Bioresource Technology 150 (2013) 1–8

Contents lists available at ScienceDirect

Bioresource Technology

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

# Microbial recycling of glycerol to biodiesel

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## HIGHLIGHTS

- *De novo* biodiesel production using glycerol as sole carbon source.
- Genetic engineering of fatty acid ethyl ester (FAEE) biosynthetic pathway to improve biodiesel production.
- High production level of FAEEs (813 mg $L^{-1}$) from glycerol by fermentation optimization.

## ARTICLE INFO

*Article history:*
Received 7 July 2013
Received in revised form 13 September 2013
Accepted 17 September 2013
Available online 2 October 2013

*Keywords:*
Biodiesel
Glycerol
*Escherichia coli*
Genetic engineering
Fermentation

## ABSTRACT

The sustainable supply of lipids is the bottleneck for current biodiesel production. Here microbial recycling of glycerol, byproduct of biodiesel production to biodiesel in engineered *Escherichia coli* strains was reported. The KC3 strain with capability of producing fatty acid ethyl esters (FAEEs) from glucose was used as a starting strain to optimize fermentation conditions when using glycerol as sole carbon source. The YL15 strain overexpressing double copies of *atfA* gene displayed 1.7-fold increase of FAEE productivity compared to the KC3 strain. The titer of FAEE in YL15 strain reached to 813 mg $L^{-1}$ in minimum medium using glycerol as sole carbon source under optimized fermentation conditions. The titer of glycerol-based FAEE production can be significantly increased by both genetic modifications and fermentation optimization. Microbial recycling of glycerol to biodiesel expands carbon sources for biodiesel production.



## 1. Introduction

Worldwide increasing petroleum consumption induces economical and environmental problems at present time (Tarabet et al., 2012). Considerable attention was focused on the development of alternative fuel sources (Hansen et al., 2005). Biodiesel, fatty acid alkyl ester, is considered to be an alternative for petroleum-based diesel fuel. The most common technique for producing biodiesel is transesterification, which refers to a catalyzed chemical reaction of triacylglycerides (TAGs) with alcohols (Ma and Hanna, 1999; Rottig et al., 2010). Despite of possessing attractive prospects, the traditional biodiesel production still faces some bottlenecks as follows: the limited supply of lipid feedstock which generally relies on geographical and seasonal conditions, and the chemical transesterification which is energy consumption intensive and needs further waste treatment processes (Du and Liu, 2012; Yousuf, 2012). Glycerol as a low-price byproduct is inevitably produced in current biodiesel industry (Hoydonckx et al., 2004; Posada et al., 2012).

The global biodiesel market is estimated to reach 37 billion gallons by 2016 with an average annual growth of 42%, which means about 4 billion gallons of raw glycerol will be generated (Anand and Saxena, 2012; Yang et al., 2012). Glycerol stemming from biodiesel production flooding the market led to a dramatic decrease in prices of glycerol (Anand and Saxena, 2012; Dharmadi et al., 2006; Durnin et al., 2009). Thus, the process converting low value glycerol into high value products via biological or chemical routes currently attracts increasing interest (Chatzifragkou et al., 2011; Gungormusler et al., 2011; Posada and Cardona, 2010; Posada et al., 2012).

In terms of the issues on feedstock supply, waste cooking oils, oils accumulated by the heterotrophic microbes and photosynthetic microalgae have been used as feedstock for biodiesel production (Chisti, 2007; Ratledge and Cohen, 2008; Thanh le et al., 2010). Especially, oil from microalgae has been considered to be one of the most promising TAGs resources which provide a clue to produce biodiesel with environmental benefits and large net

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http://dx.doi.org/10.1016/j.biortech.2013.09.073

energy gains (Schubert, 2006; Wijffels and Barbosa, 2010). Nevertheless, the high costs for feedstock and high energy consuming in the downstream processes render the production uneconomical.

Distinct from approaches utilizing the oily fraction of biomass, novel strategies for biodiesel production from sugars hydrolyzed from abundant lignocellulosic biomass were developed through constructing non-native biosynthetic pathways of biodiesel molecules in microbial hosts. This promising concept was initially put into practice in genetically engineered *Escherichia coli* strains co-expressing genes encoding enzymes for ethanol production from *Zymomonas mobilis* and the *atfA* gene encoding acyl-coenzyme A: diacylglycerol acyltransferase (WS/DGAT) from *Acinetobacter baylyi* strain ADP1 (Kalscheuer et al., 2006). Additionally, a thioesterase for releasing feedback inhibition of fatty acid biosynthesis pathway as well as an endoxylanase and a xylanase for utilization of hemicellulose were introduced, which lead to the direct biosynthesis of FAEE with a yield of 11.6 mg $L^{-1}$ from the mixture of glucose (0.2%) and xylan (2%) (Steen et al., 2010).

