Engineering of a Xylose Metabolic Pathway in \textit{Rhodococcus} Strains

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ABSTRACT

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 control of the *tac* promoter with an *E. 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 sugars 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 demonstrated that it was feasible to produce lipid from the sugars including xylose derived from renewable feedstock by genetic modification of the rhodococci strains.
INTRODUCTION

Lipid consisting of fatty acids can be transformed through hydrotreatment into a range of hydrocarbons including green diesel, or by transesterification into biodiesel as 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 high content of lipids mainly including triacylglycerol (TAG) under imbalanced nutrition conditions (11, 46, 57). In contrast, most bacteria were able to accumulate the lipophilic storage products such as polyhydroxyalkanoic acid (PHA) or poly(3-hydroxybutyric acid) (PHB) (12, 42). However, a few bacteria, mainly belonging to the genera Acinetobacter, Mycobacterium, Streptomyces, Nocardia and Rhodococcus can accumulate TAG during cultivation under nitrogen limited conditions (4, 54). Especially, the Gram-positive bacteria Rhodococcus strains are of biotechnological importance due to their broad catabolic diversity of enzymatic capabilities (37, 51). It was reported that the metabolically versatile bacterium, Rhodococcus jostii RHA1 isolated from lindane-contaminated soil, which is initially known for its outstanding capability to degrade polychlorinated biphenyls (PCBs) (37), could accumulate up to 48.4% and 56.9% of lipid of the cell dry weight (CDW) growing on glucose and gluconate under nitrogen starving conditions, respectively (23). Another oleaginous rhodococci strain, R. opacus PD630, as a super single cell oil (SCO) producer with the lipid content of 76% of CDW, has been thoroughly studied including 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, 57). Clearly the strains R. opacus PD630 and R. jostii RHA1
showed the ability of production of high content of lipids, which main components are TAGs, from the carbon sources including glucose, gluconate and sucrose (23, 32, 53).

The feedstocks of lignocellulosic biomass are the most abundant renewable resources in the world (48). Fuels derived from lignocellulosic biomass will improve the energy security, decrease greenhouse gas emissions and move our industrial society toward sustainability (13, 50). The availability of the genetic tools and complete genome sequences offer the opportunities to investigate the metabolic traits of the two *Rhodococcus* strains by genetic perturbation and functional genomics study (2, 23, 24, 35). Furthermore, the metabolic engineering approach can be used to improve microorganisms for utilization of carbohydrates from the biomass (56). As discussed above, the strains *R. opacus* PD630 and *R. jostii* RHA1 can efficiently convert the carbon sources such as glucose into lipids, but there has been no report on the 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 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 were expressed in the *Rhodococcus* strains to provide them with 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 fatty acid profiles of the cells grown on xylose were studied.
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 H₃BO₄ and 5.13×10⁻² mL concentrated HCl. The stock solutions of the sugars including glucose and D-xylose were sterilized with 0.22-μm-pore-size filter and added to the autoclaved medium. The growth of the strains on other carbon sources including 30 g/L of D-arabinose, glycerol, glucose, galactose, lactose, L-arabinose, fructose, mannose, sucrose and gluconate was also tested, respectively. The 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 the 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 the nitrogen limited conditions (23). The Rhodococcus cells were grown in LB and harvested by centrifugation with 8,000 g, at 4°C and 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/L of glucose or xylose, and 0.5 g/L (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. CA, USA) according to the manual provided by the manufacturer. Plasmid DNA was extracted from *E. coli* by using a QIAprep Miniprep kit from QIAGEN (Valencia, CA). Restriction endonucleases were purchased from NEB (MA, USA). PCR was performed with the 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 in Invitrogen (Grand Island, NY). The sequence and generated restriction sites of the primers are listed in Table S1 in the supplemental material. After running the agarose gel, the digested PCR products and plasmids by restriction endonucleases were recovered and purified with 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 by water, and treated with 10% glycerol, and then electroporated with the plasmids as previously described (47, 59). After electroporation, the cell was 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). The DNA fragment containing poly-histidine (His) tag was generated with the primers HisF and HisR by PCR using plasmid pNV18 as the template (Table S1 in Supplementary Materials). The PCR product was digested with XhoI and EcoRI, and inserted into these two restriction sites of plasmid pNV18 to obtain the plasmid pNVHis18. The tac promoter P_tac containing
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 the plasmid pNVHis18 was treated by 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 *Streptomyces* sp. TK23 as the template. The PCR product was digested with the restriction endonucleases PstI and KpnI. The digestion product was inserted the plasmid pTACHis18 to generate the *xylA* expression vector, pXYLA. Similarly, the recombinant expression vector bearing 1.5-kb *xylB* from *Streptomyces* sp. TK23 was constructed and designated as pXYLB. The expression cassette of *xylB* including the coding sequence of *xylB* and tac promoter was amplified with primers xyl-tac1 and xyl-tac2 using the plasmid pXYLB as the template by PCR. The PCR product was digested with HindIII and inserted into the plasmid pXYLA. The resulting 7.6-kb plasmid, pXYLAB contained both *xylA* and *xylB*, and their expression was driven by P<sub>tac</sub> individually (see Fig. S1 in the Supplemental Material).

