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Overexpression of acetyl-CoA carboxylase in *Aspergillus terreus* to increase lovastatin production

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ABSTRACT

The present work describes the application of homologous recombination techniques in a wild-type *Aspergillus terreus* (**ATCC 20542**) strain to increase the flow of precursors towards the lovastatin biosynthesis pathway. A new strain was generated to overexpress acetyl-CoA carboxylase (ACCase) by replacing the native ACCase promoter with a strong constitutive P*adhA* promoter from *Aspergillus nidulans*. Glycerol and a mixture of lactose and glycerol were used independently as the carbon feedstock to determine the degree of response by the *A. terreus* strains towards the production of acetyl-CoA, and malonyl-CoA. The new strain increased the levels of malonyl-CoA and acetyl-CoA by 240% and 14%, respectively, compared to the wild-type strain. As a result, lovastatin production was increased by 40% and (+)-geodin was decreased by 31% using the new strain. This study shows for the first time that the metabolism of *Aspergillus terreus* can be manipulated to attain higher levels of precursors and valuable secondary metabolites.

Introduction

Lovastatin is a naturally derived drug commonly prescribed to patients suffering from heart-related diseases [1]. Lovastatin binds competitively to the reaction site of hydroxymethylglutaryl-CoA reductase (HMGCR), which catalyses the rate-limiting step in the conversion of hydroxylmethylglutartyl-CoA (HMG-CoA) to mevalonate, an essential precursor for the production of cholesterol [2]. Conversion of HMG-CoA to mevalonate is thereby suspended, and the production of cholesterol ceases. The ability of lovastatin to bind to HMGCR is attributed to its structural similarity to HMG-CoA [3]. Lovastatin can also exert pleiotropic effects that indirectly affect other non-cardiovascular diseases such as cancer, Alzheimer's disease, Parkinson's disease, multiple sclerosis, rheumatoid arthritis and osteoporosis [4]. Lovastatin is produced as a secondary metabolite during the fermentation of sugars by *Aspergillus terreus* (**ATCC 20542**) [4]. This is accompanied by the production of (+)-geodin, a metabolite that is formed from similar precursors to lovastatin [5–7]. While (+)-geodin has fibrinolytic activity [8] and can act as an antimicrobial and antiviral agent [8,9], its production is detrimental to lovastatin yield and purity.

Appropriate manipulation of the *A. terreus* metabolism network may enable improved yields of lovastatin, while raising the efficiency of substrate utilization and its growth. Limited information exists on the *A. terreus* metabolic network, despite its importance in the pharmaceutical industry. Recent developments to improve lovastatin production from *A. terreus* have concentrated mainly on fermentation and environmental optimization [4,10,11]. Strain modification has been limited to random mutation techniques and gene cluster perturbations [12–14]. Metabolic engineering techniques have already shown great potential to enhance valuable compound production by various microorganisms, including in *Aspergillus oryzae* for the production of L-malic acid [15]. To date, while the use of metabolic engineering for enhanced production of lovastatin from *A. terreus* is limited, recent work points to its potential [16].

Fig. 1 depicts a proposed composite metabolic pathway for *A. terreus* that has been assembled from individual pathways produced by various studies that include substrate metabolism [17], lovastatin [18] and (+)-geodin [19] biosynthesis and acyl-CoA metabolism in *Aspergillus* species and other closely related organisms [20]. The carbon flux from glycolysis of the substrate is ultimately directed to acetyl-CoA, a central intermediate precursor in the tricarboxylic acid (TCA) cycle, originating from pyruvate through the action of pyruvate dehydrogenase. A portion of acetyl-CoA is directed towards a subsequent reaction by acetyl-CoA carboxylase (ACCase) to malonyl-CoA. The cellular levels of these two

