Journal of Biotechnology 192 (2014) 156–160

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Short communication

Engineering of *Corynebacterium glutamicum* for minimized carbon loss during utilization of D-xylose containing substrates

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ARTICLE INFO

Article history:
Received 19 August 2014
Received in revised form
24 September 2014
Accepted 30 September 2014
Available online 7 October 2014

Keywords:
Corynebacterium glutamicum
Metabolic engineering
D-Xylose
Weimberg pathway
α-Ketoglutarate

ABSTRACT

Biomass-derived D-xylose represents an economically interesting substrate for the sustainable microbial production of value-added compounds. The industrially important platform organism *Corynebacterium glutamicum* has already been engineered to grow on this pentose as sole carbon and energy source. However, all currently described *C. glutamicum* strains utilize D-xylose via the commonly known isomerase pathway that leads to a significant carbon loss in the form of CO_2, in particular, when aiming for the synthesis of α-ketoglutarate and its derivatives (e.g. L-glutamate). Driven by the motivation to engineer a more carbon-efficient *C. glutamicum* strain, we functionally integrated the Weimberg pathway from *Caulobacter crescentus* in *C. glutamicum*. This five-step pathway, encoded by the *xylXABCD*-operon, enabled a recombinant *C. glutamicum* strain to utilize D-xylose in D-xylose/D-glucose mixtures. Interestingly, this strain exhibited a tri-phasic growth behavior and transiently accumulated D-xylonate during D-xylose utilization in the second growth phase. However, this intermediate of the implemented oxidative pathway was re-consumed in the third growth phase leading to more biomass formation. Furthermore, *C. glutamicum* pEKEx3-*xylXABCD*$_{Cc}$ was also able to grow on D-xylose as sole carbon and energy source with a maximum growth rate of $\mu_{max} = 0.07 \pm 0.01\ h^{-1}$. These results render *C. glutamicum* pEKEx3-*xylXABCD*$_{Cc}$ a promising starting point for the engineering of efficient production strains, exhibiting only minimal carbon loss on D-xylose containing substrates.

Introduction

The pentose D-xylose is a key building block of most hemicelluloses and thus a main constituent of lignocellulosic biomass (5–20%) available for biotechnological production processes (Aristidou and Penttila, 2000). For the microbial production of amino acids, organic acids or alcohols with *Corynebacterium glutamicum*, the hexoses D-glucose (from starch hydrolysates), D-fructose and sucrose (from molasses) are traditionally used (Blombach and Seibold, 2010). Naturally, the substrate spectrum of *C. glutamicum* also includes the disaccharide maltose or the pentose ribose, and this bacterium has been genetically modified to use starch, cellobiose, D-galactose and L-arabinose as carbon and energy source (Schneider et al., 2011; Kotrba et al., 2003; Barrett et al., 2004; Seibold et al., 2006).

Similarly, *C. glutamicum* has been also engineered for growth on D-xylose (Kawaguchi et al., 2006; Meiswinkel et al., 2013; Kang et al., 2014). In this case, heterologous expression of a xylose isomerase XLI (encoded by *xylA*) from *Escherichia coli* is sufficient to convert D-xylose to D-xylulose, which is then phosphorylated by an endogenous xylulokinase XLK (encoded by *xylB*) to yield xylulose-5-phosphate as intermediate of the pentose phosphate pathway (Fig. 1, left). It could be also shown that an overexpression of xylose isomerases from other sources in combination with the endogenous xylulokinase resulted in a doubled growth rate on D-xylose and allowed the microbial synthesis of amino acids such as L-lysine, L-glutamate and L-ornithine as well as the diamine putrescine from this carbon source (Meiswinkel et al., 2013). However, for the latter three products, which are all derived from the TCA-cycle intermediate α-ketoglutarate, the introduction of this isomerase pathway for D-xylose assimilation in *C. glutamicum*

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http://dx.doi.org/10.1016/j.jbiotec.2014.09.026
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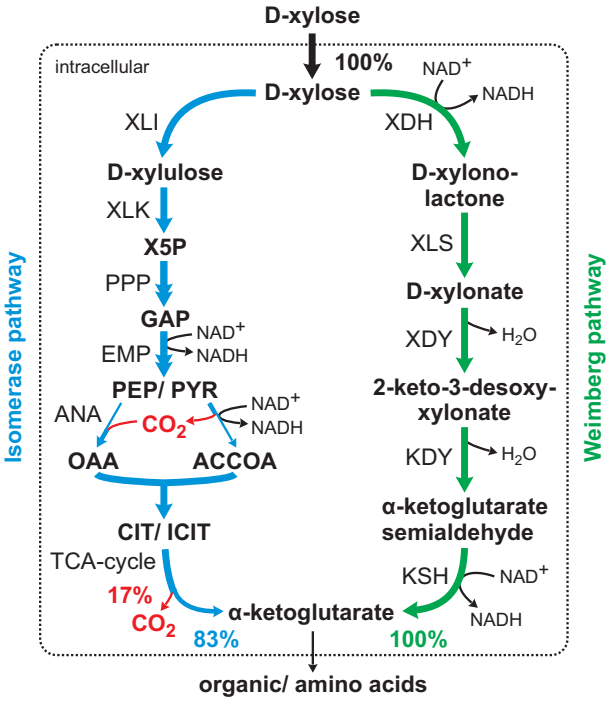

Fig. 1. Two alternative metabolic routes from D-xylose to α-ketoglutarate in *C. glutamicum*. Whereas the isomerase pathway leads to a significant carbon loss in the TCA-cycle (left), does the newly established Weimberg pathway allow for a complete carbon conversion (right).

has the drawback that a significant fraction of the D-xylose-derived carbon is lost in the form of CO_2 during product synthesis, lowering the overall product yield (cf. Fig. 1, left).

