REDUCING COD AND BOD, AS WELL AS PRODUCING TRIACYLGLYCEROL BY LDS5 GROWN IN CTMP EFFLUENT

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Both the energy shortage and pollution tend to slow down economic development and affect our daily lives. Some microorganisms not only can digest pollutants, but also can convert pollutant metabolites to triacylglycerol (TAG) that can be used to produce biodiesel. Here, we present results showing that the bacterium strain LDS5, a mutant of *Rhodococcus* sp. RHA1 (RHA1) generated in our lab, could grow well in chemithermomechanical pulping (CTMP) effluent, a type of paper mill wastewater, reduce chemical oxygen demand (COD_{Cr}) and biochemical oxygen demand (BOD_5) significantly, and produce TAG. Our data suggest that this strain has the potential to be used in paper mill wastewater treatment as well as in the development of biodiesel using biomass from paper mills.

Key words: Rhodococcus sp. RHA1; LDS5; CTMP effluent; Triacylglycerol; Biodiesel

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INTRODUCTION

With the improvements in living standards, petroleum is playing a more and more important role in our daily lives. The increasing consumption of energy means that the world supply of such resources will more rapidly reach its limit, and the resulting prospect of an energy shortage becomes one of our most serious problems. According to the International Energy Agency (IEA) report, the world oil production demand is expected to increase 1.1% per year and will reach 105 million barrels per day (Mb/d) in 2030 (IEA 2008). Fossil fuels are non-renewable resources, and the remaining accessible reserves are consumed more rapidly each year. The decline of petroleum reserves is predicted at 2% to 3% per year from 2010 (Campbell 2006). Solving the energy shortage by finding some new energy resources is becoming a hot topic around the world. A consensus of opinion has grown favoring the development of bioenergy. Biofuel is one of the most widely used forms of transportable energy. Bioethanol and biodiesel currently make up about 90% of the biofuel market (Rottig et al. 2010). Biodiesel is composed of fatty acid alkyl esters and can be synthesized by chemical, biochemical, or other methods mainly from renewable resources. The raw material for biodiesel is triacylglycerol (TAG)

that is stored in a cellular organelle called lipid droplet (Martin and Parton 2006; Zhang et al. 2010). Numerous microorganisms have the ability to accumulate neutral lipids in their lipid droplets under specific culture conditions; such organisms include algae, bacteria, and fungi (Meesters et al. 1996; Patil et al. 2008).

Furthermore, finding biomass for renewable energy from waste has attracted worldwide interest. The wastewater from the pulp and paper making industry can be considered not only as one of the most serious pollution problems, but also a source of substantial biomass. Chemithermo-mechanical pulping (CTMP) is a variety of high-yield pulping that entails heating and pre-treating spruce chips with mild chemicals in refining machinery (Roffael et al. 2001). The CTMP effluent has a complicated composition with a dark color and high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) (Kudo et al. 1991). On the other hand, the organic substances contained in CTMP effluent can be converted and utilized. Mere treatment of CTMP effluent by traditional methods no longer fully meets today's needs.

Rhodococcus sp. RHA1 (RHA1) is a bacterium that is isolated from lindane-contaminated soil (Seto et al. 1995). It is a strain of aerobic, nonsporulating, nonmotile gram-positive bacterium. RHA1 appears to have evolved to simultaneously catabolize a diverse range of compounds in an O_2-rich environment (Masai et al. 1995; McLeod et al. 2006). We modified RHA1 and obtained a new strain-LDS5 that could accumulate triacylglycerols in a nitrogen-deficient environment more effectively than the wild type when they were both cultured in CTMP. In addition, the mutant could reduce organic pollution more efficiently than RHA1 in CTMP effluent. These results indicate that the LDS5 could be a suitable bacterium that not only produces raw material for biodiesel, but also can reduce contaminant levels in pulping wastewater.

