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Preliminary Study on the Production of Extracellular Protease from a Newly Isolated *Bacillus* sp. (No.1) and the Physical Factors Affecting Its Production

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ABSTRAK

Satu bakteria termofil yang dipencil dari tempat buangan sampah-sarap di Sri Petaling, Kuala Lumpur, yang dikenali sebagai *Bacillus* sp. (No.1), berupaya tumbuh pada suhu 60°C. Penghasilan protease oleh bakteria ini dan kesan beberapa faktor fisikal keatas penghasilannya telah dikaji. Aktiviti proteolitik mula dikesan selepas 16 jam eraman dan mencapai maksimum pada 30 jam eraman dengan goncangan 200 ppm pada suhu 50°C dalam media asas. Penghasilan protease oleh bakteria ini berlaku serentak dengan akhir fasa eksponential atau permulaan fasa pegun kelok pertumbuhan dan seiring dengan sporulasi. pH, kadar goncangan dan suhu optimum untuk penghasilan protease oleh *Bacillus* sp. (No.1) ialah pH 8, 150 rpm dan 50°C, masing-masing.

ABSTRACT

A thermophilic bacterium isolated from a dumping ground in Sri Petaling, Kuala Lumpur, identified as *Bacillus* sp. (No.1), was able to grow at 60°C and showed proteolytic activity on skim milk agar. The protease production by this bacterium and some physical factors affecting its production were investigated. Significant protease activity was detected in the basal medium after incubation for 16 h at 50°C with shaking at 200 rpm and reached its peak after 30 h. The protease production coincided with the late exponential or early stationary phase of bacterial growth and corresponded with the sporulation of this bacterium. The optimum pH, agitation rate and temperature for protease production by *Bacillus* sp. (No.1) were pH 8, 150 rpm and 50°C, respectively.

Keywords: extracellular protease, production, physical factors, *Bacillus* sp. (No. 1)

INTRODUCTION

Proteases are enzymes which catalyze the hydrolysis of peptide bonds (Stenish 1975). Industrially, proteolytic processes are important for the production and processing of protein and proteases which are widely used in cheese manufacturing, tanning, food production, the detergent industry and also for medical purposes based on their elastolytic activity, caseinolytic activity, milk clotting activity, transpeptidation and condensation properties (Chaloupka *et al.*

1987; Tsuru and Yoshimoto 1987). Microbial proteases are currently receiving more attention (Aunstrup 1980) due to their short generation time, ability to be produced in bulk at a lower cost and because microbial cells are easily manipulated and can be genetically engineered to enhance protease production. The industrial production of most microbial proteases is further enhanced by the advancement in fermentation technologies.

Bacteria are by far the largest group of protease producing microorganisms. Among the proteolytic bacteria are *Bacillus* spp., *Clostridium* spp., *Lactobacillus* spp., *Pseudomonas* spp. and *E. coli* (Tsuru and Yoshimoto 1987). In our screening program for local proteolytic bacteria, we have isolated a thermophilic bacterium, designated *Bacillus* sp. (No.1) which secreted extracellular proteases. In this study, we investigate the physical factors affecting the production of protease by this bacterium.

MATERIALS AND METHODS

Bacteria Source

The bacterium was isolated from a dumping ground at Sri Petaling, Kuala Lumpur. It is able to grow at temperatures of up to 60°C and produce protease at 50°C. Partial identification of this bacterium showed that it is gram positive, rod-shaped and endospore forming and is designated *Bacillus* sp. (No.1). The pure culture was stored in 20% (v/v) glycerol at -80°C.

Preparation of Inoculum

The inoculum was prepared by inoculating a loopful of bacteria from the stock culture into 10 ml tripticase soy broth (TSB) in universal bottle and incubated by shaking at 200 rpm in a horizontal shaker bath (Hotech Instrument Corp.) for 18 h at 50°C. The cells were harvested by centrifugation at 10,000 rpm for 10 min. The bacterial pellet was dissolved in physiological saline (0.85% NaCl) to give an absorbance reading of 0.5 at 670 nm.

Time Course Study on Protease Production

One millilitre of bacterial inoculum was inoculated into 250-ml conical flasks containing 50 ml of basal medium and shaken at 200 rpm in a horizontal shaker bath at 50°C. The basal production medium used was according to Whooley *et al.* (1983) with slight modification. The medium consisted of (%, w/v) bacto-soytone: 1.00; NaCl: 0.01; K_2HPO_4: 0.02; $CaCl_2.H_2O$: 0.10%; $MgSO_4.7H_2O$: 0.05%. Samples (2.5 ml) were removed at 4-h intervals from 0-48 h for determination of (a) bacterial growth using both viable count and absorbance reading at 670 nm using a spectrophotometer (b) percentage sporulation assessed visually using a compound microscope (Laboval 4, Carl Zeiss Jena) (c) pH and (d) protease activity.

