Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Effect of temperature on batch elastase production by *Bacillus* sp. EL31410*

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Received Jan. 30, 2004; revision accepted July 7, 2004

Abstract: The production of elastase by *Bacillus* sp. EL31410 at various temperatures was investigated. In order to study the effect of temperature on elastase fermentation, different cultivation temperatures, ranging from 39 °C to 28 °C, were evaluated in shake flask. The result indicated that 37 °C was best for cell growth at earlier stage; while maximum elastase activity was obtained when the cells were cultivated at 30 °C. This result was verified by batch fermentation in 5-L bioreactor under 37 °C and 30 °C temperature, respectively. The specific cell growth rate at 37 °C was higher than that at 30 °C during earlier stage of cultivation. The maximum value [5.5 U/(h·g DCW)] of elastase formation rate occurred at 24 h at 30 °C compared to 4.6 U/(h·g DCW) at 30 h at 37 °C. Based on these results, two-stage temperature shift strategy and oscillatory temperature cultivation mode were evaluated in the next study. When compared to single temperature of 37 °C or 30 °C, both two-stage temperature shift strategy and oscillatory temperature strategy improved biomass but did not yield the same result as expected for elastase production. The maximum biomass (both 8.6 g/L) was achieved at 30 h at 37 °C, but at 42 h using two-stage temperature cultivation strategy. The highest elastase production (652 U/ml) was observed at 30 °C in batch process. It was concluded that cultivation at constant temperature of 30 °C was appropriate for elastase production by *Bacillus* sp. EL31410.

Key words: *Bacillus* sp. EL31410, Elastase, Temperature, Batch fermentation, Temperature-shift strategy
doi:10.1631/jzus.2004.1583 **Document code:** A **CLC number:** Q939

INTRODUCTION

Elastase is a protease capable of catalyzing the hydrolysis of elastin (Morihara, 1967); and can be extracted from pancreas or obtained by fermentation technology. Elastase production by microorganism is relatively more promising due to its low cost, high production rate, and readily controlled conditions. Recently it has attracted more and more interest because of the shortage of its sole source (pancreas) and potential application in biochemical medicine, meat tenderizers, cosmetics, and environment protection (Ke and Xiao, 2002). Until now, efforts have been mainly focused on screening the elastase producing strains, on studying its pathogenic effect and on its characterizations (Tsai *et al*., 1988; Shibata *et al*., 1993; Zins *et al*., 2001). There are few reports on elastase production with fermentation technology.

* Project (No. 20276064) supported by the National Natural Science Foundation of China

To achieve highest economy benefit, how to attain maximum production was considered first and then how to reduce the fermentation cycle duration (Feng *et al*., 2003; Mei *et al*., 1999). Environmental conditions, such as temperature and pH, are important factors affecting fermentation wherein microorganism metabolism is catalyzed by various kinds of enzymes. Under optimal conditions, enzymes are capable of maximum activity. For obtaining high specific cell growth rate, specific elastase formation rate, elastase activity and productivity, it is necessary to control environmental parameters to optimal conditions throughout the course of cultivation. Little information, however, is available on the optimum temperature conditions in shake flask or bioreactor, which is important for large-scale production of elastase. In our previous study, the cultivation kinetics of *Bacillus* sp. EL31410 was studied (Chen *et al*., 2004). The objective of this paper was to investigate the effect of temperature on cell growth of, and elastase formation by, *Bacillus* sp. EL31410 and to find the desired temperature cultivation mode through comparing different temperature cultivation condition in 5-L bioreactor.

MATERIALS AND METHODS

Microorganism and culture media

The elastase producing strain *Bacillus* sp. EL31410 was screened and preserved by our lab. The stock culture was maintained at 4 °C on agar slant containing (g/L) beef extract, 4; peptone, 6; yeast extract, 2; NaCl, 5 and agar, 20 (initial pH 7.5). The growth medium composition for seed culture was the same as that of the stock culture except agar. The fermentation medium optimized by Chen *et al*.(2002) consisted of (g/L) glucose, 74; casein, 11.3; corn steep flour, 6.16; K_2HPO_4, 2.06 and $MgSO_4{\cdot}7H_2O$, 0.34 (initial pH 7.5). All media were autoclaved at 121 °C for 20 min, and cooled to room temperature prior to use.