Compared with traditional biodiesel production, the novel strategy for *de novo* FAEE biosynthesis from hemicellulose possesses obvious advantages due to the abundant feedstock together with simple and shortened downstream processing. Although *de novo* FAEE biosynthesis from hemicellulose could achieve the direct conversion to biodiesel, the yield is considerably low. Lignocellulosic biomass as feedstock for biodiesel production is not available for commercial application currently because of the high costs resulting from pretreatment and enzymatic hydrolysis. So the supply of feedstock is the dominant bottleneck existing in the current biodiesel production. As mentioned previously, the glycerol has recently become cheap and abundant coproduct in traditional biodiesel industry (Almeida et al., 2012). It is a suitable carbon source for *E. coli* in the *de novo* biodiesel production.

In this work, *de novo* biodiesel production with glycerol as sole carbon source was investigated (Fig. 1). Different fed-batch feeding strategies using glycerol as a carbon source for the improvement of FAEE production were compared. In addition, the influence of minimal media on the production of FAEE was examined. Metabolic engineering strategies were also applied to modify *E. coli* for the efficient production of FAEE.

## 2. Methods

### 2.1. Enzymes, DNA kits and strains

*Taq*, *Pfu* DNA polymerase and T4 DNA ligase were purchased from Fermentas (Burlington, Canada) and all restriction enzymes were from Takara (Kyoto, Japan). Plasmid mini kits, PCR purification kits and gel extraction kits were ordered from Omega (Norcross, USA). *E. coli* strain BL21 (DE3) and DH5α were obtained from Takara (Kyoto, Japan).

### 2.2. Construction of strains and plasmids

Detailed information of plasmids was shown in Table S1. The *E. coli fadD* gene with $P_{BAD}$ promoter was excised from pMD18-T-$P_{BAD}$-*fadD* by *Nco*I, and inserted into pMSD15 at *Hind*III site, creating plasmid pKC14, so was the formation of pKC15 with *atfA* gene from *A. baylyi* ADP1. The plasmid pMD18-T-$P_{BAD}$-*atfA* was digested by *Nco*I and *atfA* gene with $P_{BAD}$ promoter was ligated into pMSD15 at HindIII site blunt ended. *FAA2* gene was amplified from *Saccharomyces cerevisiae* INVSc1 with primers faa2-S1-CATATG GCCGCTCCAGATTATGCAC and faa2-A1-GCATGCCTAAAGCTTTTC TGTCTTGACTAC. To construct 5 kb cassette $P_{BAD}$-rbs-*Nde*I, the fragment excised from pXL49 by *Xba*I and *Spe*I was inserted into pBAD33 at *Xba*I site and digested by enzymes *Nde*I and *Sal*I. Restriction enzymes *Nde*I and *Xho*I were used to insert FAA2 into the cassette $P_{BAD}$-rbs-*Nde*I by *Nde*I and *Sal*I to generate pKC20. The *Kpn*I–*Sph*I double-digested 2 kb fragment of pKC20 was cloned into pMSD15 by *Hind*III blunt-ended to generate pKC16.

### 2.3. Cell transformation

BL21 (Δ*fadE*) competent cells were transformed with pMSD8 and pXT11 plasmid, and the BL21 (Δ*fadE*) (pMSD8/pXT11) mutant was screened on solid LB plates containing carbenicillin (25 μg $ml^{-1}$) and kanamycin (25 μg $ml^{-1}$). Plasmid pMSD15 was transformed into competent cells of BL21 (Δ*fadE*) (pMSD8 pXT11) to generate KC3 mutant. The plasmid pKC14 harboring *fadD* gene, pKC15 overexpressing two-copy of *atfA* gene and pKC16 harboring *FAA2* gene was co-transformed into KC2 strain together with pMSD8 and pXT11 to generate YL14, YL15 and YL16 strain respectively, which were screened on solid LB plates containing carbenicillin (25 μg $ml^{-1}$), kanamycin (25 μg $ml^{-1}$) and chloramphenicol (17 μg $ml^{-1}$).