**Disruption of the 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 ORFs, RHA1_ro02812 (*xylB1*) and RHA1_ro02901 (*xylB2*) exhibited significant homologies to the xylulokinase (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 chromosome and the disrupted ORFs in the introduced plasmids (Table 1). The suicide vector pK18mobsacB was used to develop the plasmids for the deletion of the potential *xylB* genes from *R. jostii* RHA1 as described below.
The two DNA fragments, xylB1 and xylB2 containing two possible xylB genes of R. jostii RHA1 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, the plasmid pKXYLB1 was treated with Sall, and then self-ligated. The resulting plasmid was pKB1-Sall (Table 1). The plasmid pKB2-PstI containing PstI digested DNA fragment was constructed to disrupt the xylB2. The strain, R. jostii RHA1 was transformed with the derivative plasmids of pK18mobsacB, pKB1-Sall and pKB2-PstI by electroporation, respectively. The integration of introduced plasmids into the chromosome by first crossover was selected on LB plates containing kanamycin. The antibiotic-resistant cells were picked up and cultured overnight in LB medium and spread on LB plates containing 10% (w/v) of sucrose without antibiotics. The second crossover of chromosomal DNA led to the 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 for the amplification of the target genes. The single knockouts of xylB1 and xylB2 were designated R. jostii ΔxylB1 and R. jostii ΔxylB2. The xylB2 of the strain with disrupted xylB1 was further deleted to construct the double knockout strain, R. jostii ΔxylB1B2 (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 Streptomyces sp. TK23 in the recombinants, the enzyme activities of xylose isomerase and xylulokinase were determined by using the cell-free extract. The chemicals used in the enzymatic activity determination were obtained from Sigma (St. Louis, MO). The cell-free extracts of
Rhodococcus were prepared by using an ultrasonic homogenizer (Misonix Sonicator 3000, CT), and all of the operations were performed on ice (29). The activity of xylene isomerase of the whole-cell lysate was carried out in a 1-ml reaction volume containing 70 mM D-xylene, 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 added 0.5 M HClO₄ to stop the reaction. The formed D-xylulose was quantified by using the cysteine-carbazole-sulfuric acid assay with measuring the absorbance at 540 nm (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 media. Glucose and D-xylene were analyzed using a Dionex ICS-3000 ion chromatography system equipped with a CarboPac TM PA 20 (4 × 50 mm) analytical column, and CarboPac TM PA 20 (3 × 30 mm) guard column (Dionex Corporation, CA) (61, 64). The cell growth was quantified by measuring the absorbance at 600 nm (A₆₀₀) of the culture using the Shimadzu UV-Vis spectrophotometer, UV-2550. The standard curve of the absorbance at 540 nm (A₅₄₀) vs. the concentration of xylulose 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 method of fatty acid methyl ester preparation and gas chromatography (GC) analysis were the same as our previous publications (55, 61, 64).