Protease Assay

The protease activity was assayed according to the method of Keay and Wildi (1970) with slight modification. The reaction mixture consisted of 1.0 ml diluted enzyme (enzyme:water 1:3) preincubated at 50°C for 5 min. The reaction was started by the addition of 1 ml casein 2.0% (w/v), pH 7.0. The mixture was incubated in the water bath at 50°C for 10 min and terminated by the addition of 2.0 ml 0.4 M trichloroacetic acid. The mixture was further incubated at 50°C for 20 min, followed by centrifugation at 13,000 rpm for 10 min. To 1.0 ml supernatant, 5.0 ml of 0.4M Na_2CO_3 and 1.0 ml folin ciocalteau reagent:water (1:3, v/v) were added to give a blue colour. The coloured mixture was incubated in a water bath at 37°C for 20 min before the absorbance was read at 660 nm. One unit (U) of protease is equivalent to 0.5 micrograms tyrosine liberated by 1.0 ml enzyme solution under the assay conditions. The amount of tyrosine was determined from the Tyrosine Standard Curve.

Protease Production

The bacterium was cultivated as follows: 1.0 ml of 18-h inoculum (OD 670 = 0.5) was inoculated into 50 ml basal medium (pH 7) and agitated at the rate of 200 rpm for 30 h at 50°C. Samples were harvested and assayed for protease activity by quantitative assay method, and bacterial growth was determined by measuring absorbance at 670 nm. Unless otherwise stated, the cultivation parameters and assay procedures remained the same. The effect of initial pH of the basal medium was studied between the initial pH values ranging from 4 to 10. pH 7 was used as the control and expressed as 100%. The effect of agitation rate was studied at the shaking rates of 0, 100, 150, 200 and 250 rpm in a horizontal shaker. A shaking rate of 200 rpm was used as the control and expressed as 100%. The effect of temperature (45, 50, 55 and 60°C) was studied using 50°C as the control.

RESULTS AND DISCUSSION

Time Course Study on Protease Production

Protease production by microbes varies with the stages of bacterial growth. *Fig. 1* shows that there were two peaks of protease activity, the first peak appeared after 4 h incubation and reached its peak at 8 h incubation. The second peak, which was more significant than the first, was detected after 16 h of incubation and reached its maximum at 30 h. Since the two peaks of protease activities occurred at different incubation times, this might suggest that this bacterium produces two different types of proteases with different functions. However in this study, the second protease peak was chosen since it produced greater amounts than the first. Thus, for subsequent work, the protease was harvested after 30 h of incubation at 50°C.

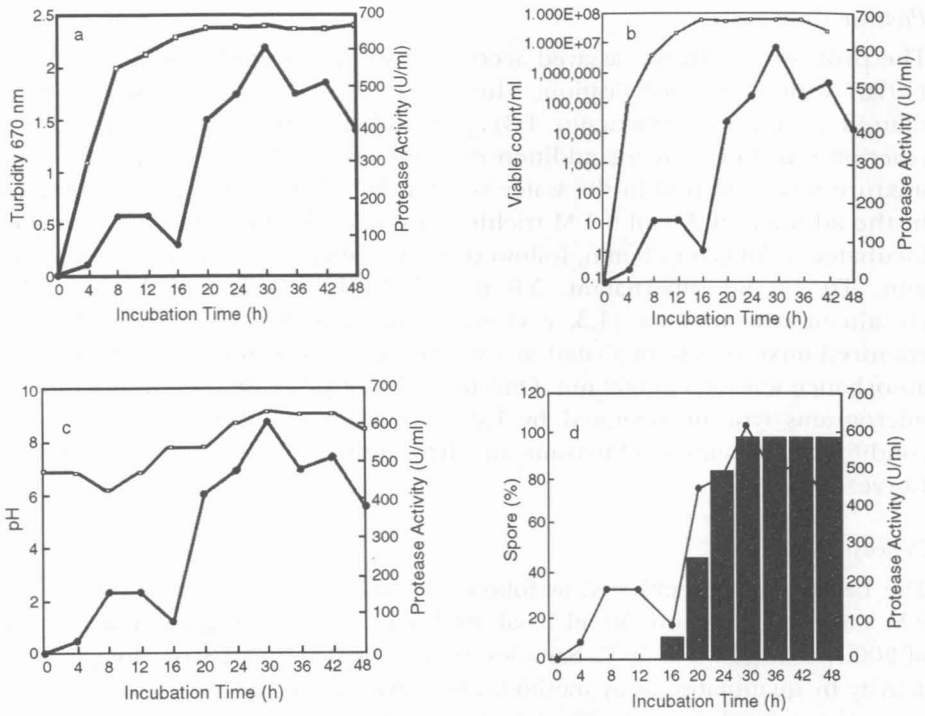

Fig. 1. Growth and protease production by Bacillus sp. (No.1). (●—●) Protease Activity

