Saccharification of Cellulose by Recombinant *Rhodococcus opacus* PD630 Strains

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The noncellulolytic actinomycete *Rhodococcus opacus* strain PD630 is the model oleaginous prokaryote with regard to the accumulation and biosynthesis of lipids, which serve as carbon and energy storage compounds and can account for as much as 87% of the dry mass of the cell in this strain. In order to establish cellulose degradation in *R. opacus* PD630, we engineered strains that episomally expressed six different cellulase genes from *Cellulomonas fimi* ATCC 484 (*cenABC*, *cex*, *cbhA*) and *Thermobifida fusca* DSM43792 (*cel6A*), thereby enabling *R. opacus* PD630 to degrade cellulosic substrates to cellobiose. Of all the enzymes tested, five exhibited a cellulase activity toward carboxymethyl cellulose (CMC) and/or microcrystalline cellulose (MCC) as high as $0.313 \pm 0.01 \text{ U} \cdot \text{ml}^{-1}$, but recombinant strains also hydrolyzed cotton, birch cellulose, copy paper, and wheat straw. Cocultivations of recombinant strains expressing different cellulase genes with MCC as the substrate were carried out to identify an appropriate set of cellulases for efficient hydrolysis of cellulose by *R. opacus*. Based on these experiments, the multicellulase gene expression plasmid pCellulose was constructed, which enabled *R. opacus* PD630 to hydrolyze as much as $9.3\% \pm 0.6\%$ (wt/vol) of the cellulose provided. For the direct production of lipids from birch cellulose, a two-step cocultivation experiment was carried out. In the first step, 20% (wt/vol) of the substrate was hydrolyzed by recombinant strains expressing the whole set of cellulase genes. The second step was performed by a recombinant cellobiose-utilizing strain of *R. opacus* PD630, which accumulated 15.1% (wt/wt) fatty acids from the cellobiose formed in the first step.

Growing concerns about the environmental impact, global supply, and security of fossil energy carriers have led to a high demand for alternative liquid transportation fuels (1). One potential alternative is the biological production of fuels from renewable biomass, so-called biofuels (2). Currently, the dominant biofuels are ethanol made from corn or sugar cane and biodiesel generated by transesterification of vegetable oils (3). Owing to the limited growth potential of agricultural production and the projected increase in the world's energy consumption and population, future biological fuels will have to originate from inedible, abundant, and renewable lignocellulosic biomass (so-called second-generation biofuels) (4). Despite extensive research in the recent past, no production process has left the proof-of-concept stage. Costs for the production of cellulosic biofuels are still twice those for starch-based fuels, and no commercial-scale production has been established (4). One of the major challenges to economic and competitive fermentation processes is the large amount of enzyme needed for efficient saccharification of lignocellulosic biomass (5). The conversion of lignocellulosic biomass requires breakdown into its components: cellulose, hemicellulose, and lignin (6). The major component is cellulose (40 to 50%), composed of β -1,4-linked D-glucose residues (7). The key step in cellulose degradation and its subsequent fermentation is the saccharification of the polymeric substrate into simple sugars, usually mediated by the action of at least three synergistically acting enzymes: endoglucanase (EG) (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21) (8). At present, these enzymes are usually produced in a dedicated process and represent the second highest expense after the feedstock (5). Consolidated bioprocessing (CBP), also known as simultaneous saccharification and fermentation (SSF), is regarded as a potential alternative to dedicated enzyme production, combining saccharification and the production of commodity chemicals in a single microorganism (9).

Many advances with regard to (cellulosic) biofuels have been made, improving the ethanol yields of microbial fermentations, mostly by employing industrial production strains such as *Saccharomyces cerevisiae* or *Escherichia coli*, but also strains of *Zymomonas* (10, 11). However, ethanol is not an ideal substitute for today's petro-fuels, although it currently dominates the biofuel market, especially in the United States and Brazil. The energy density of ethanol is only about 70% that of gasoline, and most existing engines are limited to fuel blends with no more than 10% ethanol (E10). Furthermore, the corrosiveness and hygroscopicity of ethanol hinder its distribution via the existing infrastructure, and its recovery from fermentation broth consumes large amounts of energy (12).

