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An efficient and large-scale preparation process for polysialic acid by *Escherichia coli* CCTCC M208088

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ABSTRACT

Polysialic acid (PSA) is a novel pharmaceutical material used in control release for protein drugs or in biomedical applications as scaffold. An efficient pilot production process for bacterial PSA was developed. Our PSA fermentation process by *Escherichia coli* CCTCC M208088 was optimized in a 500 L fermenter using a novel strategy by controlling pH with ammonia water feeding coupled with sorbitol supplementation. The resulting PSA level increased to 5500 mg/L as compared with the 1500 mg/L of the control. Furthermore, the process for the PSA purification from the fermentation broth was also established. PSA was isolated from the broth by ethanol precipitation, filtration with perlite as filter aid, followed by cetyl pyridinium chloride (CPC) precipitation and lyophilization. The final PSA product obtained had 98.1 ± 1.6% purity at 56.1 ± 1.7% recovery rate. Infrared spectroscopy and NMR spectroscopy analysis indicated that the structure of resulting PSA was identical to the published α-2,8 linked polysialic acid.

1. Introduction

Polysialic acid (PSA) is a polymer of sialic acid with the degree of polymerization usually between 8 and 200 residues linked by α-2,8- and/or α-2,9-glycosidic (ketosidic) bonds which exist mostly in the terminal location of the glycoconjugates or the cell membrane surface of mammals and some bacteria [1,2]. As a result of its exterior surface location in certain biomolecules, PSA plays important roles in a variety of vital biological processes such as embryogenesis, neural cell growth, differentiation, cell–cell mediation and membrane transport [3].

PSA can be mainly used in the control release of drugs and as scaffold material in biomedical applications. PSA is a poor immunogen in humans and other mammals. It does not trigger the formation of antibodies required for phagocytic removal of the invasive organism [4]. On that basis, polysialylation of protein-based drug leads to significant improvement of biological activity and prolongs the residence time of the conjugate in the blood circulation [5]. Furthermore, based on the fact that the biosynthesis of PSA in the adult brain induces and supports neuronal regeneration, combined with its trait of self-aggregates to form solids, PSA is suggested to be an ideal scaffold material for brain operation [6,7].

Due to limited availability, the market price of PSA is as high as US$200 per gram [8]. Bacteria such as *Neisseria meningitides*, *Escherichia coli*, *Haemophilus ducreyi* and *Pasteurella haemolytica* have been found to produce capsular PSA in their culture broth [9,10]. Several groups have been working on the production of PSA with different bacterial strains. However, their yields were rather low and could not meet commercial demand [11–13]. Rodriguez-Aparicio et al. optimized the physical and chemical conditions on the production of PSA by *E. coli* K235 and their PSA production was 1350 mg/L [11]. Rode et al. optimized the culture medium for the production of PSA by *E. coli* K1 and the PSA production was higher than 1500 mg/L [12]. Recently, Kapre and Shaligram developed a process for bacterial PSA production in 30 L fermenter, but their resulting PSA production only reached 3000 mg/L [13].

During PSA production by *E. coli*, the fermentation medium pH has a significant effect on the biosynthesis of the PSA. Rodriguez-Aparicio et al. found the environmental pH had a significant influence on the activity of key enzymes regulating the biosynthesis of PSA in *E. coli* [11]. The optimal pH for the PSA synthesis was determined in our previous work [14]. In addition to the medium pH, the nutritional composition of the medium also influences the PSA biosynthesis significantly, particularly the carbon and nitrogen sources. Sorbitol is the most commonly used carbon source in the PSA fermentation with *E. coli*. Previously, we demonstrated that the biosynthesis of the PSA was inhibited as sorbitol level higher than 40 g/L [15]. Furthermore, it was observed that ammonia concentration could severely affect the cell growth and PSA production with *E. coli*, and a flow injection analysis (FIA) system was employed by Honda et al. to monitor and control the ammonia in an optimal level of 0.3 g/L, which resulted in a maximum PSA production of 1900 mg/L [16].

Hitherto, few reports on the isolation of PSA from the fermentation broth have been published impacting the industrial production of PSA. To the best of our knowledge, there were only two reports

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on the PSA purification from the fermentation broth. Kapre and Shaligram developed an entire process for the PSA purification, but their process needed a series of intricate and expensive column chromatographic processing [13]. Rode et al. also developed a process for PSA purification, but it worked in a small scale with only a 20% recovery yield [12].

In our previous report, we obtained a high-yield PSA strain *E. coli* by conventional mutation. Subsequently, higher PSA production was achieved in the lab level through optimization of the physical and chemical condition for the PSA fermentation [14,15,17]. In the present work, a complete pilot production process for bacterial PSA was developed. PSA fermentation process was optimized and verified in a 500 L fermenter. The process for the purification of bacterial PSA in pilot scale was also established. Our new, efficient and complete process has been shown to be capable of large scale production of high-purity PSA.

2. Materials and methods

2.1. Microorganism and culture media

The parent strain *E. coli* K235 is an original clinical isolate from a patient with urinary tract infection. In order to increase the PSA production, the parent strain *E. coli* K235 was mutated by various approach successively. It was firstly mutated by ultraviolet and then ^{60}Co radiation and subsequently underwent N-methyl-N-nitro-N-nitrosoguanidine (NTG) treatment as well as nitrogen ion implantation.