The onset of protease production (second peak) by this bacterium corresponded to the late exponential or early stationary phase of bacterial growth (*Fig 1a & 1b*). Similar results have been observed in *Bacillus subtilis* (Doi 1972; Murao *et al.* 1979), *B. brevis* ATCC 9999 (Piret *et al.* 1983), *B. subtilis* strain 168 (Hageman *et al.* 1984) and *Enterococcus faecalis* subsp. *liquefaciens* (Fernando *et al.* 1991); their protease production also occurred at the late exponential or early stationary phase of growth. Since protease was released into the medium at the late exponential or early stationary phase of growth, it might have accumulated within the cells and only been released when it was no longer needed to hydrolyze protein for the growth of the bacterium, as suggested by Fernando *et al.* (1991) for *Enterococcus faecalis* subsp. *liquefaciens*, or else this protease was needed for autolysis, as suggested by Keen and Williams (1966).

There was a slight decrease in pH to 6.2 after 8 h cultivation (*Fig. 1c*). This might be due to the secretion of acidic metabolites during exponential growth of the bacterium. However, for longer incubation periods, pH increased and became slightly alkaline. The reason for this increment is not known, but it might be due to the secretion of alkaline metabolites or/and products of protein breakdown in the autolysis stage of bacterial growth (James and Natalie 1992).

Spores of *Bacillus* sp. (No.1) were first detected after 16 h incubation and by 30 h all cells had formed endospores (*Fig. 1d*). Cells lysis was observed after 30 h incubation. This result suggests that protease production by this bacterium is related to sporulation since both the endospore formation and protease production began after 16 h of cultivation. Similar results were reported by Murao *et al.* (1979) for *Bacillus subtilis* and Chaloupka *et al.* (1987) for *B. megaterium*; their protease production also occurred during sporulation. On the other hand, Doi (1972) showed that protease production by *B. cereus* and *B. megaterium* was not related to sporulation, or *vice versa*.

Effect of Physical Factors

Effect of Initial pH of the Medium. Fig. 2 shows that both bacterial growth and protease production occurred over a wide range of pH, from 5 to 8, with 8 being the optimum pH for protease production, and 7 being the best pH for bacterial growth. At pH of below 5 and above 8, although growth was observed no protease activity was detected. It can therefore clearly be seen that the protease production by *Bacillus* sp. (No.1) is not growth dependent. The pH range for protease production and growth of *Bacillus sp.* (No.1) was similar to most protease-producing bacteria such as *Pseudomonas myxogenes* sp. (Morihara 1959) and *Bacteroids amylophilus* strain H18 (Blackburn 1968), which produce proteases over broad ranges, from pH 4 to 9 and from pH 5.5 to 9.5, respectively. The slightly alkaline pH needed for optimum protease production is a common phenomenon for most protease producing bacteria

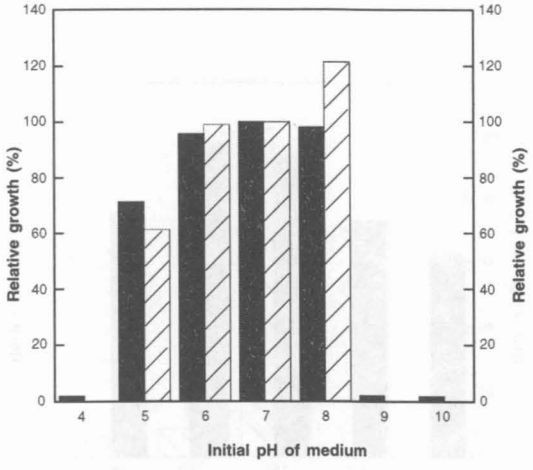

Fig. 2. Effect of medium initial pH on protease production by Bacillus sp. (No.1). Growth and protease activity at pH 7.0 were taken as 100%. The shaking rate and the temperature were 200 rpm and 50 °C, respectively. Results are means of triplicate. Relative growth (■); Relative activity (□).