Consequently, alternative fuels with properties comparable to those of petro-fuels have attracted more and more interest over the years (13). Among others, fatty acids are regarded as one potential alternative, and currently, fatty acids derived from plant oils are transesterified to fatty acid methyl ester (FAME) or fatty acid ethyl ester (FAEE), as it occurs in so-called biodiesel. The fatty acids already provide carbon chain lengths that are compatible with current engine technologies (14). Besides plants, several microorganisms are known to produce large amounts of fatty acids, stored either as triacylglycerols (TAG) or as wax esters (WE) in

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i>		
XL10-Gold	<i>endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173</i> Tet ^r F' [<i>proAB lacI^qZΔM15 Tn10</i> (Tet ^r Amy Cm ^r)]	Stratagene
Mach1-T1 ^R	F [−] ϕ80(<i>lacZ</i>)Δ <i>M15 ΔlacX74 hsdR</i> (r _K [−] m _K ⁺) Δ <i>recA1398 endA1 tonA</i>	Invitrogen
<i>R. opacus</i> PD630	TAG-producing strain	42
<i>C. fimi</i> ATCC 484	Cellulose utilization	43
<i>T. fusca</i> DSM43792	Cellulose utilization	44
Plasmids		
pEC-K18 <i>mob2</i>		22
pJAM2		23
pEC-K18 <i>mob2::cenA</i>	<i>cenA</i> as EcoRI/BamHI fragment	This study
pEC-K18 <i>mob2::cenB</i>	<i>cenB</i> as EcoRI fragment	This study
pEC-K18 <i>mob2::cenC</i>	<i>cenC</i> as XbaI fragment	This study
pEC-K18 <i>mob2::cex</i>	<i>cex</i> as EcoRI fragment	This study
pEC-K18 <i>mob2::cbhA</i>	<i>cbhA</i> as BamHI/XbaI fragment	This study
pEC-K18 <i>mob2::cel6A</i>	<i>cel6A</i> as SacI/KpnI fragment	This study
pEC-K18 <i>mob2::cenA-SP</i>	<i>cenA-SP</i> as EcoRI/BamHI fragment	This study
pEC-K18 <i>mob2::cenBA</i>	<i>cenBA</i> as EcoRI/BamHI fragment	This study
pCellulose	<i>cenA</i> , <i>cex</i> , and <i>cel6A</i>	This study
pJAM2:: <i>cenC::cex::cbhA</i>	<i>cenC</i> , <i>cex</i> , and <i>cbhA</i> as XbaI/Clal fragment	This study
pEC-K18 <i>mob2::bglABC</i>	<i>bglABC</i> as EcoRI/XbaI fragment	45

intracellular inclusions (15). *Rhodococcus opacus* strain PD630 is the model oleaginous prokaryote with regard to the accumulation and biosynthesis of lipids, which serve as carbon and energy storage compounds and can account for as much as 87% of the dry mass of the cell in this strain (16). It has been considered the production strain for high-value TAG (i.e., monoalkyl esters of short-chain alcohols and long-chain fatty acids) used in the production of biodiesel, due to its high substrate tolerance, high-density culturing, and rapid growth, which make it more favorable than other production organisms (17).

The aim of the present study was to enable the noncellulolytic, lipid-accumulating actinomycete *R. opacus* PD630 to degrade cellulose to the dimeric sugar cellobiose so as to provide the basis for the production of TAG-derived biofuels from lignocellulosic biomass by SSF. Here we report on the successful establishment of cellulose degradation based on the episomal expression of different cellulase-encoding genes by recombinant strains of *R. opacus* PD630.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and cultivation conditions. All bacteria and plasmids used in this study are listed in Table 1; the primers used are listed in Table S1 in the supplemental material. Cells of *R. opacus* PD630 were cultivated at 30°C in mineral salts medium (MSM) as described in reference 18. Carbon sources were added to liquid MSM as indicated below. Liquid cultures in Erlenmeyer flasks were incubated on a horizontal rotary shaker at an agitation of 110 rpm. Solid media were prepared by the addition of 1.5% (wt/vol) agar. *Escherichia coli* cells were cultivated at 37°C in lysogeny broth (LB); *Thermobifida fusca* DSM43792 cells were grown in Czapek peptone medium at 42°C (19); and cells of *Cellulomonas fimi* ATCC 484 were grown in Standard I medium at 30°C (Carl Roth, Karlsruhe, Germany). Antibiotics were used according to reference 20 and as indicated below.

Isolation, analysis, and modification of DNA. Plasmid DNA was prepared from crude lysates by the alkaline extraction method (21). Total

DNA of *C. fimi* ATCC 484 and *T. fusca* DSM43792 was prepared by using the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Restriction endonucleases (Fermentas, St. Leon Rot, Germany) were applied under the conditions recommended by the manufacturer. All other genetic procedures and manipulations were conducted as described in reference 20.

