African Journal of Biotechnology Vol. 6 (18), pp. 2130-2134, 19 September 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity

Chuan-chao Dai*, Jie Tao, Feng Xie, Yi-jun Dai and Mo Zhao

Jiangsu Key Laboratory for Biological Diversity and Biotechnology, College of Life Science, Nanjing Normal University, Nanjing, Jiangsu Province, 210046, China.

Accepted 15 August, 2007

This study explored a strategy to convert agricultural and forestry residues into microbial lipid, which could be further transformed into biodiesel. Among the 250 yeast strains screened for xylose assimilating capacity, eight oleaginous yeasts were selected by Sudan Black B test. The lipid content of these 8 strains was determined by soxhlet extraction method. One strain (T216) was found to produce lipids up to 36.6%, and it was identified as *Rhodotorula glutinis*. The optimal fermentation conditions were obtained as follows: glucose as carbon source 100 g/L; yeast extract and peptone as nitrogen sources at, respectively, 8 and 3 g/L; initial pH of 5.0; inoculation volume of 5%; temperature at 28°C, shaking speed of 180 r/min, cultivated for 96 h. Under these conditions, *R. glutinis* accumulated lipids up to 49.25% on a cellular biomass basis and the corresponding lipid productivity reached 14.66 g/L. Experiments with a 5-L bioreactor under the optimal culture conditions showed that *R. glutinis* accumulated lipids up to 60.69%, resulting in 23.41 g/L in lipid productivity. More encouraging results were observed for the lipid production with alternative carbon sources. Corn stalk and *Populus euramevicana* leaves hydrolysate could be used to substitute glucose. Chemical analysis indicated that biodiesel obtained by transesterification possessed similar composition to that from vegetable oil, one of the widely used feedstock for biodiesel.

Key words: Oleaginous yeast, *Rhodotorula glutinis*, culture optimization biodiesel.

INTRODUCTION

Negative environmental consequences of fossil fuels combustion and concerns about petroleum supplies have spurred the research for renewable biofuels. Biodiesel can be an interesting alternative for energy resource and may be used as substitute for petroleum-based diesel. Increasing in its production, biodiesel has attracted a broad public interest. The conventional method for biodiesel production is to transesterify plant oil with methanol (Krawczyk, 1996; Ma and Hanna, 1999).

However, the cost of biodiesel is currently more expensive than that of conventional diesel due to high cost share (70 - 85%) of the raw material. Increasing interest is generated to explore ways to reduce the high cost of biodiesel, especially the cost of the raw materials (Wu and Miao, 2006).

Lignocellulosic materials such as corn stalk, *Populus euramevicana* leaves and rice straw provide abundant and renewable energy sources. Lignocellulosics contain sugars that are polymerized to cellulose and hemicellulose, and they can be liberated by hydrolysis and subsequently converted to biofuel. Rice straw and corn stalk, especially, are characterized by variety, huge quantity and wide distribution, although great amount of these materials are burned in fields. This practice not only increases the air pollution and consequently affects the public health, but also is a great waste of energy materials.

If biodiesel can be produced from agricultural and fores-try residues, the environmental benefits can be much more significant than the economic benefits (Hill et al., 2006). One option is to exploite carbohydrate-based microbial oil, because it consists of similar fatty acids to that of plant oil (Tao et al., 2006). If we could isolate olea-

*Corresponding author. E-mail: taojie198305@yahoo.com.cn, daichuanchao@njnu.edu.cn. Tel: (0)86-025-85891382(0). Fax: 025-85891526(0).

Table 1. Medium composition used in this study.

Component	Enrichment medium	Isolation medium	YPD	Inoculum medium	Nitrogen-limited medium	Hydrolyzate medium
Glucose (g/L)	40	20	20	40	100	----
Hydrolysate sugar (g/L)	----	----	----	----	----	100
Yeast extract (g/L)	10	----	20	15	8	8
Peptone (g/L)	10	----	20	5	3	3
$(NH_4)_2SO_4$	----	2	----	----	----	----
$MgSO_4.7H_20$	----	0.2	----	----	----	----
KH_2PO_4	----	0.5	----	----	----	----
$CaCl_2.2H_2O$	----	0.1	----	----	----	----
Agar (g/L)	----	20	20	----	----	----
pH	4.0	5.0	6.0	6.0	5.0	5.0

ginous microbes with xylose assimilating capacity (xylose and glucose are the main compositions in the hydrolyzates of lignocellulosic materials), it would be of great significance in the utilization of agricultural and forestry residues.

