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ORIGINAL RESEARCH PAPER

Improved cellobiose utilization in *E. coli* by including both hydrolysis and phosphorolysis mechanisms

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Abstract Cellobiose is a major intermediate from cellulase hydrolysis of pretreated plant biomass. Engineering biocatalysts for direct use of cellobiose could eliminate the need for exogenous β-glucosidase. Additionally, rapid removal of cellobiose in a simultaneous saccharification and fermentation facilitates enzymatic hydrolysis as cellobiose is a potent inhibitor for cellulases. We report here improved cellobiose utilization by engineering *Escherichia coli* to assimilate the disaccharide both hydrolytically and phosphorolytically (shorter fermentation time). Additionally, we demonstrate that engineering intracellular cellobiose utilization circumvents catabolite repression allowing simultaneous fermentation of xylose and cellobiose. Using *meso*-2,3-butanediol as model product, we further demonstrate that the accelerated carbon metabolism led to improved product formation (higher titers and shorter fermentation times), illustrating the utility of the engineered biocatalysts in biorefinery applications.

Keywords 2,3-Butanediol · Biorefinery · Catabolite repression · Cellobiose · Cellobiose phosphorylase · Cellodextrinase

Introduction

To utilize lignocellulosic biomass requires a combination of pretreatment and enzymatic hydrolysis to overcome recalcitrance of the material (Alvira et al. 2010; Carere et al.2008; Kumar et al. 2009). This generates a mixture of sugars containing cello-oligosaccharides and monosaccharides, dominated by glucose, cellobiose, and xylose (Barr et al. 1996; Coughlan and Ljungdahl 1998; Fendri et al. 2009; Lavarack et al. 2002; Moxley et al. 2008). While wild type *Escherichia coli* readily metabolizes many types of monosaccharides, including xylose (Kim et al. 2007; Lawford and Rousseau 1993), *E. coli* strains are not able to use cellobiose and other cello-oligosaccharides. Engineering *E. coli* cells for direct use of cellobiose is of interest as the disaccharide is a major intermediate from enzymatic hydrolysis. Direct use of cellobiose by a biocatalyst in a fermentation process could eliminate the need for exogenous β-glucosidase. Additionally, rapid removal of cellobiose in a simultaneous saccharification and fermentation facilitates enzymatic hydrolysis as cellobiose is a potent inhibitor for cellulases (Bezerra and Dias 2005; Gruno et al. 2004; Zhao et al. 2004).

Engineering *E. coli* for direct use of cellobiose has been attempted in the past. By surface display of a

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β-glucosidase, cellobiose was hydrolyzed to glucose which was then taken up by cells and metabolized intracellularly (Tokuhiro et al. 2008). Recently, we showed that a periplasmic expression of a *Saccharophagus* cellodextrinase was also successful in generating a strain capable of utilizing cellodextrin including cellobiose (Rutter et al. 2013). Alternative to surface display or periplasmic expression of a hydrolase where the disaccharide is hydrolyzed outside of cytoplasm, cellobiose could be transported into cells by utilizing a transporter, such as LacY (Sekar et al. 2012). Once inside the cytoplasm, cellobiose could be hydrolyzed into glucose molecules by a recombinant hydrolase such as β-glucosidase (Ait et al. 1979; Saha and Bothast 1996). We have recently demonstrated that cellobiose could be alternatively metabolized via a phosphorolysis mechanism (Sekar et al. 2012) and, in this approach, instead of a hydrolase, a cellobiose phosphorylase is used that splits cellobiose into glucose and glucose-1-phosphate using inorganic phosphate as a donor.

The present study investigates whether a combination of hydrolysis and phosphorolysis could improve cellobiose utilization. We show that engineered *E. coli* cells with both hydrolysis and phosphorolysis mechanisms could readily convert cellobiose into *meso*-2,3-butanediol with high yield and conversion rate, demonstrating the utility of the improved biocatalyst in biorefinery.

Materials and methods

Strains and plasmids

Strains and plasmids used in this study are listed in Supplementary Table 1. *E. coli* MGLAP is a derivative of MG1655, previously constructed to overproduce pyruvate (Shin et al. 2012). Three expression plasmids used in this study, pBBDO, pSTCED, and pQECEP harbors genes for production of *meso*-2,3-butanediol (BDO), the cellodextrinase (CED), and cellobiose phosphorylase (CEP), respectively (supplementary Table 1).

