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# Isolation and characteristics of one marine psychrotrophic cellulase - generating bacterium Ar/w/b/75°/10/5 from Chuckchi Sea, Arctic

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**Abstract** Microorganisms living in polar zones play an important part as the potential source of organic activity materials with low temperature characteristics in the biotechnological applications. A psychrotrophic bacterium (strain Ar/w/b/75°/10/5), producing cellulase at low temperatures during late-exponential and early-stationary phases of cell growth, was isolated from sea ice-covered surface water in Chuckchi Sea, Arctic. This bacterium, with rod cells, was Gram-negative, slightly halophilic. Colony growing on agar plate was in black. Optimum growth temperature was 15℃. No cell growth was observed at 35℃ or above. Optimum salt concentration for cell growth was between 2 and 3 % of sodium chloride in media. Maximal cellulase activity was detected at a temperature of 35℃ and pH8. Cellulase was irreversibly inactivated when incubated at 55℃ within 30 min. Enzyme can be kept stable at the temperature no higher than 25℃. Of special interest was that this bacterium produced various extracellular enzymes including cellulase, amylase, agar hydrolase and protease, at low or moderate temperature conditions, which is certainly of it potential value for applications.

**Key words** psychrotrophic, bacterium, cellulase, Chuckchi Sea, Arctic.

## 1 Introduction

Cellulase is one of the important industrial enzymes and one of the most extensively multicomponent enzyme systems because of their ability to decompose the cellulosic biomass into glucose, which in turn can be converted to other valuable chemicals and energy (Mukataka *et al.* 1998). Cellulases are widely used in the food and feed industries, and also in the textile and pulp and paper industries (Nakari-Setala *et al.* 1995). Using cellulase enzymes for denim garment treatment has become a widely used practice (Tyndall 1992).

Many different cellulases have been described. Cellulase actually is a group of enzymes which, acting together, hydrolyze cellulose. Complete cellulase enzyme systems generally consist of three kinds of enzymes: (1) cellobiohydrolase (CBH, also known as exocellulase, or filter paper activity, EC 3.2.1.91), (2) endo-1,4-β-D-glucanase (carboxymethyl cellulase or CMCase, EC 3.2.1.4), and (3)1,4-β-D-glucosidase (EC 3.2.1.21). Endo glucanase (CMCase) attacks randomly in the interior of cellulose structure.

It is not very active against crystalline cellulose, but they are capable of hydrolyzing substituted celluloses, such as carboxymethyl cellulose. CMCase produces cellulodextrins (also known as cellulooligosaccharides). In fact, alkaline cellulases applied widely in detergent industry mainly are CMCase. Cellobiohydrolase (CBH) attacks crystalline cellulose from the non-reducing end. It produces cellobiose. CBH is very important for the degradation of microcrystallline cellulose. Beta-glucosidase hydrolyzes cellobiose into glucose.

Microorganisms living in constantly low temperature environments including polar zones, high altitudes and deep-sea areas, can generate low – temperature enzymes, PUFA (Polyunsaturated fatty acids), carotenoids and other organic activity materials adapting to cold temperature. Low temperature enzymes are characterized by having a high catalytic efficiency at low and moderate temperatures as low as 30 °C (Feller *et al.* 1996). Along with the enzyme – producing microorganisms, such low temperature enzymes have some advantages for food and feed industries, detergents pulp and paper industries, and environment protection industry, for example, to improve the waste treatment at ambient temperature in cold and temperate climates, and to save energy and to cut down expenses. In the present study, we report the isolation and characteristics of one marine psychrotrophic bacterium capable of producing cellulase (CMCase) with an optimum activity temperature of 35 °C.

