

Engineering of a Xylose Metabolic Pathway in *Rhodococcus* Strains

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The two metabolically versatile actinobacteria *Rhodococcus opacus* PD630 and *R. jostii* RHA1 can efficiently convert diverse organic substrates into neutral lipids mainly consisting of triacylglycerol (TAG), the precursor of energy-rich hydrocarbon. Neither, however, is able to utilize xylose, the important component present in lignocellulosic biomass, as the carbon source for growth and lipid accumulation. In order to broaden their substrate utilization range, the metabolic pathway of D-xylose utilization was introduced into these two strains. This was accomplished by heterogenous expression of two well-selected genes, *xylA*, encoding xylose isomerase, and *xylB*, encoding xylulokinase from *Streptomyces lividans* TK23, under the control of the *tac* promoter with an *Escherichia coli*-*Rhodococcus* shuttle vector. The recombinant *R. jostii* RHA1 bearing *xylA* could grow on xylose as the sole carbon source, and additional expression of *xylB* further improved the biomass yield. The recombinant could consume both glucose and xylose in the sugar mixture, although xylose metabolism was still affected by the presence of glucose. The xylose metabolic pathway was also introduced into the high-lipid-producing strain *R. opacus* PD630 by expression of *xylA* and *xylB*. Under nitrogen-limited conditions, the fatty acid composition was determined, and lipid produced from xylose by recombinants of *R. jostii* RHA1 and *R. opacus* PD630 carrying *xylA* and *xylB* represented up to 52.5% and 68.3% of the cell dry weight (CDW), respectively. This work demonstrates that it is feasible to produce lipid from the sugars, including xylose, derived from renewable feedstock by genetic modification of rhodococcus strains.

Lipid consisting of fatty acids can be transformed through hydro-treatment into a range of hydrocarbons, including green diesel, or by transesterification into biodiesel as a renewable fuel replacement (11, 33, 43). As an alternative feedstock for the hydrocarbon, lipid produced by microorganisms has attracted much attention (34). It was reported that a variety of eukaryotic microorganisms, such as microalgae, yeasts, and fungi, accumulate a high content of mainly lipids, including triacylglycerol (TAG), under imbalanced nutrition conditions (11, 46, 55). In contrast, most bacteria are able to accumulate lipophilic storage products, such as polyhydroxyalkanoic acid (PHA) or poly(3-hydroxybutyric acid) (PHB) (12, 42). However, a few bacteria, mainly bacteria belonging to the genera *Acinetobacter*, *Mycobacterium*, *Streptomyces*, *Nocardia*, and *Rhodococcus*, can accumulate TAG during cultivation under nitrogen-limited conditions (4, 54). Strains of the Gram-positive bacterium *Rhodococcus* are especially of biotechnological importance due to the broad catabolic diversity of their enzymatic capabilities (37, 51). It was reported that the metabolically versatile bacterium *Rhodococcus jostii* RHA1, which was isolated from lindane-contaminated soil and which was initially known for its outstanding ability to degrade polychlorinated biphenyls (PCBs) (37), could accumulate up to 48.4% and 56.9% of the lipid of the cell dry weight (CDW) when it was growing on glucose and gluconate under nitrogen-starving conditions, respectively (22). Another oleaginous rhodococcus strain, *R. opacus* PD630, a super single-cell oil (SCO) producer with a lipid content of 76% of CDW, has been thoroughly studied, including by lipid body characterization, lipid content determination, fatty acid composition analysis, high cell density culture, pilot-scale fermentation, and whole-genome sequencing (3, 24, 32, 53, 55). Clearly, strains *R. opacus* PD630 and *R. jostii* RHA1 have shown the ability to produce a high content of lipids, the main components of which are TAGs, from carbon sources, including glucose, gluconate, and sucrose (22, 32, 53).

The feedstocks of lignocellulosic biomass are the most abun-

dant renewable resources in the world (48). Fuels derived from lignocellulosic biomass will improve energy security, decrease greenhouse gas emissions, and move our industrial society toward sustainability (13, 50). The availability of genetic tools and complete genome sequences offers the opportunity to investigate the metabolic traits of the two *Rhodococcus* strains by genetic perturbation and functional genomics study (2, 22, 24, 35). Furthermore, the metabolic engineering approach can be used to improve microorganisms for utilization of carbohydrates from biomass (57). As discussed above, strains *R. opacus* PD630 and *R. jostii* RHA1 can efficiently convert carbon sources such as glucose into lipids, but there has been no report on improvement of their capabilities for utilization of the diverse sugars, including xylose, present in lignocellulosic biomass. The purpose of this work is to fill this gap by expanding the substrate range of *Rhodococcus* through implementation of the D-xylose metabolic pathway. In this study, the two well-characterized genes *xylA* and *xylB*, encoding D-xylose isomerase and xylulokinase from *Streptomyces lividans* TK23, respectively, were expressed in the *Rhodococcus* strains to provide them with a xylose utilization capability (20). The *Rhodococcus* recombinants were characterized for their enzyme activities, growth performance, and sugar consumption. The role of the genes encoding endogenous xylulokinase in *R. jostii* RHA1 was also assessed. Finally, the lipid contents of recombinants of *R. opacus* PD630 and *R. jostii* RHA1 and the fatty acid profiles of the cells grown on xylose were studied.

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MATERIALS AND METHODS

Strains, plasmids, and cultivation conditions. The *Rhodococcus* strains *R. opacus* PD630 and *R. jostii* RHA1 were grown at 30°C in W minimal salt medium containing sugar as the carbon source (41). The composition of W medium (per liter) included 0.85 g KH₂PO₄, 4.90 g Na₂HPO₄ · 12H₂O, 0.50 g (NH₄)₂SO₄, 0.10 g MgSO₄ · 7H₂O, 9.50 mg, FeSO₄ · 7H₂O, 10.75 mg MgO, 2.00 mg CaCO₃, 1.44 mg ZnSO₄ · 7H₂O, 1.12 mg MnSO₄ · 4H₂O, 0.25 mg CuSO₄ · 5H₂O, 0.28 mg CoSO₄ · 7H₂O, 0.06 mg H₃BO₃ and 5.13 × 10⁻² ml concentrated HCl. The stock solutions of the sugars, including glucose and D-xylose, were sterilized with a 0.22-μm-pore-size filter and added to the autoclaved medium. The growth of the strains on other carbon sources, including 30 g/liter of D-arabinose, glycerol, glucose, galactose, lactose, L-arabinose, fructose, mannose, sucrose, and gluconate, was also tested. *Rhodococcus* cells grown in lysogeny broth (LB) medium were harvested for electroporation (47). *Escherichia coli* TOP10 grown at 37°C was used as the host strain for gene cloning. LB medium supplemented with 50 μg/ml kanamycin or 100 μg/ml ampicillin was used for culture of *E. coli* carrying plasmids. For the cultivation of *Rhodococcus* recombinants, 50 μg/ml of neomycin was used.