All transformations were performed by heat shock at 42 °C for 30 s, followed by incubation in SOC media for 1 h and then plated on LB containing an appropriate antibiotic (ampicillin 100 μg/ml or chloramphenicol 25 μg/ml or kanamycin 50 μg/ml).

Cultivation and expression conditions

Single colonies of plasmid bearing strains were inoculated into LB medium supplemented with an appropriate antibiotic and cultivated overnight. This overnight seed culture was used to inoculate up to 100 ml LB in Erlenmeyer flasks to OD_{600} of 0.1. When cell density reached between 0.3 and 0.4, IPTG was added to 0.2 mM and flasks were incubated for 16 h to induce the expression of recombinant proteins. MGLAP strains were induced at 18 °C.

Enzyme assays

For verification of activity of crude lysates of strains expressing Ced3A and Cep94A, cells were harvested 16 h after induction (induction condition as above) and lysed by ultrasonication in 50 mM MES buffer (pH 6.0). Hydrolysis of cellobiose was determined by monitoring glucose formation using a glucose (GO) assay kit (Sigma). Reaction mixtures (100 μl) contained 10 μl crude lysate and 90 μl phosphate-buffered saline (PBS) containing 1 % w/v cellobiose. Assays were incubated at 25 °C for 30 min and the GO reagent was added 1:1 (v/v) to the reaction mixture. The mixture was then incubated at 37 °C for 30 min and absorbance at 540 nm was measured.

Fermentation conditions

Cells harvested from induced cultures (as above) were washed with PBS (pH 7.0) and resuspended into LB medium containing 1 % (w/v) substrate along with the appropriate antibiotics to an initial OD_{600} of 0.05. Anaerobic cultivation was carried out at 37 °C and 250 rpm with 0.2 mM IPTG in capped 20 ml scintillation vials with at least 10 ml medium. Samples were collected periodically and residual sugar, 2,3-butanediol and acetoin concentrations determined by HPLC. Cell densities were calculated from OD_{600} values.

Analytical method

The concentrations of cellobiose, glucose, xylose, acetoin, and 2,3-butanediol were measured by HPLC equipped with an Aminex HPX-87H column (Bio-Rad) using 5 mM H_2SO_4 as eluant at 0.4 ml/min.

Results and discussion

Cellobiose metabolism in engineered strains

Previously, we constructed an *E. coli* strain capable of growth on cellobiose for lactic acid production by expressing, in its periplasm, a cellodextrinase, Ced3A, from *Saccharophagus degradans* (Rutter et al. 2013). In this strain, cellobiose was split into two glucose molecules in the periplasm from where they were taken up for intracellular metabolism. We also constructed a strain that metabolizes cellobiose via phosphorolysis mechanism by expressing a cellobiose phosphorylase, Cep94A, from *S. degradans* (Sekar et al. 2012). This strain could grow on cellobiose and additionally convert cellobiose to ethanol with high yield (Sekar et al. 2012). The goal of the present study was to investigate whether cellobiose metabolism could be accelerated by engineering a strain with both hydrolysis and phosphorolysis mechanisms. Additionally, we aim to show that the potential acceleration of cellobiose metabolism could lead to an enhanced production of a biorefinery product.

We chose to use *meso*-2,3-butanediol (BDO) as a model product. To this end, a MG1655 derivate, designated as MGLAP (Supplementary Table 1), was used in this study. This strain was previously engineered to eliminate production of lactic acid and acetate production by knockout of genes associated with these two metabolites. As a result, the host strain, transformed with a plasmid (pBBDO) containing enzymes for BDO production, produced (from glucose) 2,3-butanediol as the major fermentation product (Shin et al. 2012). The strain was further modified by transforming it with pSTCED and pQECEP, expressing both the cellodextrinase (CED) and the cellobiose phosphorylase (CBP), respectively (Supplementary Table 1). The resulting strain, capable of metabolizing cellobiose using both hydrolysis and phosphorolysis mechanisms, is designated CED+CBP. Similarly, the control strains, expressing either cellodextrinase or cellobiose phosphorylase, are designated as CED and CBP strains, respectively. Finally, an empty vector strain, designated as empty vector control, was also included in this fermentation study (Supplementary Table 1). The four strains were compared with respect to their ability to utilize cellobiose (Fig. 1a) and their ability to produce BDO (Fig. 1b).

