Metabolic Engineering 12 (2010) 499–509

Contents lists available at ScienceDirect

Metabolic Engineering

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

Metabolic impact of the level of aeration during cell growth on anaerobic succinate production by an engineered *Escherichia coli* strain

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ARTICLE INFO

Article history:
Received 9 May 2010
Received in revised form
7 September 2010
Accepted 21 September 2010
Available online 29 September 2010

Keywords:
Succinate
Aeration effect
E. coli
Transition

ABSTRACT

The metabolic impact of two different aeration conditions during the growth phase on anaerobic succinate production by the high succinate producer *Escherichia coli* SBS550MG (pHL413) was investigated. Gene expression profiles, metabolites concentrations and metabolic fluxes were analyzed. Different oxygen levels are known to induce or repress transcription, synthesis of different enzymes, or both, affecting cell metabolism and thus product yield and productivity. The succinate yield was 1.55 and 1.25 mol succinate/mol glucose, and the productivity was 1.3 and 0.9 g L^{-1} h^{-1}) for the low aeration experiment and high aeration experiment, respectively. Changes in the level of aeration during the cells growth phase significantly modified gene expression profiles and metabolic fluxes in this system. Pyruvate was accumulated during the anaerobic phase in the high aeration experiment, which could be explained by a lower *pflAB* expression during the transition time and a lower flux towards acetyl-CoA during the anaerobic phase compared to the low aeration case. The higher PflAB flux and the higher expression of genes related to the glyoxylate shunt (*aceA*, *aceB*, *acnA*, *acnB*) during the transition time, anaerobic phase, or both, improved succinate yield in the low aeration case, allowing the system to attain the maximum theoretical succinate yield for *E. coli* SBS550MG (pHL413).

1. Introduction

Facultative organisms are able to grow under aerobic and anaerobic conditions by changing their cell physiology and metabolic pathways to adapt to the new environment. Changes in cell metabolism are controlled by sensing and regulatory systems that sense oxygen levels and transmit a signal to modify gene expression accordingly (Sawers, 1999). *Escherichia coli* has several sensing mechanisms, among them the Fnr and Arc regulator systems have been studied (Becker et al., 1996; Shalel-Levanon et al., 2005a, 2005b; Spiro and Guest, 1990). In anaerobic conditions *E. coli* metabolism generates several fermentation products such as acetate, lactate, formate, ethanol and succinate in various yields. Efforts to genetically modify *E. coli* and carefully control growth conditions to generate high production of a single product have been subjects of many investigations in applied microbiology.

Succinate is widely used in industry as additive in food and pharmaceuticals, as well as a precursor of biodegradable polymers, surfactants, synthetic resins, among other uses (Hong and Lee, 2002; Lee et al., 2004; Zeikus et al., 1999). *E. coli* does not naturally accumulate succinate; its natural production is rather low (maximum theoretical yield of 1 mol succinate per mol of glucose). In the past few years, several engineered *E. coli* strains have been created to produce succinate from glucose at various yields. Some approaches include the deletion of genes encoding enzymes involved in competing pathways such as LdhA (Jantama et al., 2008b; Mat-Jan et al., 1989; Sanchez et al., 2005b), AdhE (Jantama et al., 2008b; Sanchez et al., 2005b), PflAB (Jantama et al., 2008b) and AckA-Pta (Jantama et al., 2008b; Sanchez et al., 2005b); the overexpression of enzymes that channel the carbon towards succinate through the formation of oxaloacetate, such as Ppc and Pyc (Gokarn et al., 1998, 2000; Lin et al., 2005; Millard et al., 1996; Vemuri et al., 2002a; Wang et al., 2006), or through malate formation by the malic enzyme (Hong and Lee, 2001; Kwon et al., 2007; Stols and Donnelly, 1997; Stols et al., 1997). An *E. coli* strain with a deletion in the isocitrate lyase repressor gene (*iclR*) (Sanchez et al., 2005b) and an *Aspergillus niger* strain overexpressing the isocitrate lyase gene (*icl*) have also been constructed to potentially increase the flux through the glyoxylate shunt and therefore increase succinate production. However, *iclR* deletion in *E. coli* did not show significant difference in succinate production and *icl* overexpression in *A. niger* produced an increase in fumarate instead of succinate (Meijer et al., 2009). On the other hand, the deletion of transporter related genes, such as the glucose-specific permease of the phosphotransferase system, Ptsg

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doi:10.1016/j.ymben.2010.09.002

(Chatterjee et al., 2001; Donnelly et al., 1998) and the formate transporter, FocA (Jantama et al., 2008a) have been performed to increase succinate production.

The strain used in this work was SBS550MG (pHL413), created by Sánchez and collaborators (Sanchez et al., 2005b). This strain has the following genes deleted *adhE*, *ldhA*, *iclR* and *ackA-pta* and overexpresses the gene *pyc* encoding for pyruvate carboxylase from *Lactococcus lactis*. This strain has shown to produce succinate at high yields, 1.6 mol succinate/mol glucose in a dual-phase system; where the first phase was aerobic and the cells were grown in shake flasks followed by an anaerobic phase where succinate was produced in shake flasks or batch bioreactor (Sanchez et al., 2005b, 2006). This yield value is also the theoretical maximum for this strain (Cox et al., 2006; Sanchez et al., 2006). In this study we developed the entire process for succinate production by *E. coli* strain SBS550MG (pHL413) in a bioreactor; where the strain was grown aerobically and then the condition was switched to anaerobic in the same bioreactor for succinate production. The centrifugation step was then eliminated saving time and resources, which is desirable for scale up purposes.