### 2.4. Culture medium

For shake flask seed cultivation, LB media supplemented with 25 mg $L^{-1}$ ampicillin, 25 mg $L^{-1}$ kanamycin, and 17 mg $L^{-1}$ chloramphenicol were used. LB media consisted of 1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl. The detailed information of the fermentation media used here was shown in Table S2 of the additional files. Complex medium and chemically defined medium were evaluated as the initial medium, complex medium contained 2% w/v tryptone, 1% w/v yeast extract and 1% w/v sodium chloride, and chemically defined medium contained (%, w/v): $Na_2SO_4$ 0.2, $(NH_4)_2SO_4$ 0.268, $NH_4Cl$ 0.05, $K_2HPO_4$ 1.46, $Na_2HPO_4 \cdot 12H_2O$ 0.802, $(NH_4)_2$H-citrate 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, $VB_1$ 0.001, glycerol 2 and 3 ml trace element solution ($CaCl_2$ 0.05, $ZnSO_4 \cdot 7H_2O$ 0.018, $MnSO_4 \cdot H_2O$ 0.01, $Na_2$-EDTA 1, $FeCl_3$ 0.8, $CuSO_4 \cdot 5H_2O$ 0.016, $CoCl_2 \cdot 6H_2O$ 0.018). Appropriate antibiotics were added (see above). The chemically defined medium used as feed solution contained 7% glycerol, 0.2% $MgSO_4 \cdot 7H_2O$ and 0.1% $(NH_4)_2SO_4$ with the exception of the complex medium for the feed solution with 7% yeast extract.

### 2.5. Shake flask cultures and seed cultures

Recombinant strains of *E. coli* were streaked onto LB agar plates with antibiotics (see above) and incubated at 30 °C overnight. Single colonies were picked and inoculated into 10 ml of LB media in 50 ml flasks, and the flasks were incubated at 30 °C in a rotary shaker at 200 rpm for 12 h. For seed cultures, the cells were collected by centrifugation at 5000 rpm for 1 min, resuspended into 150 ml of sterilized LB in 500 ml flasks and were incubated at 30 °C and 200 rpm for 12 h. For shake flask fermentation cultures, the cells were collected by centrifugation at 5000 rpm for 1 min, resuspended into 50 ml of sterilized chemically defined medium as mentioned above with exception for 5 g $L^{-1}$ glycerol instead of 20 g $L^{-1}$ in 250 ml flasks, and shaken until an $OD_{600}$ of 1.5–2 was reached. Arabinose was subsequently added into the culture to a final concentration of 0.4% for induction of the *araBAD* promoter. One hour later, IPTG was added to a final concentration of 0.5 mol $L^{-1}$ for induction of the T7 promoter. At about 20 h after induction, the culture was extracted for GC–MS analysis.

### 2.6. Fed-batch fermentation

Fed-batch fermentation was performed in a 5 L fermentor (Biostat Bplus, Sartorius) with a working volume of 3 L. The pH

was controlled at 7.5 by automatic addition of 2 mol $L^{-1}$ HCl or 5 mol $L^{-1}$ NaOH. To maintain the dissolved oxygen level around 20% of air saturation, the agitation speed was varied from 200 to 600 rpm. The flow rate of air was maintained at 2 L $min^{-1}$. Inoculum was 5% (v/v) of overnight cultures (see above). Cultures were fed at a constant rate of 0.9, 0.6, 0.3, or 0.15 g $L^{-1}$ $h^{-1}$ when the dissolved oxygen rose sharply, in the case of two-stage feeding strategy, the feeding rate of 0.3 g $L^{-1}$ $h^{-1}$ shifted to 0.15 g $L^{-1}$ $h^{-1}$ while the induction was began. Cells were induced at $OD_{600}$ values of 8–10 by L-arabinose (final concentration of 0.4%) for pMSD15, and pKC15. After 1 h, IPTG (final concentration of 0.5 mmol $L^{-1}$) was added to induce genes coded on pMSD8 and pXT11. Fermentation broth samples (~20 ml) were collected at a series of time points and immediately kept at −80 °C for fatty acid ethyl ester analysis.

### 2.7. Analysis method

Optical density was measured at 600 nm and used as an estimate of cell mass. The identities of fatty acid ethyl ester in the GC MS analysis have been previously described (Duan et al., 2011). Ethanol concentration was determined using a biosensor (SBA-40C) from Biology Institute of Shandong Academy of Science (Jinan, China).

