Metabolic Engineering ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Regular Article

Use of pantothenate as a metabolic switch increases the genetic stability of farnesene producing *Saccharomyces cerevisiae*

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ARTICLE INFO

Article history:
Received 17 April 2014
Received in revised form
27 June 2014
Accepted 17 July 2014

Keywords:
Genetic stability
Pantothenate
Metabolic switch
Farnesene
Media development
Yeast

ABSTRACT

We observed that removing pantothenate (vitamin B_5), a precursor to co-enzyme A, from the growth medium of a *Saccharomyces cerevisiae* engineered to produce β-farnesene reduced the strain's farnesene flux by 70%, but increased its viability, growth rate and biomass yield. Conversely, the growth rate and biomass yield of wild-type yeast were reduced. Cultivation in media lacking pantothenate eliminates the growth advantage of low-producing mutants, leading to improved production upon scale-up to lab-scale bioreactor testing. An omics investigation revealed that when exogenous pantothenate levels are limited, acyl-CoA metabolites decreased, β-oxidation was reduced from unexpectedly high levels in the farnesene producer, and sterol and fatty acid synthesis are likely limiting the growth rate of the wild-type strain. Thus pantothenate supplementation can be utilized as a "metabolic switch" for tuning the synthesis rates of molecules relying on CoA intermediates and aid the economic scale-up of strains producing acyl-CoA derived molecules to manufacturing facilities.

1. Introduction

Expanding the population of a genetically engineered microorganism is a necessary facet of realizing commercially relevant quantities of its product molecule. The population must double between 45 and 70 times in order to reach a population size sufficient for industrial scale production ($\geq 1\ m^3$ bioreactor volumes). This requires that each base in the genome must be replicated on the order of 10^{14}–10^{19} times. Given that mutation rates in microorganisms are on the order of 10^{-9}–10^{-11} mutations/bp/replication (Drake, 1991), it is a statistical certainty that every base in the genome will have mutated at least once in the population by the time the target cell density is reached. Some fraction of these mutations will be detrimental to production of the target product. If the product is anabolic in nature (i.e., it requires a net consumption of ATP or NADPH for its synthesis) or is toxic to the cell, there may also be a positive selective pressure for that mutation to increase its frequency in the overall population. By separating fermentation conditions into two distinct phases, a minimum-productivity biomass propagation phase and a longer maximum-productivity production phase, we may reduce the severity of the selection in long fermentations. This is useful since the effect of low producing mutants may only be damaging to production in sufficiently long fermentations (Douma et al., 2011; Gravius et al., 1993; Birch et al., 1989; Nielsen, 2013; Ferea et al., 1999).

One strategy to remove the fitness advantage of low-producing mutants during the biomass propagation phase and thusly reduce their impact during production phase may be accomplished by means of a "genetic switch" where gene expression is transcriptionally reduced by means of an extracellular signal. Industrially robust methods off triggering transcriptional changes include phosphate or nitrogen starvation (Meadows et al., 2010; Cardwell, 2013). Alternatively, we define a "metabolic switch" as a switch based on limiting a vitamin or enzyme cofactor to specifically limit the overall rate of product formation, with minimal impact on pathway gene expression. Such a switch would not trigger protein synthesis but rather alter intracellular metabolite concentrations, and the time required for activation of the switch would depend only on transport of the vitamin inside the cell and the metabolic conversion to the limiting cofactor.

In this paper, we investigate how pantothenate can be used as a metabolic switch to regulate the production rate of β-farnesene in genetically engineered *Saccharomyces cerevisiae*. Farnesene, a sesquiterpene isoprenoid polymer of acetyl-CoA, has many potential applications as a renewable feedstock for diesel fuel, polymers, and cosmetics. (Gronenberg et al., 2013; Rude and Schirmer, 2009). The first committed metabolic step in farnesene synthesis via the

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http://dx.doi.org/10.1016/j.ymben.2014.07.006
1096-7176/

so-called mevalonate pathway (Martin et al., 2003) begins with a condensation of two acetyl-CoA molecules and a third acetyl-CoA is also consumed at the second reaction step of the pathway (Maury et al., 2005). The overall stoichiometry of farnesene synthesis via the mevalonate pathway is:

$$9\ \text{acetyl-CoA} + 9\ \text{ATP} + 3H_2O + 6\ \text{NADPH} + 6H^+ \rightarrow \text{farnesene} + 9\text{CoA} + 6\ \text{NADP}^+ + 9\ \text{ADP} + 3\ P_i + 3\ PP_i + 3CO_2$$

When *S. cerevisiae* metabolizes sugars, the acetyl group of cytosolically synthesized acetyl-CoA is primarily derived from pyruvate decarboxylation and oxidation of acetaldehyde to acetate, which is in turn condensed with coenzyme-A to yield acetyl-CoA (Fig. 1a) (Pronk et al., 1996). The carbon backbone of coenzyme A is synthesized from one molecule of L-cysteine, R-pantothenate (vitamin B_5), and adenosine-triphosphate (Fig. 1a and b). L-cysteine and adenosine-triphosphate are synthesized readily by *S. cerevisiae* for other biosynthetic needs, whereas pantothenate has no other known role in yeast other than for CoA synthesis.