Construction of plasmids and transfer into *E. coli*. The coding regions of *cenA* (accession no. M15823.1), *cenB* (accession no. M64644.1), *cenC* (accession no. X57858.1), *cex* (accession no. M15824.1), and *cbhA* (accession no. L25809.1) from *C. fimi* ATCC 484 and of *cel6A* (accession no. M73321.1) from *T. fusca* DSM43792 were amplified by PCR using oligonucleotides FcenA and RcenA for *cenA*, FcenB and RcenB for *cenB*, FcenC and RcenC for *cenC*, Fcex and Rcx for *cex*, FcbhA and RcbhA for *cbhA*, and Fcel6A and Rcel6A for *cel6A* (Table 1). For PCR, Hercules II DNA polymerase (Agilent, Santa Clara, CA, USA) was used according to the manufacturer’s instructions. PCR products were extracted from the gel after separation using the PeqGOLD gel extraction kit (Peqlab, Erlangen, Germany). For expression experiments in *R. opacus*, the *E. coli*-*Corynebacterium glutamicum* shuttle vector pEC-K18*mob2* containing the *lac* promoter (22) and the *E. coli*-*Mycobacterium-Rhodococcus* shuttle vector pJAM2 containing the *ace* promoter (23) were used for the cloning of *cenA*, *cenB*, *cenC*, *cex*, *cbhA*, *cel6A* and *cenA-SP* with the respective restriction enzymes (see Table S1 in the supplemental material). These plasmids conferred resistance to kanamycin (50 to 75 μg/ml), for the purpose of selection, on *E. coli* and *R. opacus* strain PD630. All plasmids were transferred to *E. coli* strain XL10-Gold by transformation (24).

In-Fusion construction of plasmids. For the simultaneous cloning of multiple genes, the In-Fusion HD EcoDry cloning kit (Clontech, Otsu, Shiga, Japan) was used according to the manufacturer’s instructions. For PCR of *cenA*, *cenC*, *cex*, and *cbhA* from *C. fimi* ATCC 484 and of *cel6A* from *T. fusca* DSM43792, oligonucleotides IFcenA and IFRcenA for *cenA*, IFcenC and IFRcenC for *cenC*, IFcbhA and IFRcbhA for *cbhA*, IFcex and IFRcex for *cex*, and IFcel6A and IFRcel6A for *cel6A* were used (see Table S1 in the supplemental material).

Transfer of DNA into *R. opacus* PD630 by electroporation. Plasmids pEC-K18*mob2*, pEC-K18*mob2::cenA*, pEC-K18*mob2::cenB*, pEC-K18*mob2::*

cenC, pEC-K18mob2::*cbhA*, pEC-K18mob2::*cex*, pEC-K18mob2::*cel6A*, pEC-K18mob2::*cenA*::*cel6A*, pEC-K18mob2::*cenA*-SP, pJAM2, and pJAM2::*cenC*::*cex*::*cbhA* (Table 1) were transferred by electroporation using the protocol described previously (25).

Preparation of soluble fractions of *R. opacus* PD630 cells. A 50-ml culture of *R. opacus* PD630 was incubated for 24 h at 30°C. Cells were harvested by centrifugation ($4,000 \times g$) for 15 min, washed twice with sterile saline (0.85% [wt/vol] NaCl), and suspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.4). Cells were lysed by a 10-fold passage through a precooled French pressure cell at 1,000 MPa. The lysates obtained were centrifuged as described above in order to remove residual cells, and the soluble and membrane fractions were prepared by a 1-h centrifugation of the supernatant at $100,000 \times g$ and 4°C.

Preparation of cellulosic substrates. Microcrystalline cellulose (MCC) and carboxymethyl cellulose (CMC; Carl Roth, Karlsruhe, Germany) were used directly without prior processing. Bleached birch cellulose and cotton battings were cut into pieces of approximately 0.5 cm². Wheat straw was washed with distilled H₂O until the wash fluid was clear and was subsequently dried at 70°C. All substrates were added to flasks and were sterilized by autoclaving with no medium added.

Phosphoric acid swollen cellulose (PASC) was prepared as follows. Five grams of MCC was moistened with distilled H₂O, and 150 ml of ice-cold *ortho*-phosphoric acid (85%) was added. The mixture was stirred in an ice bath for 1 h. Then 100 ml of ice-cold acetone was added, and the suspension was sucked off, washed 3 times with 100 ml acetone, and again washed twice with 500 ml distilled H₂O. The PASC was suspended with 100 ml of distilled H₂O and was stored in a refrigerator for as long as 1 month. Before use, PASC was harvested by centrifugation, washed twice with sterile mineral salts medium, added to flasks, and sterilized by autoclaving.

Qualitative cellulase activity assay. Qualitative analysis of cellulase activity was carried out as described by Béguin (26). In brief, recombinant strains harboring plasmids with cellulase genes were incubated on MSM plates containing 0.5% (wt/vol) CMC and 0.1% (wt/vol) glucose at 30°C for 3 days. Directly thereafter, the plates were stained with a 0.1% (wt/vol) Congo red solution for 5 min. The plates were destained with a 1 M NaCl solution until clear zones were visible.