The physiological state of the cells is critical in a dual-phase system where the enzymes produced during the first phase (aerobic-growth phase) will influence the performance of the cells in the anaerobic-production phase. In the present study, we analyzed the metabolic effect of two different aeration conditions during the growth phase on metabolite and gene expression profiles, as well as the metabolic fluxes, during the production phase to better understand the differences in cell metabolism that originate differences in product distribution and, elucidate potential bottle necks and possible strain improvements.

2. Material and methods

2.1. Strain

The *E. coli* strain SBS550MG (pHL413) was used for the experiments. This strain contains the following gene deletions: *adhE ldhA iclR ackpta*::*Cm* (Sanchez et al., 2005b) and harbors the pHL413 plasmid containing the pyruvate carboxylase gene (*pyc*) from *L. Lactis* in pTrc99A, Ap^R (Lin et al., 2004).

2.2. Batch cultures

The fermenter system used was a 1.0 L Bioflo 110 fermenter (New Brunswick Scientific) equipped with a glass pH electrode and a polarographic dissolved oxygen (DO) electrode Ingold InPro 6000 (Mettler Toledo) to monitor and control pH and dissolved oxygen conditions, respectively. The medium used for the aerobic phase contained 20 g/L tryptone, 10 g/L yeast extract, 0.9 g/L $K_2HPO_4 \times 3H_2O$, 1.1 g/L KH_2PO_4, 3 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \times 7\ H_2O$, 0.25 g/L $CaCl_2 \times H_2O$, 200 mg/L ampicillin, 30 μL/L antifoam sigma #204, 1 mg/L thiamine, 1 mg/L biotin, 2 g/L glucose. Another 2 g/L of glucose were added when the initial glucose was depleted. No pH control was applied during aerobic growth. After the additional glucose was depleted and the pH increased to 7.4–7.5, the air flow was stopped and a 0.2 L/min CO_2 flow was established. At this time 20 g/L glucose were added to the culture. Samples were taken at different times and their metabolite concentrations were determined by HPLC.

2.3. Extracellular metabolites analysis

Extracellular metabolites such as glucose, succinate, formate and pyruvate among others were analyzed by HPLC as describer earlier (Sanchez et al., 2005b). In brief, the HPLC system (Shimadzu-10A System, Shimadzu, Columbia, MD, USA) was equipped with a cation-exchange column (HPX-87H, Biorad Labs, Hercules, CA, USA), a differential refractive index (RI) detector (Waters 2410, Waters, Milford, MA, USA) and an ultraviolet (UV) detector (Shimadzu SPD-10A). The mobile phase used was a 2.5 mM H_2SO_4 solution at a 0.6 mL/min flow rate. The column was operated at 55 °C.

2.4. Metabolic fluxes analysis

The metabolic network considered for the analysis, was adapted from the network described by Sánchez and collaborators (Sanchez et al., 2006). In brief, 17 fluxes were considered, v_1–v_{17}, including glycolysis, glyoxylate shunt, anaerobic fermentation reactions, heterologous conversion of pyruvate into oxaloacetate, catalyzed by the *L. lactis* pyruvate carboxylase (Pyc), and malate, succinate and pyruvate excretion. The redox balance for NADH was also included assuming no NADH accumulation (Sanchez et al., 2006). *E. coli* strain SBS550MG (pHL413) did not show growth during the anaerobic phase, thus the flux to biomass (v_2) was assumed to be zero. In the present work, the high aeration experiment showed pyruvate accumulation; then pyruvate excretion was also included in the metabolic flux analysis. The stoichiometric matrix including the above fluxes and the NADH balance was based on the pseudo-steady-state hypothesis (PSSH) for the intracellular intermediate metabolites and the law of mass conservation (Stephanopoulos et al., 1998). The description of the metabolic flux determination is presented in Appendix A.

2.5. Gene expression analysis

2.5.1. RNA preparation

Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Samples that were not extracted immediately were treated with Protect reagent from Qiagen (Valencia, CA, USA), frozen using liquid nitrogen and stored at −80 °C until extraction. The isolated RNA was treated with DNaseI (Promega, Madison, WI, USA) and RNase Inhibitor (Promega, Madison, WI, USA) according to manufacturer protocol. The reaction was incubated at room temperature for 15 min. The RNA was then extracted with Phenol/CIA extract solution (CIA=24:1 chloroform:isoamyl alcohol) once and then with chloroform once. The RNA was mixed with 1/10 volume of 3 M NaCl and 2 volumes of ethanol and incubated at −80 °C for 20 min. Then, the mixture was centrifuged and washed with 75% ethanol (Shalel-Levanon et al., 2005a). The precipitated RNA was resuspended in RNase-free water (Sigma, St. Louis, MO, USA). The concentration of RNA was quantified by measuring the absorbance at 260 nm and applying the formula, concentration (μg/mL)=$A_{260} \times 40 \times$ dilution factor.

2.5.2. cDNA synthesis and quantitative PCR amplification

The cDNA was synthesized using the Promega Reverse Transcriptase System (Promega, Madison, WI, USA) and the RNA extracted as described above. The reaction was carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA, USA). The cDNA was synthesized in a total reaction mixture volume of 60 μL containing 1 μg of RNA as template. The reaction mixture was incubated for 10 min at room temperature for primer extension, 30 min at 50 °C for reverse transcription, and then 5 min at 95 °C and 5 min at 6 °C for inactivation of the reverse transcriptase. Non-amplification controls were prepared by not adding reverse transcriptase to the mixture. The synthesized cDNA was then