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Full Length Research Paper

A new strategy to enhance polysialic acid production by controlling sorbitol concentration in cultivation of *Escherichia coli* K235

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Polysialic acid (PSA) is a new pharmaceutical material used in control release of protein drugs and as scaffold material in biomedical applications. It is also a vital source of sialic acid and its derivatives. In this paper, we demonstrated that the substrate sorbitol has significant effect on bacterial growth and PSA formation in cultivation of *Escherichia coli* K235. Lower sorbitol concentration favored bacterial growth but retards PSA formation in the fed-batch fermentation. Higher sorbitol concentration stimulated PSA formation but inhibited bacterial growth in the batch fermentation. Sorbitol concentration gradient experiment revealed that PSA formation was seriously limited as sorbitol concentration was lower than 20 g/L, while bacterial growth was obviously inhibited as the sorbitol concentration higher than 40 g/L. A new strategy of controling sorbitol concentration in a range of 20 - 40 g/L in the broth during the fermentation process increased PSA yield by 20%.

Key words: Polysialic acid, fed-batch, sorbitol concentration.

INTRODUCTION

Polysialic acid (PSA) is a polymer of sialic acid with degree of polymerization usually between 8 to 200 residues linked by α-2,8- and/or α-2,9-glycosidic (ketosidic) bonds (Troy, 1996) which exists mostly in the terminal location of the glycoconjugates or the cell membrane surface of mammals and a few bacteria (Angata et al., 2002). As a result of its exterior surface location in certain biomolecules, PSA play important roles in a variety of vital biological processes such as embryogenesis, neural cell growth, differentiation, cell-cell mediating and membrane transport (Vimr et al., 2004). PSA and its derivatives have attracted increasing attention for their potential as innovative pharmaceutical products. In additional to being precursor of sialic acid derivatives, PSA is also used in control release of drugs and as scaffold material in biomedical applications (Wunder et al., 1994; Gregoriadis et al., 2005; Stark et al., 2008; Bruns et al., 2007).

PSA was mainly produced by microbial fermentation. Several research groups worked on the production of PSA with different bacterial strains (Uchida and Tsukada, 1973; Camino et al., 1990; Rodriguez-Aparicio et al., 1988; Rode et al., 2008; Kapre and Shaligram, 2008; Zhan et al., 2002). Rodriguez-Aparicio et al. (1988) found that PSA biosynthesis in bacteria was regulated by temperature as well as influenced by pH-value and aeration of the growth media. Rode et al. (2008) optimized the nutrition requirement for PSA production with *Escherichia coli* K-1 with PSA yield higher than 1500 mg/L. Kapre and Shaligram developed a process for the production of high purity polysialic acid with high molecular weights. A high-yield PSA mutant of *E. coli* K235 had been screened by our laboratory, and its optimum pH for PSA synthesis was pH 6.4 in the previous work (Zhan et al., 2002).

In the production of PSA from *E. coli*, sorbitol is usually chosen as the carbon source. In this work, we found that sorbitol titer in the broth triggered different effect on bacterial growth and PSA synthesis in the

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Abbreviations: PSA, Polysialic acid; **UDP-GlcNAc,** uridine diphophate- N-acetylglucosamine.

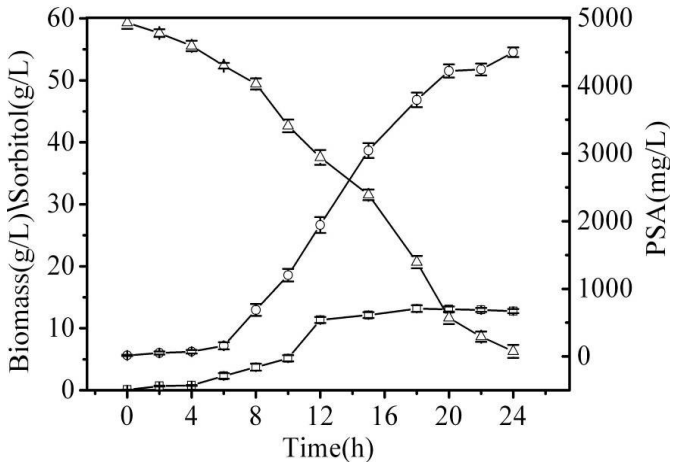

Figure 1. The kinetics of batch cultivation of *E.coli* K235. Biomass (□), PSA (○), and sorbitol (▲).

cultivations of *E. coli* K235. In order to further increase PSA yield, a novel strategy of controlling sorbitol in a suitable level was adapted.