## 3. Results and discussion

### 3.1. Production of FAEEs in the mutant E. coli strain KC3 under the optimized fed-batch fermentation

#### 3.1.1. Optimization of feeding rates

*De novo* biosynthesis of FAEE from glucose in the mutant *E. coli* strain KC3 was reported previously (Duan et al., 2011). To further investigate the capability of KC3 mutant converting glycerol to FAEE, fed-batch fermentation with glycerol as the main carbon source in both initial and feed medium were performed in three different low constant feeding rates (0.6, 0.3 and 0.15 g $L^{-1}$ $h^{-1}$). Different feeding rates resulted in remarkably different cell growth rates during the feeding period. The cell growth evaluated by optical density ($OD_{600}$) was much better at the feeding rate of 0.3 g $L^{-1}$ $h^{-1}$ than that of 0.6 g $L^{-1}$ $h^{-1}$. Compared with the above two feeding rates, the cell growth at the feeding rate of 0.15 g $L^{-1}$ $h^{-1}$ was relatively slow during the early feeding phase. However, the growth increased rapidly in the later phase of cultivation, an $OD_{600}$ of 37.6 was reached at the end of the fermentation, which was the highest $OD_{600}$ of the fed-batch fermentation at three different feeding rates (Fig. 2A).

Many studies have reported the optimization of ethanol production from glycerol in *E. coli* (Yazdani and Gonzalez, 2008). The ethanol production of KC3 mutant with the feeding rate of

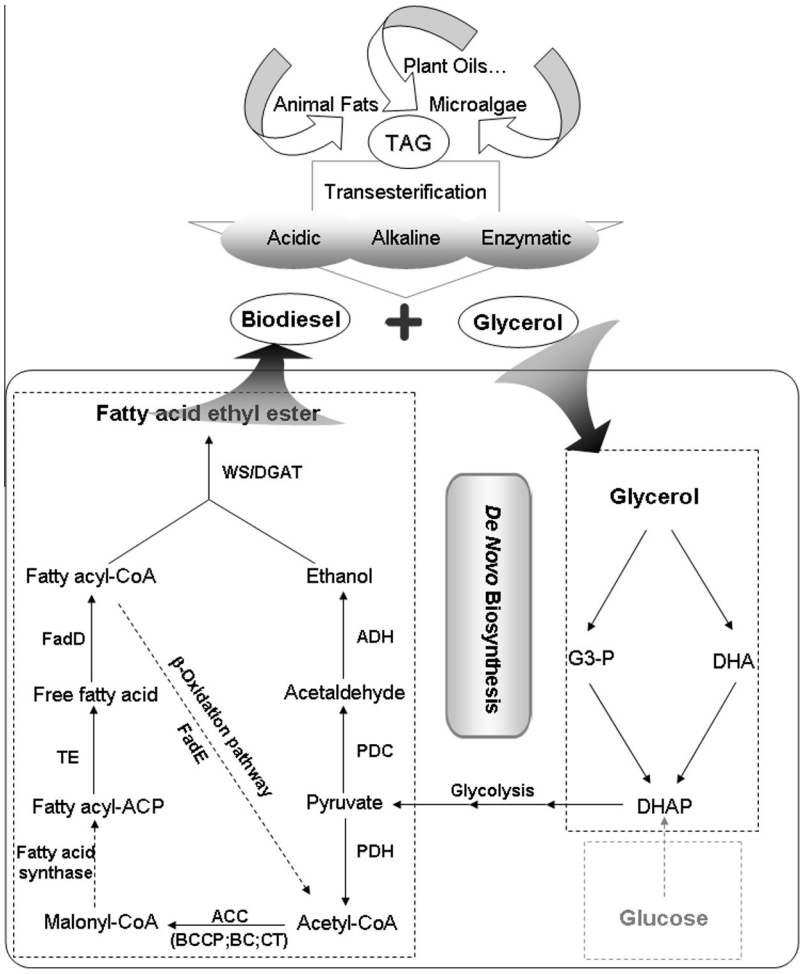


**Fig. 1.** Schematic overview of chemical transesterification for biodiesel and *de novo* FAEE biosynthesis pathway from glycerol in engineered *E. coli* strains. ACC, acetyl-CoA carboxylase; ADH, alcohol dehydrogenase; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CT, carboxyltransferase; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; FadD, fatty acyl-CoA synthase; FadE, acyl-CoA dehydrogenase; G3-P, glycerol 3-phosphate; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; TAG, triacylglyceride; TE, thioesterase; WS/DGAT, wax synthase/acyl-coenzyme A: diacylglycerol acyltransferase.