DNA sequencing and analysis. All of clones were sequenced by GENEWIZ, Inc (NJ, USA).
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 *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 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 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 and glycerol as the sole carbon source for the growth. It also indicated that both two strains could use glucose or gluconate as a sole carbon source for growth, and A_{600} of *R. jostii* RHA1 and *R. opacus* PD630 reached 6.35 and 7.06 when 30g/L glucose was present in the media. 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 the strain *R. jostii* RHA1 could degrade a broad range of natural and xenobiotic compounds including the 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 of 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, respectively. 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 in the recombinant was not repressed by glucose present in the media. As shown in Fig. 2A, the result showed that the recombinants R. jostii XYLA and R. jostii XYLAB could grow on 30 g/L of xylose, whereas the control, wild-type strain R. jostii RHA1 bearing the empty vector pNV18 could not grow on this C5 sugar. The recombinant R. jostii XYLAB expressing both xylA and xylB from Streptomyces sp. TK23 had higher biomass yield than R. jostii XYLAB only carrying xylA. However, according to the time course of the growth, these two strains demonstrated a similar growth rate on xylose. The specific growth rate, $\mu$ of R. jostii XYLA and R. jostii XYLAB were 0.052 h$^{-1}$ and 0.054 h$^{-1}$ on 30g/L of xylose, 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 two strains was 40 g/L, and the maximum $A_{600}$ of the strain harboring the genes xlyA and xylB reached 5.5 at 120 h. The result showed that higher content of sugars inhibited the growth of the strains. It also manifested that R. jostii XYLAB showed 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, $A_{600}$ of the culture grown on D-xylose and glucose was determined, respectively. 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 the strain *R. jostii* pNV18 grown on glucose. The specific growth rate of the strain grown on D-xylose and glucose was 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 inoculum were cultured on glucose (29).

**Endogenous xylB genes from *R. jostii* RHA1.** The growth profiles of *R. jostii* RHA1 recombinants suggested that there was some endogenous enzyme with the activity of the xylulokinase in the wild-type *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 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* ΔxylB1_xylA grew more efficiently than xylB1-deleted strain *R. jostii* ΔxylB2_xylA when xylA was coordinately expressed in these two strains. The double knockout of xylB1 and xylB2 carrying the expression vector pXYLA showed minimal growth in the medium containing 10 g/L of D-xylose (Fig. 3).

**Consumption of mixture sugars consisting of glucose and D-xylose.** The results showed that both sugars could be consumed in the media containing the sugar mixtures of about 4 g/L glucose and 4 g/L D-xylose by the cells of *R. jostii* XYLAB bearing plasmid pXYLAB (Fig. 4), and there was no apparent diauxic shift during the cultivation. However, it 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 the strain *R. jostii* XYL AB was about 0.18 h⁻¹, which was comparable to that of the wild type grown on 4 g/L glucose, 0.19 h⁻¹. However, the biomass yield was remarkably improved 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* XYL A and *R. opacus* XYL AB were developed by electroporation of the strain *R. opacus* PD630 with the plasmids pXYLA and pXYLAB, respectively (Table 1). As shown in Table 2, the enzyme activity of xylulokinase in the wild-type *R. opacus* PD630 could be detected. It also showed that the specific activity of xylose isomerase was observed in the recombinant strains bearing xylA whereas little activity was detected with the wild-type strain of *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 growth rate of the strains cultivated on 30 g/L 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 A₆₀₀ of *R. opacus* XYL AB was approximately 4.3, which was lower than that of *R. jostii* XYL AB. It also showed that unlike the growth of the *R. jostii* RHA1 recombinants, expression of xylB from *Streptomyces* sp. TK23 could neither enhance 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 total fatty acid was detected. To allow the cells to accumulate lipids, the cells were cultured on glucose or D-xylose under nitrogen limited conditions (23). As shown in Table 3, the lipids present in the cells consisted of saturated and unsaturated fatty acids.
acids, and other than even-carbon fatty acids, there were a certain amount of odd-carbon ones, which peculiarly existed in some bacteria (14). Like most of the organisms, the fatty acids with a chain length more than 14 predominantly existed in the cells. Although all of the strains grown in different carbon sources contained the same prominent fatty acids including pentadecenoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1n7), and oleic acid (C18:1n9), the relative portion of the fatty acids was different. Furthermore, the lipid content of the cells grown in D-xylose was a little higher compared with lipid produced from glucose. As also demonstrated by the previous studies, the results showed that the lipid content of *R. opacus* PD630 was higher than that of *R. jostii* RHA1 (23, 24, 57). The recombinants *R. opacus* PD630 and *R. opacus* XLAB grown in D-xylose could accumulate up to 68.3% and 52.5% of lipid (CDW), whereas the lipid content of the natural strain cultivated in glucose was about 61.2% and 42.63% of CDW (Table 3). However, the recombinants *R. jostii* XLAB and *R. opacus* XLAB grown on W medium containing 5 g/L of xylose and 1 g/L of (NH₄)₂SO₄ with a carbon to nitrogen ratio of 5:1 only produced 5.2% and 8.8% of lipid, respectively. The results verified that culture conditions of high carbon to nitrogen ratio like 40:1 in this study could lead to accumulation of high contents of lipid in the recombinant *R. jostii* RHA1 and *R. opacus* PD630 (32).