The present study was to isolate oleaginous yeast strains with xylose assimilating capacity in *Rose centifoli*a. The production of microbial oil was optimized and scaled up to a 5-L bioreactor. Biodiesel was obtained by transesterifying fatty acids, and the composition of final products was analyzed.

MATERIALS AND METHODS

Culture media

The Culture media used in this study is shown in Table 1.

Yeast strains that could assimilate xylose isolation

Flower samples were milled in sterilized mortar and 0.5 g was enriched in 50 mL enrichment medium for 24 h at 28°C. The culture was diluted in a series of 10-fold dilutions in order to achieve a countable number of cells in 0.1 mL. Then 0.1 mL diluted culture was inoculated onto solid isolation medium using spread-plate technique for 24 h at 28°C.

Total 250 yeast strains were isolated from *R. centifolia* samples collected at Nanjing, China, in October 2005. The strains were stored at -80°C and then sub cultured on YPD agar slopes.

Screening of oleaginous yeast

The isolated 250 yeast strains were further screened using a rapid and sensitive technique. The cells were stained with Sudan Black B so that the absorbance measured at 580 nm gave lipid concentration in the fermentation broth using unstained cells as control (Thakur, 1989). The total lipid concentration of the selected 8 strains was determined by Soxhlet extraction method (Dai et al., 2005). The 8 oleaginous yeast strains were precultured in inoculum medium, and then 24 h old preculture was inoculated on nitrogen-limited medium for 3 days at 28°C with shaking at 140 r/min in 2000 mL Erlenmeyer flasks containing 500 mL media.

Identification of oleaginous red yeast

An integrated approach including morphological, biochemical and physiological characterization (Barnett et al., 1990) and genotypic (sequencing of D1/D2 domain of 26S rRNA encoding gene) methods (Botes et al., 2006; Lopandic et al., 2006) was used for the identification of the selected oleaginous yeast.

Optimization of culture conditions for lipid production by *Rhodotorula glutinis*

The influence of inoculation volume (10, 15, 20, 25, and 30%), carbon sources (glucose, sucrose, malt sugar, and xylose), nitrogen sources [urea, $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3, yeast extract, and peptone] on the lipid content was investigated (Jang et al., 2005; Li et al., 2006). Cultures were grown in 250 mL Erlenmeyer flasks containing 50 mL media, and the experiments were done with triplicates.

Fermentation in a 5-L bioreactor

Fermentation was carried out with the optimal fermentation medium in a 5-L automatic airlift bioreactor (Zheng Jiang Oriental Biology Equipment Corporation, China), equipped with disc impeller, oxygen and pH electrodes. The equipment also monitored temperature, agitation speed, gas purging flow rate, pumping rates, antifoam addition and the vessel level. The pH value and temperature were kept constant throughout the experimental time (Dai et al., 2006).

Preparation of hemicellulosic hydrolysis from corn stalk, *Populus euramevicana* leaves and rice straw

Corn stalk, *Populus euramevicana* leaves and rice straw were obtained from Jiangsu Province in China. Materials were ground, and sieved to obtain consistent particle sizes within 40 meshes. Hemicellulosic hydrolysis was prepared by using sulfuric acid hydrolysis at a ratio of solid: liquid ratio of 1:10 in boiling water bath for 8 h. The suspension was then centrifuged to remove the unhydrolyzed residue. The residue was washed with water at 80 ± 0.5°C to extract sugars. The supernatant and washings were then pooled together, and total reducible sugars were determined by spectro-

Table 2. The lipid content of 8 oleaginous yeast strains.

Strain No.	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Lipid coefficient
T8	9.86	17.11±1.14	1.69	1.69
T39	10.56	21.08±1.09	2.23	2.23
T86	13.32	21.50±2.21	2.86	2.86
T107	11.76	20.25±0.42	2.38	2.38
T169	6.90	17.07±0.18	1.18	1.18
T212	14.94	25.07±0.80	3.75	3.75
T216	15.16	36.63±1.23	5.55	5.55
T236	12.10	21.65±0.79	2.62	2.62

Lipid coefficient: the amount of lipid produced per 100 g glucose.