EXPERIMENTAL

Strains and Culture Conditions

The *Rhodococcus* sp. RHA1 strain was kindly provided by Prof. Lindsay D. Eltis from the University of British Columbia, Vancouver, Canada. Both RHA1 and the mutants were incubated aerobically in a nutrient broth (0.8% NB in H_2O) medium at 30^{o}C, 200 rpm/min. After 36 h, 75 mL cells (OD600≈2.0) were harvested by centrifugation (2,500 g, 10 min) and then inoculated into 150 mL effluent or mineral salt medium (MSM) (Alvarez et al., 2008) at 30^{o}C.

Construction of Knockout Mutant

The gene ro01152 was deleted in frame using the *sacB* counter selection system, and the mutant was denoted as LDS5. First, the 284 up fragment and 324 down fragment were amplified separately. After ligation with each other, the flanking sequences were cloned into pK18*mobsacB*, generating mutagenic plasmid. In the next step, the plasmid was transferred into *E.coli* S17-1, which served as the donor cell, then into *Rhodococcus* sp. RHA1 after conjugation. The LDS5 mutants were selected on LB plates containing 30 μg/mL nalidixic acid and 50 μg/mL kanamycin followed by *sacB* counter selection. Final confirmation of the removal of the gene ro01152 in kanamycin-sensitive, sucrose-

resistant colonies was conducted by colony PCR using a pair of primers that match sequences flanking the target gene. The plasmid pK18*mobsacB* and the bacterium *E.coli* S17-1 were kindly provided by Ping Xu (Shanghai Jiao Tong University, China).

Transmission Electron Microscopy

The bacteria were examined by transmission electron microscopy (TEM) through ortho staining. The sample was loaded on carbon-coated copper grids. Then 2% (w/v) phosphotungstic acid was used to stain the sample for 2 min. Then the grid was washed with deionized water three times before visualization with a FEI Tecnai20 (FEI company) electron microscope.

Thin Layer Chromatography (TLC)

Lipids in bacteria were extracted by a mixture of chloroform: methanol: medium (1:1:1, v/v/v) twice. The organic phase was collected and then evaporated under high purity nitrogen. The lipids were dissolved in 100 μL of chloroform and then were subjected to 60Å silica gel plate of 250 μm thickness (Whatman), then dried by air. The samples were developed by applying the solvent system hexane: diethyl ether: acetic acid (80:20:0.8, v/v/v) to separate neutral lipids. The plate was put into an iodine tank for 30 min to visualize the lipid fractions. A mono-, di-, and triacylglycerol mix was used as the lipid standard (Sigma, 1787-1AMP).

TAG Quantification Assay

The same amount of LDS5 and wild type bacteria were inoculated into CTMP wastewater after they were grown in NB for 36 h. Then 1 mL of medium was drawn from each sample at 0 h, 24 h, 48 h, 72 h, 96 h, and 120 h. The bacteria were washed twice by normal saline, and then sonicated in 400 μL of 1% TritonX-100 for 6 sec, 10 times at 200 Watts. The whole cell lysate was then centrifuged at 10,000 g for 5 min at room temperature. 10 μL supernatant of each sample was added into 96-well plate mixed with buffer-mix (Buffer I: Buffer II, 4:1, v/v) in triacylglycerol GPO-POD assay kit (Applygen, E1003), incubated for 20 min at 30^{o}C. Color intensity was measured using a Multiskan (Thermo) microplate reader at 565 nm.

COD_{Cr} and BOD_5 Test

The COD_{Cr} of CTMP effluent was detected by the dichromate method, and BOD_5 was detected by dilution and the seeding method. Both the detections were carried out according to the National Standard of China (GB 11914-89 and 7488-87).

RESULTS AND DISCUSSION

Rhodococcus sp. RHA1 Could Accumulate TAG in MSM and Pulp Mill Effluent

Rhodococcus sp. RHA1 was originally isolated from pesticide-contaminated soil and has the ability to accumulate triacylglycerol (TAG) in lipid droplets. To obtain more TAG that is converted from exogenous carbon sources, the growth media for TAG

production were optimized, similar to previous reports in other members of *Rhodococcus* sp. (Alvarez et al. 1996). The TLC (thin layer chromatography) result in Fig. 1a showed that RHA1 produced more TAG in the MSM medium (nitrogen limit medium) than in the NB medium (nutrient broth medium). The electron microscopy pictures also showed that RHA1 had larger lipid droplets in MSM than in NB (Fig. 1b). The main difference between NB and MSM is that NB contains a higher concentration of nitrogen than MSM, indicating that the bacteria could produce more TAG in response to nitrogen source-limiting circumstances, in agreement with previous reports.