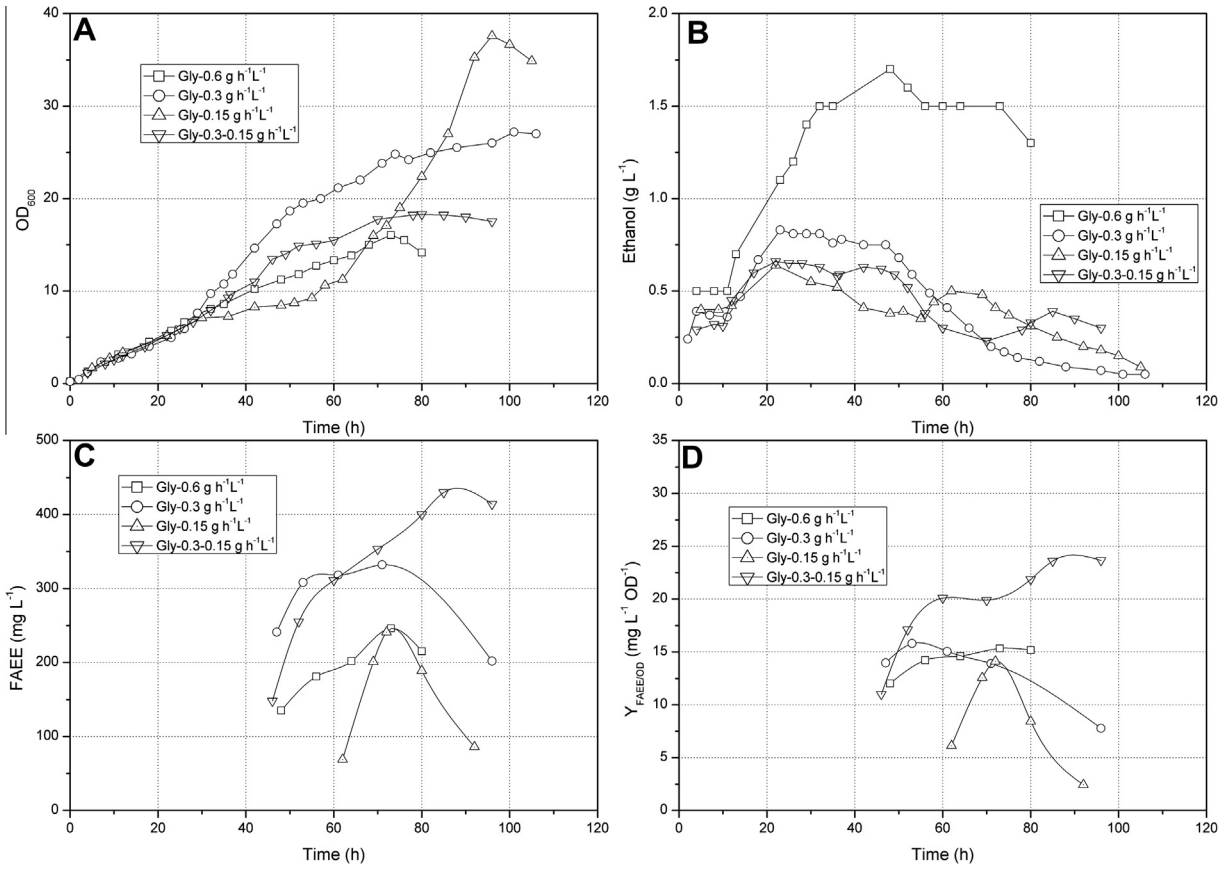


**Fig. 2.** Comparison of the fed-batch fermentation profiles for (A) cell growth, (B) ethanol accumulation, (C) FAEE production, and (D) $Y_{FAEE/OD}$ of the *E. coli* mutant strain KC3 applying three glycerol feeding rates and a two-stage feeding strategy in the complex medium. The experiments were conducted in fully controlled 5-L fermentor with a working volume of 3 L. The pH was controlled at 7.5 by automatic addition of 2 mol $L^{-1}$ HCl or 5 mol $L^{-1}$ NaOH. The flow rate of air was maintained at 2 L $min^{-1}$, the agitation speed was varied from 200 to 600 rpm maintaining the dissolved oxygen level around 20% of air saturation. Cells were grown to $OD_{600}$ of 8–10 at which point 0.4% arabinose was added with further cultivation for 1 h. Then 0.5 mmol $L^{-1}$ IPTG was added with further cultivation.

