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Effect of Fermentation Temperature on Mannatide Production by α-*hemolytic Streptococcus*

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Temperature is one of the most important environmental factors for cell growth and product formation. Batch microbial mannatide fermentations by α-*hemolytic Streptococcus* H1S-33 at various temperatures, ranging from 28 to 39 °C, were studied. The lag time was shorter and the specific cell growth rate and specific mannatide production rate in the early stages of growth were higher at 30 °C or higher temperature. The average cell growth rate was higher and the biomass reached the maximum earlier than other temperature at 37 °C during fermentation 0-11 h and declined obviously after 11 h, but they remained at a higher level at 33 °C after 11 h. Therefore the two-phase temperature control strategy was presented. The temperature control strategy was that the temperature was 37 °C during 0-11 h, it was adjusted to 33 °C with the descending 1 °C an hour after 11 h. The biomass (dry cell weight) and mannatide production yield reached 9.24 g·L^{-1} and 1.16 g·L^{-1} at the variable temperature, respectively, which were higher than those at other temperatures.

Key words: α-*hemolytic Streptococcus*, fermentation, temperature, mannatide.

α-*hemolytic streptococci*, or viridans group streptococci (VGS) are variable pathogenic potential and under certain conditions are responsible for some serious diseases[1]. The pathogenicity and virulence of α-*hemolytic streptococci* have been reported as conditioned pathogens[2-4]. Howerver, a polymannopeptide (mannatide) is isolated from fermentation broth of cultured buccal *a-hemolytic streptococci* strain. Mannatide (Polyactin A, PAA) is a glycopeptide with a variety of biologically active compounds, which is a new immune enhancer developed by China independently[5]. Mannatide has a good efficacy in breast cancer, non-small cell lung cancer, stomach cancer, colon cancer, liver cancer treatment, which was suggested by numerous clinical observations[6]. It can reduce the toxic and side effects of chemotherapy and radiotherapy by improving and enhancing the stress function of body as a tumor radiotherapy chemotherapy adjuvant drugs[7,8]. And it can improve short-term efficacy by improving and regulation immune function of patients and increase white blood cells and platelet count[9]. It also has a good efficacy in aplastic anemia[10], idiopathic thrombocytopenic purpura[11], pancytopenia[12], infectious allergic arthritis[13], a variety of oral mucosal disease[14], a children's recurrent respiratory tract infections[15] and other non-neoplastic diseases. As an important bio-active substances, mannatide are used in clinical medicine, poultry feed and functional foods with more features and properties of its being found[5]. The demand for mannatide will also continue to increase. However, the yield of mannatide is low used α-*hemolytic streptococci*

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produced by submerged fermentation. So, it is a top priority issue to increase the yield of mannatide. Several articles in the literature have discussed production mannatide on strain screening, media, the optimization of fermentation conditions and other aspects[16,17]. However, there is little report about the influence of the cultivation environment such as temperature on production mannatide by α-*hemolytic streptococci* fermentation. In fact, temperature is one of the important environmental factors that affect cell growth and fermentation production formation[18]. It has a great influence on the fermentation process. It mainly affects on various enzyme reaction rates, microbial metabolism regulation mechanism, changing the direction of the synthesis of bacterial metabolites and the physical and chemical properties of the fermentation broth[19-21]. And it affects the fermentation kinetics and the biosynthesis of products[22,23]. Therefore, the purpose of this study was to investigate the effect of fermentation temperature and its control strategy on growth, mannatide production by α-*hemolytic streptococci*.

MATERIALS AND METHODS

Microorganism and culture media

The strains of α-*hemolytic Streptococcus* H1S-33 were provided by the microbiological laboratory of College of Life Yangtze University (Jingzhou, China).

Broth medium ($g \cdot L^{-1}$): beef extract 5.0, peptone 10.0, NaCl 5.0 (pH 7.2).

Blood Agar medium were provided by Beijing AOBOX biological technology co., LTD.

Liquid culture medium ($g \cdot L^{-1}$): glucose 5.0, tryptone 5.0, peptone 5.0, yeast extract 3.0, beef extract 5.0, NaCl 5.0 (pH 7.2).

Seed medium ($g \cdot L^{-1}$): glucose 20.0, peptone 10.0, yeast extract 10.0, beef extract 5.0, NaCl 10.0 (pH 7.5).

Fermentation broth ($g \cdot L^{-1}$): beef extract 10.0, peptone 3.0, yeast extract 7.0, NaCl 5.0 (pH 7.2).

All media were prepared with distilled water and sterilized in autoclave (121 °C, 30min).