Batch fermentation in shake flask

The inoculum was prepared by transferring a loopful of cells from slant into 25 ml seed medium in 250-ml Erlenmeyer flasks and cultivated for 18 h on a rotary shaker at 200 rpm for 18 h at 37 °C. Elastase production was carried out in incubator shakers. One ml of the seed culture was inoculated into 25 ml fermentation medium (in 250-ml Erlenmeyer flasks) autoclaved at 121 °C for 20 minutes previously. The seed culture was cultivated using incubator shaker operating at 37 °C and 200 rpm. Temperature was controlled to different required temperatures in the range of 28 °C–39 °C.

Batch fermentation in bioreactor

Seed preparation was the same as that for batch fermentation in shake flask. Batch fermentation was carried out in 5-L bioreactor with rotational speed of 300 rpm, inoculation size of 4%, loading coefficient of 0.6, air aeration of 1 vvm, initial pH of 7.5, and required temperature.

Two-stage temperature shift cultivation experiment

37 °C was used from the beginning to 18 h of cultivation, then shifted from 37 °C to 30 °C at 18 h. Other conditions were the same as those in single constant temperature experiments.

Oscillatory temperature cultivation experiment

The cultivation temperature was fixed at 37 °C during the first 3 h, and changed to 30 °C 3 h later, then changed back to 37 °C after 3 h, thus completing a cycle which was repeated till the end of the experiment.

Assays

Biomass was determined by dry cell weight (DCW, g/L). The 10 ml culture broth was centrifuged for 20 min at 3000 rpm, and the pellet was washed twice with distilled water and then dried at 80 °C to constant weight.

Samples was taken from the fermentation broth every 6 h and centrifuged at 6000 rpm for 15 min. Then the supernatant was suitably diluted. Elastase activity (EA) was assayed by the colorimetric method of Sachar (1955). Enzyme preparation was incubated with 20 mg of Congo-red elastin

in 2 ml of 0.2 mol/L boric acid buffer (pH 7.4) with shaking for 20 min at 37 °C and 100 rpm. The reaction was stopped by adding 2 ml of 0.7 mol/L sodium phosphate buffer (pH 6.0), and filtered immediately. Absorbency of the filtrate was read at 495 nm against a control (no enzyme). One unit of elastase activity was defined as the amount of enzyme required to solubilize 20 mg elastin-congo red under the test conditions. Reducing sugar concentration (RSC) was measured according to DNS method (Zhang *et al*., 1997).

Statistical analysis

Every experiment was replicated thrice. Data of interest were analyzed with LSD method.

RESULTS AND DISCUSSIONS

Elastase production at different temperatures in shake flask

In order to study the influence of temperature on *Bacillus* sp. EL31410 cell growth and elastase production, five different temperature (39 °C, 37 °C, 35 °C, 30 °C, 28 °C) experiments were performed in shake flask. Time-course of elastase batch fermentation at the different temperatures is shown in Fig.1. With rising operating temperature, the time taken to reach maximum DCW (Fig.1a) and maximum EA (Fig.1b) value was shortened. During the first 24 h, faster cell growth was observed at higher temperatures (39 °C, 37 °C and 35 °C). After 24 h cultivation, the biomass declined at higher temperatures but kept on increasing at the lower temperatures (30 °C and 28 °C). Maximum DCW appeared at lower temperatures, but lagged that during higher temperatures. During cell cultivation under higher temperatures, the elastase biosynthesis lagged cell growth by about 6 h and then increased obviously. But the period when EA rapidly increased was extended beyond 12 h for 30 °C and 28 °C. At the former stage, the EA of higher temperatures were higher than those of lower ones, but during mid- and later-stage the EA of cells cultivated at 30 °C was the highest. The differences of maximum EA under the five tested temperatures were significant (P=0.05). The consumption of reducing sugar at 39 °C was the fastest and the lowest at 28 °C throughout the process. In addition, a longer stabilization period for the target product appeared also at 30 °C. The reducing sugar at higher temperatures ceased to be used and seem to increase slightly, while at lower temperatures glucose continued to be utilized.

