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Process optimization with alternative carbon sources and modulation of secondary metabolism for enhanced ansamitocin P-3 production in *Actinosynnema pretiosum*

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ABSTRACT

Ansamitocin P-3 (AP-3), synthesized by *Actinosynnema pretiosum*, is a microtubule disruptor with significant antitumor activity. Although efforts have been made for the study of ansamitocin biosynthetic gene clusters and its fermentation improvement, the yield and productivity of AP-3 are still limited. In this study, fructose was found to be more beneficial to AP-3 production than glucose, and the culture condition was optimized via single-factor experiments and response surface method. The AP-3 concentration in the Erlenmeyer flasks reached 144 mg/L with the optimized medium containing fructose 9.36 g/L, glycerol 26.79 g/L and soluble starch 3.03 g/L, increased by ninefold compared with that before optimization. The result of medium optimization showed that fructose was an important element for effective increase in AP-3 production. Transcription of genes involved in primary metabolism and ansamitocin biosynthetic pathway was investigated to elucidate metabolic responses of cell metabolism to the substitution of fructose for glucose. It was demonstrated that using fructose as the major carbon source could relieve glucose repression and therefore result in flux rearrangement in primary metabolism for better providing biosynthetic precursors and stimulating the secondary metabolism in *A. pretiosum*. The results obtained might be of particular benefit to further enhancement of ansamitocin productivity.

1. Introduction

Ansamitocins, a group of maytansinoid antibiotics which have extraordinary antitumor activity in vitro via blocking the assembly of tubulin forming into functional microtubules, are produced by *Actinosynnema pretiosum* subsp. *auranticum* ATCC31565 (Cassady et al., 2004; Liu et al., 1996). The antibody-conjugated drug using maytansinoid as the active compound was approved by the United States Food and Drug Administration recently for treating breast cancer (Dhillon, 2013), which presents an extraordinary value to human health and potentially an asset to the commercial market.

Biosynthesis of AP-3 is a complex process (Fig. 1) with the starter unit of UDP-glucose (Yu et al., 2002). Next, UDP-glucose is converted into 3-amino-5-hydroxybenzoic acid (AHBA) with multiple steps of reactions, which is subsequently used for the biosynthesis of proansamitocin through polyketide synthase (PKS) (Kang et al., 2012; Kim et al., 1996; Yu et al., 2002). AP-3 is finally synthesized after a series of post-PKS modifications. During post-PKS modifications, isobutyryl is introduced to the macrocyclic lactam at C-3 position (Spiteller et al., 2003). UDP-glucose, as a connection node between primary metabolism and AP-3 biosynthesis, is formed from glucose-1-phosphate (G1P), an intermediate metabolite reversibly converted from an important primary metabolite of glucose-6-phosphate (G6P) via phosphoglucomutase (pgm) (Gao et al., 2014). The metabolic node of G6P therefore plays an important role in controlling carbon flux distributions into either AHBA biosynthesis through an important intermediate of G1P or several central metabolic pathways including the Embden-Meyerhof (EM) pathway, the pentose phosphate (PP) pathway and the Entner–Doudoroff (ED) pathway. Even though most published studies have focused on ansamitocin biosynthetic gene clusters in recent decades (Bandi et al., 2006; Floss, 2006; Moss et al., 2002; Wu et al., 2011; Yu et al., 2002), it is still necessary to explore the connection between primary metabolism and secondary biosynthesis of AP-3, especially the effects of several commonly used carbon