## 2 Materials and methods

### 2.1 *Bacterial strain, medium and culture condition*

Samples were collected from sea ice area in Chukchi Sea, Arctic. Zobell 2216E agar plate containing 0.5% (w/v) sodium carboxymethylcellulose (CMC) was used to isolate marine bacteria at an incubation temperature of 5℃ for 7 days. Bacteria that grew on the plate were reinoculated on CMC-Zobell 2216E agar plate containing Congo red as indicator, by incubating at 5℃ for 5 days ( Teather and Wood 1982 ). Isolates that showed a clear zone of hydrolysis of CMC on agar plate were selected for further research. One bacterial strain showing CMCase activity was obtained, and designated Ar/W/b/75°/10/5.

The selected strain was cultured in CMC-Zobell 2216E medium at 15℃ with vigorous shaking of 220 rounds per min for 72 h. The cell growth of bacterium was estimated by measuring absorbance of cultured broth at 540 nm.

Basic medium for carbon source utilization of the bacterium was composed of 10g/l NaCl, 5g/l various carbon source, 3.2g/l $Na_2HPO_4$, 1.3g/l $KH_2PO_4$, 1g/l $NaNO_3$, 0.8g/l $Na_2SO_4$ and 0.5g/l $(NH_4)_2HPO_4$. And nitrogen source utilization medium was composed of the following: 10g/l NaCl, 7g/l $K_2HPO_4$, 5g/l glucose, 5g/l various nitrogen source, 3g/l $KH_2PO_4$, 0.5g/l sodium citrate and 0.1g/l $MgSO_4 \cdot 7H_2O$. Luria-Bertani medium containing different concentration of sodium chloride was used to examine the effect of salinity on cell growth and cellulase production of bacterium (Sambrook *et al.* 1989).

### 2.2 *Assay of enzyme activity*

The cultured broth was directly used as crude enzyme preparation for total enzyme activity assaying, and supernatant fluid after centrifugation (4000 rpm, 10min, at 4℃) for

removing cells was used as crude extracellular enzyme preparation.

Cellulase activity was measured by analyzing reducing sugars released as a result of enzyme reaction with substrate of CMC. 0.5 ml of crude enzyme preparation were added into 1.5 ml of 50 mmol $l^{-1}$ phosphate buffer (pH 7.0) containing 0.5% (w/v) CMC. After 30 min of incubation at 35 ℃, reaction was stopped by incubation in boiled water for 10 min. Reducing sugars released from the reaction were determined using method of 3,5 – dinitrosalicylic acid (DNS) (Miller 1959), with glucose as standard. Absorbance at 530 nm was measured. One unit of enzyme activity was defined as 1 μg of glucose liberated per 1 min at 35℃.

Similar methods were taken to assay enzyme activities of amylase or agar hydrolase, using starch or agar, respectively, instead of CMC, as corresponding substrate.

Protease activity was assayed by the method of Folin phenol ( Zeng *et al.* 2001). One unit of protease activity was defined as the amount of enzyme releasing 1 μg of tyrosin per 1 min at 40℃.

## 3 Results and discussion

### *3.1 Isolation and characteristics of cellulase-producing bacteria*

One bacterium showing CMC hydrolytic activity was isolated from sea ice-covered surface water from Chukchi Sea, Arctic, and designated Ar/W/b/75°/10/5. Colonies growing on agar plate were in humid and lustrous facial status with disk shape, smooth edge, and flat carina. Ripe colonies, encircled with a thin milky ring, were in black. For ability of hydrolyzing agar, colonies were obviously sunken on agar plate.

In shape cells of this bacterium were rod – like in size of (0.2 – 0.4) × (0.6 – 1.8) μm. Some cells showed slightly arc shape. This bacterium was negative by Gram staining, positive of oxidase and catalase, and showing enzyme activities of protease, amylase, agar hydrolase besides CMCase. Without motility, this strain was unsensitive to ampicillin, as well as 2,4 – diamino – 6,7 – diisopropyl teridine. It could grow in spite of the existing of oxygen or not, and produced weak acids with no carbon dioxide occurring in glucose fermentation. No reducing activity to produce sulfated hydrogen was detected. Further research on classification and identification of this bacterium will be carried out later.