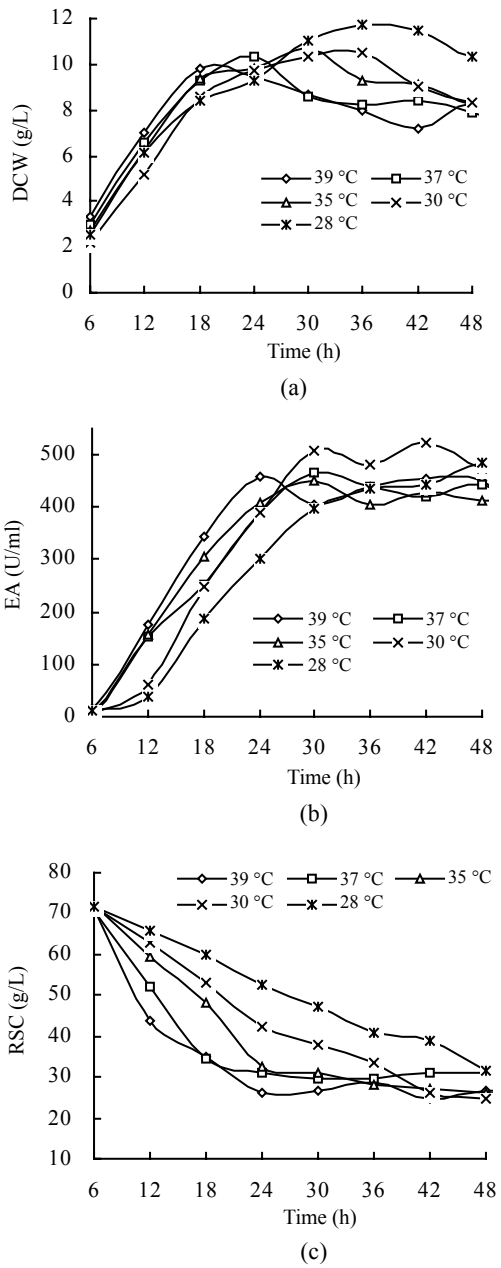

Fig.1 Time-course of elastase production at 39 °C, 37 °C, 35 °C, 30 °C and 28 °C (a) DCW; (b) EA; (c) RSC

High temperature was beneficial for cell growth while low temperature was favorable for elastase biosynthesis. Sometimes cell autolyzation and enzyme inactivity are also accelerated under too high temperature; so targeted product yield is affected negatively (Chu and Li, 2002). However, the operating temperature must not be too low, as biochemical reaction rate typically decreases with decreasing temperature. Thus lower operating temperature would decrease enzyme production and prolong the required fermentation course as well. In addition, it is not easy to control industry-scale fermentation when the temperature is too much lower than the ambient temperature (Feng *et al*., 2003). Based on the discussion above, 37 °C and 30 °C were considered to be beneficial for *Bacillus* sp. cell growth and elastase production, respectively. In order to further verify these results gained from shake flask, more experiments were carried out in 5-L fermenter and some dynamics data was also collected and analyzed.

Effect of temperature on specific growth rate

Fig.2 shows the results of batch fermentation at 37 °C and 30 °C in 5-L bioreactor. Specific growth rate (μ_{cell}) at 37 °C and 30 °C showed similar tendency before 30 h cultivation, although μ_{cell} at 37 °C was higher than that at 30 °C; after 30 h, when μ_{cell} decreased and showed obviously different behavior. μ_{cell} was down to minus at 32 h and 45 h at 37 °C and 30 °C, respectively. The curve fluctuated, especially during the later stage of experiment. It was thought that the microorganism reused the by-products in the broth and grew again during the later stage of cultivation.

Effect of temperature on the specific elastase production rate

Fig.2 shows that during the period of 20 h to 48 h, specific elastase formation rate ($P_{elastase}$) at 30 °C was faster than that at 37 °C. After cultivation for 24 h, the maximum value of $P_{elastase}$ [5.5 U/(h·g DCW)] occurred at 30 °C, but was 4.6 U/(h·g DCW) at 30 h at 37 °C. These data implied that operation at 30 °C in bioreactor improves elastase production and prolongs the period of stable production.

