production (Fig. 5). At 200 rpm, the inhibitory activity was 47.52%, which was the highest among the various shaking speeds tested and was 7 times higher than that shows in static culture. When the cultivation was carried out at a higher shaking speed, many bubbles were created. Consequently, the shaking speed at 200 rpm was the best in this experimental system.

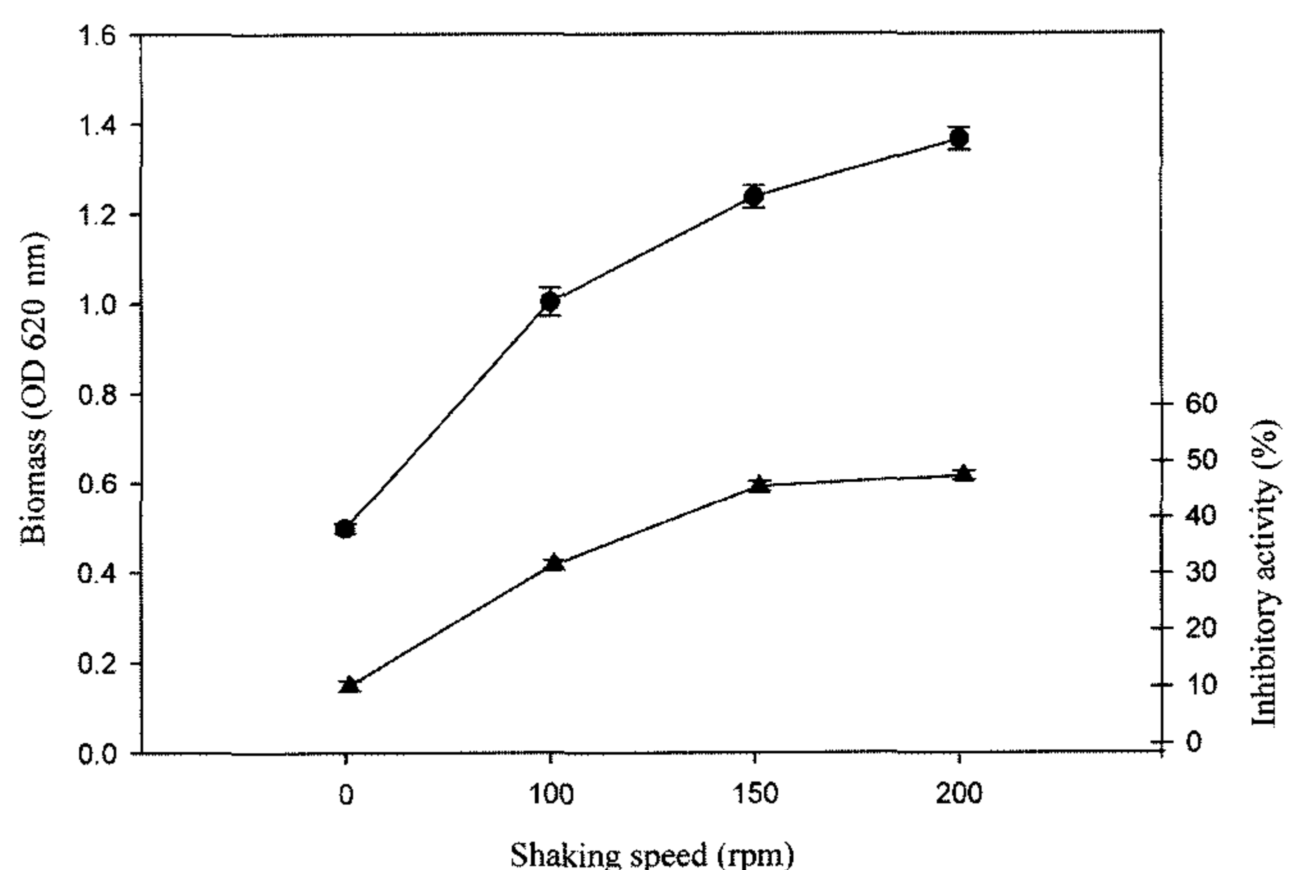

Fig. 5. Correlation between growth and inhibitory activity of strain PB01 at different shaking speeds.
All experimental data are means±SEM of triplicate determinations. (●) Biomass, (▲) Inhibitory activity.