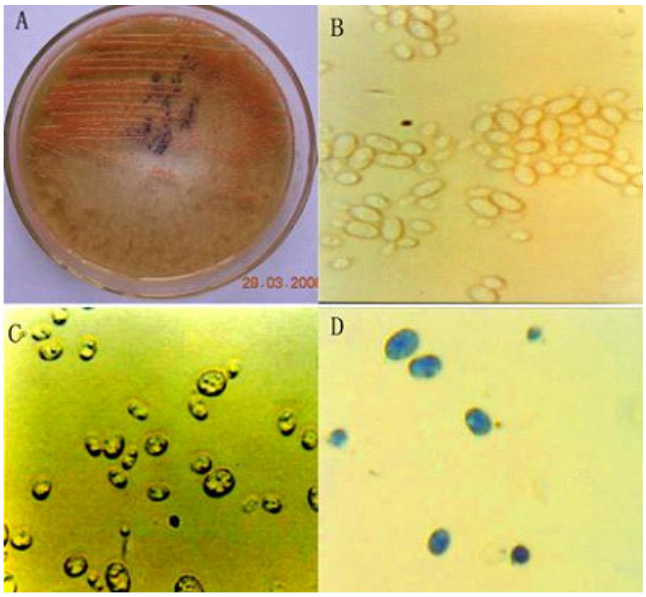

Figure 1. Photo of strain T216. (A) T216 strain on wort medium. (B) T216 cells grown on wort medium. (C) T216 grown on nitrogen-limited medium without staining. (D) T216 grown on nitrogen-limited with staining.

photometer using a dinitrosalicylic acid (DNS) reagent (Nigam, 2002). The initial sugar composition of the tree leaves hydrolytes after concentration was determined by HPLC as described by Van et al. (1988). Shake flask fermentation was conducted in triplicates with mixed sugars 100 g/L, yeast extract and peptone as nitrogen source at 8 and 3 g/L at the optimal fermentation conditions.

Production of biodiesel by transesterification

Microbial oil was extracted by Soxhlet extraction method before transesterification. The transesterfication reactions were carried out using sulfuric acid as catalyst in flasks at following conditions: 30:1 molar ratio of methanol to oil, 160 rpm, 5 h of reaction time, temperature at 55°C and 80% catalyst amount based on oil weight (Liu et al., 2004; Wu et al., 2006). The reaction mixture was cooled and undisturbed until two layers were formed in a separating funnel. The upper layer (biodiesel) was separated with petroleum ether and the final biodiesel product was obtained by evaporating the ether from the solution. The fatty acid methyl esters of biodiesel were analyzed by GC/MS (GC: VARIAN CP-3800; MS: VARIAN Saturn 2200) according to Pablo et al. (2006).

RESULTS

Screening of yeast strains

In our preliminary study, 250 red yeast strains with xylose assimilating capacity were isolated from *R. centifolia* samples. By applying Sudan Black B tests, 8 strains were identified as potential lipid biomass producer (Table 1). Although this technique did not allow precise insight about cellular lipid content, it did give, at least partially, information on the lipid accumulation ability of the yeast strains tested. In order to screen out the best strain, lipid biomass produce by the 8 selected yeast strains were further measured with GC analysis and Soxhlet extraction method. A strain T216 was found to be able to accumulate the highest lipid content (36.63%) (Table 2).

Identification of T216 yeast strain

Strain T216 was grown on solid wort medium, and observed under microscopy (10 x 100) as shown in Figure 1. Morphological, biochemical and physiological analyses indicated that T216 strain belongs to *Rhodotorula* sp. Primers used for the amplification of the D1/D2 fragment yield a fragment about 595 bp for the T216 strain. The sequence obtained (EF081370) was compared with those available in the GenBank, and the results showed high D1/D2 sequence similarity (99.2%) with the type strain of *Rhodotorula glutinis*. Therefore, T216 strain belonged to *R. glutinis.*

Optimization of culture conditions

Uniform design principles and single-factor experimental design were employed to investigate the effects of culture conditions on the lipid production by *R. glutinis*. Optimal

Table 3. Lipid production by agricultural and forestry residues.

Residue	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Lipid coefficient
Corn stalk	17.08	11.78±0.53	2.01	2.01
Tree leaves	16.56	28.59±1.17	4.73	4.73
Rice straw	3.58	5.74±0.20	0.21	0.21