The Weimberg pathway, first discovered in *Pseudomonas fragi* and later also in *Haloferax volcanii* and *Caulobacter crescentus* (Weimberg, 1961; Johnsen et al., 2009; Stephens et al., 2007), represents a promising alternative for D-xylose assimilation. In this five-step oxidative pathway the pentose D-xylose is exclusively oxidized to the C_5-compound α-ketoglutarate without carbon loss (cf. Fig. 1, right). The functional implementation of the Weimberg pathway enabled *Pseudomonas putida* to grow on D-xylose as sole carbon source with a maximum growth rate of $\mu_{max} = 0.21\ h^{-1}$ (Meijnen et al., 2009). The Weimberg pathway has not been introduced to *C. glutamicum* before. However, it offers the advantage of a direct conversion of D-xylose to α-ketoglutarate, which is a precursor of the above mentioned products and also an interesting product itself (Barrett and Yousaf, 2008; Otto et al., 2011; Stottmeister et al., 2005). In this study we functionally integrated the Weimberg pathway from *C. crescentus* into *C. glutamicum* and compared this pathway to the already established isomerase pathway in the same organism. Furthermore, we provide a detailed analysis of biomass formation and substrate utilization during growth on D-xylose and mixed substrates and identified the accumulation of the pathway intermediate D-xylonate as a bottleneck during D-xylose assimilation.

Materials and methods

Construction of strains and plasmids

Routine methods such as PCR, DNA restriction or DNA ligation were carried out according to standard protocols (Sambrook and Russell, 2001). The oligonucleotides used for cloning, colony-PCRs and DNA sequencing were obtained from EurofinsMWGOperon (Ebersberg, Germany) and are listed in the Supplementary Table S1. Bacterial strains and plasmids used or constructed in the course of this work are listed in the Supplementary Table S2. All details regarding strain and plasmid construction can be found in the Supporting Information.

Cultivations

The defined medium CGXII (Keilhauer et al., 1993) supplemented with indicated D-glucose and D-xylose concentrations was used for all cultivations. The pH of the medium was adjusted with 4 M NaOH to 7.0. Supplements such as biotin, protocatechuate, trace elements, IPTG, spectinomycin, D-xylose, and D-glucose were added after autoclaving.

All cultivations were carried out in 48-well FlowerPlates (m2p-labs GmbH, Baesweiler, Germany) incubated in a BioLector (m2p-labs GmbH) embedded in a liquid handling station (JANUS™ working station, Perkin Elmer, Waltham MA, USA) for automated sampling and sample processing. Cultures were inoculated with 50 μl cell suspension either from cryo-stocks or fresh seed culture into 1 ml fresh medium. The cultivation settings were always kept at 1000 rpm, 95% humidity and 30 °C. Depending on the experiment the online signals for biomass (backscatter; gain 20), dissolved oxygen (pO_2; gain 35) and pH (gain 35) were measured in intervals of 9 (unsampled process) or 22 min (triggered sampling process), respectively. The FlowerPlates were covered with a gas permeable sealing foil (m2p-labs GmbH) to prevent contaminations and to allow a uniform gas exchange. Medium preparation and inoculation were performed manually under a sterile bench. For the estimation of substrate-specific biomass yields ($Y_{X/S}$) the corresponding backscatter (BS) measurements were recalculated to cell dry weight (CDW) data using the calibration model $CDW\ [g\,l^{-1}] = 0.048 \times BS\ g\,l^{-1} - 0.78\ g\,l^{-1}$ (Rohe et al., 2012).

GC–ToF–MS analysis

Untargeted metabolome screening in culture supernatants was performed via an Agilent 6890N gas chromatograph coupled to a Waters Micromass GCT Premier high resolution time of flight mass spectrometer. For details regarding sample preparation, MS operation and peak identification the reader is referred to Paczia et al. (2012).

Determination of D-glucose and D-xylose in culture supernatants

For substrate analytics two enzymatic assays were transferred into an automated 384-well microtiter plate format using the JANUS™ working station and an integrated plate reader (EnSpire™ Multimode Plate Reader, Perkin Elmer, Waltham MA, USA). D-glucose was measured with a standard protocol (Peterson and Young, 1968) and D-xylose with an enzymatic D-xylose assay kit (Megazyme, Wickow, Ireland). In both cases the NADH increase at 340 nm was monitored.

Results & discussion

Expression of the Weimberg pathway from C. crescentus for D-xylose assimilation in C. glutamicum

C. crescentus metabolizes D-xylose through the five-step Weimberg pathway yielding α-ketoglutarate (Fig. 1, right). All five enzymes of this catabolic route are encoded in the *xylXABCD* operon in the genome of this bacterium (Stephens et al., 2007). The D-xylose catabolism is initiated by the oxidation of D-xylose to D-xylonolactone, catalyzed by a xylose dehydrogenase (XDH, encoded by *xylB*). Subsequently, a xylonolactonase (XLS, encoded by *xylC*)