Effect of temperature on specific reducing sugar consumption rate

The consumption rate of reducing sugar at 37 °C was faster than that at 30 °C during the period of the beginning to 36 h. At 37 °C more substrate was contributed to cell growth, so EA (468 U/ml) was

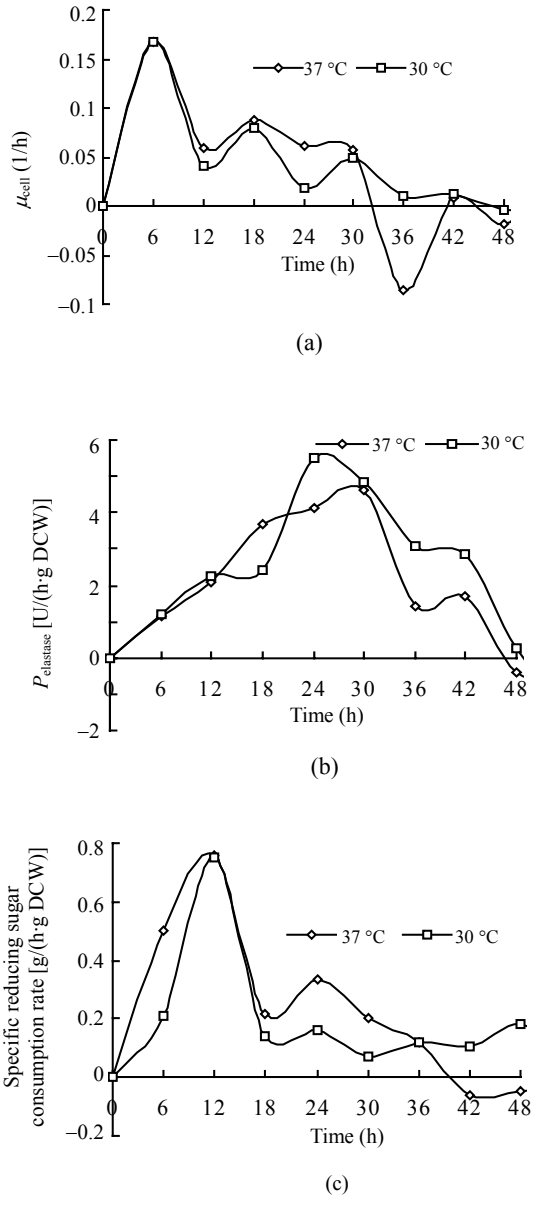

Fig.2 Batch fermentation at 37 °C and 30 °C carried out in 5-L fermenter (a) μ_{cell}, (b) $P_{elastase}$, (c) specific reducing sugar consumption rate

not high. This finding implied that the substrate metabolites may have a role in contributing to elastase production.

Temperature shift protocols

In general, the optimal cultivation conditions for cell growth and enzyme production are the same but are sometimes quite different. There are about three kinds of temperature cultivation mode for fermentation: cultivation at a single constant temperature, two-stage (or multi-stages) temperature-shift cultivation strategy (Zheng *et al*., 2001) and oscillatory temperature cultivation mode (Zhang *et al*., 2002). Based on the results above, if a relatively high temperature was adopted during earlier stage, it will be beneficial for accumulation of DCW and enhance EA indirectly. During the mid- and later-stages, however, lower temperature should not only be beneficial for cell content, but also be favorable for elastase formation and secretion. At the same time, temperature shifted periodically from high to low temperature may better induce production of some enzymes for elastase biosynthesis, so oscillatory temperature cultivation mode was also tested. In the following work, the cell cultivation was carried out in 5-L bioreactor where various parameters, namely constant 37 °C, constant 30 °C, two-stage temperature shift strategy and oscillatory temperature cultivation mode were manipulated and tried.

Effect of different temperature modes

Comparative profiles of elastase fermentation under the four temperature conditions described above are shown in Fig.3. Before 24 h, the biomass of *Bacillus* sp. EL31410 varied with the operating temperatures. As expected, the biomass of two kinds of temperature-shift strategies was higher than that of either 37 °C or 30 °C in this case. Maximum biomass (about 8.6 g/L) was achieved at 37 °C and two-stage temperature cultivation strategy at 30 h and 42 h, respectively. However, for 37 °C, after the DCW peak, it rapidly declined to lower than that of the other cultivation temperatures, for reasons still remaining unknown. And for 30 °C and two-stage temperature cultivation strategy, the cell growth still maintained a suitable rate while the other two dropped after 30 h. These results indicated that temperature shift cultivation strategies favor cell growth.