In order for the cells to accumulate lipid, the wild-type strains and the recombinants were cultured under nitrogen-limited conditions (22). The *Rhodococcus* cells were grown in LB medium and harvested by centrifugation at 8,000 × g and 4°C for 10 min. Cell pellets were washed twice with W medium without sugar and nitrogen. After washing, the cells were inoculated into 100 ml of W medium containing 20 g/liter of glucose or xylose and 0.5 g/liter (NH₄)₂SO₄. The culture was incubated at 30°C and 200 rpm in a 500-ml flask. The cells, grown for 96 h, were harvested and washed prior to lipid analysis.

DNA techniques. Genomic DNA of the bacterium was isolated by using an UltraClean microbial DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manual provided by the manufacturer. Plasmid DNA was extracted from *E. coli* by using a QIAprep mini-prep kit from Qiagen (Valencia, CA). Restriction endonucleases were purchased from NEB (MA). PCR was performed with Phusion high-fidelity DNA polymerase from NEB. For amplification of the genes *xylA* and *xylB* by PCR, GC buffer was added into the reaction mixture per the manufacturer's instruction. Oligonucleotide primers were synthesized by Invitrogen (Grand Island, NY). The sequence and generated restriction sites of the primers are listed in Table S1 in the supplemental material. After the agarose gel was run, the PCR products and plasmids digested by restriction endonucleases were recovered and purified with a QIAquick gel extraction kit (Qiagen). The competent cells of *E. coli* were prepared with CaCl₂ and transformed by the heat shock procedure (44). *Rhodococcus* cells were washed with water, treated with 10% glycerol, and then electroporated with the plasmids as previously described (47, 59). After electroporation, the cells were incubated on LB agar plates containing 50 μg/ml of neomycin.

Construction of plasmids carrying genes for xylose utilization. The *E. coli*-*Rhodococcus* shuttle vector pNV18 was used to develop the expression vector in this study (10). A DNA fragment containing a polyhistidine (His) tag was generated with the primers HisF and HisR by PCR using plasmid pNV18 as the template (see Table S1 in the supplemental material). The PCR product was digested with XhoI and EcoRI and inserted into these two restriction sites of plasmid pNV18 to obtain plasmid pNVHis18. The *tac* promoter (*P_{tac}*) containing a ribosome binding site (RBS) was amplified with the primers TacF and TacR by PCR using plasmid pTAC-MAT-Tag-1 (Sigma, St. Louis, MO) as the template. Then, plasmid pNVHis18 was treated with PstI and HindIII and ligated with the digested PCR product to produce the expression vector pTACHis18. The 1.2-kb PCR product of *xylA* was obtained using genomic DNA of *S. lividans* TK23 as the template. The PCR product was digested with the restriction endonucleases PstI and KpnI. The digestion product was inserted into plasmid pTACHis18 to generate the *xylA* expression vector pXYLA. Similarly, the recombinant expression vector bearing the 1.5-kb *xylB* fragment from *S. lividans* TK23 was constructed and designated

pXYLB. The expression cassette of *xylB*, including the coding sequence of *xylB* and *tac* promoter, was amplified by PCR with primers *xyl-tac1* and *xyl-tac2* using plasmid pXYLB as the template. The PCR product was digested with HindIII and inserted into plasmid pXYLA. The resulting 7.6-kb plasmid, pXYLAB, contained both *xylA* and *xylB*, and their expression was driven by *P_{tac}* individually (see Fig. S1 in the supplemental material).

Disruption of native RHA1 *xylB* genes. Analysis of the genome sequence of *R. jostii* RHA1 revealed that the deduced sequence of the amino acids of two putative open reading frames (ORFs), RHA1_ro02812 (*xylB1*) and RHA1_ro02901 (*xylB2*), exhibited significant homologies to the xylulokinase amino acid sequence (37). To study the roles of these two candidates in xylose catabolism in the wild-type strain, these two genes were disrupted, and the double knockout was also generated by homologous integration between the fragments of the chromosome and the disrupted ORFs in the introduced plasmids (Table 1). The suicide vector pK18mobsacB was used to develop plasmids for the deletion of the potential *xylB* genes from *R. jostii* RHA1 as described below (45, 52). Two DNA fragments containing two possible *xylB* genes of *R. jostii* RHA1, *xylB1* and *xylB2*, were amplified by PCR. The PCR products were digested with EcoRI and HindIII and cloned into plasmid pK18mobsacB to form the plasmids pKXYLB1 and pKXYLB2, respectively. To disrupt the coding region of *xylB1*, plasmid pKXYLB1 was treated with SalI and then self-ligated. The resulting plasmid was pKB1-SalI (Table 1). Plasmid pKB2-PstI, containing a PstI-digested DNA fragment, was constructed to disrupt *xylB2*. Strain *R. jostii* RHA1 was transformed with the derivative plasmids of pK18mobsacB, pKB1-SalI, and pKB2-PstI by electroporation. The integration of plasmids introduced into the chromosome in the first crossover was selected for on LB agar plates containing kanamycin. The antibiotic-resistant cells were picked up and cultured overnight in LB medium and spread on LB agar plates containing 10% (wt/vol) sucrose without antibiotics. The second crossover of chromosomal DNA led to kanamycin-sensitive cells, but some of these cells were the recovered wild-type strains. Mutants with the disrupted gene were detected by colony PCR using the same primers used for the amplification of the target genes. The single knockouts of *xylB1* and *xylB2* were designated *R. jostii* Δ*xylB1* and *R. jostii* Δ*xylB2*, respectively. *xylB2* of the strain with a disrupted *xylB1* was further deleted to construct the double-knockout strain *R. jostii* Δ*xylB1B2* (Table 1).