Cellulases originated from a wide variety of fungi and other microbes. Usually, acidic cellulases are derived from fungi, including *Phanerochaete*, *Schizophyllum*, *Irpex*, *Acremonium*, *Trichoderma*, *Sporotrichum*, *Sclerotium*, *Talaromyces*, *Thielavia*, *Chrysosporium*, *Penicillium*, *Aspergillus*, *Orpinomyces*, and *Fusarium* (Li *et al.* 1997; Walter et al. 1996). Neutral or alkaline cellulases mainly originate from actinomycetes or bacteria, including *Bacillus*, *Thermotoga*, *Clostridium*, *Ruminococcus*, *Streptomyces*, *Pseudomonas and Chryseomonas* (Bronnenmeier *et al.* 1995; Laurent *et al.* 2000; Spiridonov *et al.* 1998; Kataeva *et al.* 1999). At present, most of the alkaline cellulases for detergent industry are derived from *Bacillus* sp.

### *3.2 Characteristics of bacterium Ar/W/b/75°/10/5*

*3.2.1 Carbon source utilization of bacterium Ar/W/b/75°/10/5*

This bacterium was inoculated into basic medium containing various carbohydrates for carbon source utilization. After incubation with vigorous shaking at 15℃ for 48 h, cell growth and cellulase activity were examined.

Figure 1 showed that bacterium Ar/W/b/75°/10/5 could utilize glucose, sucrose or soluble starch as sole carbon source for cell growth, and effect of sucrose or soluble starch was much better. It also showed that this bacterium could not directly utilize CMC as sole carbon source for cell growth. Contrasting with poor cell growth in basic carbon source utilization medium containing 0.5% CMC, this bacterium grew well in the same medium with additional 0.2% agar. It suggested that this bacterium could hydrolyze agar as carbon source for cell growth. As high concentration of cells but low cellulase activity were observed in broth, it indicated that carbon source was closely connected with cell growth of this bacterium, but had little effect on cellulase production. Usually, microorganisms preferentially utilize simple monosaccharides with small molecular weight, such as glucose for cell growth. It's one interesting phenomenon for this bacterium's utilization of sucrose or starch for growth better than glucose.

This bacterium was cultivated in Zobell 2216E medium added to with different carbon source, agar or CMC, respectively, at 15℃ for 48h. Result showed that in these two media, after 48h of incubation, concentrations of monosaccharides increased 1 or 3 times, respectively. Compared to Zobell 2216E medium without additional carbohydrate, cellulase activity in these three broths had no obvious difference (data not shown). It suggested that cellulase produced by this strain did not belong to induced enzymes.

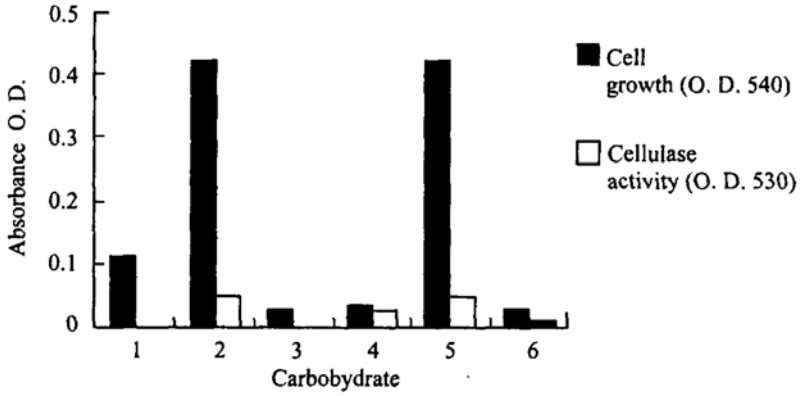


Fig. 1 Carbon source utilization of the strain Ar/W/b/75°/10/5
1. Glucose 2. Sucrose 3. Lactose 4. Maltose 5. Soluble starch 6. CMC

*3.2.2 Nitrogen source utilization of bacterium Ar/W/b/75°/10/5*

This bacterium was inoculated into various nitrogen source utilization media. After incubation with vigorous shaking at 15℃ for 72 h, cell growth and cellulase activity were examined.