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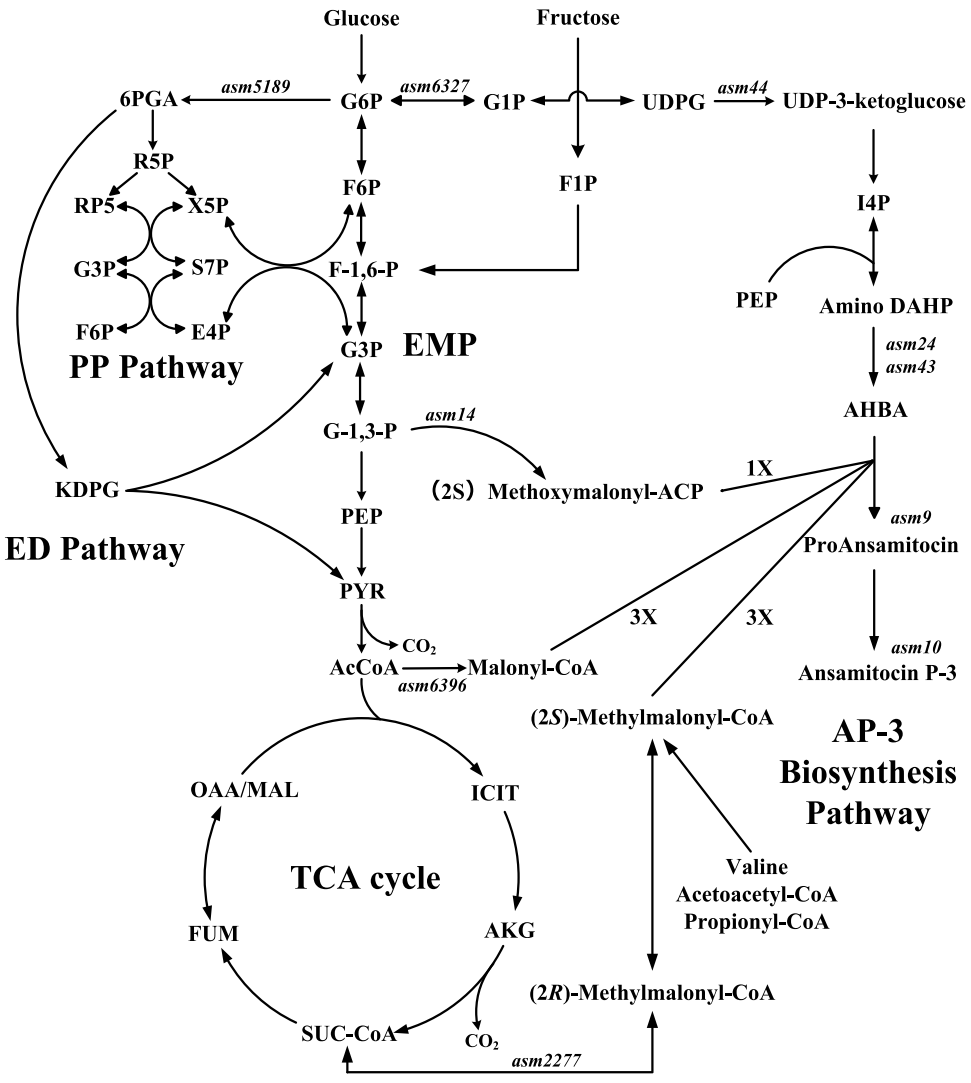

Fig. 1. Simplified metabolic network for AP-3 biosynthesis. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F-1,6-P, fructose-1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; G-1,3-P, glycerate 1,3-diphosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; ICIT, isocitrate; AKG, 2-ketoglutaric acid; SUC-CoA, succinyl-CoA; FUM, fumarate; OAA, oxaloacetate; MAL, malate; 6PGA, 6-phosphogluconate; R5P, ribulose 5-phosphate; RP5, ribose 5-phosphate; X5P, xylulose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; aminoDAHP, 3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; G1P, glucose 1-phosphate; UDPG, UDP glucose; I4P, iminoerythrose 4-phosphate; AHBA, 3-amino-5-hydroxybenzoic acid.

sources on the complicated assembling process for ansamitocin production.

Although AP-3 has shown exciting potential as an antitumor agent, the low yield has impeded its use in commercial applications. Approaches such as genetic modification (Bandi et al., 2006; Ng et al., 2009) and medium optimization (Bandi et al., 2006; Gao et al., 2014; Jia and Zhong, 2011; Lin et al., 2010, 2011; Srinivasulu et al., 2005) have been developed for the improvement of AP-3 production; however, the resulting titers do not meet the requirement of industrial production. Efficient manipulation of fermentation process is therefore still essential to enhance both the yield and productivity of AP-3. As the fundamental element for cell growth and proliferation, carbon sources provide components for cellular carbon backbone and serve as energy for cell functioning. Some of them even play noticeable roles in regulating intracellular primary and secondary metabolism. Response surface methodology (RSM), based on both mathematical and statistical methods, is an effective approach to optimize medium for improving metabolites production, especially secondary metabolites such as validamycin A production (Fan et al., 2013). In *A. pretiosum*, the medium ingredients using sucrose, dextrin, polypeptone and yeast extract (Srinivasulu et al., 2005), or using maltose, dextrin, cotton seed flour and yeast extract (Bandi et al., 2006) have been previously used as corresponding factors based on central composite design (CCD) to improve AP-3 production. The obtained AP-3 yields were still rather low (around 80 mg/L in shake flask fermentation), indicating that additional components might be further required to enhance AP-3 production potential of *A. pretiosum*.

Effects of different types and concentrations of carbon sources on the production are usually first investigated in medium optimization in order to identify the most appropriate carbon source for a specific metabolic product. Although glucose is the most commonly used carbon source for most types of microorganisms, it usually presents negative effects on the production of secondary metabolites. The actinorhodin secretion by *Streptomyces lividans* was found to be significantly inhibited when glucose was present in the medium, where the synthesis of a secondary metabolism-stimulating regulatory protein was repressed (Kim et al., 2001). Similar effects of glucose repression on the production of a number of other antibiotics have also been observed (Escalante et al., 1999; Lounes et al., 1996; Sanchez et al., 2010). Alternative carbon sources or the combination of different carbon sources were