Experimental procedure

Frozen bacterial stock of *α-hemolytic Streptococcus* were dilute with broth medium, then incubated in Blood Agar medium at 37 °C for 48h. Single colonies growing well with the green were scraped off the plating with a ring and suspended in 5 ml of liquid culture medium. The cell suspension was used to inoculate 100 ml of liquid culture medium in 250 ml erlenmeyer flasks, which were then incubated at 37 °C, 150 r/min on a rotary shaker for 24 h. The 2 ml cell suspension incubated in 198 ml of seed medium at 37 °C, 150 r/min on a rotary shaker for 18 h. This solution was used as a source of inoculums. The 2 ml inoculums (seed medium) incubated in 198 ml of fermentation broth in each bottle and covered with a cotton cap. The fermentations were incubated in duplicate at six different temperatures (28, 30, 33, 35, 37 and 39 °C) each time. The biomass was assessed by weighing dry cell weight and mannatide production was measured by absorbance in wavelength of 570 nm at 2, 4, 6, 8, 11, 14, 17, 20, 23, 27, 30, 32h, respectively.

Biomass

The biomass was determined by weighing dry cell weight. The seven 90 ml fermentation broth were centrifuged at 19,800×g for 10 min at 37 °C for seven fermentation time. The bacterial cell were collected, washed with distilled water three times and dried in the 105 °C oven to constant weight and cooled in desiccators before weighing for dry cell weight. The fermentation broth was scanned by spectrophotometer (Ledon, UT-1900A UV/V Spectrophotometer, Suzhou, China). The highest absorbance value was at 405 nm when blank fermentation broth was used as a control. The regression equation between the dry cell weight levels ($y\ \mu g \cdot ml^{-1}$) and the absorption value (x) was obtained by $y = 9.913\ x - 0.056$ ($R^2 = 0.986$), which was further used to estimate the biomass. The results were expressed in grams per liter.

Mannatide standard curve

The 10 g D-mannan (National Institutes for Food and Drug Control, Beijing, China) dried in the 105 °C oven to constant weight was dissolve and diluted with distilled water to 100 ml. The concentration of standard mannan solution is 100 $\mu g \cdot ml^{-1}$. Seven volumes (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml) of the standard solution were diluted with distilled water to 2.0 ml. Those solutions mixed immediately with 0.5 ml 2.5% (w/v) α-naphthol and put them into ice water for 5 min, then mixed with 4.5 ml concentrated sulfuric acid, and subjected to boiling water bath for 3 min, put them into ice water

for 10 min. The solution was scanned by spectrophotometer (Ledon, UT-1900A UV/V Spectrophotometer, Suzhou, China). The highest absorbance value was at 570 nm when distilled water was used as a control. The regression equation between the mannan levels (y μg·ml^{-1}) and the absorption value (x) was obtained by y = 15.083 x - 0.246 (R^2 = 0.999), which was further used to estimate the mannatide production.

Definition of mannatide production

The 1 ml of fermentation broth at each measuring time was centrifuged at 19,800×g for 6 min. The 1 ml supernatant was diluted 30 times with distilled water. The 1 ml solution and 1 ml distilled water were mixed and treated according to the mannatide standard curve method. Its absorbance at 570 nm used to calculate the mannan levels of samples by the regression equation. The mannatide productions of samples were estimated and expressed the levels multiplied by the dilution factor.

RESULTS AND DISCUSSION

Effect of temperature on growth of α-hemolytic Streptococcus

The growth rates of α-*hemolytic Streptococcus* were faster before 11 h than those after 11 h at different temperatures (Fig. 1). The maximum specific growth rate occurred in the 4th hour at 30-39 °C, and ahead of 4 h than that at 28 °C. At same time, the maximum specific growth rates increased with increasing of the temperature except 37 and 39 °C and slowed down with of fermentation time. But the biomass was gradually increased with of fermentation time under 28-39 °C (Fig. 2). The lag phase of bacteria growth continued for 4 to 6 h, and the stable phase reached at 28 and 30 °C for 27 h. The lag phase continued for 2 h at the other 4 temperature. However, the stable phase reached at 33 and 35 °C for 23 h, at 37 and 39 °C for 20 and 17 h, respectively. Although the speed of bacteria growth was faster at 39 °C than 37 and 35 °C in the early days, its largest biomass reduced 17% and 13% than that at 37 and 35 °C, respectively. These indicated that it is advantageous to the growth of bacteria with increasing temperature, but it is easy to cause rapid bacterial metabolism at the early stage to advance into the stationary and decline phase because of advanced autolysis seriously at

Table 1. Comparison of fermentation parameters of the mannatide batch fermentation process at different temperatures