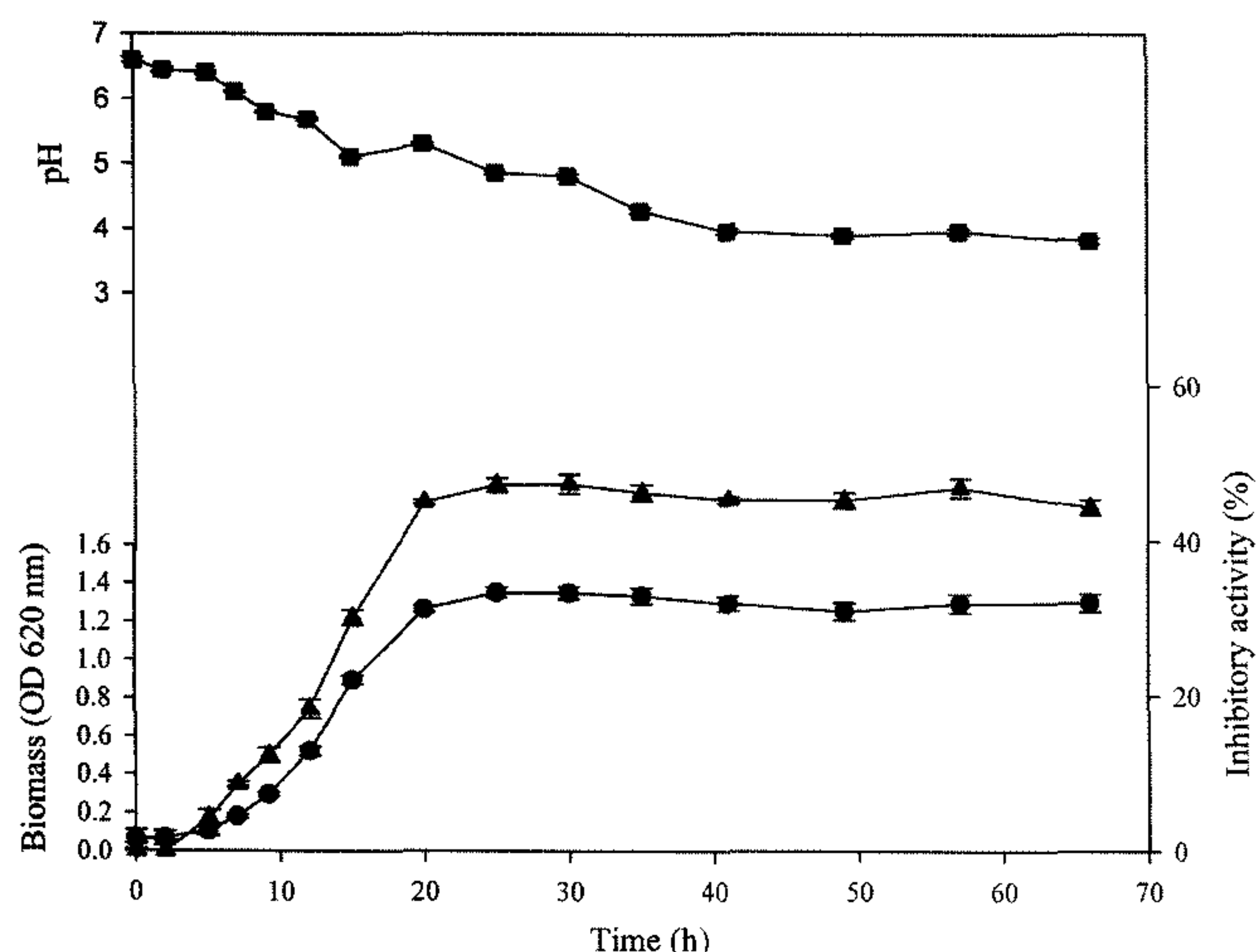

Fig. 6. Time course of growth, cathepsin B inhibitory activity, and pH variation.

All experimental data are means±SEM of triplicate determinations. (●) Biomass, (▲) Inhibitory activity, (■) pH.

Growth of Strain PB01 and Kinetics of Inhibitor Formation During Cultivation Time

The standard growth curve of strain PB01 and the inhibitor formation were performed according to previous tests fir 70 h. A typical time course of growth, cathepsin B inhibitory activity, and pH variation is shown in Fig. 6. These results revealed that the increase or decrease of inhibitory activity accompanied with biomass for each tested condition. As shown in Fig. 5, after a short lag period of 2 h, the biomass of strain PB01 increased rapidly and entered the stationary phase after 20–24 h of cultivation. During the culture time, the pH of the medium declined rapidly from 6.6 at the beginning to 4.86 after 25 h. The cathepsin B inhibitory activity paralleled the cell growth at a constant rate up to the end of the log phase. This is unusual for secondary metabolites [4], and may be closer to the so-called growth-associated type [5]. This kind of bacterium does not consume special carbon and nitrogen sources for producing cathepsin B inhibitor. Hence, the inhibitor may be a product from the growing stage and useful for bacterium growth. Optimizing the culture conditions is the first step to achieveing large-scale production, which helps to conjecture that the inhibitor is a product of the growing stage preliminarily. Purifying a potent cathepsin B inhibitor from the strain PB01 culture and exploring the cathepsin B inhibitor mechanism is an ongoing project in our laboratory.

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