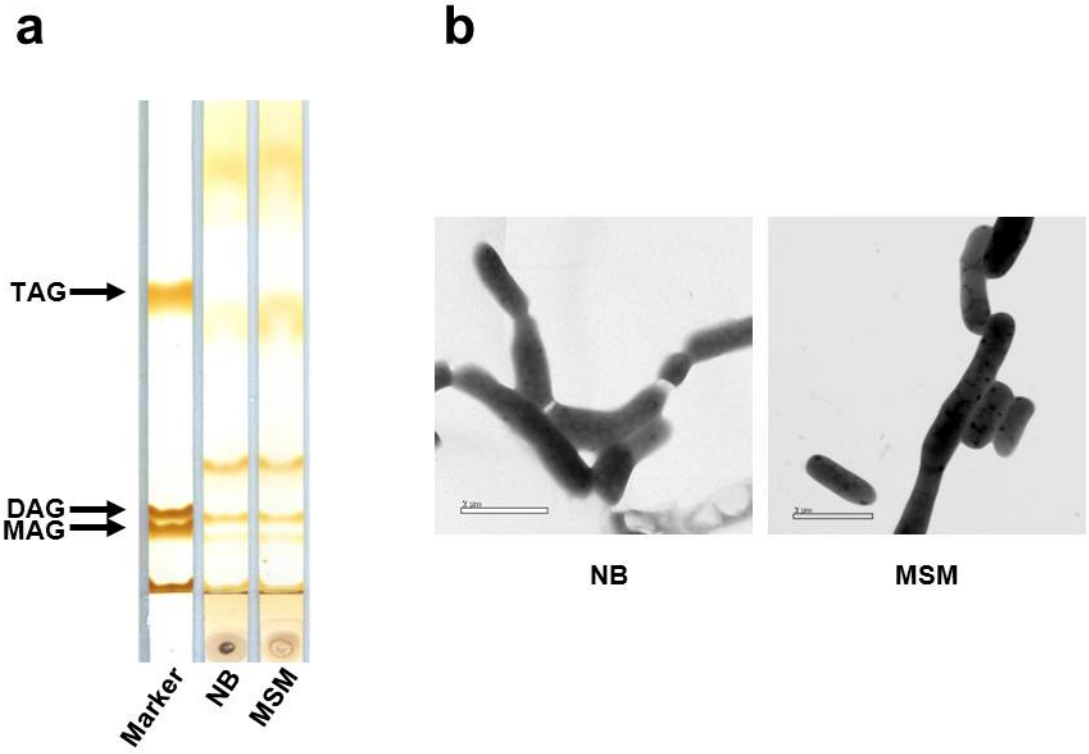

Fig. 1. TAG accumulation in *Rhodococcus* sp. RHA1. RHA1 cells were cultured in NB or MSM for 24 h. (a) Neutral lipids were extracted by methanol and chloroform, developed and analyzed by TLC. DAG, diacylglycerol; MAG, monoacylglycerol. (b) The bacteria were fixed, stained, and visualized by transmission electron microscope.

Similar to MSM, the pulping effluent also contains a low amount of N sources and high amounts of lignin, cellulose, and hemicelluloses that might be used as carbon sources by *Rhodococcus* sp. (Ahmad et al. 2010). RHA1 cells were cultured in various pulping wastewaters, and the color of CTMP effluent was lightened and cleared, indicating that the bacteria may consume some components in the wastewater (Fig. 2a). The TLC result in Fig. 2b showed that RHA1 could grow in both CTMP and alkaline peroxide mechanical pulping (APMP) effluents as well as accumulate TAG. The cells in CTMP effluent seemed to produce more TAG than in MSM and APMP effluent, suggesting that wastewater of CTMP is more suitable for RHA1 to accumulate TAG. In the future studies, the ingredients in CTMP effluent that are the major carbon sources for RHA1 will be investigated first.