Enzyme assays. To investigate the function of the potential enzymes in *Rhodococcus* strains for xylose utilization and verify the expression of *xylA* and *xylB* from *S. lividans* TK23 in the recombinants, the enzyme activities of xylose isomerase and xylulokinase were determined by using the cell extract. The chemicals used in the enzymatic activity determination were obtained from Sigma (St. Louis, MO). The cell extracts of *Rhodococcus* were prepared by using an ultrasonic homogenizer (sonicator 3000; Misonix, CT), and all of the operations were performed on ice (29). The assay for the activity of xylose isomerase of the whole-cell lysate was carried out in a 1-ml reaction volume containing 70 mM D-xylose, 20 mM MgCl₂, 5 mM MnSO₄, and 2 mM dithiothreitol in 100 mM Tris buffer (pH 7.5) (29). The reaction mixture was incubated for 30 min at 30°C, and then 0.5 M HClO₄ was added to stop the reaction. The D-xylulose formed was quantified by using the cysteine-carbazole-sulfuric acid assay with measurement of the absorbance at 540 nm (*A*₅₄₀) (17, 18). The enzyme activity of xylulose kinase was determined by the reduction of D-xylulose in the reaction mixture as described before (29). One unit of enzyme activity was defined as the amount of enzyme which generated 1 μmol of the product or consumed the substrate, D-xylulose, per minute.

Analytical procedure. The samples were collected and centrifuged, and the supernatant was used for the determination of the content of the residual sugar in the medium. Glucose and D-xylose were analyzed using a Dionex ICS-3000 ion chromatography system equipped with a CarboPac TM PA 20 analytical column (4 by 50 mm), and CarboPac TM PA 20 guard column (3 by 30 mm) (Dionex Corporation, CA) (61, 64). Cell growth was quantified by measuring the *A*₆₀₀ of the culture using a Shi-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s) ^a	Source or reference
Strains		
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>S. lividans</i> TK23	Wild type, source of <i>xylA</i> and <i>xylB</i>	20
<i>R. jostii</i> RHA1	Wild type	37
<i>R. opacus</i> PD630	Wild type	24
<i>R. jostii</i> XYLA	<i>R. jostii</i> RHA1 bearing plasmid pXYLA	This study
<i>R. jostii</i> XYLAB	<i>R. jostii</i> RHA1 bearing plasmid pXYLAB	This study
<i>R. jostii</i> pNV18	<i>R. jostii</i> RHA1 bearing plasmid pNV18	This study
<i>R. opacus</i> XYLA	<i>R. opacus</i> PD630 bearing plasmid pXYLA	This study
<i>R. opacus</i> XYLAB	<i>R. opacus</i> PD630 bearing plasmid pXYLAB	This study
<i>R. opacus</i> pNV18	<i>R. opacus</i> PD630 bearing plasmid pNV18	This study
<i>R. jostii</i> Δ <i>xylB1</i>	<i>R. jostii</i> RHA1 <i>xylB1</i> knockout	This study
<i>R. jostii</i> Δ <i>xylB2</i>	<i>R. jostii</i> RHA1 <i>xylB2</i> knockout	This study
<i>R. jostii</i> Δ <i>xylB1B2</i>	<i>R. jostii</i> RHA1 double knockout of <i>xylB1</i> and <i>xylB2</i>	This study
<i>R. jostii</i> Δ <i>xylB1_xylA</i>	<i>R. jostii</i> RHA1 <i>xylB1</i> knockout bearing plasmid pXYLA	This study
<i>R. jostii</i> Δ <i>xylB2_xylA</i>	<i>R. jostii</i> RHA1 <i>xylB2</i> knockout bearing plasmid pXYLA	This study
<i>R. jostii</i> Δ <i>xylB1B2_xylA</i>	<i>R. jostii</i> RHA1 double knockout of <i>xylB1</i> and <i>xylB2</i> bearing plasmid pXYLA	This study
Plasmids		
pMAT-TAG	Amp ^r ; <i>E. coli</i> expression vector, source of <i>tac</i> promoter	Sigma
pNV18	Km ^r ; <i>E. coli</i> - <i>Rhodococcus</i> shuttle vector	10
pTACHis18	Km ^r ; expression vector containing the <i>tac</i> promoter, derived from pNV18	This study
pXYLA	Km ^r ; pTACHis18 containing <i>xylA</i> from <i>S. lividans</i> TK23	This study
pXYLB	Km ^r ; pTACHis18 containing <i>xylB</i> from <i>S. lividans</i> TK23	This study
pXYLAB	Km ^r ; pTACHis18 containing <i>xylA</i> and <i>xylB</i> genes	This study
pK18mobsacB	Km ^r ; <i>sacB</i> ; cloning vector for mobilization into bacteria	45
pKXYLB1	Km ^r ; pK18mobsacB containing the <i>xylB1</i> fragment	This study
pKXYLB2	Km ^r ; pK18mobsacB containing the <i>xylB2</i> fragment	This study
pKB1-SalI	Km ^r ; pKXYLB1 containing disrupted <i>xylB1</i>	This study
pKB2-PstI	Km ^r ; pKXYLB2 containing disrupted <i>xylB2</i>	This study

^a Amp^r, ampicillin resistance; Km^r, kanamycin resistance; *sacB*, gene encoding levansucrase.

madzu UV-visible spectrophotometer (UV-2550). A standard curve of the A_{540} versus the concentration of xylose was also generated by using this spectrophotometer. For quantification of the lipid and fatty acid profiles of the strains, the cells were harvested and freeze-dried overnight. The methods used for fatty acid methyl ester preparation and gas chromatography (GC) analysis were the same as those described in our previous publications (56, 61, 64).

DNA sequencing and analysis. All of clones were sequenced by GENEWIZ, Inc. (NJ). The sequence of the *Streptomyces xylA* and *xylB* genes was obtained from GenBank (accession no. AF184899) (39). To elucidate the pathway for D-xylose metabolism, the database Kyoto Encyclopedia of Genes and Genomes (KEGG) was used (39). The genes from *R. jostii* RHA1 involved in D-xylose metabolism were retrieved from KEGG or the *Rhodococcus* Genome Project (<http://www.rhodococcus.ca/>) (37). The genome sequence of *R. opacus* PD630 was available at the following website: <http://www.broadinstitute.org> (24). The sequence was analyzed by using the BLAST server of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>), when needed (36).