Parameters	Temperature (°C)								
	39	37	35	33	30	28	37-33	37-35	
Culture time (h)	32	32	32	32	32	32	32	32	
Maximum biomass culture time (h)	17	20	23	23	27	27	27	27	
Maximum mannatide culture time (h)	27	27	27	27	23	27	27	23	
Maximum biomass (g·L^{-1})	7.58±0.38	9.15±0.46	8.56±0.43	7.92±0.40	7.63±0.38	7.32±0.37	9.24±0.46	9.23±0.46	
Maximum mannatide production (g·L^{-1})	0.95±0.05	0.97±0.05	1.07±0.05	1.12±0.06	0.76±0.04	0.62±0.03	1.16±0.06	1.09±0.05	
Average specific growth rate (h^{-1})	0.091±0.035	0.100±0.027	0.098±0.032	0.098±0.027	0.097±0.015	0.094±0.021	0.097±0.022	0.097±0.017	
Average specific mannatide production rate (g·L^{-1}·h^{-1})	0.096±0.012	0.099±0.015	0.109±0.032	0.114±0.036	0.098±0.035	0.09±0.025	0.108±0.034	0.106±0.026	
Biomass productivity (g·L^{-1}·h^{-1})	0.237±0.012	0.286±0.014	0.268±0.013	0.248±0.012	0.238±0.012	0.229±0.011	0.289±0.013	0.288±0.014	
Mannatide productivity (g·L^{-1}·h^{-1})	0.030±0.002	0.030±0.001	0.033±0.002	0.035±0.002	0.024±0.001	0.019±0.001	0.036±0.002	0.034±0.002	

Table 2. Comparison of average specific growth rate and average specific mannatide production rate at different temperatures

Temperature	Average specific growth rate (h^{-1})		Average specific mannatide production rate ($g \cdot L^{-1} \cdot h^{-1}$)	
(°C)	Within 11 h	After 11 h	Within 11 h	After 11 h
39	0.166±0.008	0.016±0.008	0.183±0.009	0.024±0.001
37	0.179±0.009	0.020±0.001	0.191±0.009	0.022±0.001
35	0.178±0.009	0.017±0.008	0.202±0.011	0.030±0.012
33	0.175±0.009	0.022±0.001	0.208±0.002	0.040±0.002
30	0.161±0.008	0.033±0.002	0.190±0.009	0.022±0.001
28	0.145±0.007	0.042±0.002	0.190±0.009	0.005±0.002

high temperature. Therefore, the higher was the temperature, the shorter was the lag phase, the higher was the maximum specific growth rate, and the shorter reached the growth peak time. To α-*hemolytic Streptococcus*, the maximum specific growth rate, the fastest speed of bacteria growth and reproduction and the largest number of cells all were at 37 °C than the other 5 temperature. Hence, 37 °C is the optimum growth temperature of α-*hemolytic Streptococcus*.

Effect of temperature on the mannatide production

The concentration of mannatide increased gradually along with the growth of cells in the early stage of the fermentation (Fig. 3). The production speed of mannatide accelerated with the increase of temperature. The mannatide production still increased entering growth stability phase and the maximum production occurred at 28 and 30 °C for 20 h and at 33, 35, 37 and 39 °C for 27 h, respectively. The maximum yield of mannatide was 0.95 $g \cdot L^{-1}$ at 39 °C, decreased 16% and 12% than that at 33 and 35 °C, respectively. The mannatide production significantly decreased at the late phase of fermentation because mannatide might be used as nutrient by the bacteria. Therefore, the high temperature is beneficial to the growth of cells and relatively low temperature is more suitable for the synthesis of mannatide at 33~37 °C (Fig. 1, Fig. 2 and Fig. 3).

To better understand the effect temperature on cell growth and mannatide production, the fermentation parameters of the mannatide batch fermentation process at different temperatures were listed in table 1. The biomass, the average specific growth rate and biomass productivity reached the maximum at 37 °C for 20

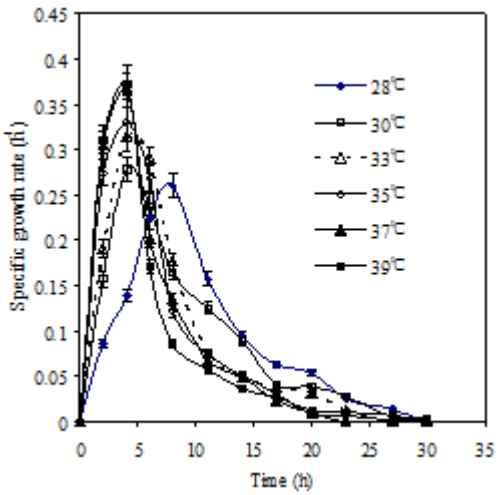

Fig. 1. Effects of different temperatures on specific growth rate of α-*hemolytic Streptococcus*