MATERIALS AND METHODS

Microorganism and culture media

A high-PSA strain, *E. coli* K235 was utilized in this study. Fresh slant culture was incubated at 37℃ for 12 h before inoculation.

The seed medium contained (g/L): NaCl 5, peptone 10, and beef extract 3.0 at pH 7.0. The seed culture was prepared in a flask on a reciprocal shaker at 250 rpm and 37℃ for 8 - 10 h.

The medium for batch fermentation contained (g/L): sorbitol 60, ammonium sulfate 5, K_2HPO_4 2.5 and $MgSO_4$ 0.9 and peptone 1.5 at pH 7.8.

Batch and fed-batch cultivation of *E. coli* K235

In the following batch and fed-batch cultivations of *E. coli* K235, the total addition concentration of the sorbitol was 60 g/L.

In the batch cultivation of *E. coli* K235, the initial sorbitol concentration was 60 g/L and 4 % (v/v) seed culture was inoculated into a 30 L fermenter (NLF 30 L, Bioengineering AG, Switzerland) with a working volume 18 l. The aeration and agitation was 1.5 vvm and 600 rpm, respectively. The pH was controlled at 6.4 by adding 14% NH_4OH through a computer-controlled peristaltic pump when the pH dropped to 6.4. The temperature was maintained at 37℃ during the entire fermentation process.

In the fed-batch cultivation of *E. coli* K235 for PSA production, pulse fed-batch, constant feeding rate fed-batch and exponential feeding rate fed-batch cultivations were carried out. Except for sorbitol titer, all the condition in fed-batch cultivations was the same to that of the batch cultivation. All the fed-cultivations were initiated with an initial sorbitol concentration of 10 g/L. In the pulse fed-batch cultivation, 225 g of sorbitol was added into the fermenter at 5, 10, 15, and 20 h of cultivation. In the constant feeding rate fed-batch cultivation, sorbitol solution (800 g/L) was pumped into the fermenter at 5 h at a rate of 120 ml/h. In exponential fed-batch cultivation, the sorbitol feeding rate is determined by Equation 1, which is derived from a mass balance with the assumption of a constant cell yield on sorbitol throughout the fermentation (Ding and Tan, 2006).

$$F = \mu V_0 X_0 \exp(\mu t) / Y_{X/S}(S_i - S) \quad (1)$$

where F is the feeding rate (L/h), V_0 is the initial culture volume (L), μ is the specific growth rate (0.2 h^{-1}), X_0 is the initial cell concentrations (g/L), $Y_{X/S}$ is the theoretical cell yield on substrate (0.25 g biomass/g sorbitol), S_i and S are sorbitol concentrations in the feeding solution and in the fermenter (g/L), t is the culture time (h). The feeding started from the beginning of the exponential phase. Sorbitol was fed into fermenter using a computer-controlled peristaltic pump in accordance with the feeding rate determined by Equation 1.

All the above cultures were carried out in triplicate, and the mean value with standard deviation was presented.

Analytical methods

A 0.5 ml sample was taken out at interval, followed by centrifuged at 10000×g for 10 min. The amount of polysialic acid in the supernatant of the culture was determined by using the resorcinol method (Pesez and Bartos, 1974), and expressed as micrograms of N-acetylneuraminic acid. The amount of sorbitol was determined using the periodic acid-chromotropic acid colorimetric method (Svennerholm, 1957). Biomass was determined by drying the cells at 80℃ to a constant weight.

RESULTS

The kinetics of batch cultivation of *E. coli* K235 was shown in Figure 1. After a 4 h lag, cell growth entered into the exponential growth phase until the end of 12 h. The biosynthesis of PSA started from the early stage of the exponential-phase and lasted until at the end of the fermentation. It seemed to be semi-coupled to the cell growth. Maximum PSA yield reached 4499 mg/L at the end of the cultivation. It can be seen from the Figure 1, residual sorbitol was kept in a high level (40 - 60 g/L) during the exponential growth phase (1 - 12 h), which may retard bacterial growth. This can be alleviated by utilizing the strategy of fed-batch cultivation (Huang et al., 2004). Therefore, different fed-batch cultivations were experimented in the following study.