**DISCUSSION**

Biomass including crop residues, woody biomass, and organic wastes can be served as a renewable source for biofuel production (48). Therefore, to utilize these various feedstocks including the sugars derived from lignocellulosic biomass, a strain with a broad substrate
growth range 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 carbohydrates transportation
and catabolism will provide the foundation for a better understanding of the metabolic
pathways and regulatory networks of the diverse substrates utilization in these organisms (8,
24, 37). The results showed that these two strains initially could not utilize the pentose
d-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 be subsequently converted into D-ribulose-5-phosphate
and further metabolized through pentose phosphate pathway (PPP) (6, 20, 27). To develop the
xylose-utilizing strains, most of the effort was toward expression of the genes *xylA* and *xylB*
encoding these two enzymes from *E. coli* in the different hosts including *C. 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 broad optimum pH (20). Furthermore, the genes
from *Streptomyces* will be more readily expressed in rhodococci because both belong to the
high GC-content actinomycetes and thus possess similar codon usage bias and regulation for
the gene expression (21). Therefore, in this study, the genes *xylA* and *xylB* encoding enzymes
for catabolism of D-xylose were cloned from *Streptomyces* sp. TK23. After expression of *xylA*
from *Streptomyces* sp. TK23 under *tac* promoter in *R. jostii* RHA1, the recombinant could
grow in the media containing D-xylose as the sole carbon source. The growth of the
recombinant *R. jostii* RHA1 on D-xylose was further improved by additional expression of
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 only required the expression of *xylAB* for xylose utilization (38). Several xylose transporters were found in bacteria including both the ABC-type transporter and xylose-proton symporter (58). However, there were only two sugar transporters, major facilitator superfamily (MFS) glucose transporter GlcP, and phosphotransferase system (PTS) PtsH responsible for the uptake of fructose were characterized in the 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. The enzyme assay of xylulokinase of the wild-type *R. jostii* RHA1 and *R. opacus* PD630 showed that the strains possessed 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 *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 the wild-type *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. 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 regulatory
protein for regulation of xylose metabolism in *Rhodococcus* strains are unknown. The gene
*xylA* was also expressed alone and co-expressed 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). But 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 xylulokinase encoded by *xylB*
after expression of *xylA* gene in the strain *R. opacus* PD630.

In this study, the recombinants of *R. jostii* RHA1 demonstrated sequential consumption of
glucose and xylose even the genes *xylA* and *xylB* were expressed constitutively by P<sub>lac</sub> (15). It
also found that many other wild-type and engineered bacteria such as *E. coli*, *Pseudomonas* sp.
and *Zymomonas* sp. could not co-utilize D-xylose and glucose, either (28, 38). This drawback
prevents the strains from efficient utilization of the complex sugar mixtures while extending
the cultivation time in the fermentor (30). Therefore, development of the strains with the
capability of simultaneous utilization of both C5 and C6 sugars is critical for biofuel
production from the lignocellulosic biomass. The previous studies suggested that this
sequential carbohydrate utilization in the microorganisms resulted from the carbon catabolite
repression or the allosteric competition of the sugars during sugar transport (16, 29). In the
recombinant *C. glutamicum*, the results showed that additional expression of the L-arabinose
transporter gene, *araE* enhanced its capability of pentose utilization, and simultaneous
consumption of pentose and glucose was mainly observed under oxygen-deprived conditions
(28). The *E. coli* mutants devoid of the phosphotransferase system (PTS) were developed to
avoid this regulatory system, but the problems still remained to be resolved (22). Whereas, recently it reported that overexpression of the xylose transporter, xylT and other catabolism genes in the glucose-PTS-deficient Clostridium acetobutylicum allowed the strain to utilize glucose and xylose simultaneously (58). Our previous studies showed that the oleaginous yeast and fungi were capable of using all forms of sugars and even other degradation products of the lignocellulosic biomass from pretreatment for cell biomass and lipid production (61). However, most of them also preferred 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 C5 sugar in the sugars mixture, either (27). Until this bottleneck was overcome by expression of an intracellular β-glucosidase and cellodextrin transporter to bypass the glucose repression for D-xylose fermentation, and the recombinant strain co-utilized 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 genetically engineering oleaginous strains for pentose utilization.