Quantitative enzyme activity assays. The activities of cellulases were determined with azo-CMC (Megazyme, Dublin, Ireland). For this purpose, 1 ml of the corresponding *R. opacus* PD630 culture in the late-exponential-growth phase, grown in liquid MSM with 1% (wt/vol) glucose as the carbon source, was harvested by centrifugation, and 125 µl of the supernatant was mixed with 125 µl of 50 mM Tris-HCl buffer (pH 7.4) and was preincubated for 10 min at 30°C. The reaction was started by the addition of 250 µl unbuffered azo-CMC solution and 10 s of mixing with a vortex mixer. The mixture was incubated at 30°C for 30 min, and the reaction was finally stopped by the addition of 1.25 ml precipitant solution (80% [vol/vol] ethanol with 0.29 M sodium acetate and 22 mM zinc acetate [pH 5]). The remaining nonhydrolyzed substrate was removed by 10 min of centrifugation at $1,000 \times g$, and the absorbance of the supernatant at 590 nm was measured and compared with that of the reaction blank in a spectrophotometer. Activities were calculated by reference to a standard curve with an EG from a *Trichoderma* sp. (catalog no. E-CELTR; lot 80701; Megazyme International Ireland Ltd., Bray, Ireland).

Quantitative analysis of cellobiose contents. The cellobiose contents of the medium were analyzed by high-performance liquid chromatography (HPLC). The culture medium was centrifuged for 10 min at $18,000 \times g$ to remove cells. Supernatants were filtered using 0.2-µm Spartan filters (Whatman, Dassel, Germany) and were applied to a Eurokat Pb column (order no. 30GX350EKN; Knauer, Berlin, Germany) using water-acetonitrile (95:5) as the eluent at 75°C with a flow rate of 0.5 ml · min⁻¹. The HPLC system used comprises a Kontron system 522 pump, an HPLC 560 autosampler (Kontron, Munich, Germany), and a Sedex 80 low-temperature evaporative light-scattering (LT-ELS) detector (Sedere, Alfortville, France).

Analysis of fatty acid contents of recombinant *R. opacus* PD630 cells by gas chromatography (GC). The fatty acid contents of recombinant *R. opacus* PD630 cells were determined as described in detail elsewhere (17).

RESULTS

Search for genes encoding cellulase enzymes in *R. opacus* PD630. Although the wild type *R. opacus* PD630 utilizes a variety of different sugars, including D-glucose and L-rhamnose, both of which are constituents of plant hemicellulose, as well as starch-derived sugars, such as maltotriose or maltose, it cannot degrade cellulose and its dimer cellobiose. It was concluded earlier by Holder and coworkers, on the basis of the genome sequence of *R. opacus* PD630, that the lack of suitable hydrolases capable of cleaving the β-1,4 linkage is most likely the reason for this deficiency (27). In agreement with that study (27), we were not able to detect cellulase activity in *R. opacus* PD630.

Strategies to establish cellulose degradation in *R. opacus* PD630. For heterologous expression in *R. opacus* PD630, six cellulases from two different cellulolytic Gram-positive bacteria, *Cellulomonas fimi* ATCC 484 (CbhA, CenA, CenB, CenC, and Cex) and *Thermobifida fusca* DSM43792 (Cel6A), were chosen. These cellulases exhibit high activities toward cellulose and possess an inherent signal peptide, which should allow the secretion of these cellulases by *R. opacus* PD630. The corresponding genes have a high G+C content, matching the codon usage of *R. opacus* PD630. All genes were already heterologously expressed in *E. coli*, and the combination of endocellulases (CenA [28], CenB [29], CenC [30], Cel6A [31] [EC 3.2.1.4]), one cellobiohydrolase (CbhA [32] [EC 3.2.1.91]), and an exocellulase (Cex [33] [EC 3.2.1.8]), which could act synergistically, was considered to enhance cellulose degradation, thereby increasing the overall cellobiose yields of recombinant strains.

An expression system encoded by the *E. coli*-*Mycobacterium*-*Rhodococcus* shuttle vector pJAM2 (23, 34) was chosen. In addition, the *E. coli*-*Corynebacterium* shuttle vector pEC-K18mob2 was tested for replication in *R. opacus* PD630. After 3 days, transformants appeared on selective medium, and plasmid DNA was isolated from three randomly chosen transformants. Restriction analysis with specific nucleases confirmed the autonomous replication of pEC-K18mob2 in *R. opacus* PD630. The plasmid copy numbers of pEC-K18mob2 and pJAM2 in *R. opacus* PD630 grown in MSM were calculated as 39 ± 4 and 6 ± 1 copies per chromosome, respectively (35).

