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Establishment of Cellobiose Utilization for Lipid Production in *Rhodococcus opacus* PD630

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***Rhodococcus opacus* PD630, which is known for its ability to accumulate large amounts of triacylglycerols (TAG), was metabolically engineered, and a cellobiose utilization pathway was introduced. Activities of β-glucosidases were determined, and recombinant strains accumulated fatty acids up to 39.5 ± 5.7% (wt/wt) of cell dry mass from cellobiose.**

Today's first-generation biofuels are mainly derived from edible plant parts, such as sugar cane and corn, and their production is associated with numerous economical, ecological, and social problems (1). The microbial conversion of abundant lignocellulosic biomass into so-called second-generation biofuels in an economically competitive process is regarded as a key challenge for sustainable fuel production (2, 3). Cellulose, the homopolymer of 1,4-β-linked D-glucose, is the major component of lignocellulose (40 to 50%). The key steps in cellulose degradation and subsequent fermentation include the saccharification of the polymeric substrate into simple sugars, usually mediated by the action of at least three enzymes (endoglucanase [E.C. 3.2.1.4], exoglucanase [E.C. 3.2.1.91], and β-glucosidase [E.C. 3.2.1.21]) that act in a synergistic manner (4, 5). For fermentation of cellulosic materials, these enzymes are usually produced in a separate process and thereby represent the second highest raw material expense factor in such fermentation process after the feedstock itself (6). The process involving simultaneous saccharification and fermentation (SSF), also known as consolidated bioprocessing (CBP), is regarded as potential alternative to dedicated enzyme production, as it combines both saccharification of lignocellulosic materials and fermentation of the released sugars in one microorganism (4, 7). However, several challenges must still be overcome to achieve economically viable production processes (8), and to date no production of cellulosic biofuels on a commercial scale has been established (9).

Rhodococcus opacus strain PD630 is the model oleaginous prokaryote for accumulation and biosynthesis of lipids, which serve as carbon and energy storage and can account for up to 87% of the cell dry mass in this strain (10–12). It has been considered as a production strain for high-value triacylglycerols (TAGs) from renewable resources for the production of biodiesel, monoalkyl esters of short-chain alcohols, and long-chain fatty acids, due to its high substrate tolerance, its ability to be cultivated to high densities, and its rapid growth, which make it favorable over other production organisms (12–14). Unfortunately, this strain does not use cellobiose (1,4-β-D-glucopyranosyl-D-glucopyranose), the main product of cellulases, as the sole carbon and energy source (15, 16).

Strategies to establish cellobiose utilization in *R. opacus* PD630. All bacteria, plasmids, and primers used in this study are listed in Tables 1 and 2. Previous studies concluded that the cellobiose deficiency of *R. opacus* PD630 is due to a missing active glycoside hydrolase enzyme (15). In order to establish cellobiose utilization in *R. opacus* PD630, three different strategies were applied. The first strategy, employing two extracellular β-glucosidases (BGL) from *Rhodococcus erythropolis* DSM43066 and *Gordonia polyisoprenivorans* VH2, which can both use cellobiose as the sole carbon and energy source, aimed at the extracellular cleavage of cellobiose and subsequent uptake of the generated glucose. The second strategy attempted to complement the lack of a suitable cytoplasmic β-glucosidase. Despite being noncellulolytic, *Escherichia coli* is known to possess cryptic genes for cellobiose utilization (21) and also a characterized periplasmic β-glucosidase enzyme (BglX) (22), which was chosen for this study. The third strategy conferred both a transporter and a β-glucosidase, using the *bglABC* operon of *Thermobifida fusca*, to *R. opacus*. This operon, first described and partially characterized by Spiridonov and Wilson (23), comprises genes encoding two ABC sugar transport proteins (BglA and BglB) and a cytoplasmic β-glucosidase (BglC).

