Effects of Temperature on Growth and Protease Production of *Cytophaga psychrophila*

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Our previous study¹⁾ demonstrated that the optimal temperatures for the protease production were lower than those for the growth of a mesophilic bacterium, *Aeromonas hydrophila*. As protease is one of the pathogenic factors, the previous results provoked us into making a similar work with the low temperature-loving bacterium, *Cytophaga psychrophila* (syn. *Flexibacter psychrophilus*). This bacterium is known as the causative agent of bacterial coldwater disease in salmonids and some other freshwater fishes²⁾.

Materials and Methods

Bacterial strains: Six *C. psychrophila* isolates of fish origin were included in this study. The details of these bacterial strains are shown in Table 1. After collecting the strains, these were stored at -80°C for long term preservation.

Media and culture conditions: Stock cultures of the organisms were maintained on tryptone yeast-extract (TYE) broth at 18°C . One loopful of cell suspension was inoculated in 18 ml of each TYE broth in a L-shaped tube and subsequently incubated at different points of a temperature gradient incubator (TGI: Toyo Co., Tn-3), over a linear temperature gradient from 2 to 32°C . The shaking speed was about 30/min. Media composition: TYE ; tryptone 0.4%, yeast extract 0.05%, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.02%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05% and pH 7.2.

Table 1. *Cytophaga psychrophila* strains used in this study

Strain	Isolation date	Host species	Location
NCMB1947 ^T	Unknown	Coho salmon	Washington, USA
FPC816	1991	Rainbow trout	Tokyo, Japan
FPC825	Jul. 1990	Coho salmon	Oregon, USA
FPC828	Mar. 1990	Coho salmon	Miyagi, Japan
FPC927	May 1993	Ayu	Wakayama, Japan
FPC946	Jan. 1994	Oikawa	Hiroshima, Japan

^T = Type strain.

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Determination of growth: Measurement of growth of the organisms was performed by a spectrophotometer (Hitachi, U-2000) at 480 nm and expressed as optical density (OD). Similar pattern in time course effect for bacterial growth was found among the all 6 strains up to around 26 h. So, samples were collected for the determination of growth after around 22 h of incubation period (within log phase).

Determination of protease production: In the log phase culture, no considerable amount of protease was detected. In 56 h culture every point of protease production curve remarkably differed at different temperatures. So, protease production was measured after 56 h when the culture at temperature near optimal growth reached stationary phase. Quantitative protease assay for the supernatants with azocasein (Sigma, USA) as a substrate was performed according to the method of Sarath *et al.*³⁾ with a slight modification. A 2% (weight/volume) solution of azocasein in 0.1M tris-HCl, pH 7.2, was used. Incubation time for digestion mixture at 25°C was fixed for 4 h. After addition of trichloroacetic acid to terminate the assay, total mixture was allowed to stand for 20 min to ensure complete precipitation of the remaining azoprotein and azoprotein fragments. Intensity of the orange color was measured as optical density (OD) in Hitachi U-2000 spectrophotometer at 440 nm. Triplicate data were averaged and analyzed with t-test. A reference standard curve for trypsin (Difco, USA; 5, 10, 20, 30, 40, and 50 mg/ml in 0.1M tris-HCl, pH 7.2) was prepared.

Results

Growth and protease production of all the strains were profoundly affected by incubation temperatures (Table 2). A representative result is shown in Fig. 1. The growth optimal temperature was found at $19.6 \pm 0.5^{\circ}\text{C}$. The growth temperature ranges were 5.5 to 24°C for 56 h incubation period. At all growth temperatures studied, considerable amount of protease production was not observed until the late log phase or stationary phase of growth. The optimal temperature for protease production was at $13.3 \pm 1.9^{\circ}\text{C}$ (Table 2). Despite relatively good growth at 21°C , protease production was suppressed markedly at this temperature (Fig. 1). Slowly, they produced small amount of protease at higher temperatures (above 18.5°C). On

Table 2. Optimal temperatures on the growth and protease production of *C. psychrophila* strains

Strain	Growth ($^{\circ}\text{C}$)	Protease production ($^{\circ}\text{C}$)
NCMB1947	19.2	13.5
FPC816	19.5	10.8
FPC825	20.0	11.9
FPC828	20.0	12.5
FPC927	20.0	15.5
FPC946	19.0	15.5

Fig. 1. Effects of temperature on growth and protease production of *C. psychrophila* strain, FPC 816.

the other hand, at lower temperatures in comparison to growth the protease production were higher. The temperature ranges for protease production were from 5.5 to 24°C for a period of 56 h culture. Only two strains out of six produced a small amount of protease at 24°C, the highest temperature for growth. The optimum temperatures for protease production were lower than those for the growth and the differences were statistically significant ($P < 0.05$). The differences among the peaks of protease production and growth were 4 to 9°C. The optimum temperature for protease production of *C. psychrophila* was 6.3°C lower than that of the growth optimum temperature.

The amount of protease varied with growth temperature and strains of the bacterium studied. Strain NCMB 1947 yielded the maximum protease which was the double of the strain FPC 927, yielded the minimum at a fixed incubation temperature (data not shown).

Discussion

The present study on the effects of temperature on the growth

and protease production of *C. psychrophila* indicates that these processes are temperature dependent. Our findings of growth optimal temperature at $19.6 \pm 0.5^\circ\text{C}$ well coincided with the results of Pacha⁴⁾ that optimal temperature was 20°C . Holt *et al.*⁵⁾ found that optimal growth was at 15°C . Such variations may be due to the differences of strain, age of strain, source, environmental factors, setting of experiments, etc.

We took the samples for protease measurement from late log phase due to good production during this period. This idea also had been documented by McKellar⁶⁾. We observed the lower optimal temperature for protease production at $13.3 \pm 1.9^\circ\text{C}$ than growth optimum, $19.6 \pm 0.5^\circ\text{C}$. The protease production fell sharply at incubation temperatures above the apparent optimum, despite extensive growth. It is known that the environmental temperature which favors disease occurrence has a tendency to be lower than the growth optimal temperature of the pathogen. The optimal temperature for protease production of *C. psychrophila* seems to accord with the environmental temperature favorable for outbreaks of bacterial coldwater disease. Further *in vivo* works are needed in this line.

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