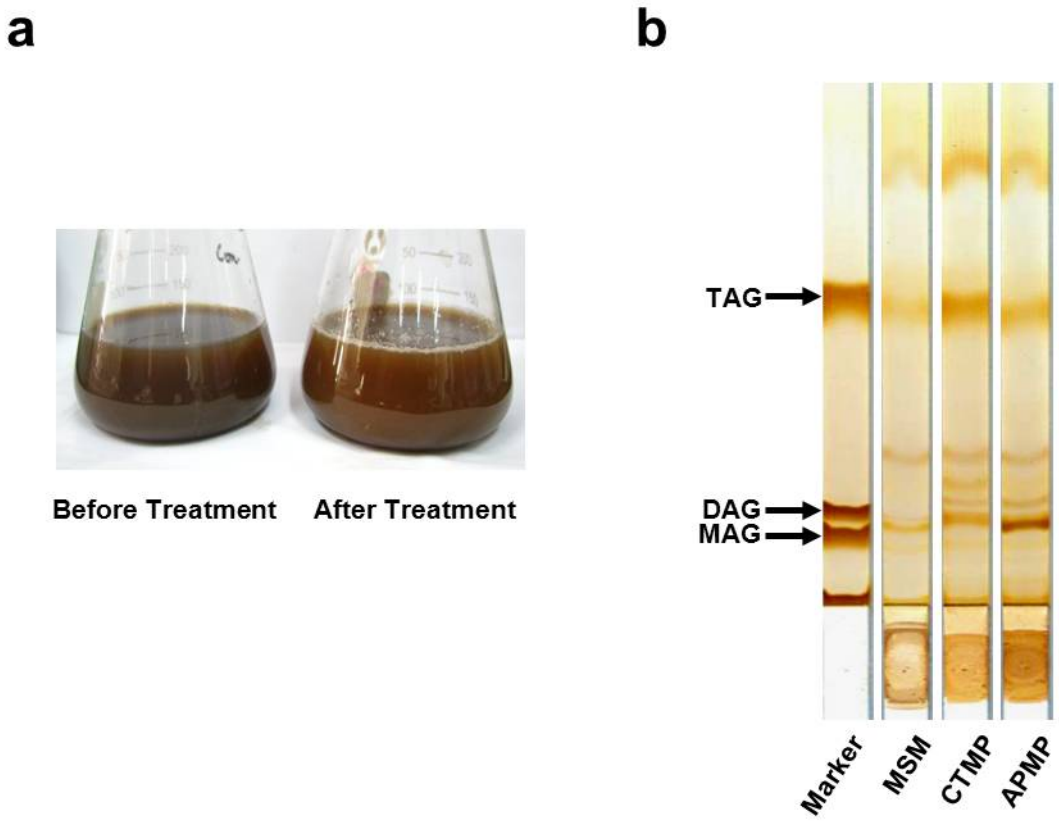

Fig. 2. TAG content of RHA1 cultured in different types of effluent. RHA1 cells were cultured in MSM or pulping wastewater for 48 h. (a) The color of CTMP effluent was lightened after RHA1 treatment. (b) Lipids were extracted from RHA1 cultured in different media, and then analyzed by TLC.

Deletion of LDS5 in *Rhodococcus* sp. RHA1 Improved Yield of TAG as well as Removal Efficiency for COD_{Cr} and BOD_5

To improve yield of TAG for future industrial applications, the genes coding lipid droplet-associated proteins were knocked out, and TAG was measured in those mutants. Figure 3b shows that all gene-knockout strains were able to accumulate TAG in CTMP effluent, and the LDS5 strain produced more TAG than the wild type and other gene-knockout strains (LDS and LDS3) in a time-dependent manner. All strains including LDS5 accumulated the maximum amount of TAG after cultivation for 48 h, indicating that 48 h is the optimal time point to harvest bacteria for the highest TAG yield and lowest cost. In fact, after 48 h, the TAG content of all strains dropped a little (Fig. 4b).