Cloning of cellulase genes in expression vectors suitable for *R. opacus* PD630. For expression experiments, all genes comprising suitable ribosome binding sites for *R. opacus* PD630 were ligated, alone or in combination, either to the pEC-K18mob2 vector under the control of the *lac* promoter or to the pJAM2 vector under the control of the acetamidase promoter, yielding plasmids pEC-K18mob2::*cbhA*, pEC-K18mob2::*cenA*, pEC-K18mob2::*cenB*, pEC-K18mob2::*cenBA*, pEC-K18mob2::*cenC*, pEC-K18mob2::*cel6A*, pEC-K18mob2::*cex*, pJAM2::*cenC*::*cex*::*cbhA*, and pCellulose (Table 1; Fig. 1).

Control of gene expression and detection of cellulolytic activity. All plasmids were transferred to *E. coli* Mach1-T1^R for qualitative activity assays. It was shown previously that the heterologous expression of cellulase genes from Gram-positive bacteria in *E. coli* led to the accumulation of the enzymes in the cytoplasm and periplasm and that the increased level of expression resulted in nonspecific leakage of the premature but active enzymes into the

FIG 1 Physical maps of the plasmids constructed. Relevant cleavage sites and structural genes are indicated. Km^r, kanamycin resistance cassette; *rep*, origin of replication; *per*, positive effector of replication. The structural genes (with accession numbers given in parentheses) encode the following enzymes: *cenA* (M15823), endocellulase A; *cenB* (M64644.1), endocellulase B; *cenC* (X57858.1), endocellulase C; *cbhA* (L25909.1), cellobiohydrolase A; *cex* (M15824), exocellulase from *C. fimi*; *cel6A* (M73321), endocellulase 6A from *T. fusca*.

medium (36). All strains harboring endocellulase genes exhibited clear-zone formation, indicating that cellulose was hydrolyzed by recombinant endocellulases. In contrast, the empty vectors pEC-K18mob2 and pJAM2 did not confer the ability to hydrolyze cellulose on the cells.

Transfer of plasmids to *R. opacus* PD630 and establishment of cellulose degradation. All plasmids were transferred to *R. opacus* PD630 by electroporation. All recombinant strains were transferred to MSM plates containing 0.1% (wt/vol) glucose and 0.5% (wt/vol) CMC, incubated at 30°C for 3 days, and stained with Congo red. In agreement with the results of the investigation of CMC degradation in recombinant *E. coli* strains, all plasmids, except for plasmids pEC-K18mob2::*cbhA*, pEC-K18mob2::*cex*, and the endocellulase-containing plasmids pEC-K18mob2::*cenB* and pEC-K18mob2::*cenA*, conferred the ability to degrade CMC on *R. opacus* PD630, whereas endocellulase activity was absent in the vector control strains (see Fig. S1 in the supplemental material). All enzymes were also shown to exhibit activity toward PASC after 4 days of incubation, although their activities, visible by the formation of small halos around the colonies, were dramatically lower than that of CMC. However, no clear-zone formation was observed after the staining of MSM plates containing a microcrystalline cellulose (MCC) overlay instead of a CMC or PASC overlay.

Localization of cellulolytic activity. To determine whether the cellulases are translocated through the membrane by their Sec

translocon or if the cellulase activity in the medium is the result of cell lysis and subsequent leakage of the enzyme, the signal peptide sequence, as predicted by SignalP (37) and previous determinations (28) for *cenA*, was omitted by PCR. The product was ligated to the pEC-K18mob2 vector, yielding plasmid pEC-K18mob2::*cenA*-SP. This plasmid was transferred to *E. coli* Mach1-T1^R and *R. opacus* PD630, and the cellulase activity in the culture medium was determined. Neither recombinant *E. coli* supernatants nor *R. opacus* PD630 supernatants exhibited activity on MSM plates containing 1% (wt/vol) CMC after 2 days of incubation, in contrast to the control strains harboring pEC-K18mob2::*cenA*. To check whether the truncated *CenA* enzyme was active or whether it had lost its activity completely as a result of the modification, the soluble cell and membrane fractions of disrupted *R. opacus* PD630/pEC-K18mob2::*cenA*-SP cells were screened for activity. It was found that cellulase activity was present only in the soluble cell fraction, indicating that the truncated enzyme was no longer translocated through the cell membrane and that, vice versa, the native enzyme was indeed secreted into the culture medium and was not only leaked during cell lysis.