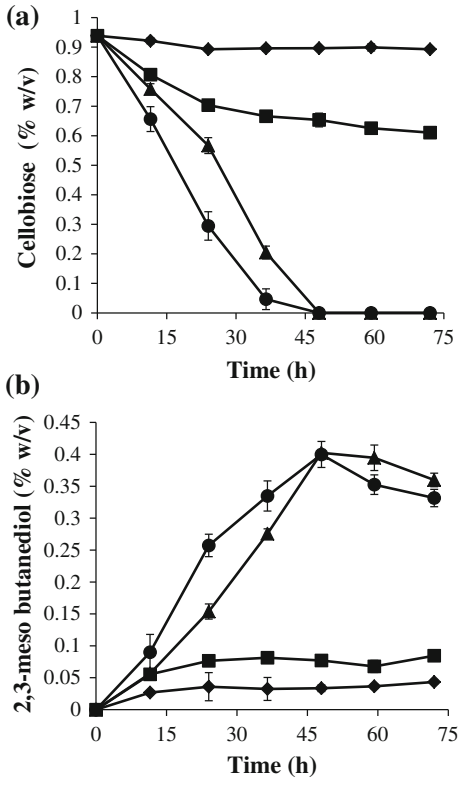

Fig. 1 Cellobiose (**a**) and 2,3-butanediol (**b**) concentrations during the fermentation of LB with 1 % cellobiose by MGLAP/pSTV28+pQE80L (*filled diamond*), MGLAP/pSTCED+pQE80L (*filled square*), MGLAP/pSTV28+pQECEP (*filled triangle*), and MGLAP/pSTCED+pQECEP (*filled circle*)

As shown, during the 72 h anaerobic fermentation of 1 % (w/v) cellobiose, minimal consumption of cellobiose was observed for the empty vector control. In contrast, significant consumption of cellobiose was observed for other three strains, with fast consumption evident for strains expressing CBP alone or both CBP and CED. By 36 h, the cellobiose concentrations for the two strains expressing single enzyme were 0.7 and 0.2 % for the strain expressing CED and for the strain expressing CBP, respectively, indicating that the phosphorylase-expressing cells consumed cellobiose faster than the cellodextrinase-expressing cells. The lowest residual cellobiose concentration at 36 h was with the strain expressing both cellodextrinase and phosphorylase, with about 0.05 % (w/v) cellobiose remaining.

These results show that hydrolysis and phosphorolysis are synergistic and cells with both mechanisms metabolize cellobiose much more rapidly. It can be

seen in Fig. 1a that CED+CBP and CEP were able to completely utilize cellobiose in about 50 h. In comparison, the CED strain expressing only cellodextrinase was able to utilize only 40 % of the cellobiose by the end of the fermentation (72 h).

Time profiles of *meso*-2,3-BDO production from cellobiose are shown in Fig. 1b. Significant product formation was only observed with the strain expressing cellobiose phosphorylase and the strain expressing both cellobiose phosphorylase and cellodextrinase (Fig. 1b), with the latter outpaced the former before the product concentration peaked at 48 h. Both strains reached the same maximum, 0.4 % (w/v), at 48 h, representing an 80 % of theoretical yield. The CED expressing strains produced slightly more BDO than the empty vector control strain to a maximum of 0.07 % w/v, suggesting that expressing Ced3A alone is not sufficient for significant cellobiose metabolism and BDO production.