RHA1 has been reported to be able to degrade materials in waste (Masai et al. 1997). With respect to remove pollution from pulping wastewater, we measured COD_{Cr} and BOD_5 in CTMP effluent after 48 h of cultivation of RHA1 and the mutant LDS5. The COD_{Cr} removal efficiency of LDS5 was about twice as high as the wild type RHA1 in CTMP effluent (47.72±0.32% vs. 22.07±11.62%, Fig. 4). Also LDS5 decreased 69.95±13.22% BOD_5 in CTMP. Meanwhile wild type RHA1 only decreased BOD_5 by 18.35±12.00%, indicating that LDS5 was more efficient than the wild type to clean up the organic pollutants in CTMP pulp mill wastewater.

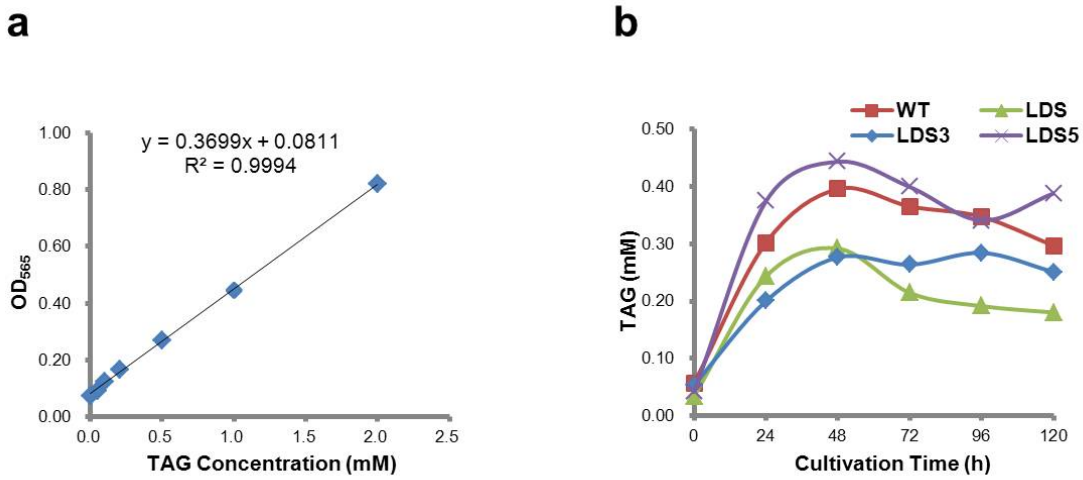

Fig. 3. Comparison of TAG accumulation between WT and KO bacteria. (a) TAG standard curve was measured by the TAG quantification kit. (b) TAG content of RHA1 and different knockout strains were compared in a time-course.

Considering that both TAG yield and the ability of reducing pollutants in CTMP effluent were higher in LDS5 than in RHA1, we assume that LDS5 utilizes more organic materials and converts them into endogenous lipids, resulting in higher TAG production and reduction of oxygen-demanding pollutants. One should determine if LDS5 grows faster, or it has more or larger lipid droplets than the wild type RHA1 in CTMP in future studies.

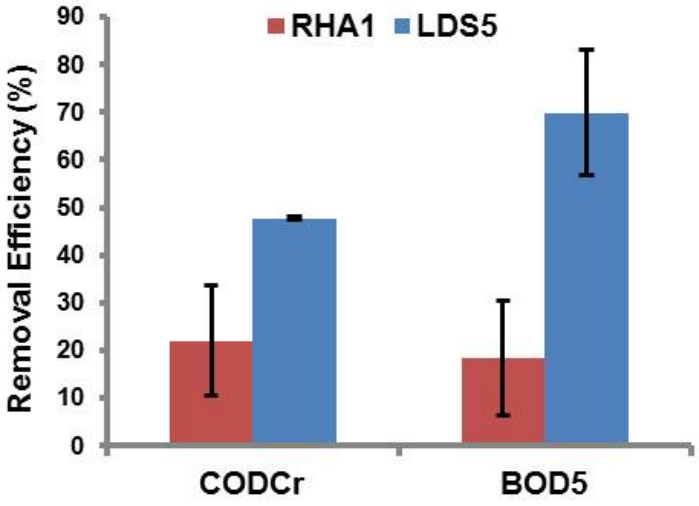

Fig. 4. LDS5 is more efficient than WT to remove COD_{Cr} and BOD_5. WT RHA1 and the mutant LDS5 were cultured in CTMP effluent for 48 h, respectively, and then the COD_{Cr} and BOD_5 were measured in the conditioned medium (bacteria removed).