Quantitative determination of endocellulase activities in culture supernatants of *R. opacus* PD630. The quantitative activities of the EGs of four recombinant strains of *R. opacus* expressing different cellulase genes were determined with azo-CMC (Megazyme, Ireland) as the substrate (Fig. 2). In general, the activities determined for the supernatants of the four

FIG 2 Endocellulase activity of recombinant *R. opacus* PD630 in the culture medium. Activity was determined with azo-CMC (Megazyme, Ireland) at 30°C. Bar 1, *R. opacus*/pEC-K18mob2::cenA; bar 2, *R. opacus*/pEC-K18mob2::cenB; bar 3, *R. opacus*/pEC-K18mob2::cenC; bar 4, *R. opacus*/pEC-K18mob2::cel6A.

strains tested were relatively low, ranging from $0.01 \text{ U} \cdot \text{mL}^{-1}$ to $0.313 \pm 0.01 \text{ U} \cdot \text{mL}^{-1}$ for strains carrying pEC-K18mob2::cenB and pEC-K18mob2::cenA, respectively. No endocellulase activity was measured in the soluble cell fraction or in the periplasm of these strains.

Quantitative determinations of cellulase activity toward MCC in *R. opacus* PD630. To quantify the cellulase activities of recombinant *R. opacus* strains in the culture medium, cells were cultivated in liquid MSM containing 1% (wt/vol) glucose as the carbon source plus 1% (wt/vol) MCC as the substrate, and the concentrations of the main cellulase product, cellobiose, were determined by HPLC after several days of incubation (Fig. 3). All cellulases tested exhibited activity toward MCC in liquid culture, whereas no activities were detected in the vector control strains. Among recombinant strains, those expressing *cenA* exhibited the highest MCC conversion rates, amounting to $2.2\% \pm 0.07\%$ (wt/vol) of MCC converted after 35 days. To further investigate the synergistic actions of endo- and exocellulases, the recombinant strains were also cocultivated so as to determine the optimal enzyme set for the efficient hydrolysis of cellulose by *R. opacus* PD630. For this purpose, all precultures were adjusted to an optical density of 15, and flasks were inoculated with equal volumes of the corresponding strains. As expected, cultures with combinations of exo- and endocellulases exhibited conversion rates higher than those for single- or double-endocellulase cultures. The highest cellobiose contents in the medium were found when all available cellulases were used ($3.7\% \pm 0.03\%$ [wt/vol]), followed by those for pEC-K18mob2::cenA/pJAM2::cenC::cex::cbhA and pEC-K18mob2::cel6A/pJAM2::cenC::cex::cbhA cultures ($3.6\% \pm 0.05\%$ [wt/vol] and $3.2\% \pm 0.05\%$ [wt/vol], respectively). The additional expression of *cenC* by the high-copy-number vector pEC-K18mob2 increased the MCC conversion rate by 60% (from $2.7\% \pm 0.01\%$ [wt/vol] to $4.3\% \pm 0.08\%$ [wt/vol], respectively). The highest activity was observed when 2% MCC was used ($3.3\% \pm 0.16\%$ [wt/vol]).

FIG 3 Quantitative cellulase enzyme assay. Recombinant strains of *R. opacus* PD630 were cultivated alone (experiments 1 to 3) or in combination (experiments 4 to 7) in liquid MSM containing 1% (wt/vol) MCC plus 1% (wt/vol) glucose. Cellobiose contents were determined after 16 (light shaded bars), 25 (dark shaded bars), and 35 (filled bars) days of incubation. Experiments: 1, *R. opacus*/pEC-K18mob2::cenA; 2, *R. opacus*/pJAM2::cenC::cex::cbhA; 3, *R. opacus*/pEC-K18mob2::cel6A; 4, *R. opacus*/pEC-K18mob2::cenA and *R. opacus*/pJAM2::cenC::cex::cbhA; 5, *R. opacus*/pEC-K18mob2::cenA and *R. opacus*/pEC-K18mob2::cel6A; 6, *R. opacus*/pEC-K18mob2::cel6A and *R. opacus*/pJAM2::cenC::cex::cbhA; 7, *R. opacus*/pEC-K18mob2::cenA, *R. opacus*/pEC-K18mob2::cel6A, and *R. opacus*/pJAM2::cenC::cex::cbhA; 8, vector control strain *R. opacus*/pEC-K18mob2. Error bars indicate standard deviations of triplicate measurements.

TABLE 2 Rates of conversion of different cellulosic materials by recombinant cellulases

Substrate	Conversion rate (%) with:	
	Cocultivation ^a	CenA reference strain ^b
Copy paper	3.3 ± 0.2	1.8
Cotton	5.3 ± 0.9	4.3
Sawdust	ND ^c	0
Hygienic paper	ND	7.2
Wheat straw	ND	1.3

^a *R. opacus* PD630/pEC-K18mob2::cenA, *R. opacus* PD630/pEC-K18mob2::cel6A, and *R. opacus* PD630/pJAM2::cenC::cex::cbhA.