For cloning of the respective genes, the coding regions of bgl_{RE} from *R. erythropolis*, bgl_{GI} from *G. polyisoprenivorans*, and $bglABC_{TF}$, $bglAB_{TF}$, and $bglBC_{TF}$ from *T. fusca* were amplified by PCR using the oligonucleotides *FbglRER_Bam*HI and *RbglRER_Xba*I for bgl_{RE}, *FbglVH2_Xba*I and *RbglVH2_Xba*I for bgl_{GI}, *FbglABC_Eco*RI and *RbglABC_Xba*I for $bglABC_{TF}$, *FbglABC-Eco*RI and *RbglAB_Sac*I for $bglAB_{TF}$, and *FbglBC_Eco*RI and *RbglABC_Xba*I *for* $bglBC_{TF}$ (Table 2). Additionally, the $bglX_{EC}$ gene from *E. coli* was amplified by PCR using the oligonucleotides *FbglX-Eco*RI and *RbglX_Kpn*I, thereby omitting the signal peptide for periplasmic location. The truncated gene was designated $bglX_{EC}$-SP. For expression experiments, all fragments were ligated to the *E. coli*/*Corynebacterium glutamicum* shuttle vector pEC-K18*mob2* (20) using the respective restriction enzymes (Table 2), yielding plasmids pEC-K18*mob2*::bgl_{RE}, pEC-K18*mob2*::bgl_{GI}, pEC-K18*mob2*::$bglX_{EC}$-SP, pEC-K18*mob2*::$bglABC_{TF}$ (Fig. 1), pEC-K18*mob2*::$bglAB_{TF}$, and pEC-K18*mob2*::$bglBC_{TF}$. All plasmids were analyzed by sequencing and later transferred to *R. op-*

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli Mach1-T1	F^- ϕ80(*lacZ*)ΔM15 Δ*lacX74 hsdR*(r_K^- m_K^+) Δ*recA1398 endA1 tonA*	Invitrogen (Karlsbad, Germany)
R. opacus PD630	TAG-producing strain	10
R. erythropolis DSM43066	Cellobiose utilization	17
T. fusca DSM43792	Cellobiose utilization	18
G. polyisoprenivorans VH2	Cellobiose utilization	19
Plasmids		
pEC-K18*mob2*		20
pEC-K18*mob2*::bgl_{RE}	bgl_{RE} as a BamHI/XbaI fragment	This study
pEC-K18*mob2*::bgl_{GI}	bgl_{GI} as an XbaI fragment	This study
pEC-K18*mob2*::$bglX_{EC}$-SP	$bglX_{EC}$-SP as an EcoRI/KpnI fragment	This study
pEC-K18*mob2*::$bglABC_{TF}$	$bglABC_{TF}$ as an EcoRI/XbaI fragment	This study
pEC-K18*mob2*::$bglAB_{TF}$	$bglAB_{TF}$ as an EcoRI/SacI fragment	This study
pEC-K18*mob2*::$bglBC_{TF}$	$bglBC_{TF}$ as an EcoRI/XbaI fragment	This study

acus PD630 by electroporation according to the previously described protocol (24).

Recombinant strains were cultivated both on solid and in liquid MSM (14) with 1% (wt/vol) glucose and/or cellobiose as the carbon source. Only recombinant strains harboring pEC-K18*mob2*::$bglABC_{TF}$ exhibited significant growth with cellobiose, whereas all other strains grew like the wild type. When *R. opacus* pEC-K18*mob2*::$bglABC_{TF}$ was cultivated in liquid MSM containing different concentrations (1, 1.7, or 4%, wt/vol) of cellobiose as the sole carbon source, similar growth for all cultures was observed ($\mu = 0.021$ to 0.025 h^{-1}) (Fig. 2). However, cultures with 1.7 and 4% (wt/vol) cellobiose exhibited a shorter lag phase and higher final optical densities than cultures with 1% (wt/vol) cellobiose.

High-performance liquid chromatography (HPLC) analysis of medium cellobiose contents was done as follows. Culture media were centrifuged at 14,000 × *g* to remove cells. Supernatants were filtered using Spartan 0.2-μm filters (Whatman, Dassel, Germany) and applied to a Eurokat Pb column (30GX350EKN; Knauer, Berlin, Germany) using water-acetonitrile (95:5) as the eluent at 75°C and a flow rate of 1 ml min^{-1}. The HPLC system used comprises a Kontron system 522 pump and an HPLC 560 autosampler (Kontron, München, Germany) as well as a Sedex 80 LT-ELS detector (Sedere, Alfortville, France). Determination of cellobiose contents of the culture supernatants revealed that cellobiose was utilized only by growing cells of strain *R. opacus* PD630 pEC-K18*mob2*::$bglABC_{TF}$. After 250 h cultivation, cellobiose was completely consumed in the 1% culture (Fig. 2), whereas in the 1.7 and 4% cultures, glucose accumulated in the medium after a cultivation period of 229 h, most likely as a result of cell death and subsequent leakage of BglC into the medium. Glucose levels reached 0.66 ± 0.01% and 2.73 ± 0.06% (wt/vol) in the 1.7 and 4% cultures, respectively. The latter was higher than what was expected; however, the low remaining volume led to accelerated evaporation of the medium. In general, growth of recombinant *R. opacus* PD630 pEC-K18*mob2*::$bglABC_{TF}$ was considerably slower with cellobiose than that of wild-type *R. opacus* PD630 with sucrose or glucose ($\mu = 0.088$ h^{-1} and $\mu = 0.072$ h^{-1}, respectively [25]). Taking into account the fact that low growth rates could also be observed in complex media, this provides evidence that expression of $bglABC_{TF}$ represents a strong metabolic burden to the cells, thereby decreasing consumption and growth rates. Interestingly, the additional stabilization of the plasmid by kanamycin resulted in a significantly shorter lag-phase of recombinant strains (data not shown).