During the mutation, the agar plate with 1% Bromothymol Blue indicator solution was used as the primary screening for the high-yield PSA strain. The fast-growing colonies with relatively large-size yellow zones were selected for further confirmation in the flask culture. Finally, a mutant strain, *E. coli* CCTCC M208088 was isolated. It demonstrated higher PSA productivity in shake flask cultivation, which was registered by the China Center for Type Culture Collection.

Slant culture medium consisted of (g/L): NaCl 5, peptone 10, beef extract 3.0, agar 20 at pH 7.0. The seed medium-1 contained (g/L): NaCl 5, peptone 10, and beef extract 3.0 at pH 7.0. The seed medium-2 contained (g/L): sorbitol 20, ammonium chloride 0.5, K_2HPO_4 2.5, $MgSO_4$ 0.9 and peptone 1.5 at pH 7.8.

The medium for batch fermentation contained (g/L): sorbitol 40, ammonium chloride 5, K_2HPO_4 2.5, $MgSO_4$ 0.9 and peptone 1.5 at pH 7.8.

2.2. PSA production with *E. coli* CCTCC M208088 in 500 L fermenter

The production of PSA by *E. coli* CCTCC M208088 was carried out in a 500 L fermenter (Lehui Industry Company Ltd., Ningbo, China) with a working volume 300 L. One loop of cells from a fresh slant were transferred to 250 mL flask containing 30 mL seed medium-1 and grew on a rotary shaker for 8 h. The harvested seed culture was inoculated into a 3000 mL flask with 1000 mL seed medium-2 and incubated for 12 h. Then the prepared seed culture was pumped into fermenter with 4% (v/v) inoculation size. The basic fermentation parameters in 500 L fermenter were: temperature 37 °C, aeration 1.5 vvm (volume of air per volume of medium per min) and agitation 300 rpm. Variable parameters and fermentation nutrient supplementation employed are listed in Section 3.

2.3. Purification of PSA from the fermentation broth

After harvest, the fermentation broth was pasteurized at 80 °C for 30 min. The following separation of biomass was achieved by continuous centrifugation at 12,000 × *g* with a flow rate of 2.5 L/min. Ultrafiltration (MWCO 10 kDa) was subsequently conducted to reduce the liquid volume. For the purification of the PSA, a detailed treatment protocol (see Section 3) was developed.

2.4. Analytical methods

A 0.5 ml fermentation broth was sampled, followed by centrifugation at 10,000 × *g* for 10 min. The amount of PSA in the supernatant of the culture was determined by using the resorcinol method [18]. The amount of sorbitol was tested using the periodic acid–chromotropic acid colorimetric method [19]. The level of ammonium chloride was assayed by the method of Nesseler [20]. Biomass was determined by drying the pellet at 80 °C to a constant weight. Protein concentration was analyzed according to the Bradford method [21] and bovine serum albumin (BSA) (0.1 mg/ml) was used as standard. All results were the average of three independent replicate assays.

2.5. IR spectroscopy

The purified PSA was extruded to slice with potassium bromide, and then scanned by the infrared spectrometer IR-440 (Shimadzu, Japan) in the range of 4000–400 cm^{-1}.

2.6. NMR spectroscopy

The structure of the purified PSA was determined with a Bruker BioSpin Corporation (Billerica, Massachusetts, USA) DPX 400 NMR at 35 °C. The PSA was dissolved in D_2O at 80 mg/ml. The ^{13}C 1D spectra were recorded at 100 MHz with ^{13}C carrier at 90 ppm (sw 250 ppm), acquiring 64k complex points (acquisition time 1.3 s), and referenced to an internal TMS standard at 0 ppm.

3. Results and discussion

3.1. Optimization of fermentation process to enhance PSA production

Microbial fermentation for PSA production with *E. coli* CCTCC M208088 was carried out in a 500 L pilot fermenter. As shown in Fig. 1A, sorbitol and ammonium chloride (NH_4^+) were rapidly consumed for bacterial growth and PSA production. Ammonium chloride was exhausted at 16 h of the fermentation process. pH level sharply decreased from initial pH 7.2 to the low value of 4.0 due to the accumulated acidic metabolites and subsequently flatten up to a plateau level. The maximum biomass of 7.6 g/L and PSA production of 1535 mg/L were achieved by the end of the fermentation. It was noteworthy that a steep decline of the bacterial growth and PSA synthesis occurred at the moment when ammonium chloride was exhausted as well as pH decreased to its minimum level. Thus, we inferred that the exhaustion of the nitrogen source and the lower pH at the early stage of fermentation may negatively influence the bacterial growth and PSA synthesis.

Environmental pH has a significant influence on the PSA synthesis and the optimal pH level (pH 6.4) for the PSA fermentation by *E. coli* have been reported in our previous study [11,14]. In addition, constant-speed feeding of NH_4Cl solution during the PSA fermentation employed in our previous study had shown a positive effect on stimulating cell growth, but an excess accumulation of NH_4Cl was usually observed at the stationary phase, which hindered the further synthesis of PSA and caused some problems during the subsequent purification of PSA, particularly in the steps of ethanol precipitation and CPC treatment. Therefore, in order to supply adequate nitrogen source as well as control pH in an optimal level for the PSA fermentation, a novel strategy of feeding ammonia water for control pH at 6.4 was adopted. Using this method, nitrogen