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Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity

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Abstract

A novel in vivo method of producing succinate has been developed. A genetically engineered *Escherichia coli* strain has been constructed to meet the NADH requirement and carbon demand to produce high quantities and yield of succinate by strategically implementing metabolic pathway alterations. Currently, the maximum theoretical succinate yield under strictly anaerobic conditions through the fermentative succinate biosynthesis pathway is limited to one mole per mole of glucose due to NADH limitation. The implemented strategic design involves the construction of a dual succinate synthesis route, which diverts required quantities of NADH through the traditional fermentative pathway and maximizes the carbon converted to succinate by balancing the carbon flux through the fermentative pathway and the glyoxylate pathway (which has less NADH requirement). The synthesis of succinate uses a combination of the two pathways to balance the NADH. Consequently, experimental results indicated that these combined pathways gave the most efficient conversion of glucose to succinate with the highest yield using only 1.25 moles of NADH per mole of succinate in contrast to the sole fermentative pathway, which uses 2 moles of NADH per mole of succinate.

A recombinant *E. coli* strain, SBS550MG, was created by deactivating *adhE*, *ldhA* and *ack-pta* from the central metabolic pathway and by activating the glyoxylate pathway through the inactivation of *iclR*, which encodes a transcriptional repressor protein of the glyoxylate bypass. The inactivation of these genes in SBS550MG increased the succinate yield from glucose to about 1.6 mol/mol with an average anaerobic productivity rate of 10 mM/h(~0.64 mM/h-OD_{600}). This strain is capable of fermenting high concentrations of glucose in less than 24 h. Additional derepression of the glyxoylate pathway by inactivation of *arcA*, leading to a strain designated as SBS660MG, did not signicantly increase the succinate yield and it decreased glucose consumption by 80%. It was also observed that an *adhE*, *ldhA* and *ack-pta* mutant designated as SBS990MG, was able to achieve a high succinate yield similar to SBS550MG when expressing a *Bacillus subtilis* NADH-insensitive citrate synthase from a plasmid.

Keywords: *Escherichia coli*; Pathway design; Metabolic engineering; Succinate production; High productivity; Dual production pathways

1. Introduction

Succinate produced by fermentation represents a potential route to the production of commodity chemicals from renewable feedstocks (Zeikus et al., 1999; Kim et al., 2004). Succinate has drawn much interest because it has been used as a precursor of numerous chemicals including pharmaceuticals and biodegradable polymers (Hong and Lee, 2002). Many attempts have been made to metabolically engineer the anaerobic central metabolic pathway of *Escherichia coli* to increase succinate yield and productivity. Among the numerous efforts made to make succinate the major fermentation product in *E. coli* are the inactivation of genes expressing fermentative enzymes such as lactate

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dehydrogenase (LDH) (Mat-Jan et al., 1989), both the LDH and pyruvate formate lyase (PFL) (Bunch et al., 1997) and deletion of multiple enzymes including PFL, LDH and the glucose-specific phosphotransferase system permease (PTSG) (Donnelly et al., 1998; Chatterjee et al., 2001). Other studies include overexpression of different enzymes such as phosphoenolpyruvate carboxylase, (PEPC) (Millard et al., 1996), malic enzyme (Stols and Donnelly, 1997; Hong and Lee, 2000), pyruvate carboxylase (PYC) (Gokarn et al., 1998, 2000; Vemuri et al., 2002a) and coexpression of both PEPC and PYC (Lin et al., 2004b). External means have also been developed in order to increase succinate production such as utilizing a dual phase fermentation production mode which comprises an initial aerobic growth phase followed by an anaerobic production phase or/and by changing the headspace conditions of the anaerobic fermentation using carbon dioxide, hydrogen or a mixture of both gases (Nghiem et al., 1999; Vemuri et al., 2002b).

2. Pathway design to achieve high succinate yield and concentration from glucose

There are several routes that can form succinate including; the reductive branch of the TCA cycle, also known as the fermentative pathway (which is primarily active under fully anaerobic conditions), and the glyoxylate pathway, which is essentially active under aerobic conditions upon adaptation to growth on acetate (Gui et al., 1996).

The fermentative pathway converts oxaloacetate (OAA) to malate, fumarate and then succinate and this pathway requires 2 moles of NADH per mole of succinate produced. One major obstacle to high succinate yield through the fermentative pathway is due to NADH limitation. This is because 1 mole of glucose can provide only 2 moles of NADH through the glycolytic pathway; however, the formation of 1 mole of succinate through the native fermentative pathway requires 2 moles of NADH. Therefore, the maximum theoretical yield (molar basis) of succinate from glucose is limited to one assuming that all the carbon flux will go through the native succinate fermentative pathway. Fig. 1A shows the wild-type *E. coli* anaerobic central metabolic pathway (the theoretical fluxes to achieve the maximum theoretical yield are also shown). The fluxes are based on 100 moles of glucose uptake. Because 200 moles of NADH are generated, only 100 moles of succinate can be formed. Due to NADH availability, any redirection of the carbon flux to OAA (for example by a heterologous PYC) will give no more than 100 moles of succinate. The overall succinate yield can be improved by increasing intracellular NADH availability. This can be accomplished either by diverting some of the carbon flux for NADH generation, or by supplying NADH through some other means. Our laboratory recently has reported a succinate production system that aims to provide higher NADH availability in order to increase succinate yield by inactivating NADH competing pathways and to benefit from potential NADH generating pathways (Sánchez et al., 2005). However, another approach to surpass this limitation is to employ a succinate production pathway that does not involve NADH or with a reduced stoichiometric NADH/succinate molar ratio.

In this study a genetically engineered *E. coli* strain has been constructed to possess a dual succinate synthesis route to produce succinate under fully anaerobic conditions (Fig. 1B). A high succinate yield recombinant *E. coli* strain was created by deactivating four different enzymes, three of these enzymes are involved in the central anaerobic pathway, the fourth one, the IclR transcriptional repressor (encoded by the gene *iclR*), regulates the expression of the *aceBAK* operon involved in the induction of the glyoxylate pathway upon growth on acetate under aerobic conditions (Gui et al., 1996). Additionally a plasmid encoding pyruvate carboxylase (PYC) from *Lactococcus lactis* has been transformed into the quadruple mutant to further improve succinate production.

The glyoxylate pathway converts 2 moles of acetyl-CoA and 1 mole of OAA to 1 mole of succinate and 1 mole of malate (Kornberg, 1966). However, the malate can be further converted to succinate using only 1 mole of NADH (Fig. 1). In sum, 2 moles of succinate can be formed from 2 moles of acetyl-CoA, 1 mole of OAA and 1 mole of NADH. Since the NADH production and consumption needs to be balanced, there should be appropriate flux proportioning among the routes to succinate. Hence, through metabolic engineering it is possible to activate the glyoxylate pathway concomitantly with the traditional fermentative pathway to achieve the highest possible succinate yield under fully anaerobic conditions.

Our current strategy increases succinate levels and yield by efficiently diverting carbon present in glucose towards succinate and by recapturing the reducing equivalents necessary for its synthesis.

In essence, the rationale behind the implementation of these mutations in *E. coli* MG1655 strain is (see Fig. 1B):

1. Deletion of *ldhA*. The main objective of knocking out the lactate pathway is to conserve both NADH and carbon atoms and help channel carbon to the acetyl-CoA pool.
2. Deletion of *adhE*. The ethanol pathway deletion is designed to conserve both NADH for further succinate formation through the native fermentative pathway; and carbon atoms in the form of