These results suggest that cellobiose phosphorolysis is a more effective mechanism than cellodextrinase-mediated hydrolysis mechanism. These two mechanisms appear to be synergistic in terms of cellobiose consumption. While earlier and faster production of BDO was observed, cells with both mechanisms did not result in higher product concentration over the fermentation cycle. Apparently, product yield is determined by factors more than the rate of cellobiose consumption. However, product yield of 80 % from cellobiose (Qin et al. 2006), is close to what

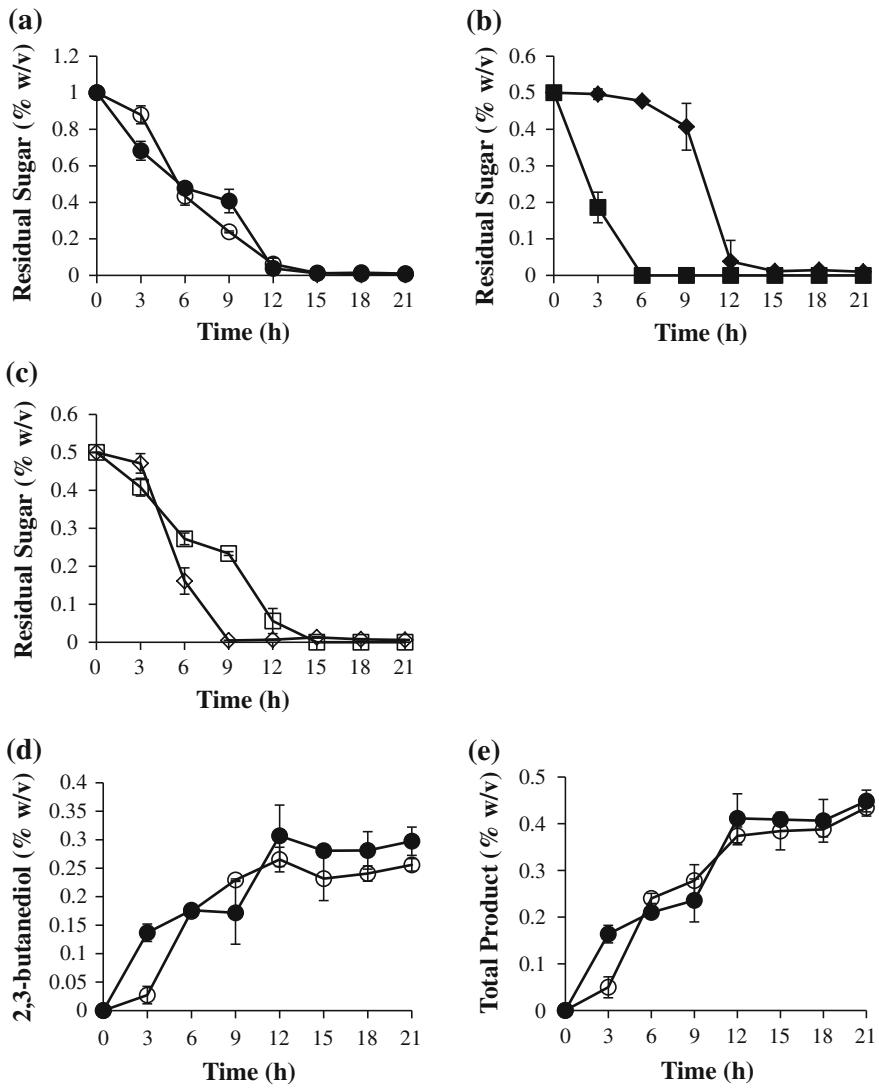

Fig. 2 **a** Total residual sugar concentration with MGLAP/pSTCED+pQECEP strains grown on 0.5 % glucose/0.5 % xylose (*filled circle*) or 0.5 % cellobiose/0.5 % xylose (*open circle*). **b** Residual glucose (*filled square*) and xylose (*filled diamond*) during fermentation of 0.5 % glucose/0.5 % xylose by MGLAP/pSTCED+pQECEP. **c** residual cellobiose (*open square*) and xylose (*open diamond*) during fermentation of 0.5 % cellobiose/0.5 % xylose by MGLAP/pSTCED+pQECEP. **d** Total 2,3-butanediol concentration with MGLAP/pSTCED+pQECEP strains grown on 0.5 % glucose/0.5 % xylose (*filled circle*) or 0.5 % cellobiose/0.5 % xylose (*open circle*). **e** Total 2,3-butanediol and acetoine concentration with MGLAP/pSTCED+pQECEP strains grown on 0.5 % glucose/0.5 % xylose (*filled circle*) or 0.5 % cellobiose/0.5 % xylose (*open circle*)

was achieved with glucose [87 % by Shin et al. (2012)], indicating that cellobiose could be used as effectively as glucose for BDO production.