Pantothenate supplementation is common in yeast media, with concentrations of calcium D-pantothenate salt (CAS number: 137-08-6) varying from 0.4 mg/L (Yeast Nitrogen Base (YNB), Wickerham formula) to 1 mg/L (Verduyn et al., 1990), and 12 mg/L (Westfall et al., 2012). Until relatively recently, it was believed that *S. cerevisiae* could not synthesize the molecule de novo and required supplementation to the growth medium (Stolz and Sauer, 1999; Leonian and Lilly, 1942). However, in 2001 it was demonstrated that *S. cerevisiae* has a functional pantothenate synthesis pathway with the Fms1 enzyme shown to catalyze the rate limiting step, although the kinetics of synthesis are not rapid enough to support maximum growth rates on glucose (White et al., 2001). It is also known that pantothenate is transported into the cell by Fen2p, which is reported to have K_m of 3.5 μM (Stolz and Sauer, 1999).

Since pantothenate is the limiting precursor to CoA in *S. cerevisiae* and the transport of pantothenate contributes significantly to its availability to the cell, its concentration in growth media may impact acetyl-CoA levels in the cell and the metabolic rates dependent on those levels. In this paper, we characterized the physiological response of CEN.PK2 yeast engineered to produce high levels of farnesene in different pantothenate concentrations. We found that adjusting the extracellular availability of pantothenate to levels significantly below the reported K_m of Fen2p leads to decreased rates of production of farnesene, improved cell viability and has minimal impact on the maximum growth rate of the engineered yeast. Furthermore, we show that propagating the producer population in a cell culture medium

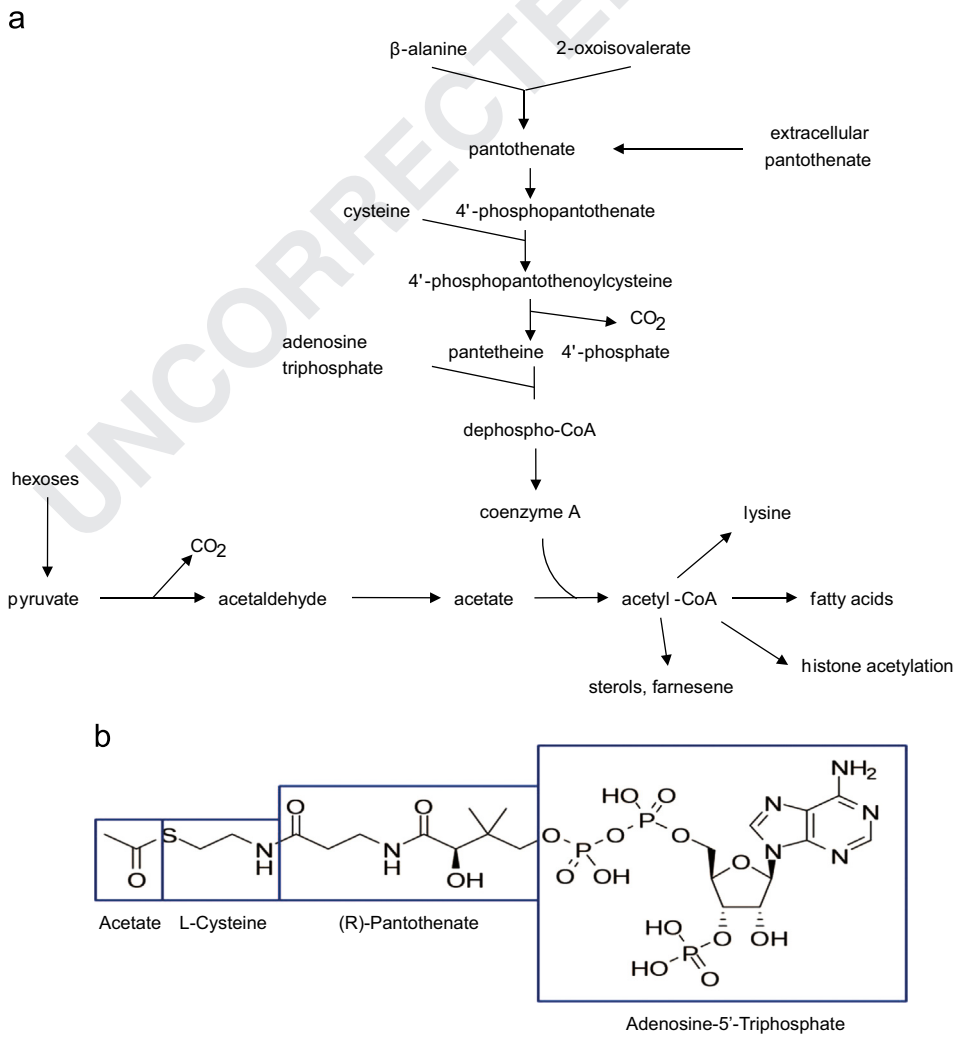

Fig. 1. (a) Primary route of biosynthesis of acetyl-CoA in yeast cytoplasm. (b) Structure of acetyl-CoA, with the metabolic origins highlighted. (R)-pantothenate is the only molecule supplied in minimal yeast media, although it can be synthesized de novo at a rate suboptimal for maximum growth rate.

Please cite this article as: Sandoval, C.M., et al., Use of pantothenate as a metabolic switch increases the genetic stability of farnesene producing *Saccharomyces cerevisiae*. Metab. Eng. (2014), http://dx.doi.org/10.1016/j.ymben.2014.07.006