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Production of validamycin A from hemicellulose hydrolysate by *Streptomyces hygroscopicus* 5008

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HIGHLIGHTS

- Corncob hydrolysate was used as a low-cost feedstock for antibiotic fermentation.
- Xylose in corncob hydrolysate had a major contribution to VAL-A biosynthesis.
- Higher VAL-A production from corncob hydrolysate was achieved by an engineered strain.

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ABSTRACT

Validamycin A (VAL-A) is an important agricultural antibiotic produced by *Streptomyces hygroscopicus* 5008, which uses starch as carbon source occupying about 20% of total production cost. To reduce the medium cost, corncob hydrolysate – a hemicellulose hydrolysate was applied as a low-cost substrate to VAL-A fermentation. It was found that three major sugars in corncob hydrolysate including D-glucose, D-xylose and L-arabinose could all be utilized by *S. hygroscopicus* 5008 to produce VAL-A while D-xylose was the main contributor. A higher VAL-A production titer from D-xylose was achieved by using a genetically engineered strain TC03 derived from *S. hygroscopicus* 5008, which resulted in 1.27-fold improvement of VAL-A production from the medium containing 13% (v/v) corncob hydrolysate compared to that by its original strain. A medium cost analysis was done and compared with previous reports. This work indicates a great potential of the hemicellulose hydrolysate as substrate for antibiotic fermentation.

1. Introduction

Validamycin A (VAL-A) is a well-known and important C_7N-aminocyclitol antibiotic, which is produced by *Streptomyces hygroscopicus* var. *jinggangensis* 5008 (hereafter *S. hygroscopicus* 5008). It has been used to efficiently prevent and treat sheath blight disease of corps including rice, and its current market in China is around 30–50 thousand ton per year with annual revenue of billions of RMB (Chinese Yuan). Recently this antibiotic is also recognized as an important raw material for production of an antidiabetic drug, voglibose (Yu et al., 2005). Because of its commercial significance, enhancing the VAL-A production has been the focus of many investigations during past decades (Wei et al., 2012; Zhou et al., 2014, 2011). In another aspect, it is evident that reducing the VAL-A production cost is critical to its large-scale production due to its rather low price. The medium in industrial-scale VAL-A production contains more than 100 g L^{-1} of starch (like rice and corn powder) as carbon source, which takes about 70% of the total medium cost (Wei et al., 2012). Therefore, developing a cheap culture medium by using biomass feedstock as substrates could save the industrial production cost, reduce the competition of the starch based food consumption, and also contribute to the sustainable growth of society and economy.

Hemicellulosic biomass is the second most available renewable resource in nature which has great potential as a substrate for microbial fermentation (Wang et al., 2010). D-Xylose is the most abundant pentose sugar in hemicellulosic biomass and can be utilized as an inexpensive substrate by many microorganisms (Wong et al., 1991; Wu et al., 2014; Ye et al., 2013). In bacteria, D-xylose is usually first isomerized by D-xylose isomerase (encoded by *xylA*) to form D-xylulose, then phosphorylated to generate D-xylulose 5-phosphate by D-xylulose kinase (encoded by *xylB*), and

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subsequently metabolized through the pentose phosphate (PP) pathway (Wong et al., 1991). According to the genome sequencing result of *S. hygroscopicus* 5008 (GenBank accession: CP003275) from our State Key Laboratory of Microbial Metabolism at Shanghai Jiao Tong University (Wu et al., 2012), two putative D-xylose utilizing genes – *xylA* and *xylB* were identified in the genome. These two genes in *S. hygroscopicus* 5008 are clustered and show 91% amino acid identity to the reported genes in *S. rubiginosus* (Wong et al., 1991), implying the former could also utilize D-xylose as carbon source. On the other hand, sedoheptulose-7-phosphate (S-7-P), the key intermediate metabolite of the PP pathway, is the direct precursor for VAL-A biosynthesis. It has been identified that biosynthesizing 1 mol VAL-A requires 2 mol S-7-P (Bai et al., 2006). Consequently, it seems straightforward to use D-xylose as carbon source for the VAL-A biosynthesis since it could be a direct precursor supply.

Corncob hydrolysate (or called corncob molasses, xylose mother liquid) is a typical kind of lignocellulosic biomasses and widely available in China. It is an acid hydrolysate waste which is generated during the industrial-scale production of D-xylose from corncob (Wang et al., 2010). Corncob hydrolysate contains a high concentration of mixed sugars (including D-xylose, L-arabinose and D-glucose) and D-xylose occupies their two-third amount (Cheng et al., 2011). Recently, this low cost and high carbohydrate content feedstock has attracted increasing attention for the production of industrially important chemicals such as acetone, butanol and ethanol (Xiao et al., 2012), propionic acid (Liu et al., 2012), L-lactic acid (Wang et al., 2010), and xylitol (Cheng et al., 2011). However, there is still lack of reports on application of such cheap feedstocks to produce commercially interesting antibiotics like VAL-A.