Co-fermentation of cellobiose/xylose for BDO production

As shown above, cellobiose utilization could be improved by inclusion of both hydrolysis and phosphorylase mechanisms. To further investigate its utility in biorefinery, additional experiments were carried out under the condition of mixed sugar fermentation with cellobiose and xylose. We hypothesize that fast intracellular metabolism of cellobiose by cellobiose phosphorylase may reduce the extracellular glucose concentration sufficiently low to remove catabolite repression. If this is the case, simultaneous consumption of cellobiose and xylose will result and

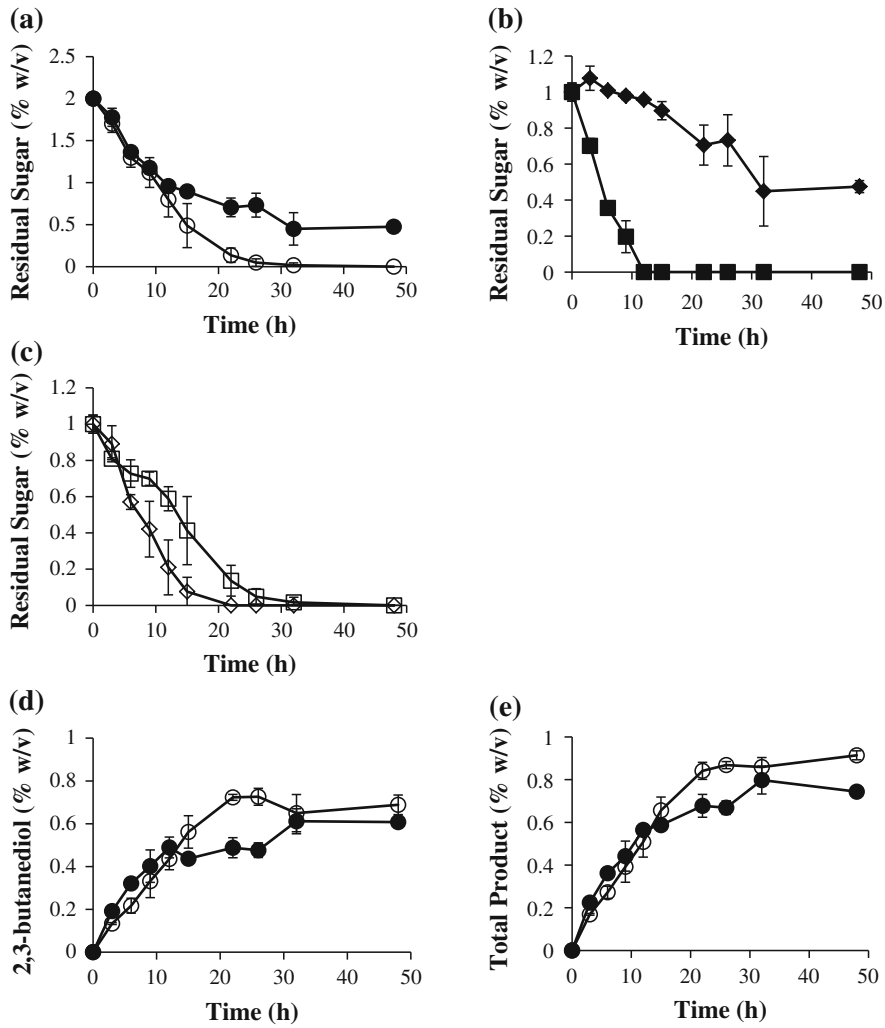

Fig. 3 **a** Total residual sugar concentration with MGLAP/pSTCED+pQECEP strains grown on 1 % w/v glucose/1 % (w/v) xylose (*filled circle*) or 1 % (w/v) cellobiose/1 % (w/v) xylose (*open circle*). **b** Residual glucose (*filled square*) and xylose (*filled diamond*) during fermentation of 1 % (w/v) glucose/1 % (w/v) xylose by MGLAP/pSTCED+pQECEP. **c** Residual cellobiose (*open square*) and xylose (*open diamond*) during fermentation of 1 % (w/v) cellobiose/1 % (w/v) xylose by MGLAP/pSTCED+pQECEP. **d** Total 2,3-butanediol concentration with MGLAP/pSTCED+pQECEP strains grown on 1 % (w/v) glucose/1 % (w/v) xylose (*filled circle*) or 1 % (w/v) cellobiose/1 % (w/v) xylose (*open circle*). **e** Total 2,3-butanediol and acetoine concentration with MGLAP/pSTCED+pQECEP strains grown on 1 % (w/v) glucose/1 % (w/v) xylose (*filled circle*) or 1 % (w/v) cellobiose/1 % (w/v) xylose (*open circle*)

this should improve the overall carbon metabolism. To investigate this possibility, anaerobic mixed sugar fermentation (0.5 % cellobiose and 0.5 % xylose) were run for *E. coli* cells expressing both CED and CBP. This is compared to mixed monosaccharide fermentation of the same concentration (0.5 % glucose and 0.5 % xylose).

Figure 2a shows that the strain exhibited similar total sugar (cellobiose plus xylose or glucose plus xylose) consumption rates. However, consumption of each individual sugar was considerably different. In mixed monosaccharide fermentation, the utilization of glucose and xylose is biphasic (Fig. 2b). The consumption of xylose began only when glucose was exhausted. In contrast, in the case of cellobiose and xylose, a clear co-metabolism was evident from Fig. 2c. Interestingly, xylose was apparently metabolized faster than cellobiose. Despite the differences in the dynamics of sugar utilization, little differences were observed in final BDO titers. In both cases, the final 2,3-butanediol concentration was 0.3 % (w/v) after 12 h fermentation (Fig. 2d), representing a 60 % yield. This is lower than the 80 % yield on cellobiose (Fig. 1b). HPLC analysis showed that a precursor molecule, acetoin, was accumulated in both cases.