BGL enzyme assays. Activity of β-glucosidases was determined as described by Adin et al. (26). Enzyme assays of the soluble protein fractions obtained from cells of the recombinant strain of *R. opacus* PD630 harboring pEC-K18*mob2*::$bglABC_{TF}$ or pEC-K18*mob2*::$bglX_{EC}$-SP demonstrated the presence of active BGL (0.881 ± 0.011 U mg^{-1} and 0.003 ± 0.0001 U mg^{-1}, respectively, at 30°C), whereas no activity was detected in the soluble protein fraction of the control strain PD630 harboring plasmid pEC-K18*mob2*. This finding confirmed the assumption, made by

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5′–3′)[a]
*FbglRER_Bam*HI	AAAGGATCCGGGAGCTCCTTGATGGCACTGACGTGCCTGCT
*RbglRER_Xba*I	AAATCTAGATCATCGAGTAGCCGTACAGCTGCG
*FbglVH2_Xba*I	AAATCTAGAGGAAGAGGACCCCATGAGCCGACCTCACCACC
*RbglVH2_Xba*I	AAATCTAGACTAGAGCTGTGCCCGCGGCC
*FbglX_Eco*RI	AAAGAATTCGGGAGCTCCTTGATGGATTTATTCGGCAACCATCCATTAA
*RbglX_Kpn*I	AAGGTACCTTACAGCAACTCAAACTCGCCTTTCTTAACG
*FbglABC_Eco*RI	AAAGAATTCGGCCGTCCTCTCTTCCATCTGACATCTGACCTCTC
*RbglABC_Xba*I	AAATCTAGAGCCGCCGGGACGGCGAGATTTTGACCTATC
*RbglAB_Sac*I	AAAGAGCTCTCACTTAATGGCACCTTCCATGATCC
*FbglBC_Eco*RI	AAGAATTCGGGAGCTCCTTGATGGCTGCGACTTCGACCCC

[a] Restriction sites are underlined.

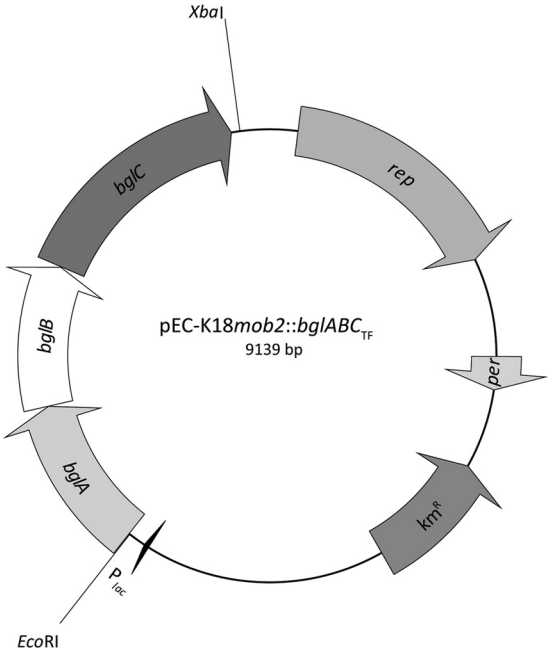

FIG 1 Physical map of the constructed plasmid pEC-K18*mob2*::*bglABC*$_{TF}$. Relevant cleavage sites and structural genes are indicated (*rep*, origin of replication; *per*, positive effector of replication; km^R, kanamycin resistance cassette; P$_{lac}$, *lac* promoter; *bglABC* operon encoding two sugar transporters (*bglAB*, accession no. YP_288996 and YP_288997) and a β-glucosidase (*bglC*, accession no. YP_288998) from *T. fusca*.