RESULTS

Growth of *Rhodococcus* strains with various carbon sources. The growth performance of *R. jostii* RHA1 and *R. opacus* PD630 on diverse carbon sources in terms of the A_{600} of the culture at 120 h is shown in Fig. 1. The results showed that neither of the strains could utilize arabinose, xylose, or glycerol as the sole carbon source for growth. They also indicated that both strains could use glucose or gluconate as the sole carbon source for growth, and the

A_{600} s of *R. jostii* RHA1 and *R. opacus* PD630 reached 6.35 and 7.06, respectively, when 30 g/liter glucose was present in the medium. As shown in Fig. 1, the strain *R. opacus* PD630 could grow on galactose. However, *R. jostii* RHA1 could not utilize this sugar as the carbon source. The results showed that although strain *R. jostii* RHA1 could degrade a broad range of natural and xenobiotic compounds, including aromatic compounds, carboxylate compounds, and lignin, the capability of the strain for carbohydrate catabolism needs to be further improved (9, 37). Furthermore, our results also confirmed that there was a significant difference in catabolic phenotypes, including galactose utilization, in the closely related species *R. jostii* RHA1 and *R. opacus* PD630 (24).

Construction and growth of *xylA* and *xylB* recombinants of *R. jostii* RHA1 on xylose. The resulting vectors pXYLA and pXYLAB were introduced into *R. jostii* RHA1. The results of enzyme assay showed that D-xylose isomerase and xylulokinase were actively expressed in the recombinants *R. jostii* XYLA and *R. jostii* XYLAB (Table 2). Especially, the expression of these two enzymes by the recombinant was not repressed when glucose was present in the medium. As shown in Fig. 2A, the result showed that the recombinants *R. jostii* XYLA and *R. jostii* XYLAB could grow on 30 g/liter of xylose, whereas the control, wild-type strain *R. jostii* RHA1 bearing the empty vector pNV18 could not grow on this C₅ sugar. Recombinant *R. jostii* XYLAB, expressing both *xylA* and *xylB* from *S. lividans* TK23, had a higher biomass yield than *R.*

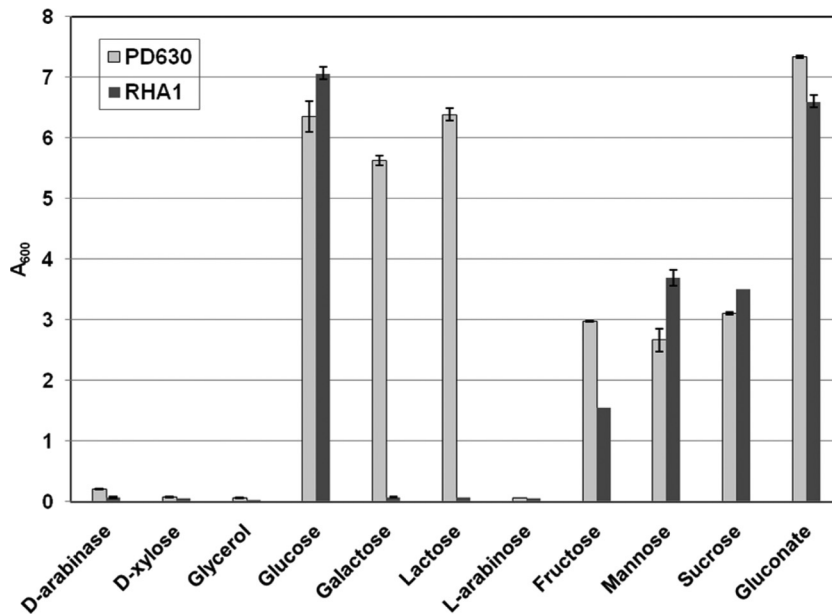


FIG 1 Growth of *R. jostii* RHA1 and *R. opacus* PD630 in W medium containing 30 g/liter of different sugars at 120 h. The cultures were first incubated in LB medium at 30°C and 200 rpm until late log phase. The cells were harvested by centrifugation, washed to remove the metabolites, and inoculated into 100 ml of W medium in a 500-ml flask. The initial A_{600} was adjusted to about 0.1. Incubation was carried out at 30°C and 200 rpm.

jostii XYLAB, carrying only *xylA*. However, according to the time course of growth, these two strains demonstrated similar growth rates on xylose. The specific growth rates (μ 's) of *R. jostii* XYLA and *R. jostii* XYLAB on 30 g/liter of xylose were 0.052 h^{-1} and 0.054 h^{-1} , respectively. Additionally, the biomass yield on different contents of D-xylose was assessed. As indicated in Fig. 2B, the optimal content of xylose for both strains was 40 g/liter, and the maximum A_{600} of the strain harboring the *xylA* and *xylB* genes reached 5.5 at 120 h. The result showed that a higher content of sugars inhibited the growth of the strains. It also manifested that *R. jostii* XYLAB showed a higher biomass yield than *R. jostii* XYLA on D-xylose under the same culture conditions. To compare the growth of cells on different carbon sources, the A_{600} of the culture grown on D-xylose and glucose was determined. As indicated in Fig. 2C, although the final biomass yields of these two strains were similar, *R. jostii* XYLAB grew much slower on D-xylose than strain *R. jostii*/pNV18 grew on glucose. The specific growth rates of the

TABLE 2 Specific activities of xylose isomerase and xylulokinase in *R. jostii* RHA1, *R. opacus* PD630, and their recombinants^a

Strain (carbon source)	Sp act (U/mg protein)	
	Xylose isomerase	Xylulokinase
<i>R. jostii</i> RHA1 (glucose)	ND ^b	0.16 ± 0.02
<i>R. jostii</i> XYLA (glucose)	0.27 ± 0.01	0.17 ± 0.04
<i>R. jostii</i> XYLA (xylose)	0.39 ± 0.02	0.53 ± 0.10
<i>R. jostii</i> XYLAB (xylose)	0.40 ± 0.05	4.89 ± 0.61
<i>R. opacus</i> PD630 (glucose)	ND	0.21 ± 0.01
<i>R. opacus</i> XYLA (glucose)	0.20 ± 0.01	0.23 ± 0.02
<i>R. opacus</i> XYLA (xylose)	0.42 ± 0.05	0.46 ± 0.03
<i>R. opacus</i> XYLAB (xylose)	0.37 ± 0.02	2.96 ± 0.30

^a The data were calculated from triplicate measurements, and data are represented as the average \pm standard deviation. Glucose or xylose at 20 g/liter was used as the carbon source in this study.