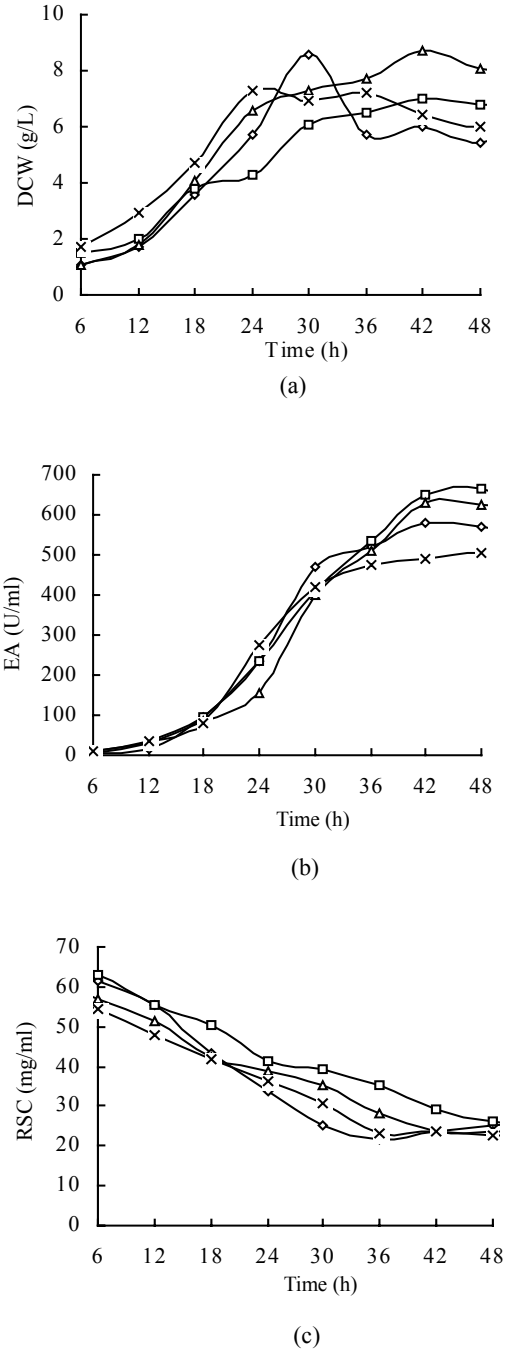

Fig.3 Elastase production by *Bacillus* sp. EL31410 at different temperature mode: (◊) 37 °C, (□) 30 °C, (△) two-stage temperature-shift cultivation strategy, (×) oscillatory temperature mode (a) DCW; (b) EA; (c) RSC

Similar time-course profiles of elastase fermentation were observed among these four temperature cultivation protocols as shown in Fig.3. When cultivated for 24 h, EA of two-stage temperature-shift cultivation strategy was the lowest (P=0.01). The reason was considered that temperature shift from 37 °C to 30 °C at 18 h caused the microorganism to readjust its metabolism system activities. The highest value (652 U/ml) was obtained at 30 °C in the bioreactor. The differences of maximum EA under the four tested temperature conditions were significant (P=0.05). These results suggested that both two-stage temperature cultivation strategy and oscillatory temperature cultivationmode did not significantly improve elastase production level as expected, although it was true that temperature-shift strategies favored to cell growth during the earlier stage of cultivation. The possible reason was that the mRNA of some enzymes for biosynthesis and secretion of elastase was not stable within a certain temperature range. An appropriate decrease in temperature would enhance the stability of the mRNA and prolong the duration of enzyme production. Another reason was that the high concentrations of metabolites and by-products accumulated in culture system probably inhibited enzyme production during the cultivation process (Beg *et al*., 2002).

Before 18 h of cultivation, the reducing sugar was consumed at a high rate under the two-stage temperature shift strategy. After 18 h, a rapidly declining tendency was observed just at 37 °C. During the cell cultivation, glucose was consumed very slowly at 30 °C, RSC was not significant different (P=0.05) at 48 h.

CONCLUSION

This study indicated that the influence of temperature on elastase fermentation was very significant. 37 °C was favorable for cell growth at earlier stage. Maximum elastase activity was achieved when the cells were cultivated at 30 °C. This result was verified when the cells were cultivated at 37 °C or 30 °C in the reactor. Comparison of four different cultivation temperature modes showed that both two-stage temperature shift strategy and oscillatory temperature cultivation mode enhanced biomass but did not have the same result as expected for elastase production. It was concluded that cultivation at constant temperature of 30 °C was appropriate for elastase batch fermentation by the strain of *Bacillus* sp. EL31410. This study's results provide useful information to applied and industrial microbiologists, and process engineers for designing scale-up strategies for process optimisation.

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