Figure 2 showed that this strain only utilized yeast extract as nitrogen source for cell growth well. It suggested that this bacterium required some complicated and organic nutrition conditions for cell growth and enzyme production.

*3. 2. 3 Effect of temperature on cell growth of bacterium Ar/W/b/75°/10/5*

This bacterium was cultivated in Zobell 2216E medium at different temperatures for 46h. Cell growth and cellulase activity in the broth were observed.

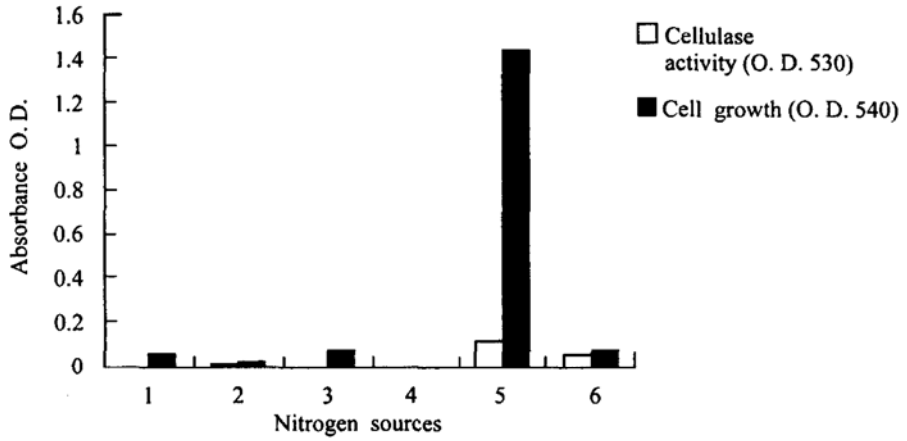


Fig. 2. *Nitrogen source utilization of the strain Ar/W/b/75°/10/5.*
1. $NH_4NO_3$ 2. $KNO_3$ 3. $(NH_4)_2SO_4$ 4. Urea 5. Yeast extract 6. Peptone

Result (See Fig. 3) showed that optimum temperature for cell growth was 15℃. No cell growth was observed at 35℃ or above. According to the definition (Morita 1975; Russell 1990; Bowman *et al.* 1997) psychrophiles as organisms have the upper growth limits below 20℃, and psychrotrophs are capable to grow at 0℃ but show the upper limit up to 40℃, so this bacterium was classified as psychrotrophic. Temperature of sampling site was −1. 1℃, and time for sampling was on Aug. 25, the warmest season in Chuckchi Sea, Arctic. For the constantly cold environments, microorganisms living in Arctic not only are adaptable to the low temperatures, but also are potential producers for organic activity materials with low temperature characteristics. At the same time, scientists have mentioned and proved that in polar regions as well as in deep-sea waters, the psychrotrophs are relatively much more abundant than the psychrophiles (Feller *et al.* 1996; Connell 1994; Delille 1992). It should be ascribed to the large scale of change of environmental temperatures in polar zones and frequently motion of microorganisms on the earth through air, water, and so

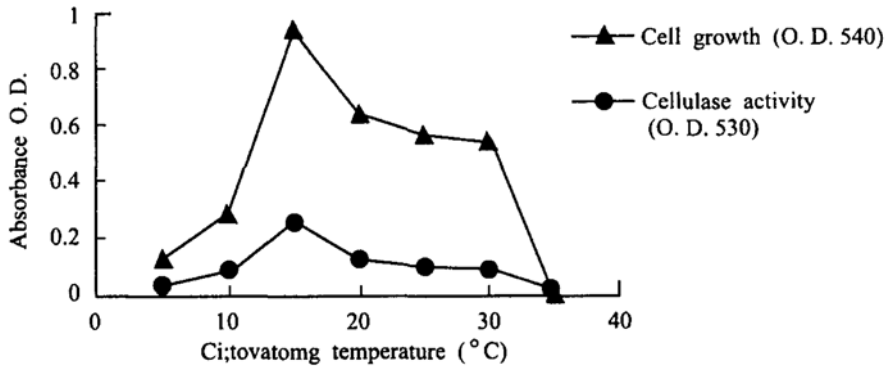


Fig. 3. Effect of temperature on cell growth of bacterium Ar/W/b/75°/10/5.

on.