^b ND, not detected.

strain grown on D-xylose and glucose were 0.07 h^{-1} and 0.19 h^{-1} , respectively. Furthermore, the lag phase of the cell incubated on D-xylose was much longer. A similar phenomenon was also observed with other recombinants grown on xylose as the carbon source, especially when the culture seeds for the inoculum were cultured on glucose (29).

Endogenous *xylB* genes from *R. jostii* RHA1. The growth profiles of *R. jostii* RHA1 recombinants suggested that some endogenous enzyme with the activity of xylulokinase was present in wild-type strain *R. jostii* RHA1. The enzyme assay of xylulokinase further confirmed this result (Table 2). Additionally, as shown in Table 2, the activity of the enzyme xylose isomerase for conversion of D-xylose into xylulose was not detectable in the wild-type strain. Two possible genes, RHA1_ro02812 (*xylB1*) and RHA1_ro02901 (*xylB2*), encoding xylulokinase were found in *R. jostii* RHA1 by homology analysis. As shown in Fig. 3, the *xylB2*-disrupted strain *R. jostii* Δ *xylB1*-*xylA* grew more efficiently than strain *R. jostii* Δ *xylB2*-*xylA* with the *xylB1* deletion when *xylA* was coordinately expressed in these two strains. The strain with the double knockout of *xylB1* and *xylB2* carrying the expression vector pXYLAB showed minimal growth in medium containing 10 g/liter of D-xylose (Fig. 3).

Consumption of a mixture of sugars consisting of glucose and D-xylose. The results showed that both glucose and D-xylose could be consumed by the cells of *R. jostii* XYLAB bearing plasmid pXYLAB when the two sugars were present in the medium as a mixture at 4 g/liter each (Fig. 4), and there was no apparent diauxic shift during the cultivation. However, the results still showed that the consumption of D-xylose did not begin until the glucose was almost depleted, at about 36 h. The specific growth rate of strain *R. jostii* XYLAB was about 0.18 h^{-1} , which was comparable to that of the wild type grown on 4 g/liter glucose, 0.19 h^{-1} . However, the biomass yield was remarkably im-

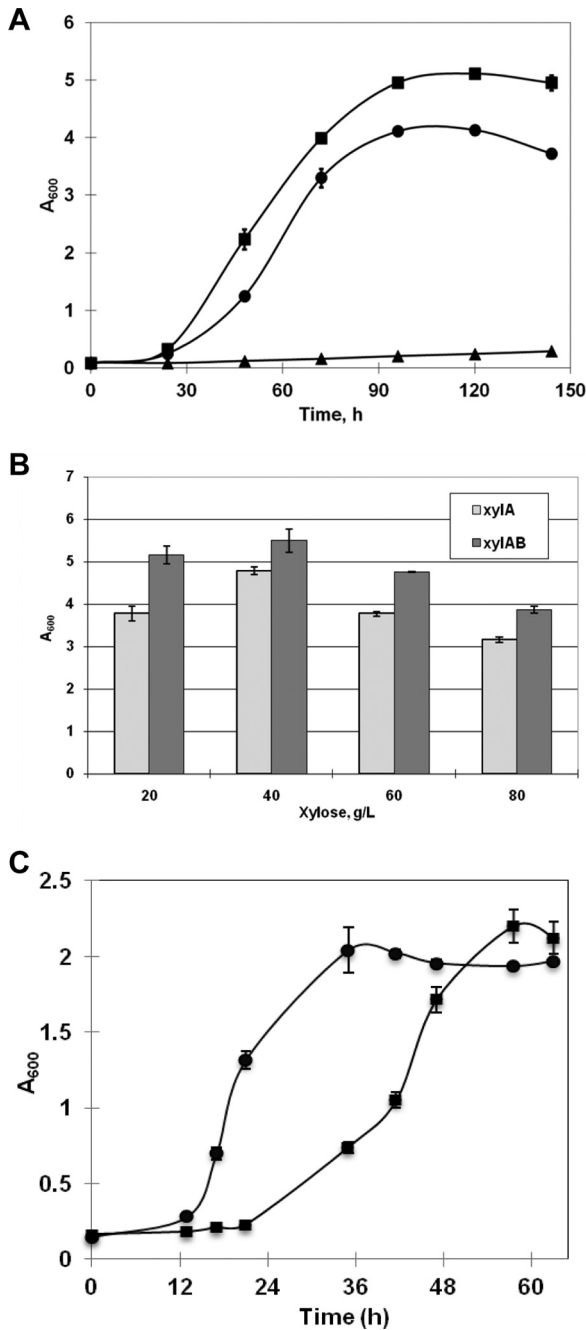


FIG 2 Growth of recombinant cells of *R. jostii* RHA1 in W medium at 30°C and 200 rpm. (A) The initial A_{600} of the culture was adjusted to about 0.1, and the cells were grown in medium containing 30 g/liter D-xylose. Samples were taken every 12 h, and the A_{600} was determined. ■, *R. jostii* XYLAB; ●, *R. jostii* XYLA; ▲, *R. jostii*/pNV18. (B) Cells were grown in medium containing different contents from 20 g/liter to 80 g/liter of D-xylose. The cell biomass yield, indicated as the A_{600} , was detected at 120 h. xylA, *R. jostii* XYLA; xylAB, *R. jostii* XYLAB. (C) Recombinant *R. jostii* XYLAB bearing *xylA* and *xylB* from *S. lividans* TK23 was incubated on 4 g/liter D-xylose (■), and *R. jostii*/pNV18 harboring the empty plasmid pNV18 was grown in W medium containing 4 g/liter glucose (●).

proved on the mixture containing both C₅ and C₆ sugars (Fig. 2 and Fig. 4).