Pulse fed-batch culture, constant feeding rate fed-batch culture and exponential fed-batch culture were conducted to assess their effect on the PSA production in *E. coli* K235. In the pulse fed-batch cultivation of *E. coli* K235 (Figure 2A), the same amount of sorbitol was added into the fermenter at 5, 10, 15 and 20 h of cultivation. Due to the lower residual sorbitol concentration, biomass increased to 16.68 g/L as compared with 13.18 g/L in the batch culture. However, the PSA yield and PSA yield per gram biomass ($Y_{P/X}$) in the pulse fed-batch culture was 4406 mg/L and 267 mg PSA/g biomass, respectively, which were lower than that of the batch culture (Table 1).

Constant feeding rate fed-batch cultivation was similar to the pulse fed-batch cultivation. It indicated an obvious increase in biomass as compared to batch culture, with a maximum value of 17.72 g/L (Figure 2B). However, its

$Y_{P/X}$ decreased to 264 mg PSA/g biomass, which was 25% lower than that of the batch culture (Table 1).

The exponential fed-batch cultivation (Figure 2C) evidenced the highest biomass of 19.76 g/L, but the lowest $Y_{P/X}$ of 219 mg PSA/g biomass in all the cultivations (Table 1). It seemed that this culture method was preferable for the bacterial growth, but not for the PSA synthesis.

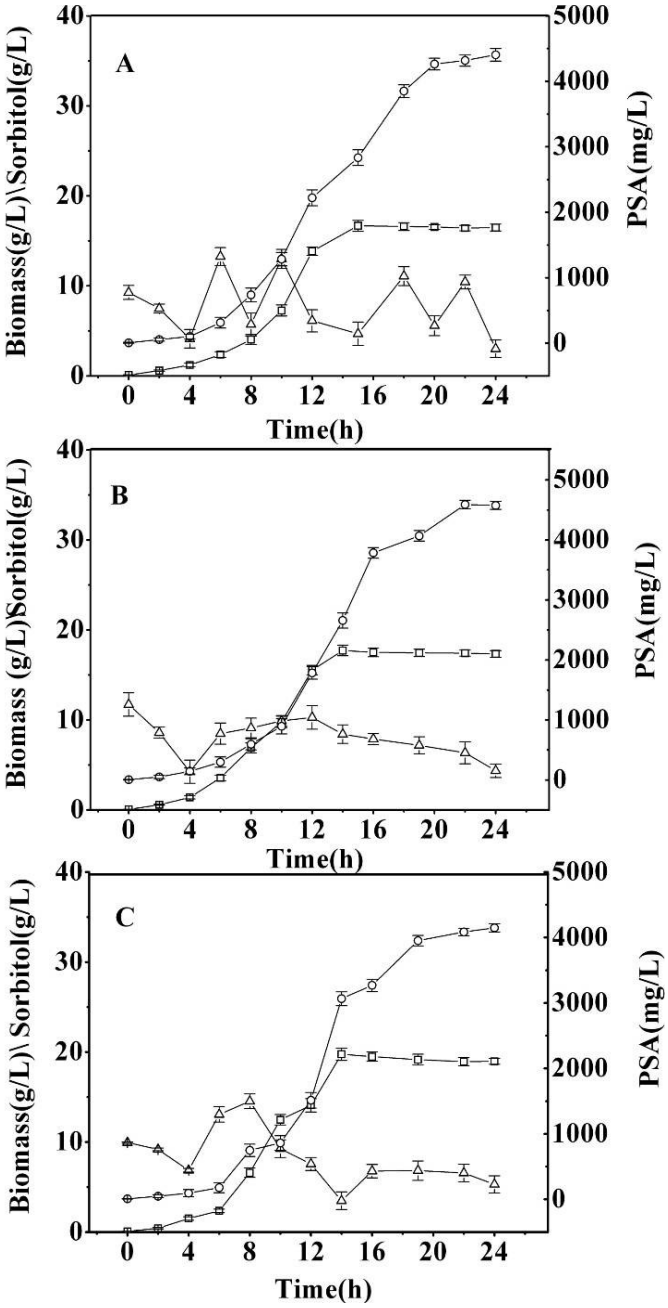

Figure 2. The kinetics of fed-batch cultivation of *E. coli* K235. Pulse feeding fed-batch, (A) constant feeding rate fed-batch, (B) exponential fed-batch (C) cultivation of *E.coli* K235. Biomass (□), PSA (○), and sorbitol (△).

The kinetic parameters from various culture methods on PSA production were shown in Table 1. Batch cultivation showed the highest $Y_{P/X}$ but the lowest biomass in all the culture methods. In the cases with fed-batch cultivations, including pulse fed-batch, constant feeding rate fed-batch, and exponential fed-batch cultivation, an evident increase in biomass but an obvious decrease in $Y_{P/X}$ was observed as compared with the batch culture.