^b *R. opacus* PD630/pEC-K18mob2::cenA.

^c ND, not determined.

Expression plasmid pCellulose. Based on the results obtained in the MCC cocultivation experiment, a pEC-K18mob2 derivative harboring a set of three endocellulase- and two exocellulase/cellobiohydrolase-encoding genes (*cenA*, *cenC*, *cbhA*, *cex*, and *cel6A*) was designed by employing the In-Fusion (Clontech, Japan) system. However, analysis of the transformants obtained revealed that the *cenA*, *cex*, and *cel6A* genes had hybridized by their ribosome binding sites and that neither *cbhA* nor *cenC* was integrated into the final construct. The resulting plasmid, pEC-K18mob2::cenA::cex::cel6A, was designated pCellulose. After its transfer to *R. opacus*, the endocellulase activity in the culture supernatant was determined to be $0.262 \pm 0.02 \text{ U} \cdot \text{ml}^{-1}$, and $9.3\% \pm 0.6\%$ (wt/vol) or $3.2\% \pm 0.1\%$ of bleached birch cellulose or MCC, respectively, was converted to cellobiose after 22 days.

Degradation of various cellulolytic materials. The relative abilities of recombinant *R. opacus* PD630 to degrade a variety of cellulosic materials, besides artificial cellulose substrates, was of special interest. In total, five different materials—softwood sawdust, shredded copy paper, wheat straw, cotton, and hygienic paper—were tested as possible substrates without further processing. One percent (weight/volume) of the material served as the substrate, whereas 1% (wt/vol) glucose was used as the carbon source. Cellulase activity was observed for all substrates tested except sawdust (Table 2). In general, cellulase activity increased with an enhanced substrate surface (e.g., hygienic paper, which became suspended more rapidly than the more rigid copy paper) and decreased with lignin content (e.g., wheat straw and softwood sawdust).

Production of lipids from birch cellulose. Birch cellulose was fermented to lipids in two steps. First, 1% (wt/vol) birch cellulose was saccharified by a coculture of *R. opacus* strains PD630/pEC-K18mob2::cenA, PD630/pECK18mob2::cenC, PD630/pECK18mob2::cel6A, and PD630/pJAM2::cenC::cex::cbhA for 18 days. At two time points, the cellobiose contents of the culture medium were determined by HPLC. After 11 days, $17.4\% \pm 1.1\%$ of the birch cellulose was converted to cellobiose, corresponding to a cellobiose concentration of 0.17% (wt/vol); at the end of the cultivation, after 18 days, $20\% \pm 0\%$ (wt/vol) was converted (cellobiose concentration, 0.2% [wt/vol]). Before the inoculation of the flasks with the cellobiose-utilizing strain *R. opacus* PD630/pEC-K18mob2::bglABC, cells were removed by centrifugation and filtration, and the cultures were incubated for another period of 4 days, until the cellobiose of the medium was completely depleted. For these cells, the fatty acid content of the dry matter was determined to be 15.1% (wt/wt) by GC analysis.

DISCUSSION

In this study, we conferred the ability to degrade cellulose on *R. opacus* PD630 by the heterologous expression of as many as three genes, which were episomally introduced by using two vector systems with differing copy numbers per cell (high and low copy numbers) and different promoters (the *lac* promoter and the acetamidase promoter) controlling gene expression. Both plasmids were stably replicated in *R. opacus*, and the copy numbers per cell of these plasmids matched those found in the literature for two other members of the phylum Actinobacteria, *Mycobacterium smegmatis* (23) and *Corynebacterium diphtheriae* (22). The strengths of the two promoters were not determined; however, induction of the pJAM2 acetamidase promoter by the addition of 1% acetamide had no effect on the cellulase activities of recombinant strains (data not shown). Therefore, we concluded that both promoters are constitutively expressed in *R. opacus*.