Construction and characterization of *R. opacus* PD630 recombinants. Two recombinants, *R. opacus* XYLA and *R. opacus*

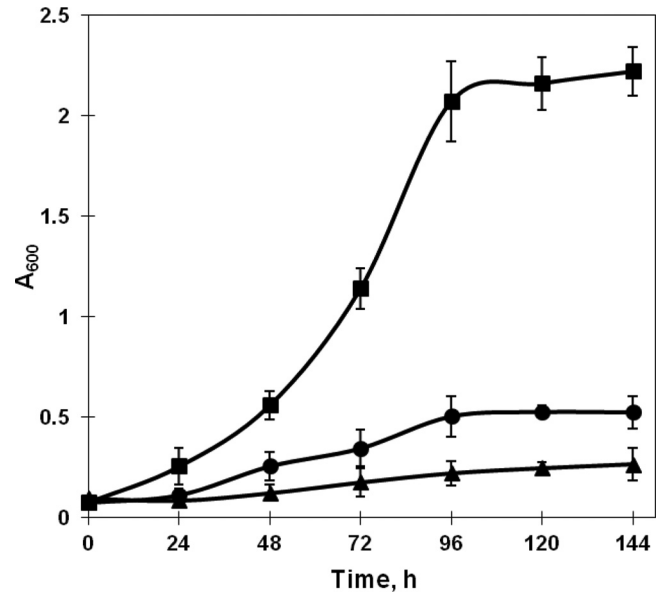


FIG 3 Growth of *xylB*-disrupted *R. jostii* RHA1 bearing *xylA* on 10 g/liter D-xylose at 200 rpm and 30°C. The samples were taken every 24 h, and the A_{600} was determined. ■, *R. jostii* Δ xylB2_xylA; ●, *R. jostii* Δ xylB1_xylA; ▲, *R. jostii* Δ xylB1B2_xylA.

XYLAB, were developed by electroporation of strain *R. opacus* PD630 with plasmids pXYLA and pXYLAB, respectively (Table 1). As shown in Table 2, the enzyme activity of xyulokinase could be detected in wild-type strain *R. opacus* PD630. It also shows that the specific activity of xylose isomerase was observed in the recombinant strains bearing *xylA*, whereas little activity was detected with wild-type strain *R. opacus* PD630 (Table 2). The result showed that both strains could utilize xylose as the sole carbon source with similar growth rates and biomass yields (Fig. 5). The specific

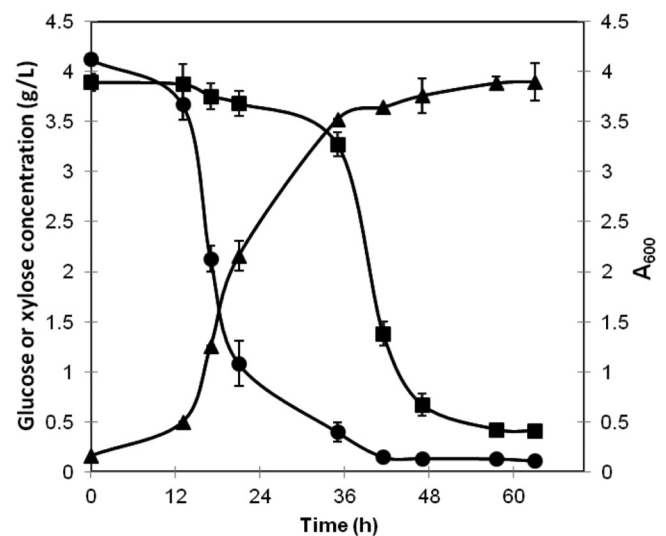


FIG 4 Growth of *R. jostii* XYLAB bearing plasmid pXYLAB and time function of sugar consumption in medium containing a sugar mixture consisting of about 4 g/liter of glucose and D-xylose. The incubation was carried out in a 250-ml flask at 200 rpm and 30°C with an initial A_{600} of 0.15. ▲, A_{600} of the culture; ●, residue glucose content (g/liter); ■, residue xylose content (g/liter).

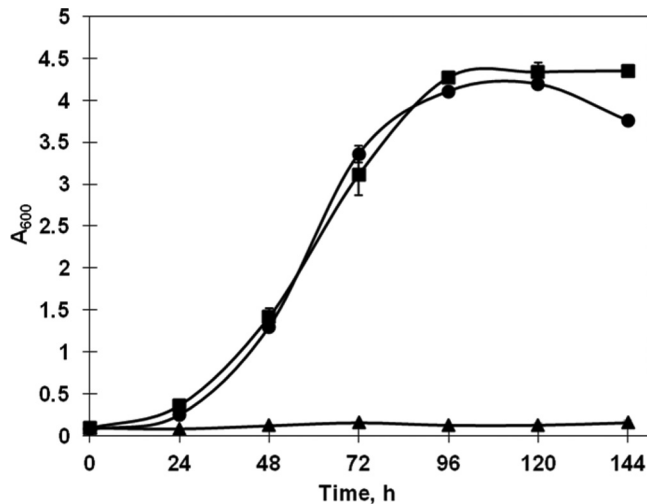


FIG 5 Growth of recombinant cells of *R. opacus* PD630 in W medium containing 30 g/liter xylose at 30°C and 200 rpm. The initial A_{600} of the culture was adjusted to about 0.1. After inoculation, samples were taken every 12 h and the A_{600} was determined. ■, *R. opacus* XYLAB; ●, *R. opacus* XYLA; ▲, *R. opacus*/pNV18.

growth rate of the strains cultivated on 30 g/liter xylose was about 0.05 h^{-1} , which was similar to the growth rate of the recombinant of *R. jostii* RHA1 (Fig. 2 and Fig. 5). However, the final A_{600} of *R. opacus* XYLAB was approximately 4.3, which was lower than that of *R. jostii* XYLAB. It also showed that unlike the growth of the *R. jostii* RHA1 recombinants, expression of *xytB* from *S. lividans* TK23 could enhance neither the cell growth rate nor the yield of biomass on xylose.