Some SCO microorganisms were 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 of nitrogen or other limiting nutrients. The profiles of fatty acids occurring in the lipids of the strains were dependent on the 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% of lipid (CDW) under the nitrogen-limited
conditions. Actually, the highest contents of the lipid for these two strains, 76% and 56.9%
were achieved by using the same carbon source, gluconic acid (3, 23). By combination of the
results in this study, it suggested that the carbon sources such as D-xylose and gluconic acid
metabolized by pentose phosphate pathway were desirable for the lipid accumulation. This is
also supported by the calculation of the maximum theoretical yield of SCO produced per
xylose consumed, which is around 0.34 g/g (40). The culture conditions for biomass yield and
lipid accumulation need to be optimized to achieve higher yield and genetic modification of
the strains can further improve the productivity (7, 32, 63). The profiles of the fatty acid of the
lipid were studied, and the straight-chain odd-carbon fatty acids were detected due to in the
strains both type 1a fatty acid synthetase (FAS) and FAS II responsible for fatty acid synthesis
(24). The results confirmed that the D-xylose could be served 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 biosynthesis of fuels,
pharmaceuticals and valuable chemicals (51). The results in this study provide an insight into
improvement of the performance of *Rhodococcus* strains by enabling them to utilize the
sugars including xylose from the renewable lignocellulosic biomass. However, our research
presented in this study was still a preliminary test. The future work will include further
improvement of the stains for substrate utilization, optimization of the cultivation conditions
for biofuel production, and evaluation and enhancement of the tolerance of the strains to the
inhibitors derived from the lignocellulosic biomass pretreatment.
ACKNOWLEDGEMENT

We thank Dr. Lindsay D. Eltis (Department of Microbiology & Immunology, University of British Columbia) for the gift of strain *Rhodococcus* sp. RHA1, Dr. Anthony Sinskey (Department of Biology, Massachusetts Institute of Technology) for the gift of strain, *Rhodococcus* sp. PD630, and Dr. Jun Ishikawa (Department of Bioactive Molecules, National Institute of Infectious Diseases, Japan) for the gift of plasmid pNV18. The authors also thank Jim O’Fallon for his assistance with the lipid analysis.
and determination of keto sugars and trioses. J. Biol. Chem. 192:583.


TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristic(s) a</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> Top10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>S. lividans</em> TK23</td>
<td>Wild type, source of xylA and xylB</td>
<td>(20)</td>
</tr>
<tr>
<td><em>R. jostii</em> RHA1</td>
<td>Wild type</td>
<td>(37)</td>
</tr>
<tr>
<td><em>R. opacus</em> PD630</td>
<td>Wild type</td>
<td>(24)</td>
</tr>
<tr>
<td><em>R. jostii</em> XYLA</td>
<td><em>R. jostii</em> RHA1 bearing plasmid pXYLA</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> XYLAB</td>
<td><em>R. jostii</em> RHA1 bearing plasmid pXYLAB</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> pNV18</td>
<td><em>R. jostii</em> RHA1 bearing plasmid pNV18</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. opacus</em> XYLA</td>
<td><em>R. opacus</em> PD630 bearing plasmid pXYLA</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. opacus</em> XYLAB</td>
<td><em>R. opacus</em> PD630 bearing plasmid pXYLAB</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. opacus</em> pNV18</td>
<td><em>R. opacus</em> PD630 bearing plasmid pNV18</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> ΔxylB1</td>
<td><em>R. jostii</em> RHA1 xylB1 knockout</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> ΔxylB2</td>
<td><em>R. jostii</em> RHA1 xylB2 knockout</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> ΔxylB1B2</td>
<td><em>R. jostii</em> RHA1 double knockout of xylB1 and xylB2</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> ΔxylB1_xylA</td>
<td><em>R. jostii</em> RHA1 xylB1 knockout bearing plasmid pXYLA</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> ΔxylB2_xylA</td>
<td><em>R. jostii</em> RHA1 xylB2 knockout bearing plasmid pXYLA</td>
<td>This study</td>
</tr>
<tr>
<td><em>R. jostii</em> ΔxylB1B2_xylA</td>
<td><em>R. jostii</em> RHA1 double knockout of xylB1 and xylB2 bearing plasmid pXYLA</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMAT-TAG</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;; <em>E. coli</em> expression vector, source of tac promoter</td>
<td>Sigma</td>
</tr>
<tr>
<td>pNV18</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; <em>E. coli-Rhodococcus</em> shuttle vector</td>
<td>(10)</td>
</tr>
<tr>
<td>pTACHis18</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; expression vector containing the tac promoter, derived from pNV18</td>
<td>This study</td>
</tr>
<tr>
<td>pXYLA</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; pTACHis18 containing xylA from <em>S. lividans</em> TK23</td>
<td>This study</td>
</tr>
<tr>
<td>pXYLB</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; pTACHis18 containing xylB from <em>S. lividans</em> TK23</td>
<td>This study</td>
</tr>
<tr>
<td>pXYLAB</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; pTACHis18 containing xylA and xylB genes</td>
<td>This study</td>
</tr>
<tr>
<td>pK18mobsacB</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; sacB; cloning vector for mobilization into bacteria</td>
<td>(45)</td>
</tr>
<tr>
<td>pKXYLB1</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; pK18mobsacB containing the xylB1 fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pKXYLB2</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; pK18mobsacB containing the xylB2 fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pKB1-Sall</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; pKXYLB1 containing disrupted xylB1</td>
<td>This study</td>
</tr>
<tr>
<td>pKB2-Pstl</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; pKXYLB2 containing disrupted xylB2</td>
<td>This study</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Amp<sup>+</sup>, ampicillin resistance; Km<sup>R</sup>, kanamycin resistance; sacB, gene encoding levansucrase.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Xylose isomerase</th>
<th>Xylulokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. jostii</em> RHA1 (grown on glucose)</td>
<td>ND b</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td><em>R. jostii</em> XYLA (grown on glucose)</td>
<td>0.27±0.01</td>
<td>0.17±0.04</td>
</tr>
<tr>
<td><em>R. jostii</em> XYLA (grown on xylose)</td>
<td>0.39±0.02</td>
<td>0.53±0.10</td>
</tr>
<tr>
<td><em>R. jostii</em> XYLAB (grown on xylose)</td>
<td>0.40±0.05</td>
<td>4.89±0.61</td>
</tr>
<tr>
<td><em>R. opacus</em> PD630 (grown on glucose)</td>
<td>ND</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td><em>R. opacus</em> XYLA (grown on glucose)</td>
<td>0.20±0.01</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td><em>R. opacus</em> XYLA (grown on xylose)</td>
<td>0.42±0.05</td>
<td>0.46±0.03</td>
</tr>
<tr>
<td><em>R. opacus</em> XYLAB (grown on xylose)</td>
<td>0.37±0.02</td>
<td>2.96±0.30</td>
</tr>
</tbody>
</table>

The data were calculated from the triplicate measurements, and data are represented as the average±standard deviation. 20 g/L of glucose or xylose was used as the carbon source in this study.

b Not detected.
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.