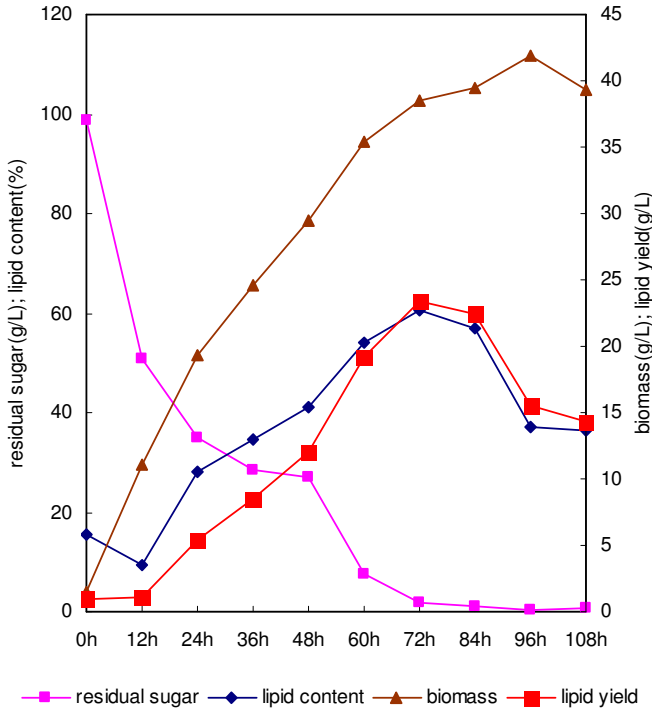

Figure 2. Growth curves of *Rhodotorula glutinis* in a 5-L bioreactor

fermentation conditions were obtained as follows: glucose as carbon source 100 g/L; yeast extract and peptone as nitrogen sources at, respectively, 8 and 3 g/L; initial pH of 5.0; inoculation volume of 5%; temperature at 28°C, shaking speed of 180 r/min, cultivated for 96 h. Under these conditions, *R. glutinis* accumulated lipids up to 49.25% on a cellular biomass basis with biomass yield of 29.77 g/L. Lipid productivity thus reached 14.66 g/L. Further experiments were performed in 5 L bioreactor under optimal culture conditions and results attained (Figure 2) showed that *R. glutinis* accumulated lipids up to 60.69% on a cellular biomass basis with biomass yield of 38.6 g/L for 72 h, which corresponding to 23.41 $g.L^{-1}$ lipid productivity.

Lipid production by agricultural and forestry residues

Encouraging results for lipid production were also observed using alternative carbon sources, corn stalk and *P. euramevicana* leaves hydrolytes as substitutes for the costly glucose. The biomass could be as high as 17.08 g/L and 16.56 g/L and the lipid contents were 11.78 and 28.59% (Table 3), respectively. *P. euramevicana* leave hydrolytes appeared to be a very good carbon sources. However, the optimization of culture conditions for this substitute should be conducted in the future. Poor growth of *R. glutinis* was observed in rice straw hydrolytes, presumably because the presence of inhibitory compounds in this hydrolytes (Table 2). Further study on the detoxification of diluted-acid lignocellulosic hydrolytes should be carried out in order to remove these inhibitors and therefore improve the efficiency of fermentation processes.

The initial sugar composition of the tree leaves hydrolyzate after concentration was determined by HPLC. The results were as following: hydrolysate, D-xylose 44.9%, glucose 26.9%, L-arabinose 18.5%, galactose 9.7%. These results showed that the selected strain could use these sugars to produce oil.

Biodiesel production from microbial lipids

After biodiesel was produced by transesterification with the yield of 81.7% and the fatty acid esters were analyzed by GC-MS. The results revealed that the composition of biodiesel was as follows: myristic acid (14:0) methyl ester 1.29%, palmitic acid (16:0) methyl ester 18.74%, stearic acid (18:0) methyl ester 1.16%, oleic acid (18:1) methyl ester 66.96%, linoleic acid (18:2) methyl ester 4.57% and low concentration of other methyl esters. The composition feature was quite similar to biodiesel from vegetable oil. Therefore, *R. glutinis* could be considered as a potential strain to convert lignocellulosic hydrolyzates into a raw material for biodiesel production.

DISCUSSION

Our results suggested that lignocellulosic biomass can be utilized to produce biodiesel. This can be a promising alternative energy source for our limited crude oil, and may even benefit the effort for reduction of accumulated carbon in atmosphere. For example, corn stover contains cellulose (40%), hemicellulose (25%), and lignin (17%). Its quantity of heat is about 17.8×10^3 $kJ.kg^{-1}$ because oleaginous microorganism can use glucose and pentaose (xylose and pectinose). In theory, corn straw can produce biodiesel 233 kg and glycerol 22.8 kg per ton (fatty acid calculates as stearic acid). As a result, the energy con-

version efficiency is about 55% (Tao et al., 2006).

However, biodiesel yield and productivity of fermentation of lignocellulosic hydrolyzate decreases as the presence of inhibiting compounds, such as weak acids, furans and phenolic compounds formed or released during the hydrolysis. The isolated *R. glutinis* has shown a great promise for industrial application because it could assimilate a wide range of sugars (xylose as the main component) present in the hydrolyzate, and withstand the inhibitors produced by the hydrolysis procedure. The results demonstrate that the new process, which combines bioengineering and transesterfication, is feasible and efficient for the production of high quality and low-cost biodiesel from microbial oil.