Lipid production by *Rhodococcus* recombinants from D-xylose. In this study, the content of lipid, indicated as the proportion of total fatty acids, was detected. To allow cells to accumulate lipids, the cells were cultured on glucose or D-xylose under nitrogen-limited conditions (22). As shown in Table 3, the lipids present in the cells consisted of saturated and unsaturated fatty acids, and other than fatty acids with even numbers of carbons, there was a certain amount of fatty acids with odd numbers of carbons, which, peculiarly, existed in some bacteria (14). Like most of the organisms, fatty acids with chain lengths of more than 14 predom-

inantly existed in the cells. Although all of the strains grown in the presence of different carbon sources contained the same prominent fatty acids, including pentadecanoic acid ($C_{15:0}$), palmitic acid ($C_{16:0}$), heptadecanoic acid ($C_{17:0}$), heptadecenoic acid ($C_{17:1n7}$), and oleic acid ($C_{18:1n9}$), the relative proportions of the fatty acids were different. Furthermore, the lipid content of the cells grown in D-xylose was a little higher than the lipid content of cells grown in glucose. As also demonstrated by previous studies, the results showed that the lipid content of *R. opacus* PD630 was higher than that of *R. jostii* RHA1 (22, 24, 55). The recombinants *R. opacus* PD630 and *R. opacus* XYLAB grown in D-xylose could accumulate lipid to up to 68.3% and 52.5% of CDW, respectively, whereas the lipid contents of the natural strain cultivated in glucose were about 61.2% and 42.63% of CDW, respectively (Table 3). However, the recombinants *R. jostii* XYLAB and *R. opacus* XYLAB grown on W medium containing 5 g/liter of xylose and 1 g/liter of $(\text{NH}_4)_2\text{SO}_4$ with a carbon-to-nitrogen ratio of 5:1 produced only 5.2% and 8.8% lipid, respectively. The results of this study verified that culture conditions consisting of a high carbon-to-nitrogen ratio like 40:1 could lead to accumulation of high contents of lipid in the recombinants *R. jostii* RHA1 and *R. opacus* PD630 (32).

DISCUSSION

Biomass, including crop residues, woody biomass, and organic waste, can serve as a renewable source for biofuel production (48). Therefore, to utilize these various feedstocks, including sugars derived from lignocellulosic biomass, a strain with the ability to grow on a broad range of substrates is desirable (1). In this study, the utilization of various carbon substrates by the two oleaginous bacteria *R. jostii* RHA1 and *R. opacus* PD630 for their growth was investigated. The further genome-scale survey of the genes for carbohydrate transportation and catabolism will provide the foundation for a better understanding of the metabolic pathways and regulatory networks of utilization of diverse substrates in these organisms (8, 24, 37). The results showed that these two strains could not initially utilize the pentose D-xylose. For utilization of D-xylose by bacteria, the enzymes D-xylose isomerase and xylulokinase are generally required to transform the transported intracellular D-xylose into D-xylulose 5-phosphate, which can subsequently be converted into D-ribulose-5-phosphate and further metabolized through the pentose phosphate pathway (PPP)

TABLE 3 Lipid content of *R. jostii* RHA1, *R. opacus* PD630, and their recombinants grown in W medium containing glucose or xylose under nitrogen-limited conditions

Fatty acid or lipid	% lipid content ^a			
	PD630 (glucose)	PD630 XYLAB (xylose)	RHA1 (glucose)	RHA1 XYLAB (xylose)
Fatty acid (total)	61.78 ± 1.2	68.30 ± 2.9	42.63 ± 1.5	52.5 ± 3.0
Myristic acid ($C_{14:0}$)	1.68 ± 0.2	0.92 ± 0.1	1.66 ± 0.1	1.38 ± 0
Pentadecanoic acid ($C_{15:0}$)	6.63 ± 0.4	11.61 ± 1.2	6.89 ± 0.1	9.79 ± 0.2
Palmitic acid ($C_{16:0}$)	25.92 ± 1.0	21.54 ± 1.7	25.33 ± 2.3	21.2 ± 1.7
Palmitoleic acid ($C_{16:1n7}$)	5.50 ± 0.9	4.37 ± 1.3	4.91 ± 1.6	4.71 ± 1.1
Heptadecanoic acid ($C_{17:0}$)	14.82 ± 2.3	19.62 ± 1.8	19.38 ± 0.7	22.39 ± 0.8
Heptadecenoic acid ($C_{17:1n7}$)	14.32 ± 0.3	18.31 ± 0.6	14.37 ± 1.9	19.37 ± 0.9
Stearic acid ($C_{18:0}$)	5.24 ± 0.1	3.38 ± 0.1	6.05 ± 0.8	3.33 ± 0.2
Oleic acid ($C_{18:1n9}$)	17.85 ± 1.5	10.60 ± 0.9	13.8 ± 1.0	7.89 ± 0.7
Linoleic acid ($C_{18:2n6}$)	1.78 ± 0.1	1.65 ± 0	1.47 ± 0.3	1.17 ± 0.1

^a Total fatty acids are expressed as a percentage of cell dry weight (CDW), and the fatty acid composition profile is shown as percent (wt/wt). The data were calculated from triplicate measurements, and data are represented as the average ± standard deviation. The carbon source is indicated in parentheses after the strain name.

(6, 20, 27). To develop the xylose-utilizing strains, most of the effort has been toward expression of the genes *xylA* and *xylB* encoding these two enzymes from *E. coli* in different hosts, including *Corynebacterium glutamicum*, *Pseudomonas putida* S12, *Zymobacter palmae*, and *Zymomonas mobilis* (29, 38, 60, 62). It was found that the first enzyme for xylose catabolism, xylose isomerase from *Streptomyces* strains, possessed high thermostability and a broad optimum pH (20). Furthermore, the genes from *Streptomyces* will be more readily expressed in rhodococci because both genera belong to the high-GC-content actinomycetes and thus possess similar codon usage bias and regulation for gene expression (21). Therefore, in this study, the genes *xylA* and *xylB*, encoding enzymes for catabolism of D-xylose, were cloned from *S. lividans* TK23. After expression of *xylA* from *S. lividans* TK23 under the *tac* promoter in *R. jostii* RHA1, the recombinant could grow in medium containing D-xylose as the sole carbon source. The growth of recombinant *R. jostii* RHA1 on D-xylose was further improved by additional expression of *xylB* (Fig. 2A). The functional expression of *xylA* and *xylB* was also confirmed by the enzyme assay. In addition, it seems that there is an efficient xylose uptake system in the wild-type strain, as *R. jostii* RHA1 required the expression of only *xylAB* for xylose utilization (38). Several xylose transporters have been found in bacteria, including both the ABC-type transporter and xylose-proton symporter (58). However, only two sugar transporters, the major facilitator superfamily (MFS) glucose transporter GlcP and phosphotransferase system (PTS) PtsH, responsible for the uptake of fructose, were characterized in strain *R. jostii* RHA1 (5). In the genome of *R. jostii* RHA1, there was a putative protein, Ro05189, annotated as the possible xylose transporter (37), but its function needs to be further identified.