(Blackburn 1968; Piret *et al.* 1983). Theoretically, the pH might affect the stability or conformation of the plasma membrane, which might be indirectly affecting the membrane bound ribosomes involved in the protease synthesis.

Effect of Agitation Rate. Hare *et al.* (1981) and Fernando *et al.* (1991) have shown that protease production is affected by agitation rate. *Fig. 3* shows that the agitation rate had a more significant effect on protease production than on bacterial growth. At 0 and 100 rpm, even though there was growth, no protease was detected and at agitation rates of 200 and 250 rpm, although good growth was observed, the production of protease was minimal. These results indicate that protease production by this bacterium is not growth dependent. The optimum agitation rate for protease production was 150 rpm; for bacterial growth it was 200 rpm. Static conditions completely inhibit protease production. Similar observations were reported for *Vibrio alginolyticus* (Hare *et al.* 1981) and *Enterococcus faecalis* subsp. *liquefaciens* (Fernando *et al.* 1991) which require shaking to stimulate protease production. In contrast, Dainty *et al.* (1978) reported that *Chromobacterium lividum* produced protease under static conditions.

Effect of Temperature. The optimum temperature for protease production was 50°C (*Fig. 4*). The yield was much lower at 45 and 55°C, and at 60°C protease production was totally inhibited even though growth was observed.

The mechanism of how temperature affects protease production is not clearly understood, but Chaloupka *et al.* (1987) suggested for *Bacillus megaterium* that at high temperatures the inhibition might be due to repression at the level of mRNA transcription or translation. On the other hand, Fernando *et*

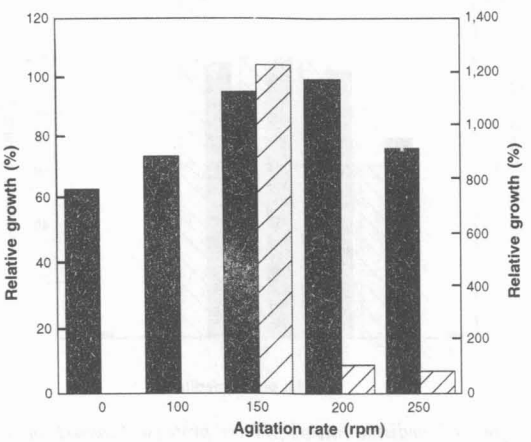

Fig. 3. Effect of agitation rate on protease pro-duction by Bacillus sp. (No.1). Growth and protease activity at 200 rpm were taken as 100%. The pH and temperature were pH7.0 and 50 °C, respectively. Results are means of triplicate. Relative growth (■); Relative activity (□).

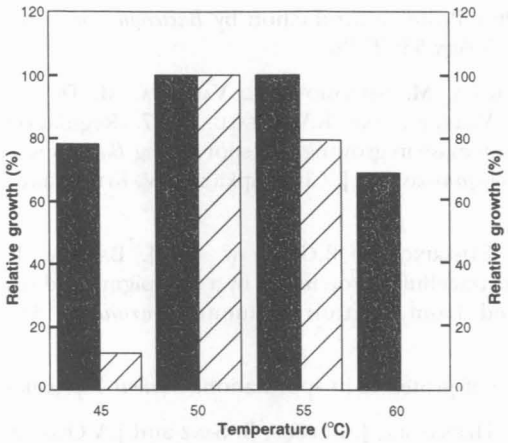

Fig. 4. Effect of temperature on protease production by Bacillus sp. (No.1). Growth and protease activity at 50 °C were taken as 100%. The shaking rate and the pH were 200 rpm and pH 7.0, respectively. Results are means of triplicate. Relative growth (■); Relative activity (□).

al. (1991) claimed that for *Enterococcus faecalis* subsp. *liquefaciens* cultivation temperature from 7 to 45°C neither affected growth nor the maximum protease production but temperature strongly affected the time necessary to attain the same protease yield. In this study, protease yield was only determined at one cultivation time, thus such comparison cannot be made.

CONCLUSION

The beginning of protease production by this bacterium coincided with the onset of sporulation; it occurred during the late exponential or early stationary phase of bacterial growth. Protease production and bacterial growth were dependent on initial pH of the medium, the rate of agitation and the cultivation temperature. Optimum pH, agitation rate and temperature for protease production by this bacterium were pH 8, 150 rpm and 50°C, respectively. Optimum bacterial growth occurred at pH 7, agitation rate of 200 rpm and at 50°C. Thus it can be concluded that protease production by this bacterium was not growth related.

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