*3. 2. 4 Effect of salinity on cell growth of bacterium Ar/W/b/75°/10/5*

Cell growth and cellulase activity were examined after inoculating this bacterium into Luria-Bertani media containing different concentrations of sodium chloride, incubating at 15℃ for 48 h.

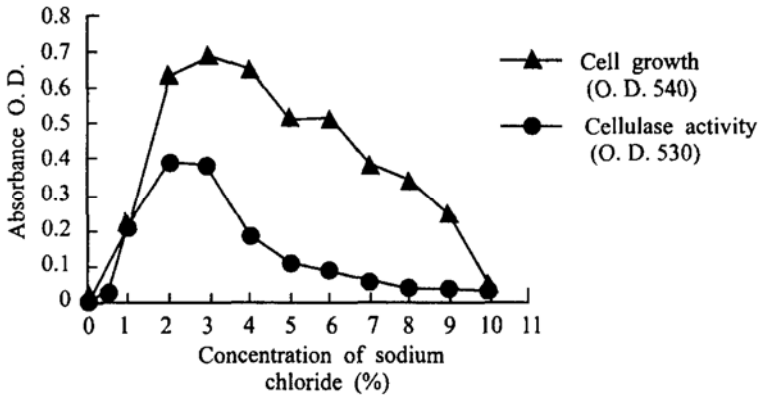


Fig. 4. Effect of salinity on cell growth of bacterium Ar/W/b/75°/10/5.

Figure 4 showed that the bacterium can grow well in medium containing salinity between 1.0 and 9.0 % sodium chloride, best when sodium chloride concentrations between 2 and 4%. No obvious cell growth occurred when salinity was zero in medium.

Generally, salinity in sea water is around 33‰, and that of the sampling water was actually 28. 1‰. Figure 4 showed that highest cellulase activities were also detected when this bacterium grew in media containing 2 to 3 % of sodium chloride, which was similar to the salinity condition of sampling site. Results suggested that this strain really originated from oceanic conditions. Keeping certain concentration of salinity in medium is necessary for cell growth and cellulase production because of this bacterium's halophilic characteristics.

*3. 2. 5 Kinetics of cell growth and cellulase production of strain Ar/W/b/75°/10/5*

Kinetics of bacterium Ar/W/b/75°/10/5 growing in Zobell 2216E medium at 15℃ was examined. Figure 5 (1) showed that this bacterium produced cellulase mainly during the late-exponential and early-stationary phases of cell growth.

## 3.3 Characteristics of cellulase produced by bacterium Ar/W/b/75°/10/5

*3. 3. 1 Ratio of extracellular cellulase to total cellulase activities of bacterium Ar/W/b/75°/10/5*

Result showed that about 74. 1% of total cellulase activities of this bacterium were extracellular enzyme. It would be beneficial for simplifying the procedure of enzyme extraction and purification in biotechnology.

Using various substrates to examine cellulase activity in the broth indicated that en-

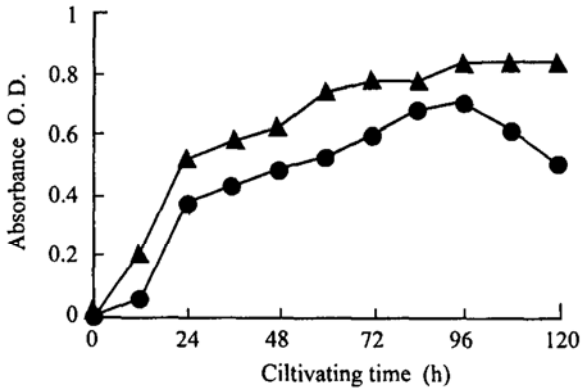


Fig. 5(1). Showed that pH value in the broth almost increased gradually along with the cultivating time. It resulted from the growth and metabolism of cells.