Except for sorbitol titer, all the conditions in fed-batch cultivations were the same to those of the batch cultivation. The total addition concentration of the sorbitol was 60 g/L in batch and fed-batch cultivations of *E. coli* K235, in the batch cultivation, the initial sorbitol concentration was 60 g/L, while in the fed-batch cultivations (pulse fed-batch, constant feeding rate fed-batch and exponential feeding rate fed-batch cultivation), they were all initiated with an initial sorbitol concentration of 10 g/L, and the remaining 50 g/L sorbitol was added into the fermenter in certain methods as described in the materials and methods. Thus, the difference between batch and fed-batch cultivations in this study lies in their residual sorbitol titter during fermentation process: batch culture maintained a relatively high residual sorbitol titter, especially at exponential growth phase with the sorbitol titer in a range of 40-60g/L, where as fed-batch cultures maintained a relatively low residual sorbitol titer around 10 g/L during the whole fermentation process (Figure 2).

As is shown in Table 1, compared to the fed-batch cultivations, batch culture displayed a enhancement of 24.1, 25.0 and 38.1% in the PSA yield per gram biomass ($Y_{P/X}$), but a decrease of 13.2, 10.0 and 24.3% in the specific cell growth rate than the pulse fed-batch, constant feeding rate fed-batch and exponential feeding rate fed-batch cultivations, respectively. It seemed that the residual sorbitol concentration in the medium had total different influence on the cell growth and PSA synthesis. Lower sorbitol titer favors the bacterial growth but limits the PSA synthesis; while higher sorbitol titer facilitates the PSA synthesis but inhibits the bacterial growth.

To further make sure the effect of the sorbitol titer on bacterial growth and PSA synthesis in detail, sorbitol concentration gradient experiment in 500 ml flask on a pH-controllable shaker was carried out. The sorbitol titer levels were set at 10, 20, 40, 60 and 80 g/L, respectively. The maximum cell specific growth rate (μ_{max}) and PSA yield per gram biomass ($Y_{P/X}$) of each level of sorbitol titer was calculated. As shown in Figure 3, on one hand, μ_{max} decreased with the sorbitol titer, especially as the sorbitol titer was higher than 40 g/L. On the other hand, $Y_{P/X}$ increased with the sorbitol titer in the range of 0 - 60 g/L. But as the sorbitol titer increased to 80 g/L, $YP_{/X}$ showed a slight decrease than that of 60 g/L.

As shown in Figure 3, cell growth was inhibited as sorbitol concentration was more than 40 g/L, while a lower sorbitol of 20 g/L would weaken PSA biosynthesis obviously. Therefore, we assumed that the sorbitol concentration in a range of 20 - 40 g/L during the fermentation process

might be a better strategy for PSA production. To achieve this, two different methods were adopted in this study. In the first trial, with an initial sorbitol concentration of 40 g/L, another 20 g/L sorbitol was added into the fermenter as sorbitol concentration was consumed less than 20 g/L. In the second trial, with an initial sorbitol concentration of 30 g/L, another 30 g/L sorbitol was added into the fermenter as the sorbitol concentration less than 20g/L. These two operations could approximately control the sorbitol concentration in the range of 20–40 g/L during the fermentation process. As a result, the former one resulted in a higher yield than the later (data not shown). Its final PSA yield reached 5399 mg/L (Figure 4), which showed a significant improvement over the former highest yield of 4499 mg/L.

Table 1. Comparison of different culture methods for PSA production.

Parameter	Batch culture	Pulse fed-batch	Constant feeding ratefed-batch	Exponential fed-batch
Biomass (g/L)	13.18 ± 0.52	16.68 ± 0.65	17.72 ± 0.58	19.76 ± 0.68
The maximal specific cell growth rate (h^{-1})	0.482 ± 0.022	0.546 ± 0.014	0.530 ± 0.019	0.599 ± 0.013
PSA (mg/L)	4499 ± 72	4405 ± 97	4574 ± 65	4148 ± 64
$Y_{P/X}$ (mg/g)	352 ± 13	267 ± 12	264 ± 9	218 ± 6