*Total fatty acids are expressed as % of cell dry weight (CDW), and fatty acid composition profile is shown as % (w/w). The data were calculated from the triplicate measurements, and data are represented as the average ± standard deviation.*

<table>
<thead>
<tr>
<th></th>
<th>PD630 (glucose)</th>
<th>PD630 (xylose)</th>
<th>RHA1 (glucose)</th>
<th>RHA1 (xylose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid content (total)</td>
<td>61.78±1.2</td>
<td>68.30±2.9</td>
<td>42.63±1.5</td>
<td>52.5±3.0</td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>1.68±0.2</td>
<td>0.92±0.1</td>
<td>1.66±0.1</td>
<td>1.38±0.0</td>
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<tr>
<td>Pentadecanoic acid (C15:0)</td>
<td>6.63±0.4</td>
<td>11.61±1.2</td>
<td>6.89±0.1</td>
<td>9.79±0.2</td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>25.92±1.0</td>
<td>21.54±1.7</td>
<td>25.33±2.3</td>
<td>21.2±1.7</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1n7)</td>
<td>5.50±0.9</td>
<td>4.37±1.3</td>
<td>4.91±1.6</td>
<td>4.71±1.1</td>
</tr>
<tr>
<td>Heptadecanoic acid (C17:0)</td>
<td>14.82±2.3</td>
<td>19.62±1.8</td>
<td>19.38±0.7</td>
<td>22.39±0.8</td>
</tr>
<tr>
<td>Heptadecenoic acid (C17:1n7)</td>
<td>14.32±0.3</td>
<td>18.31±0.6</td>
<td>14.37±1.9</td>
<td>19.37±0.9</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>5.24±0.1</td>
<td>3.38±0.1</td>
<td>6.05±0.8</td>
<td>3.33±0.2</td>
</tr>
<tr>
<td>Oleic acid (C18:1n9)</td>
<td>17.85±1.5</td>
<td>10.60±0.9</td>
<td>13.8±1.0</td>
<td>7.89±0.7</td>
</tr>
<tr>
<td>Linoleic acid (C18:2n6)</td>
<td>1.78±0.1</td>
<td>1.65±0.1</td>
<td>1.47±0.3</td>
<td>1.17±0.1</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. The growth of *R. jostii* RHA1 and *R. opacus* PD630 in the W media containing 30g/L of different sugars at 120 h. The cultures were firstly 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 media in a 500-ml flask. The initial A_{600} was regulated to about 0.1. Incubation was carried out at 30°C and 200 rpm.

Figure 2. Growth of recombinant cells of *R. jostii* RHA1 in W media 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 the media containing 30 g/L D-xylose. The samples were taken every 12 h and A_{600} was determined. ■, *R. jostii* XYLAB; ●, *R. jostii* Xyla; ▲, *R. jostii* pNV18. (B). The cells were grown in the media containing different contents from 20 g/L to 80 g/L of D-xylose. The cell biomass yield indicated as A_{600} at 120 h was detected. xylA, *R. jostii* Xyla; xylAB, *R. jostii* XYLAB. (C). The recombinant *R. jostii* XYLAB bearing xylA and xylB from *S. lividans* TK23 was incubated on 4 g/L D-xylose (■), and *R. jostii* pNV18 harboring the empty plasmid pNV18 was grown in the W media containing 4 g/L glucose (●).

Figure 3. Growth of xylB-disrupted *R. jostii* RHA1 bearing xylA on 10 g/L D-xylose at 200
rpm and 30°C. The samples were taken every 24 h and $A_{600}$ was determined. ■, *R. jostii* ΔxylB2_xylA; ●, *R. jostii* ΔxylB1_xylA; ▲, *R. jostii* ΔxylB1B2_xylA.

Figure 4. Growth of *R. jostii* XYLAB bearing plasmid pXYLAB, and time function of sugar consumption in the media containing sugar mixtures of about 4 g/L 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/L); ■, residue xylose content (g/L).

Figure 5. Growth of recombinant cells of *R. opacus* PD630 in W media containing 30 g/L xylose at 30°C and 200 rpm. The initial $A_{600}$ of the culture was regulated to about 0.1. After inoculation, the samples were taken every 12 h and $A_{600}$ was determined. ■, *R. opacus* XYLAB; ●, *R. opacus* XYLA; ▲, *R. opacus* pNV18.
Figure 1
Figure 2 (A)
Figure 2 (C)
Figure 4