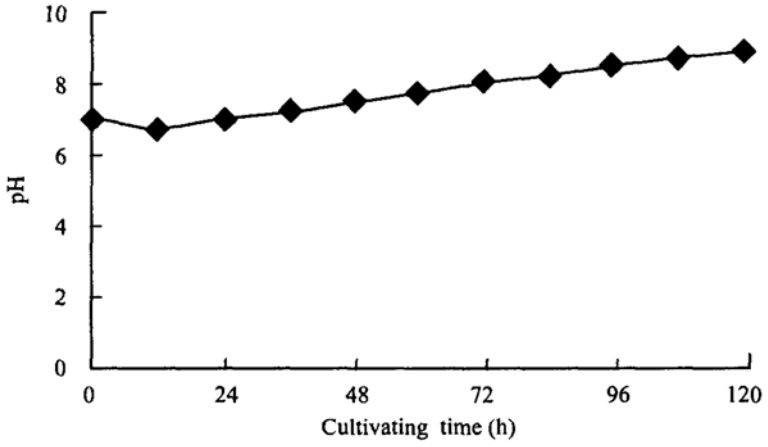


Fig. 5(2). Effect of cell growth on pH in the broth of bacterium Ar/W/b/75°/10/5.

zyme activity using CMC as substrate was about 6 times higher than that using filter paper as substrate (data not shown). It suggested that most cellulases originating from this strain belong to endoglucanase (CMCase), only a little are exocellulases. Further research would be carried out to make sure the exact sort of cellulases produced by this bacterium.

### *3.3.2 Temperature activity profile*

Enzyme reaction was detected at various temperatures ranging from 5 to 60℃ and incubating for 0.5 h with 0.5% CMC as substrate. Figure 6 showed that maximum cellulase activity of bacterium Ar/W/b/75°/10/5 occurred at 35℃. It suggests that psychrotolerant microorganisms are taken as the potential source of low – temperature cellulase or other enzymes with low temperature characteristics.

Cellulases range widely in temperature. Usually, cellulases have an optimum temperature range for activity between 45 and 65℃, and most at 55℃. Some thermostable cellulases from hyperthermophilic bacterium, such as *Thermotoga maritima*, *Thermotoga neapolitana* are optimally active at 95 or even 106°C (Jin-Duck et al. 1998; Bronnenmeier et al. 1995). On the other hand, cellulase from psychrotrophic bacterium *Chryseomonas luteola*

with optimal temperature for cellulolytic activity at 28°C has been examined (Laurent *et al.* 2000). Psychrophilic enzymes typically have maximal catalytic activity at temperature below 40°C and usually display some degree of thermolability (Nichols *et al.* 1999). Adaptation of psychrophilic proteins to low-temperature conditions determines a instability of these proteins at higher temperatures (Truong *et al.* 2001).

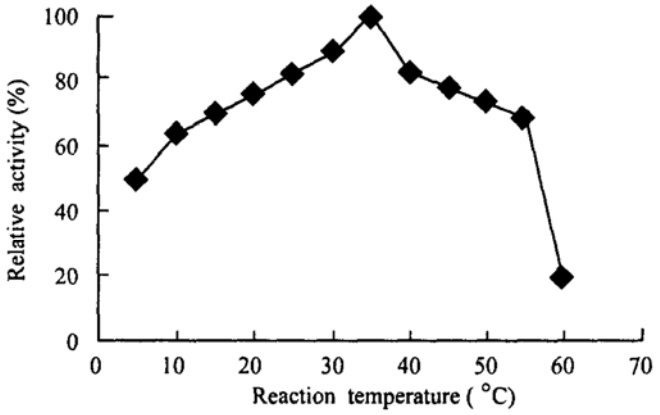


Fig. 6. Effect of temperature on cellulase activity of bacterium Ar/W/b/75°/10/5.

