Bioresource Technology 150 (2013) 266–270

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Fatty acid ethyl esters production in aqueous phase by the oleaginous yeast *Rhodosporidium toruloides*

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HIGHLIGHTS

- Fatty acid ethyl esters were produced in aqueous phase by the yeast *Rhodosporidium toruloides*.
- Up to 73% of cellular neutral glycerides were converted into fatty acid ethyl esters.
- Neutral glycerides were hydrolyzed to free fatty acids followed by esterification.
- Lipid droplets played important roles in the process.

ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form 5 October 2013
Accepted 7 October 2013
Available online 14 October 2013

Keywords:
Biodiesel
Fatty acid ethyl esters
Aqueous phase conversion
Lipid droplets
Rhodosporidium toruloides

ABSTRACT

Fatty acid ethyl esters (FAEEs) are attractive biofuel molecules. Conventional FAEEs production process uses triglycerides and ethanol as feedstocks and is sensitive to water contents. In this work, we show that the oleaginous yeast *Rhodosporidium toruloides* cells are capable of converting lipids into FAEEs intracellularly in aqueous phase. Up to 73% of cellular neutral glycerides could be converted into FAEEs when lipid-rich cells were incubated for 84 h at 35 °C, pH 6.0 in a broth containing 10 vol% ethanol. It was found that neutral glycerides were first hydrolyzed to free fatty acids followed by esterification and that lipid droplets played important roles in the process. This new process provides a novel opportunity for integration of microbial lipid production technology with bioethanol fermentation for more efficient production of drop-in biofuels from renewable resources.

1. Introduction

With increasing transportation fuel demands and environmental concerns, biofuels have been recognized as important alternatives. Biodiesel, a typical form of liquid biofuel, has attracted considerable interest in recent years (Demirbas, 2007; Fjerbaek et al., 2009; Liang et al., 2013). Biodiesel consists of monoalkyl esters of long-chain fatty acids with short-chain alcohols, primarily methanol and ethanol, resulting in fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs), respectively. In general, biodiesel is produced by transesterification of triglycerides (TAGs) with alcohols in the presence of different types of catalysts, such as bases, acids or enzymes (Cao et al., 2012; Fedosov et al., 2013; Ferella et al., 2010; Liu and Zhao, 2007; Liu et al., 2012). In these processes, water can facilitate hydrolysis of TAGs and increase the content of free fatty acids (FFAs). Base- and acid-catalyzed biodiesel production processes are particularly sensitive to water and require high-quality feedstock with a water content lower than 5 wt% (Atadashi et al., 2012). For those catalyzed by heterogeneous catalysts or lipases, biodiesel yields around 90% could be achieved for feedstocks with higher water contents (Tan et al., 2006; Yan et al., 2009). However, when water contents exceed 30%, biodiesel yields usually drop to below 60% (Atadashi et al., 2012).

Because ethanol is less toxic than methanol and can be produced at large scale by fermentation from local and renewable agricultural resources, it is more appealing to make FAEEs as a sustainable biofuel. Efforts have been made for direct production of FAEEs by engineered cell factories. For example, recombinant *Escherichia coli* strains were constructed by expressing a wax ester synthase gene and ethanol-producing genes, such that FAEEs were successfully produced at a titre of 1.28 g/L after 72 h in the presence of exogenous fatty acids (Kalscheuer et al., 2006). When it was engineered to use the endogenous fatty acid pathway, *E. coli*

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http://dx.doi.org/10.1016/j.biortech.2013.10.023

could make 0.674 g/L FAEEs directly from simple sugars such as glucose (Steen et al., 2010). To avoid the toxicity of ethanol, a dynamic sensor-regulator system was developed to substantially improve strain stability as well as FAEEs titre to 1.5 g/L (Zhang et al., 2012). Similarly, the yeast *Saccharomyces cerevisiae* has also been explored for de novo production of FAEEs at a titer of 8.2 mg/L from glucose (Shi et al., 2012). However, the efficiency of direct FAEEs production by engineered cell factories remained low.

It is tempting to develop a FAEEs production process in aqueous phase by using ethanol fermentation broth directly as the alcohol source. Ethanol fermentation broth normally contains less than 20% ethanol, plus water and byproducts such as acetaldehyde, glycerol and acetic acid. It has been known that the oleaginous yeast *Rhodosporidium toruloides* is a robust lipid producer as it can accumulate lipid over 70% with a titer of over 100 g/L (Li et al., 2007), and utilize lignocellulosic hydrolysates with excellent tolerance towards inhibitory compounds (Hu et al., 2009). Lipids in this yeast can be recovered easily by enzyme-assisted extraction (Jin et al., 2012). In this study, we show that *R. toruloides* cells are capable of converting lipids into FAEEs intracellularly in aqueous phase. This new process represents an efficient alternative approach to traditional technology using TAGs as the feedstock or engineered cell factories for FAEEs production. It also provides a novel opportunity for integration of microbial lipid production technology with bioethanol fermentation for more efficient production of drop-in biofuels from renewable resources.