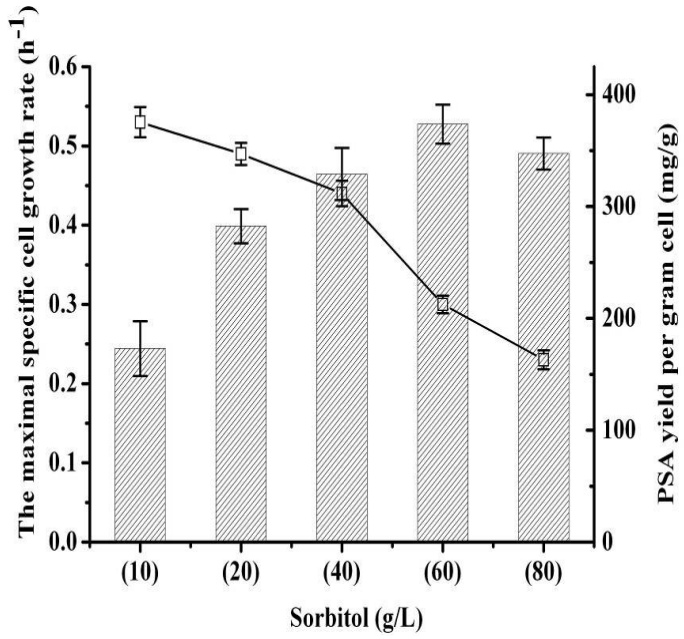

Figure 3. Effect of sorbitol titer on cell growth and PSA synthesis. μ_{max} ,Cell specific growth rate; $Y_{P/X}$, PSA yield per gram cell. μ_{max}(□) and $Y_{P/X}$(▨).

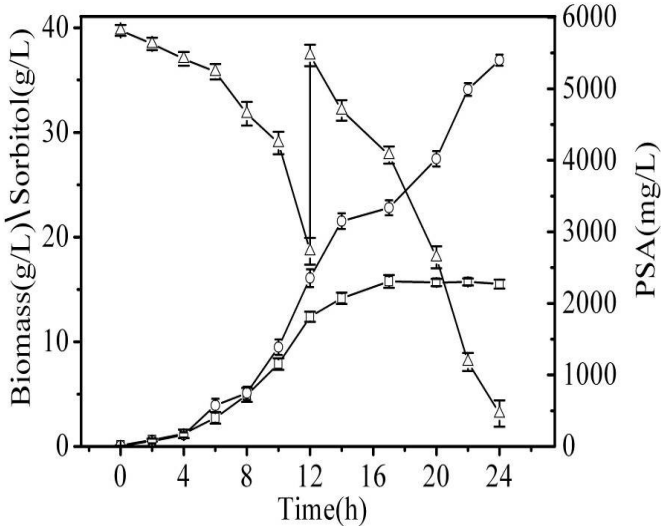

Figure 4. The kinetics of cultivation of *E.coli* K235 with the new strategy. Biomass (□), PSA (○), and sorbitol (▵).

DISCUSSION

Several bacteria such as *Neisseria meningitidis*, *E. coli*, *Haemophilus ducreyi* and *Pasteurella hemolytica* were found excreting PSA as the main components of their capsule (Mizanu and Pohl, 2008). For the report of PSA production with *E. coli*, sorbitol was the mostly utilized carbon source (Rode et al., 2008; Kapre and Shaligram, 2008; Ding and Tan, 2006). In the pathway of PSA synthesis in *E. coli*, UDP-GlcNAc derived from sorbitol is the common precursors for both the PSA and cell wall (lipopoly- saccharide and peptidoglycan) synthesis (Ringenberg et al., 2001). Therefore, as the sorbitol maintained a high level (batch culture), there was adequate UDP-GlcNAc synthesized to support the essential synthesis of cell wall while simultaneously allowing overproduction of the secondary metabolites-PSA, so the higher $Y_{P/X}$ was observed in batch culture method. However, as the sorbitol titer maintained a low level (fed-batch culture), the limited carbon source would be consumed preferably for cell growth and thus the capsular polysaccharide-PSA synthesis was inhibited, which resulted in the lower $Y_{P/X}$ in the fed batch culture methods.

In the further experiment with sorbitol concentration gradient, two critical concentrations was found: sorbitol concentration over 40 g/L would inhibit bacterial growth drastically, while less than 20 g/L would weaken PSA

biosynthesis obviously. Therefore, a new strategy that maintained the sorbitol concentration in a range of 20-40 g/L was proposed. As a result, PSA yield with 5399 mg/L

was observed, which showed a 20% enhancement compared to the former highest yield in the batch culture. This is the first report on the PSA fermentation with a yield higher than 5000 mg/L. Our research indicated that controlling the substrate concentration in a suitable level may be an efficient strategy for the bacterial secondary metabolites production.

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