The combined concentration of BDO and acetoin reached 0.4 % w/v and 0.38 % (w/v) for glucose/xylose mixture and cellobiose/xylose mixture, respectively (Fig. 2e), suggesting the lost yield in BDO is accounted for by the byproduct acetoin.

The above mixed sugar experiment was repeated with higher concentration of carbon source, 1 % (w/v) cellobiose and 1 % (w/v) xylose, compared to 1 % (w/v) glucose and 1 % (w/v) xylose. As shown in Fig. 3a, initial rates of total sugar consumption were identical for both cases but diverged after 12 h, with the monosaccharide fermentation lagged behind and about 0.5 % sugar remained at the end of the 48 h fermentation. This compares to a complete fermentation of cellobiose/xylose by 32 h, indicating the ability of use cellobiose directly in this strain improved the mixed sugar fermentation. In mixed monosaccharide fermentation, only about 50 % xylose was utilized (Fig. 3b). This contrasts with a complete utilization of xylose in mixed cellobiose and xylose fermentation (Fig. 3c). Consistent with the sugar concentration profiles, product formation during fermentation of the cellobiose/xylose mixture was more rapid than the glucose/xylose mixture with a maximum level of BDO reaching 0.72 % (w/v) at 26 h (Fig. 3d), whereas the glucose/xylose fermentation achieved a lower maximum BDO concentration of 0.61 % (w/v) 10 h later. The combined BDO and acetoin concentration reached 0.74 % (w/v) and 0.91 % (w/v) for 2 % (v/v) glucose/xylose mixture and 2 % cellobiose/xylose mixture, respectively (Fig. 3e).

These results clearly demonstrate that engineering intracellular cellobiose utilization could circumvent catabolite repression, allowing simultaneous use of xylose and cellobiose. As a result, overall carbon metabolism was improved and product concentration and yield were also improved.

Summary

We have demonstrated a significant improvement in cellobiose metabolism by including hydrolysis and phosphorolysis. Additionally, intracellular metabolism of cellobiose circumvented catabolite repression, resulting in simultaneous utilization of xylose and cellobiose. The accelerated carbon metabolism in turn led to an improved product formation, demonstrated with BDO as model product, indicating that the *E. coli* strains capable of cellobiose utilization can be advantageously used in biorefinery applications.

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