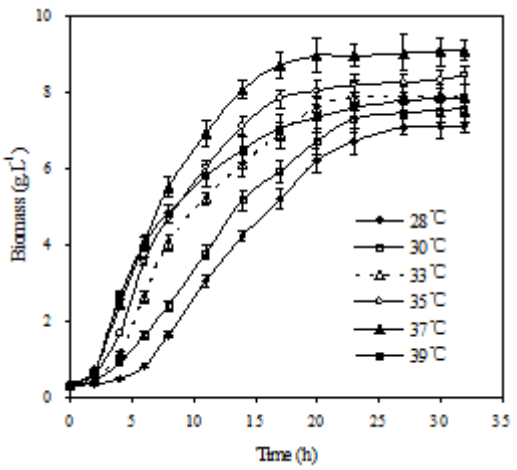

Fig. 2. Effects of different temperatures on biomass formation of α-*hemolytic Streptococcus*

h. While the mannatide production, the average specific mannatide production rate and the mannatide productivity reached the maximum at 33 °C for 27 h during the fermentation process. The relatively high temperature can promote cell growth, and low temperature is more advantageous to mannantide synthesis in batch fermentation process of α-*hemolytic Streptococcus*. Therefore, it is not enough to maintain a single temperature in the process of batch fermentation. It needs to adopt certain temperature change and the control strategy to achieve optimal cell growth and mannatide production.

Effect of two-stage fermentation temperature on growth of α-hemolytic Streptococcus and mannatide production

The average specific growth rate was higher than at the other temperature and the average specific production rate of mannatide was not significant at 37 °C at the early stage of the fermentation (0-11 h). And the average specific growth rate and the average specific production

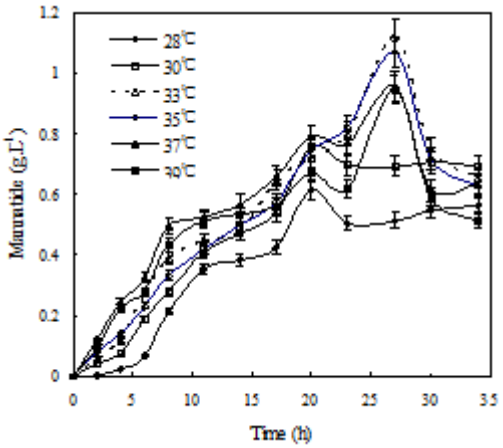

Fig. 3. Effects of different temperatures on mannatide production of α-*hemolytic Streptococcus*

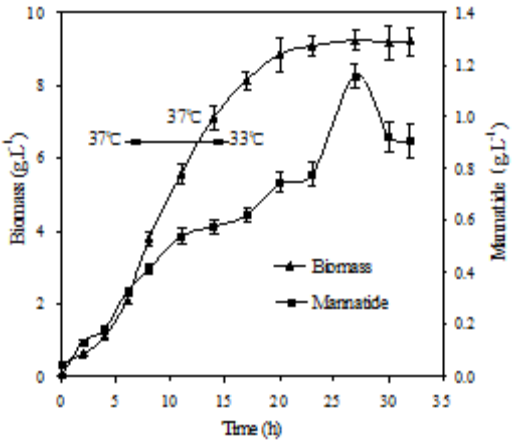

Fig. 4. Effects of different temperatures on mannatide production and biomass formation of α-*hemolytic Streptococcus*

rate of mannatide dropped significantly after 11 h at 37 °C. While when the fermentation temperature is 33 °C, the average specific growth rate and the average specific production rate of mannatide remained at a higher level (Table 2).

Two stage temperature control strategy was used to the process of α-*hemolytic Streptococcus* fermentation based on the above experimental results. The fermentation temperature is 37 °C during 0-11 h so that the bacteria cell reached into the exponential phase earlier. After 11 h, the temperature was adjusted to 33 °C with the descending 1 °C an hour. The results were shown in Table 1 and Fig. 4. The maximum of biomass and mannatide production was 9.24 (g L^{-1}) and 1.16 (g L^{-1}) at the variable temperature, and the maximum of biomass increased 16.7% than that at a single temperature of 33 °C for 27 h, and mannatide maximum production increased 19.6% than that at a single temperature of 37 °C for 27 h. The results were far above those reported in literature [16]. Therefore, Two-stage temperature control strategy balanced mannantide production with biomass.

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