In this study, the possibility of VAL-A production from corncob hydrolysate was evaluated, and the contribution of each sugar in the corncob hydrolysate to VAL-A biosynthesis by *S. hygroscopicus* 5008 was investigated. As an engineered strain of *S. hygroscopicus* 5008, which contained 3–5 copies of *val* gene clusters by tandem amplification via the zouA system, was recently constructed by our group to enhance the VAL-A production (Zhou et al., 2014), this engineered VAL-A producer was further applied to fermentation with corncob hydrolysate by fed-batch cultivation to achieve a higher production titer. This work would provide valuable information for researches on antibiotic fermentation using biomass hydrolysis as feedstock.

2. Methods

2.1. Bacterial strains

The *S. hygroscopicus* 5008 wild-type strain (Yu et al., 2005) and its engineered strain TC03, which contained 3–5 copies of *val* gene clusters by the zouA tandem amplification (Zhou et al., 2014), were used in this study. *S. hygroscopicus* 5008 was grown on solid Soya-Flour Mannitol (SFM) agar medium (20 g L^{-1} agar, 20 g L^{-1} mannitol and 20 g L^{-1} soybean powder, pH 7.2) at 30 °C for 5 days. Then the spores were collected and stored in 25% glycerol solution at −80 °C. The strain TC03 was grown in liquid Yeast Extract-Malt Extract (YEME) medium, and the mycelia were collected and suspended in 25% glycerol, then stored at −80 °C (Zhou et al., 2014).

2.2. Fermentation conditions

Corncob hydrolysate containing approximately 500 g L^{-1} D-xylose, 190 g L^{-1} L-arabinose and 150 g L^{-1} D-glucose was supplied by Jiahe Sugar Co., Ltd., Shandong, China (Cheng et al., 2011). The seed medium containing 2% (v/v) corncob hydrolysate, 2.2 g L^{-1} soybean meal, 10 g L^{-1} yeast extract, 2 g L^{-1} NaCl and 0.8 g L^{-1} KH_2PO_4 was used for corncob hydrolysate fermentation. The modified Super Optimal Broth (SOB) medium containing 20 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl and 2.5 mM KCl was applied for both single and mixed sugar fermentation. Fermentation medium contained 23 g L^{-1} soybean flour, 5 g L^{-1} yeast extract, 1 g L^{-1} NaCl and 1.5 g L^{-1} KH_2PO_4 supplemented with corncob hydrolysate (at a concentration of 7%, 10%, 13% or 15%, (v/v) or sugars.

For seed culture, 100 μl of spores or mycelia was inoculated into 500 ml shake flakes containing 100 ml seed medium at 30 °C and 220 rpm for 24 h. Then 5 ml of seed cultures was transferred to a 250 ml flask containing 50 mL fermentation medium and cultured at 37 °C and 220 rpm for 5 days. Four independent experiments were performed for each condition.

For the fed-batch cultivation with corncob hydrolysate as carbon source, two feeding modes were performed: (a) the initial corncob hydrolysate concentration was 6.5% (v/v), and then about 1.63% (v/v) corncob hydrolysate was fed at the 36th h, 48th h, 60th h and 72nd h, respectively; and (b) the initial corncob hydrolysate concentration was 13% (v/v), and then corncob hydrolysate was fed from 72nd h to 84th h with addition of 0.1% (v/v) per hour. For each condition, four independent experiments were set up.

2.3. Sampling and analytical methods

For each fermentation experiment, a total of 2 ml fermentation broth was collected from quadruplicate flasks at the designated time. Cells and supernatants were separated by centrifugation at 9000×*g* for 10 min and stored at −80 °C for further analyses. For dry cell weight, due to the insoluble medium component, total intracellular protein was used to represent cell growth as reported earlier (Wei et al., 2012). Standard Bradford method was used to determine the intracellular protein concentration released from the sonication-treated cells (Zhou et al., 2014).

All the metabolites were analyzed by high performance liquid chromatography (1200 Series HPLC, Agilent, Waldbronn, Germany) with different detectors. The supernatants were diluted 5-fold using deionized water and filtered through 0.22-μm-pore-size filters before HPLC analysis. The concentrations of D-glucose, D-xylose and L-arabinose were detected by a refractive index detector (RID) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The mobile phase (5 mM H_2SO_4) was applied with a flow rate of 0.6 ml min^{-1} at 65 °C (Fan et al., 2013). VAL-A and validoxylamine A production were determined by HPLC as previously reported (Yu et al., 2005).

2.4. Statistical analysis

The data consisted of the average of the measurements of four independent sample measurements with the error bars indicating the standard deviation. Statistical significance was analyzed using the Student's *t*-test, where $P < 0.05$ in a two-tailed analysis indicates significance.

3. Results and discussion

3.1. Effect of initial corncob hydrolysate concentration on VAL-A production

In general, a relatively high sugar concentration may lead to a higher VAL-A titer in its fermentation (Wei et al., 2012). To investigate the feasibility of using corncob hydrolysate as a low-cost raw material by *S. hygroscopicus* 5008, various initial concentrations of corncob hydrolysate were attempted for VAL-A fermentation. As