Cellulase activity was reduced by 80% when enzyme preparation was preincubated for 30 min at 50℃ (Fig. 7), and irreversibly entirely inactivated within 30 min at 55℃. It proved that cellulase from psychrotrophic bacterium has labile thermal stability in higher temperature conditions.

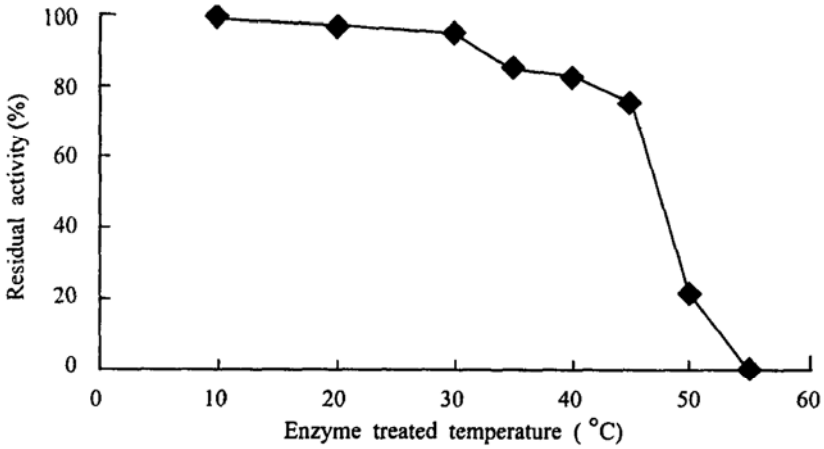


Fig. 7. Thermal stability of cellulase produced by bacterium Ar/W/b/75°/10/5(1).

Residual activity of cellulase was examined after enzyme solution was preincubated at 25, 35 or 45℃, respectively, for different hours. Result (Fig. 8) showed that cellulase activity of this strain decreased with increasing time when incubated at 35 or 45℃. Thermal

stability of cellulase produced by bacterium Ar/W/b/75°/10/5 was more labile with increasing temperatures when temperature is higher than 35℃. Cellulase of this bacterium can be kept stable when temperature is not higher than 25℃.

### *3.3.3 Effect of pH on cellulase activity of bacterium Ar/W/b/75°/10/5*

Optimum pH of cellulase activity was determined. Enzyme reaction was carried out in buffers ranging from pH 4 to 12, at 35℃ for 0.5 h. Figure 9 showed that optimum pH for cellulase activity of bacterium Ar/W/b/75°/10/5 was about 8, and the cellulase expressed fairly high enzyme activity in the range of pH 6 to 9.

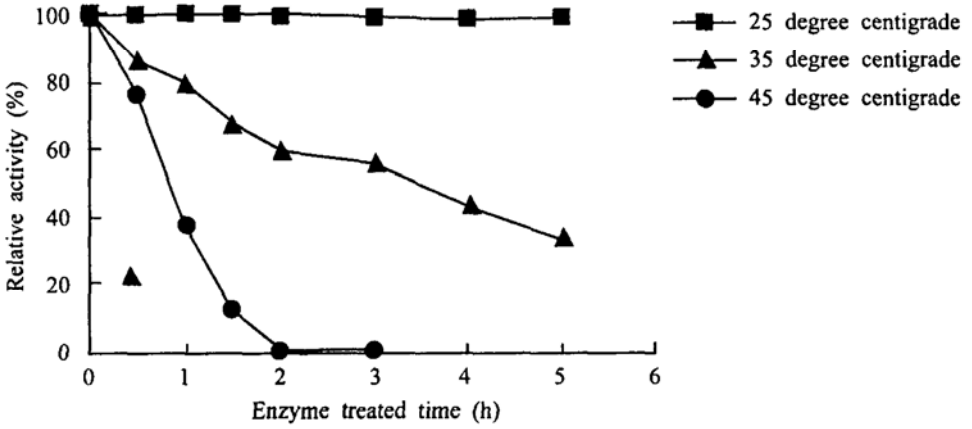


Fig. 8. Thermal stability of cellulase produced by bacterium Ar/W/b/75°/10/5(2).

