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Metabolic Engineering 7 (2005) 116–127
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield

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Received 19 August 2004; accepted 29 October 2004
Available online 8 December 2004

Abstract

The potential to produce succinate aerobically in *Escherichia coli* would offer great advantages over anaerobic fermentation in terms of faster biomass generation, carbon throughput, and product formation. Genetic manipulations were performed on two aerobic succinate production systems to increase their succinate yield and productivity. One of the aerobic succinate production systems developed earlier (Biotechnol, Bioeng., 2004, accepted) was constructed with five mutations (Δ*sdhAB*, Δ*icd*, Δ*iclR*, Δ*poxB*, and Δ(*ackA-pta*)), which created a highly active glyoxylate cycle. In this study, a second production system was constructed with four of the five above mutations (Δ*sdhAB*, Δ*iclR*, Δ*poxB*, and Δ(*ackA-pta*)). This system has two routes in the aerobic central metabolism for succinate production. One is the glyoxylate cycle and the other is the oxidative branch of the TCA cycle. Inactivation of *ptsG* and overexpression of a mutant *Sorghum pepc* in these two production systems showed that the maximum theoretical succinate yield of 1.0 mol/mol glucose consumed could be achieved. Furthermore, the two-route production system with *ptsG* inactivation and *pepc* overexpression demonstrated substantially higher succinate productivity than the previous system, a level unsurpassed for aerobic succinate production. This optimized system showed remarkable potential for large-scale aerobic succinate production and process optimization.

Keywords: Escherichia coli; Metabolic engineering; Succinate production; Aerobic fermentation; Gene inactivation; Process productivity; Theoretical yield

1. Introduction

The valuable specialty chemical succinate and its derivatives have extensive industrial applications. It can be used as an additive and flavoring agent in foods, a supplement for pharmaceuticals, a surfactant, a detergent extender, a foaming agent, and an ion chelator (Zeikus et al., 1999). Currently, succinate is produced through petrochemical processes that can be expensive and can lead to pollution problems. Much effort has shifted toward making biocatalysts a viable and improved alternative for the production of succinate. The success of microbial fermentation coupled with the use of renewable carbohydrates would significantly improve the economics of the succinate market (Schilling, 1995).

Various strains such as *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, and *Escherichia coli* have been intensively studied for their potential as biocatalysts in succinate fermentation. The obligate anaerobe *A. succiniciproducens* has shown high potential

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doi:10.1016/j.ymben.2004.10.003

for industrial scale succinate production because of its high conversion yield and productivity when fermented with whey (Lee et al., 2000). However, *A. succiniciproducens* is not practical for commercial fermentation because it is unstable due to its tendency to degenerate, and requires environments absolutely free of oxygen for cultivation (Nghiem et al., 1999). *E. coli* has also been extensively genetically engineered through the use of recombinant DNA technology in recent years to generate strains which showed promise for succinate fermentation. *E. coli* naturally produces succinate as a minor fermentation product under anaerobic conditions (Clark, 1989). Under aerobic conditions, succinate is not produced as a byproduct in *E. coli* and acetate is the main byproduct. Numerous metabolic engineering strategies to enhance succinate production in *E. coli* have met with success. Strains in which enzymatic steps involved in the succinate pathway were amplified and the organism cultured under anaerobic conditions yielded higher succinate production. An example of this was shown when phosphoenolpyruvate carboxylase (*pepc*) from *E. coli.* was overexpressed (Millard et al., 1996). Conversion of fumarate to succinate was improved by overexpressing native fumarate reductase (*frd*) in *E. coli* (Goldberg et al., 1983; Wang et al., 1998). Certain enzymes are not indigenous in *E. coli*, but can potentially help increase succinate production. By introducing pyruvate carboxylase (*pyc*) from *Rhizobium etli* into *E. coli*, succinate production was enhanced (Gokarn et al., 1998, 2000, 2001). Other metabolic engineering strategies also include inactivating competing pathways of succinate. When malic enzyme was overexpressed in a host with inactivated pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldh*) genes, succinate became the major fermentation product (Stols and Donnelly, 1997; Hong and Lee, 2001). In cultures of this *pfl* and *ldh* mutant strain, there is a large pyruvate accumulation. Overexpression of malic enzyme in this mutant strain increased succinate production driven by the high pyruvate pool toward the direction of malate formation, which was subsequently converted to succinate. An inactive glucose phosphotransferase system (*ptsG*) in the same mutant strain (pfl^- and ldh^-) had also been shown to yield higher succinate production in *E. coli* and improve growth (Chatterjee et al., 2001).

The various genetic improvements described above for succinate production have all been done under anaerobic conditions utilizing the mixed-acid fermentation pathways of *E. coli*. Unfortunately, anaerobic fermentation has inherent disadvantages that are difficult to surmount. Anaerobic conditions often cause poor cell growth and slow carbon throughput, therefore generating low production rates. Succinate formation in mixed-acid fermentation is also hampered by the limitations of NADH availability, since 2 mol of NADH are required for every mole of succinate to be formed. Strategies to overcome the anaerobic barrier have included generating enough biomass under aerobic conditions, then switching to anaerobic conditions for succinate production. This was shown to be effective using a "dual-phase" fermentation system, in which initial aerobic growth phase was started then followed by an anaerobic production phase (Vemuri et al., 2002a b).

Absolute aerobic production of succinate in *E. coli* until now has not been feasibly engineered (Lin et al., 2004). *Saccharomyces cerevisiae* has increased succinate production when succinate dehydrogenase (*sdh*) is disrupted to utilize the oxidative pathway of the TCA cycle for aerobic production (Arikawa et al., 1999). The capability to produce succinate under aerobic conditions would mean an active oxidative phosphorylation for generating energy with O_2 present as the electron acceptor. This would lead to higher biomass generation, faster carbon throughput and product formation. In this study, we seek to develop succinate production systems in *E. coli* that can function under absolute aerobic conditions. These systems would be robust and efficient with high succinate yield capability, and productivity.

Under aerobic conditions, the production of succinate is not naturally possible since it is only an intermediate of the TCA cycle. It is formed by succinyl-CoA synthetase, and then subsequently converted to fumarate by succinate dehydrogenase (SDH). Through the oxidation reaction by SDH, succinate provides electrons to the electron transport chain for oxidative phosphorylation. Because of this recycling process, succinate is never detected in aerobic cultures of *E. coli*. Acetate is the only major byproduct of *E. coli* under aerobic conditions. Previously, a novel aerobic succinate production system has been developed that can produce a substantial amount of succinate under aerobic conditions (Lin et al., 2004). This aerobic succinate production system is based on the presence of five mutations (Δ*sdhAB*, Δ*icd*, Δ*poxB*, Δ(*ackA-pta*), and Δ*iclR*) that create an active glyoxylate cycle in the host strain (Fig. 2). This pentamutant strain, with its glyoxylate cycle, can produce succinate as a major product aerobically, but there is still substantial accumulation of pyruvate and TCA cycle C_6 intermediates (citrate and isocitrate). Pathway modeling and simulation of aerobic metabolism shows that a maximum theoretical succinate yield of 1.0 mol/mol glucose consumed can be achieved. Because of the accumulation of pyruvate and TCA cycle C_6 intermediates, the pentamutant strain was hindered from achieving the maximum theoretical succinate yield.

In this study, further genetic modifications are made to the glyoxylate cycle system to improve succinate production and achieve the maximum theoretical succinate yield. Another aerobic succinate production platform was also created to examine its efficiency in