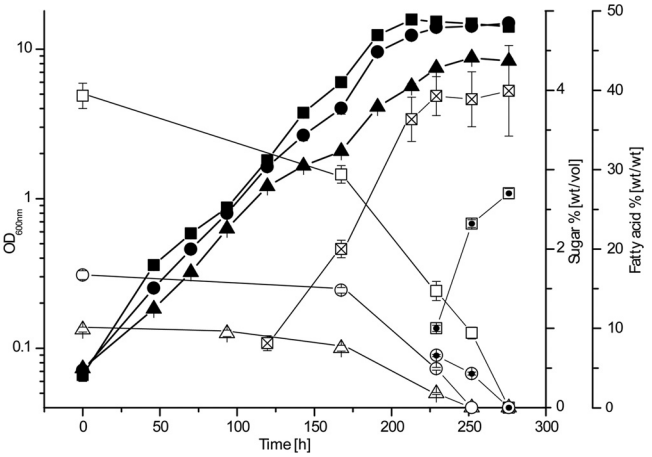

FIG 2 Growth of the recombinant strain *R. opacus* PD630 pEC-K18*mob2*::*bglABC*$_{TF}$ in the presence of different cellobiose concentrations. Cells were cultivated in liquid MSM containing 1, 1.7, or 4% (wt/vol) cellobiose as the sole carbon source. ▲, 1% (wt/vol) cellobiose; ●, 1.7% (wt/vol) cellobiose; ■, 4% (wt/vol) cellobiose. Cellobiose concentrations in the medium: △, 1% (wt/vol) culture; ○, 1.7% (wt/vol) culture; □, 4% (wt/vol) culture. Glucose concentrations in the medium: ⊙, 1.7% (wt/vol) culture; ⊡, 4% (wt/vol) culture. Fatty acid content: ⊠, 4% (wt/vol) culture. Error bars indicate standard deviations of triplicate measurements.

Holder et al., that a functional glycosyl hydrolase enzyme is lacking in *R. opacus* PD630 (15), although the experimental results further demonstrated that a suitable sugar permease is also required for growth. Interestingly, the activity of BglX$_{EC}$-SP was surprisingly low, demonstrating that the removal of the signal peptide significantly affected the activity of the enzyme. However, it must also be taken into account that the heterologous expression of a gene with no actinomycete origin and thus a different G+C content (54% for *bglX*) might negatively influence its expression level in *R. opacus* PD630. Consistent with the growth characteristics of recombinant strains, no BGL activity was detected in culture supernatants or in the soluble protein fractions of strains which expressed the genes for the extracellular β-glucosidases Bgl$_{GI}$ and Bgl$_{RE}$.

Analysis of storage lipids. Determination of the TAG content was performed as described in detail elsewhere (14, 27). Qualitative analysis by thin-layer chromatography revealed a spot corresponding to the triolein standard for all three cellobiose cultivations. In addition, the fatty acid content of cells cultivated with 4% (wt/vol) cellobiose was determined at different time points (Fig. 2), which increased from 8.13 ± 0.94% (wt/wt) at 167 h to 39.27 ± 2.45% (wt/wt) after 229 h of cultivation and remained almost constant until the cultivation ended. These values are clearly lower than the lipid levels that can be achieved with sucrose or gluconate as carbon sources, but they reflect on the one hand the basic metabolic rate for nitrogen accompanied by the long cultivation time and on the other hand the elevated nitrogen content of the medium (0.1% [wt/vol]) that was employed to stimulate cell growth.

Conclusions. In the present study, we successfully conferred the ability to utilize cellobiose to *R. opacus* PD630. However, future investigations are needed to improve both growth and lipid storage of recombinant strains. Experiments already indicate that the reduction of the *bglABC* expression level by, e.g., the addition of genes upstream of the *bglABC* operon or expression by low-copy-number vectors resulted in both faster growth and increased levels of lipid accumulation by recombinant strains (unpublished data). In addition, especially with regard to SSF, i.e., combination with cellulase genes, the integration of the cellobiose utilization genes into the genome of *R. opacus* PD630 is desirable.

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