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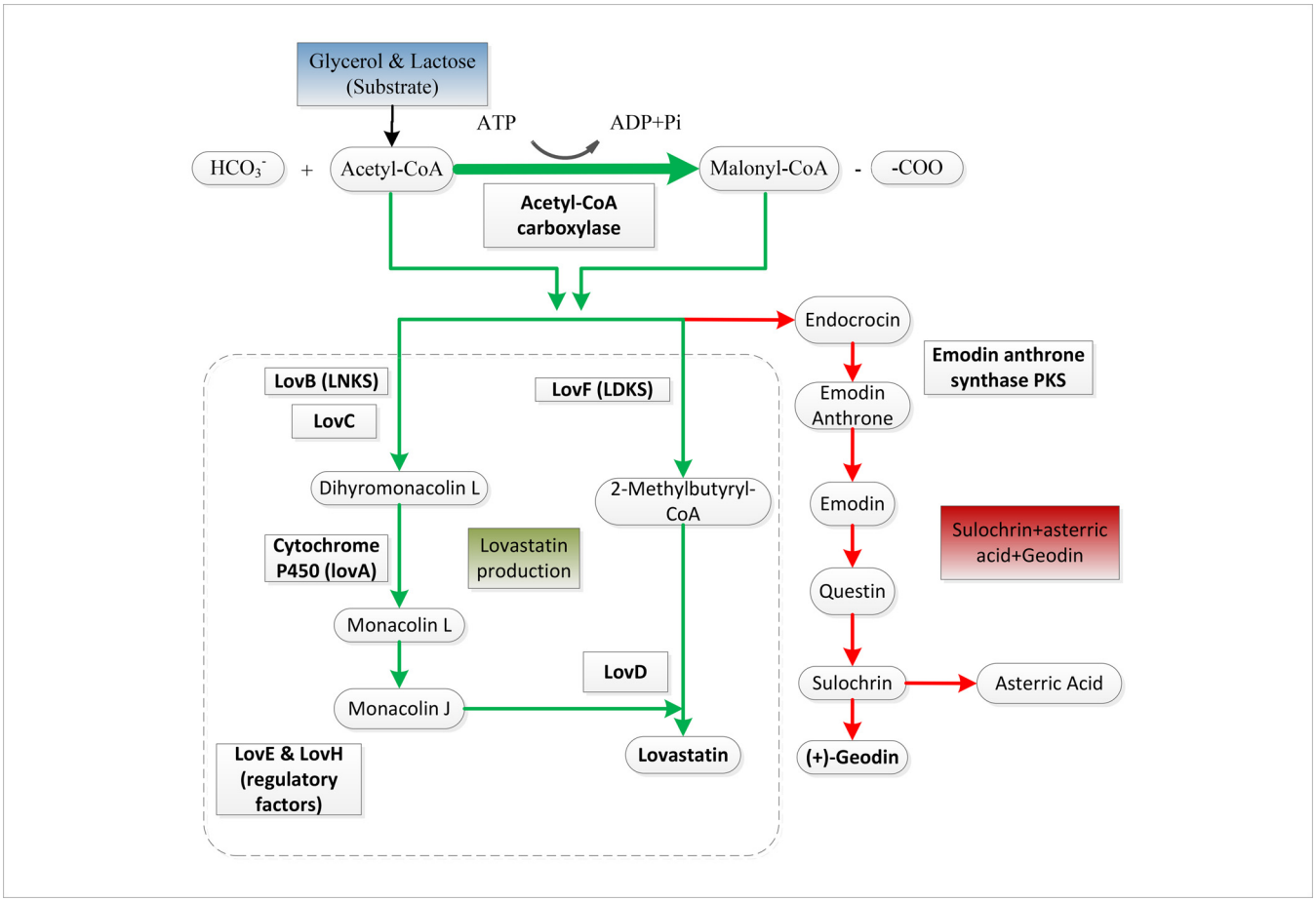

Fig. 1. Proposed metabolic pathway of *A. terreus* showing substrate metabolism, lovastatin and (+)-geodin biosynthesis highlighted in blue, green and red, respectively. The carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACCase) to malonyl-CoA is bolded representing the increase of precursor flow towards the production of lovastatin and (+)-geodin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metabolites are tightly regulated because they are constantly being utilized as the main precursors in multiple secondary metabolite biosynthetic pathways [21]. The biosynthetic pathways of (+)-geodin [19] and lovastatin [13,22] share the same precursors. Lovastatin and (+)-geodin biosynthetic pathways are initiated by the expression of *lov* and emodin anthrone polyketide synthase (PKS) genes, respectively, that utilize acetyl-CoA as a starter platform followed by continuous addition of malonyl-CoA [4]. The basic skeletal formation of both lovastatin and (+)-geodin are somewhat similar to the biosynthesis of fatty acids [18]. It is well established that lovastatin biosynthesis requires eight units of malonyl-CoA and one unit of acetyl-CoA [23], whereas (+)-geodin formation requires seven units of malonyl-CoA and one unit of acetyl-CoA [24]. Enlargement of the CoA pool to increase the desired product has already been studied and validated in specific organisms, especially in *Escherichia coli* [25–30], *Saccharomyces cerevisiae* [29,31], and *Streptomyces venezuelae* [32]. Therefore, overexpression of *A. terreus* ACCase may have a particular carbon flow effect on lovastatin and (+)-geodin productivity.

Metabolic perturbations to produce high value products are not restricted to the alteration of primary metabolism, but also extend to modifying the substrate utilization pathway, allowing modified strains to utilize low-value by-products of the chemical industry (e.g. organic wastes) as substrates to produce high value metabolites [33]. In *A. terreus*, the ability to survive in crude glycerol media and convert low-value feedstock to high-value products is hampered by the presence of impurities in the feedstock [5] and the strict regulation of primary carbon flux within the microbial genome [28]. Identifying and removing the impurities or bypassing the regulatory mechanism would allow more effective use of this type of feedstock and potentially increase the efficiency of the desired product yield. This application not only provides sustainability, but is also able to lower the cost of production. The objective of this work was to generate a new strain of *A. terreus* that can increase the precursors (malonyl-CoA and acetyl-CoA) for lovastatin biosynthesis by overexpressing the ACCase gene, and to test the metabolic response of this strain in low-value carbon feedstocks such as glycerol.

Materials and methods

Construction of acc^{ox} strain

Genetic modification to overexpress ACCase was achieved through integrative homologous recombination using 2-step overlapping extension PCR (OE-PCR) [34]. Sequences for the ACCase gene (*acc*) from *A. terreus* and the strong constitutive promoter for alcohol dehydrogenase (P*adhA*) from *A. nidulans* were retrieved from the *Aspergillus* genome online database (www.aspgd.com). Primers were designed for each gene to be amplified and were produced by IDT® (Iowa, USA) (Table 1). Velocity Taq polymerase (Bioline®, London, England) was used for all PCR reactions and the amplification of individual DNA fragments was based on the manufacturer's instructions.

The construction of a single continuous DNA (SC DNA) fragment containing the native *acc* gene under the control of a P*adhA* promoter with a nourseothricin (*nat*) gene as a selectable marker was undertaken as shown in Fig. 2. The antibiotic resistance gene for the drug nourseothricin with an associated promoter sequence was amplified from