The Optimal Culture Condition of LDS5 for Producing TAG as well as Reducing COD_{Cr} and BOD_5 in CTMP Effluent

To find the optimal culture condition for producing TAG and reducing pollutants, we first varied the amount of inoculum added into CTMP effluent. LDS5 was inoculated into NB for about 36 h until OD600≈2.0, and then diluted into CTMP effluent at the ratio of 1/10, 1/5, 1/2. By doing so, 15 mL, 30 mL, and 75 mL LDS5 in NB were respectively centrifuged at 2,500 g for 10 min and resuspended in 150 mL of CTMP effluent. As shown in Fig 5a, the TAG concentration in bacteria was increased corresponding to LDS5 inoculation ratio. COD_{Cr} and BOD_5 removal efficiency also increased from 13.20±0.75% and 41.50±0.98% to 25.34±0.77% and 44.64±10.08%, respectively, with the increasing initial bacterium amount (Fig. 5b). As the result suggested, 1/2 should be the optimal inoculating ratio for TAG accumulation in LDS5.

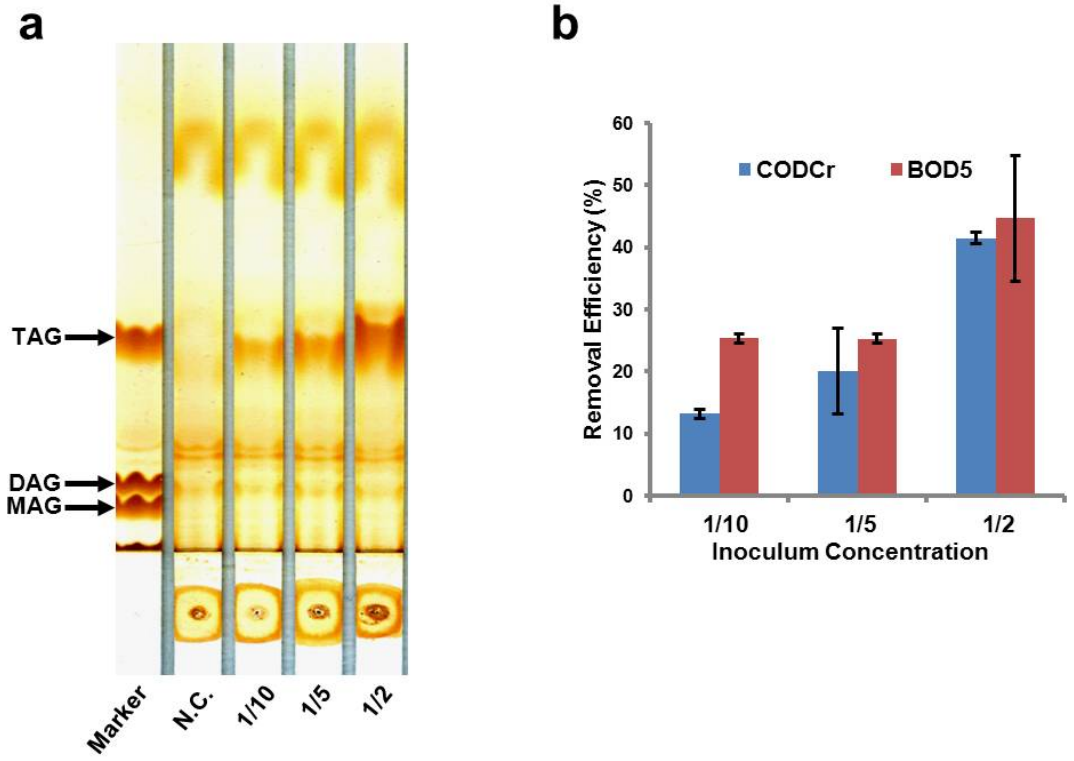

Fig. 5. TAG content and the COD_{Cr} and BOD_5 removal efficiency were increased along with LDS5 concentration. LDS5 cells were cultured in CTMP effluent with different initial inoculating concentration (1/10, 1/5, 1/2) after 36 h preculture in NB. (a) TAG content of bacteria was measured by TLC (N.C.: untreated CTMP wastewater itself only), and (b) COD_{Cr} and BOD_5 removal efficiency was detected and calculated.