In order to further reduce the cost of biodiesel production from lignocellulosic biomass, we believe that research efforts should be emphasized on the following two aspects: (i) Exogenous depolymerization enzymes used in the hydrolysis process may be replaced with plants that are capable of synthesizing these enzymes *in situ* and their cellulase and hemicellulase produced can break down cell walls just before harvest. This would greatly reduce the cost of raw material pretreatment (Arthur et al., 2006). (ii) As the accumulation of microbial oil consumes oxygen and needs a lot of energy, efforts to reduce energy consumption is a key towards the Industrialization in comparison with ethanol production.

In this study, we proposed a technology that has potential to greatly reduce the price of lipid production, which can be used to produce biodiesel and therefore relieve the potential damage of energy crisis. We are optimistic for biodiesel production from lignocellulosic materials although it appears not feasible today.

ACKNOWLEDGEMENTS

The authors are thankful to the High Technology Project BG2005326 from the Council of Science and Technology of Jiangsu Province (China) for providing financial assistance. We wish to thank Dr. Jie Zhang for his skillful technical assistance in GC/MS analysis, and we are also grateful to Dr. Hui Peng for reviewing the manuscript.

REFERENCES

Arthur JR, Charlotte KW, Brian HD (2006). The path forward biofuels and biomaterials. Science. 311(27): 484-490.

Barnett JA, Payne RW, Yarrow D (1990). Yeast - Characteristics and Identification. Cambridge, Cambridge University Press. pp. 18-226.

Botes A, Todorov SD, Dicks Leon MT (2006). Identification of lactic acid bacteria and yeast from boza. Process Biochem. 42(2): 267-270.

Dai CC, Shi Y, Wang AQ, Yuan ZL (2005). Studies on Inoculation Types of γ-linolenic Acid Fermentation by a High-producing Filamentous Fungus Strain. Chin. Food Sci. 26(4): 55-58.

Dai YJ, Yuan S, Ge F, Chen T (2006). Microbial hydroxylation of imidacloprid for the synthesis of highly insecticidal olefin imidacloprid. Appl. Microb. Cell Physiol. 71: 927-934.

Hill J, Nelson E, Tilman D, Polasky S (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. PNAS.103: 11206-11210.

Jang HD, Lin YY, Yang SS (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by Mortierella alpine. Bioresour. Technol. 96: 1633-1644.

Krawczyk T (1996). Biodiesel-Alternative fuel makes inroads but hurdles remain. Inform. 7: 801-829.

Liu GY, Yuan S, Dai CC (2004). Factors affecting γ-linolenic acid content in fermented glutinous rice brewed by *Rhizopus sp*. Food Microbiol. 21(3): 299-304.

Li YH, Liu B, Zhao ZB, Bai FW (2006). Optimization of Culture Conditions for Lipid Production by *Rhodosporidium toruloides*. Chin. J. Biotechnol. 22(4): 650-656.

Lopandic K, Zelger S, Banszky LK (2006). Identification of yeasts associated with milk products using traditional and molecular techniques. Food Microbiol. 23(4): 341-350.

Ma F, Hanna MA (1999). Biodiesel production: a review. Bioresour. Technol. 70: 1-15.

Nigam JM (2002). Bioconversion of water-hyacinth (Eichhornia crassipes) hemicellulose acid hydrolysate to motor fuel ethanol by xylose-fermenting yeast. J. Biotechnol. 97: 107-116.

Pablo C, George AS, Makram TS, Albert DV (2006). Statistical evaluation of an analytical GC/MS methods for determination of long chain fatty acids. Talanta. 68: 888-894.

Tao J, Dai CC, Dai Q (2006). The conversion efficiency and economic feasibility of microbial energy. Chin. J. Microbiol. 26(6): 48-54

Thakur MS, Prapulla SG, Karanthsuadans NG (1989). Estimation of intracellular lipids by the measurement of absorbance of yeast cells stained with Sudan Black B. Enzyme Microb. Technol. 11(4): 252-254.

Van Zyl C, Prior BA, du Preez JC (1988). Production of ethanol from sugar cane bagasse hemicellulose hydrolyzate by Pichia stipitis. Appl. Biochem. Biotechnol. 17: 357-369.

Wu Q Y, Miao XL (2006). Biodiesel production from heterotrophic microalgal oil. Bioresour. Technol. 97: 841-846.