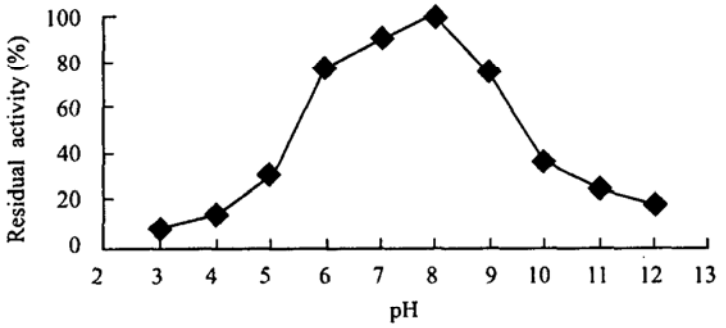


Fig. 9. Effect of pH on cellulase activity of strain Ar/W/b/75°/10/5.

### 3.4 Temperature activity profile of other enzymes produced by bacterium Ar/W/b/75°/10/5

Besides cellulase, other enzyme activities were detected in the broth of bacterium Ar/W/b/75°/10/5. Figure 10 showed optimum temperatures for activity of these enzymes, including protease, amylase and agar hydrolase, were 40, 35 and 35℃, respectively. All enzymes having been examined in the broth could been described as low – temperature type for their optimum temperature for activity is not higher than 40℃.

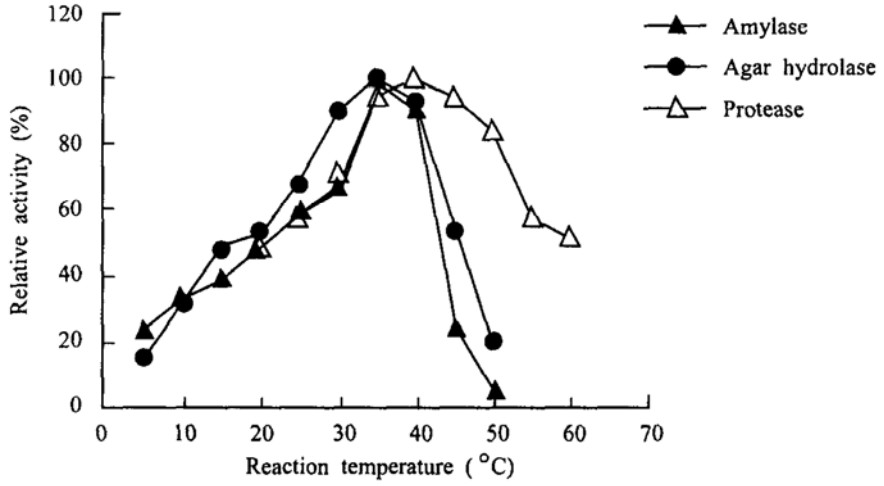


Fig. 10. Effect of temperature on enzyme activities of bacterium Ar/W/b/75°/10/5.

## 4 Conclusion

(1) As shown in the study, psychrotrophic marine bacterium Ar/W/b/75°/10/5 is Gram-negative, slightly halophilic, with cells in the shape of rod. Among carbon source nutrition, sucrose or soluble starch is much better for cell growth of this bacterium. Yeast extract is the optimal nitrogen source nutrition for cell growth. Optimum growth temperature was 15℃, and no cell growth was observed at 35℃ or above.

(2) More than 70% of total cellulases produced by bacterium Ar/W/b/75°/10/5 were extracellular enzyme. Among them, CMCase activity is much higher than exocellulase, or filter paper activity. Maximal cellulase activity was detected at a temperature of 35℃ and pH8. Producing different low – temperatue enzymes, such as cellulase, amylase, agar hydrolase and protease, psychrotrophic bacterium Ar/W/b/75°/10/5 has potential for application in biotechnology.

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