We then tested the optimal time point for COD_{Cr} and BOD_5 reduction. As shown in Fig. 3b, LDS5 reserved the most TAG at 48 h. For COD_{Cr} reduction, the removal efficiency was dramatically increased during the first 48 h and then slightly increased from 48 to 96 h (Fig. 6a). After 96 h, the efficiency dropped about 10% from 55.16±5.90% to 43.40±7.55%, which may be caused by the death of LDS5 or the decreasing of degradation activity. The reduction of BOD_5 corresponded to that of

COD_{Cr}, except that the removal efficiency dropped earlier (from 72 h, Fig. 6b). Both COD_{Cr} and BOD_5 were removed efficiently at 48 h, which was consistent with the TAG accumulation. At the same time, the yield of TAG and reduction of organic pollutants were increased with increasing the initial concentration of inoculum (Fig. 5), suggesting that BOD_5 and COD_{Cr} removal efficiency may relate to the carbon converting efficiency from an environmental carbon source to bacterial TAG.

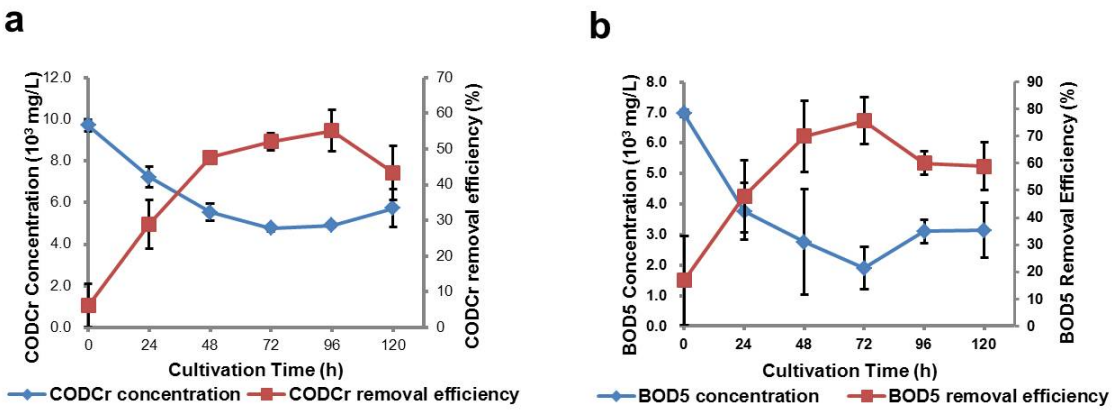

Fig. 6. COD_{Cr} and BOD_5 concentration and their removal efficiency are correlated to cultivation time. LDS5 were precultured in NB for 36 h, and then inoculated into CTMP effluent with the ratio of 1/2. 100 mL media were centrifuged to remove bacteria, and the supernatant was subjected to detect (a) COD_{Cr} and (b) BOD_5 concentration and removal efficiency at different incubation time points.

CONCLUSIONS

1. We acquired LDS5, a strain of bacterium derived from *Rhodococcus* sp. RHA1 that accumulates TAG as well as reduces pollutants from CTMP effluent.
2. LDS5 accumulated more TAG in CTMP pulping effluent than wild type RHA1. Meanwhile, it removed COD_{Cr} and BOD_5 more efficiently. TAG of LDS5 reached the highest yield at 48h, and both COD_{Cr} and BOD_5 removal efficiency rose quickly in the first 48 h, suggesting that 48 h is the optimal time point to culture LDS5 in CTMP pulping effluent for TAG acquisition, as well as reduction of pollution.
3. Compared with traditional pulping effluent treatment methods, based our laboratory-scale study, LDS5 treatment not only can remove organic pollutants from the effluent, but also can reserve TAG that can be utilized as a source of biodiesel.

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