As a noncellulolytic organism, *R. opacus* PD630 secretes neither an inherent and functional cellulolytic enzyme nor cellobiose, the main product of most bacterial cellulases (27). Therefore, five foreign genes from the actinomycetes *C. fimi* ATCC 484 and *T. fusca* DSM43792, encoding already characterized endo- and exocellulases as well as cellobiohydrolases, were chosen for the establishment of cellulose degradation in order to investigate the modulation of action and thus possible synergism, the signal translocation, and codon bias. CMC was used as the substrate for a rapid, qualitative determination of enzyme activities, but this assay is usually restricted to endocellulase activity only. For exocellulases and cellobiohydrolases, large amounts of enzyme and long incubation times are required (32), which could not be achieved in this study. However, an effort at qualitative determination of the activities of the nonendocellulase enzymes with MCC plates at various concentrations was not successful, most likely because of both low enzyme concentrations and low activity toward MCC. Endocellulase activity in the culture medium could be detected for CenA, CenC, and Cel6A. In contrast, no activity was found in the cytoplasm and periplasm of these strains, which might reflect the generally low activities detected. Interestingly, no endocellulase activity could be found for strains expressing *cenB*, and CenC exhibited little activity, whereas CenA proved superior to all other endocellulases. This is remarkable, since previous studies identified CenB and CenC as the most active carboxymethyl cellulases of *C. fimi*, but at least CenC is also known for its susceptibility to proteases, so degradation by inherent proteases of *R. opacus* cannot be excluded (38). Thus, CenB especially, since it exhibits higher activity toward crystalline cellulose than CenA and CenC (39), is still an important candidate for future experiments.

Because of this high carboxymethyl cellulase activity, CenA was selected as a model enzyme for the secretion of cellulases by *R. opacus*. The enzyme without the signal peptide was neither secreted nor leaked by *R. opacus* in the time course of the cultivation. Its activity was restricted to the cytoplasm, in contrast to the control harboring native *cenA*. In case of a leakage, the activities would have been nearly identical, since both the premature and mature enzymes are active. Consequently, we concluded that the cellulases employed are actively secreted by *R. opacus*, although the signal peptides of the cellulases are not identical. The secretion efficiencies of the cellulases might therefore differ.

Natural cellulose exhibits a crystallinity of about 70%, and the crystalline regions are more resistant to enzymatic as well as chem-

ical hydrolysis (40). Efficient bacterial cellulase systems must therefore always include exocellulase and cellobiohydrolase enzymes, which preferentially attack the more crystalline regions of the cellulose chain. The resulting decrease in the crystallinity of the substrate increases susceptibility to endocellulases, which, in turn, produce chain ends for cellobiohydrolases. Hence, this synergism is not usually found between endocellulases but is commonly observed between endo- and exocellulases as well as between different exocellulases (39). To detect the (synergistic) activities of the exocellulase employed and the cellobiohydrolase, we measured the activities of these enzymes together with the endocellulases in cocultivation experiments by determining the amounts of cellobiose formed. This approach has the advantage that exocellulase and cellobiohydrolase activities and synergisms between the three cellulase types can be analyzed. Furthermore, it gives a realistic impression of the hydrolysis efficiency to be expected later. However, it also has disadvantages, such as the accumulation of high cellobiose levels, which might be inhibitory to the cellulases; the unbalanced growth of the different strains employed, which makes it impossible to draw conclusions about the optimal stoichiometric ratio of the cellulases; and the fact that the decrease in the degree of polymerization by the action of endocellulases is not detected, because only the formation of the end product cellobiose is analyzed. As expected, the expression of *cex* and *cbhA* greatly enhanced the MCC conversion rates both with CenA and CenC and with Cel6A and CenC, respectively, whereas the combination of the endocellulases CenA and Cel6A exhibited no synergistic effect. Accordingly, high cellulose conversion rates could be achieved with plasmid pCellulose. The results obtained in the quantitative endocellulase enzyme assay, where a combination of endocellulases also exhibited no beneficial effect, were thereby corroborated. The cocultivation of three strains also yielded higher cellobiose concentrations with different cellulosic substrates, since these contained crystalline cellulose.

The conversion rates and lipid quantities obtained in this study should be regarded as a proof of concept rather than as indicating a viable production process. The long incubation time needed for saccharification of cellulose correlates with the relatively low cellulase activities determined. Hence, it is imperative for future investigations to improve the overall yields and activities of these cellulases. This could be achieved by the utilization of stronger *R. opacus* or xenogeneic promoters and vectors with even higher copy numbers than that of pEC-K18*mob2*, which was employed in this study. The further broadening of the cellulase enzyme set by additional, so far underrepresented cellulase families, such as Cel48A of *T. fusca* (41), may enhance the hydrolysis performance of recombinant strains. In addition, improvement of the accessibility of the cellulose by common preprocessing techniques, i.e., the removal of the hemicellulose and lignin portions and efficient milling of the cellulosic substrates to increase the available surface area prior to cultivation, should be considered. This would greatly improve cellulose accessibility and overall hydrolysis efficiency.

With regard to lipid production directly from cellulose (SSF) by recombinant strains, it would be desirable to join the cellulase genes and the cellobiose utilization genes in one plasmid.

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