Assay of the enzyme xylulokinase of wild-type strains *R. jostii* RHA1 and *R. opacus* PD630 showed that the strains possessed an intrinsic enzyme(s) giving xylulokinase activity (Table 2). To study its function, the two candidates of the native *xylB* in *R. jostii* RHA1, RHA1_ro02812 (*xylB1*) and RHA1_ro02901 (*xylB2*), were cloned and disrupted. After transformation of the expression vector pXYLA, only one of them, the *xylB2*-disrupted strain *R. jostii* Δ *xylB2_xylA*, showed adequate growth on D-xylose (Fig. 3). It revealed that the first one, *xylB1*, played the major role in D-xylose metabolism, according to the growth performance on D-xylose. Furthermore, in wild-type strain *R. jostii* RHA1, the enzyme activity of xylulokinase was induced by D-xylose and repressed by glucose (Table 2). This phenomenon was observed in many bacteria, including *E. coli*, *Streptomyces* sp. strain TK24, and *Bacillus subtilis* (31). There was a regulatory protein, XylR, present in some microorganisms, including natural D-xylose-utilizing and non-D-xylose-utilizing strains (20, 31, 49). Generally, expression of the related genes was induced by D-xylose but strictly repressed by glucose due to the control of this regulator (20, 49). However, the existence and function of the potential protein used for regulation of xylose metabolism in *Rhodococcus* strains are unknown. The gene *xylA* was also expressed alone and coexpressed with *xylB* in the high-lipid-producing strain *R. opacus* PD630. The recombinants were able to utilize D-xylose as the carbon source for their growth (Fig. 5). However, unlike the recombinants of *R. jostii* RHA1, the two recombinants of *R. opacus* PD630 showed similar growth characteristics. This implied that the rate-limiting step for xylose utilization in this strain was not controlled by the xylulokinase encoded by *xylB* after expression of the *xylA* gene in strain *R. opacus* PD630.

In this study, the recombinants of *R. jostii* RHA1 demonstrated sequential consumption of glucose and xylose, and even the genes *xylA* and *xylB* were expressed constitutively by P_{tac} (15). It was also found that many other wild-type and engineered bacteria, such as *E. coli*, *Pseudomonas* spp., and *Zymomonas* spp., could not cointilize D-xylose and glucose either (28, 38). This drawback prevents the strains from efficient utilization of complex sugar mixtures while extending the cultivation time in the fermentor (30). Therefore, the development of strains with the capability of simultaneous utilization of both C₅ and C₆ sugars is critical for biofuel production from lignocellulosic biomass. Previous studies suggested that this sequential carbohydrate utilization in microorganisms resulted from carbon catabolite repression or allosteric competition of the sugars during sugar transport (16, 29). In a recombinant *C. glutamicum* strain, the results showed that additional expression of the L-arabinose transporter gene *araE* enhanced its ability to utilize pentose, and simultaneous consumption of pentose and glucose was mainly observed under oxygen-deprived conditions (28). *E. coli* mutants devoid of the phosphotransferase system (PTS) were developed to avoid this regulatory system, but problems still remain to be resolved (23), although it was recently reported that overexpression of the xylose transporter *xylT* and other catabolism genes in the glucose PTS-deficient organism *Clostridium acetobutylicum* allowed the strain to utilize glucose and xylose simultaneously (58). Our previous studies showed that oleaginous yeast and fungi were capable of using all forms of sugars and even other degradation products of lignocellulosic biomass from pretreatment for cell biomass and lipid production (61). However, most of them also preferred the glucose in the sugar mixtures as their carbon source (25). The ethanol-producing yeast *Saccharomyces cerevisiae* was intensively engineered to use D-xylose, and most of the resulting strains could not efficiently use the C₅ sugar in the sugar mixture either (27). This bottleneck was overcome by expression of an intracellular β -glucosidase and cellodextrin transporter to bypass glucose repression for D-xylose fermentation, and the recombinant strain cointilized the transported cellobiose and D-xylose (19). Although the metabolic pathways of D-xylose utilization among bacteria, fungi, and bacteria are diverse (26), the results are instructive for the further genetic engineering of oleaginous strains for pentose utilization.

Some SCO-producing microorganisms are capable of accumulating substantial amounts of oil, sometimes up to 70% of their dry weight biomass (34). The lipid accumulation in such microorganisms was triggered by imbalanced culture conditions, such as starvation for nitrogen or other limiting nutrients. The profiles of fatty acids occurring in the lipids of the strains were dependent on cultivation factors such as temperature and carbon source (34). In this study, the recombinants of *R. opacus* PD630 and *R. jostii* RHA1 grown in D-xylose could accumulate up to 68.3% and 52.5% lipid (CDW) under nitrogen-limited conditions. Actually, the highest contents of the lipid for these two strains, 76% and 56.9%, respectively, were achieved by using the same carbon source, gluconic acid (3, 22). By combination of the results with those of this study, it is suggested that carbon sources such as D-xylose and gluconic acid metabolized by the pentose phosphate pathway are desirable for lipid accumulation. This is also supported by the calculation of the maximum theoretical yield of SCO produced per xylose molecule consumed, which is about 0.34 g/g (40). The culture conditions for biomass yield and lipid accumulation need to be optimized to achieve higher yields, and

genetic modification of the strains can further improve productivity (7, 32, 63). The profiles of the fatty acid of the lipid were studied, and the straight-chain odd-carbon-number fatty acids were detected in the strains due to both type 1a fatty acid synthetase (FAS) and FAS II, responsible for fatty acid synthesis (24). The results confirmed that the D-xylose could serve as one of the desirable carbon sources for lipid production by the recombinants developed in this study.

Rhodococcus has been serving as an important platform organism for biodegradation, biotransformation, and biocatalysis, such as xenobiotic degradation, and for biosynthesis of fuels, pharmaceuticals, and valuable chemicals (51). The results in this study provide insight into a means of improvement of the performance of *Rhodococcus* strains by enabling them to utilize sugars, including xylose, from renewable lignocellulosic biomass. However, our research presented in this study is still preliminary. Future work will include further improvement of the strains for substrate utilization, optimization of the cultivation conditions for biofuel production, and evaluation and enhancement of the tolerance of the strains to inhibitors derived from the lignocellulosic biomass pretreatment.

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