2. Methods

2.1. Strain, media and chemicals

R. toruloides Y4, a variant of *R. toruloides* AS 2.1389 obtained from the China General Microbiological Culture Collection Center, was grown in YPD liquid medium (containing 20 g/L glucose, 10 g/L yeast extract, and 10 g/L peptone, pH 6.0) at 30 °C and 200 rpm. The lipid production medium contained: 40 g/L glucose, 0.4 g/L KH_2PO_4, and 1.5 g/L $MgSO_4{\cdot}7H_2O$, pH 6.0. After sterilized at 121 °C for 15 min, it was supplemented with 10 mL/L of a trace element solution contained (per liter): 4.0 g $CaCl_2{\cdot}2H_2O$, 0.55 g $FeSO_4{\cdot}7H_2O$, 0.52 g citric acid·H_2O, 0.10 g $ZnSO_4{\cdot}7H_2O$, 0.076 g $MnSO_4{\cdot}H_2O$, and 100 μL 18 M H_2SO_4 (Wu et al., 2011).

Lipid-rich culture was prepared according to a published procedure (Lin et al., 2011). *R. toruloides* Y4 cells were incubated in YPD liquid medium at 30 °C, 200 rpm for 24 h, collected by centrifugation, and washed twice with sterile 0.9 wt% NaCl solution. Subsequently, they were cultured in lipid production medium at 30 °C, 200 rpm until the glucose was exhausted. The final cell mass and lipid titer were 17.5 g/L and 7.3 g/L, respectively.

Anhydrous ethanol (⩾99.8% purity) was obtained from Tianjin Hengxing Chemical Co., Ltd., Tianjin, China. Ethyl laurate (⩾99% purity), ethyl myristate (⩾98% purity), ethyl *cis*-9-Hexadecenoate (⩾95% purity), ethyl oleate (⩾98% purity), ethyl stearate (⩾99% purity), ethyl linolenate (⩾99% purity) and $CDCl_3$ (99.8% D with 0.03% (vol/vol) tetramethylsilane (TMS)) were obtained from J&K Scientific Ltd., Beijing, China. Ethyl palmitate (⩾99% purity), ethyl linoleate (⩾99% purity), Nile red (chemical pure) and *p*-nitrobenzaldehyde (*p*-NBD, 98% purity) were obtained from Sigma, Shanghai, China. All other chemicals and reagents were analytical pure and obtained commercially.

2.2. FAEEs production by R. toruloides Y4 cells

The typical procedure was performed as follows: aliquots of 6 mL of *R. toruloides* Y4 lipid-rich culture were mixed with 2 mL of 40 vol% ethanol solution in screw-capped vials sealed with parafilm. The reaction mixture was incubated at 35 °C, pH 6.0 and 200 rpm for 48 h. Ethanol concentration and water content of the reaction system were 10 vol% and 91.3 wt%, respectively. When one of these factors including temperature, pH, ethanol concentration and reaction time was investigated, the levels of others were held constant.

2.3. FAEEs production catalyzed by R. toruloides Y4 cell homogenates

R. toruloides Y4 cells from 6 mL of lipid-rich culture were lyophilized, milled to powder and extracted by cold acetone (Gu et al., 2011). Extracts and cell residues were mixed again, dried by N_2, and then resuspended with 6 mL of deionized water. Then the cell homogenates were mixed with 2 mL of 40 vol% ethanol solution and incubated at 35 °C, pH 6.0 and 200 rpm for 96 h.

2.4. Lipid extraction

Cells were homogenized with glass beads and extracted with chloroform–methanol (2:1, vol/vol) at room temperature (Folch et al., 1957). The organic extracts were washed with 0.1 wt% NaCl solution, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give the total lipids.

2.5. Lipid fractionation

About 50 mg of the lipid sample was loaded on a silica gel column, eluted by 120 mL of 1,2-dichloroethane, 80 mL of 1,2-dichloroethane/acetone (1:10, vol/vol), and 50 mL methanol in sequence (Wu et al., 2010). The solvents from each fraction were evaporated under reduced pressure. The fractions, in the order of elution, were neutral lipids (N), glycolipids plus sphingolipids (G + S), and phospholipids (P). The neutral lipids included TAGs, diglycerides (DAGs), monoglycerides (MAGs), FFAs and FAEEs. Lipid fractions were also analyzed by thin layer chromatography (TLC) using a mixture of n-hexane, diethyl ether and acetic acid (80:20:1, vol/vol/vol) as the developing agent (Nojima et al., 1995).

2.6. Analytical methods

Glucose concentration was monitored with a SBA-50B glucose analyzer (Shandong Academy of Sciences, Jinan, China).

The lipids were analyzed quantitatively by nuclear magnetic resonance (NMR) spectroscopy using a Bruker AVANCE III spectrometer (Bruker Co., Germany) operating at 400 MHz (Anderson and Franz, 2012). Lipids extracted from 8 mL of sample were dissolved in 1 mL of $CDCl_3$ and transferred to an NMR tube. Spectra were recorded at room temperature with *p*-NBD as internal standard. NMR Data were processed using MestReNova 6.1 (Mestrelab Research, Spain). Proton chemical shifts were assigned as shown in Table 1. The amounts of fatty acyl group (n_{FA}) in a lipid sample were quantified based on the amount of internal standard *p*-NBD (n_{NBD}, 66.2 μmol) according to Eq. (1):

$$n_{FA} = [2A_b/(A_f + A_g)] \times n_{NBD} \quad (1)$$

A_b, A_f, and A_g are the integration areas associated with the proton of H_b in fatty acyl, H_f and H_g in *p*-NBD, respectively. The contents of the lipid components were determined by the ratio of the amounts of their fatty acyl group to those of the fatty acyl group of total lipids (TL). The equations for neutral lipid content are shown in Table 1. The contents of neutral lipids (N), glycolipids plus sphingolipids (G + S) and phospholipids (P) were calculated according to Eq. (2):

$$C_i = n_{FA(i)}/n_{FA(TL)} \quad (2)$$