0.6 g $L^{-1}$ $h^{-1}$ was distinct from that of the other two feeding rates. The ethanol concentration rose sharply when the feeding started, and the maximum concentration of 1.7 g $L^{-1}$ was obtained in this feeding strategy. After induction, ethanol production decreased while FAEE content kept increasing. The lower ethanol accumulation correlated with the lower feeding rate. The ethanol accumulation dropped to nearly zero during the post-induction period under the fed-batch fermentation at the other two lower feeding rates (Fig. 2B).

As to FAEE production, the yields at three feeding rates differed significantly. During the post-induction phase at the feeding rate of 0.3 g $L^{-1}$ $h^{-1}$, the FAEE production reached 308 mg $L^{-1}$ after an 18 h induction, and the maximum concentration was 332 mg $L^{-1}$. In contrast, the FAEE production increased faster before they reached the maximal concentration in the other two fed-batch fermentations. After reaching the stationary phase, the FAEE production dropped rapidly in all three fed-batch fermentations at different feeding rates. The maximum concentration of FAEE in the fermentation at the feeding rate of 0.6 g $L^{-1}$ $h^{-1}$ or 0.15 g $L^{-1}$ $h^{-1}$ was only 73% of that at the feeding rate of 0.3 g $L^{-1}$ $h^{-1}$. It was found that fermentation with the lower feeding rate has the higher yield coefficients of the product FAEE to the limiting substrate glycerol ($Y_{FAEE/Gly}$).

Feeding strategy was critical because it affected the cell growth and the specific productivity of recombinant proteins (Fang et al., 2011; Norsyahida et al., 2009). The best cell growth and the highest FAEE production can be obtained at the constant feeding rate of 0.3 g $L^{-1}$ $h^{-1}$ among three different feeding rates. However, the highest value of yield coefficient of $Y_{FAEE/Gly}$ was obtained at feeding rate of 0.15 g $L^{-1}$ $h^{-1}$ as mentioned before. In addition, the culture time when the FAEE production reached the maximum was shorter at feeding rate of 0.15 g $L^{-1}$ $h^{-1}$ (Fig. 2C).

In order to maximize FAEE production and yield coefficient of the product FAEE to substrate glycerol while shortening the fermentation time, a two-stage glycerol feeding strategy was employed in this work. During the pre-induction phase, the glycerol was fed at a constant rate of 0.3 g $L^{-1}$ $h^{-1}$. When the cell culture reached an $OD_{600}$ of 8–10, the post-induction phase began and the glycerol feeding rate was shifted to 0.15 g $L^{-1}$ $h^{-1}$. The performance of the fed-batch fermentation for the two-stage feeding strategy was shown in Table 1. The ethanol accumulation was similar to that of the two constant feeding rates mentioned above. The FAEE production with the two-stage feeding rate increased faster than that with a constant feeding rate. The maximum concentration of FAEE, $Y_{FAEE/Gly}$ and the FAEE productivity reached 430 mg $L^{-1}$, 28.2 mg $g^{-1}$ and 5.06 mg $L^{-1}$ $h^{-1}$ respectively, which were the highest values observed in these fed-batch fermentations with the main carbon source of glycerol (Fig. 2C).

The efficiency of FAEE fermentation can also be characterized by calculation of the production selectivity, i.e., the yield coefficient of FAEE production on the amount of biomass produced ($Y_{FAEE/OD}$, FAEE production of per liter per $OD_{600}$). A comparison of the selectivity on fed-batch processes with different feeding strategies was shown in Table 1. It was indicated that the strategy of two-stage feeding favored the formation of FAEE over biomass (Fig. 2D).

**Table 1**
Comparison of parameters for FAEE fermentation under diverse fed-batch cultivations by two mutant strains.

| Strains | Media types | Varying feeding rate ($g\ L^{-1}\ h^{-1}$) | $FAEE_{MAX}$ ($mg\ L^{-1}$) | $OD_{MAX}$ | $Ethanol_{MAX}$ ($g\ L^{-1}$) | $Y_{FAEE/OD}$ ($mg\ L^{-1}\ OD^{-1}$) | $Y_{FAEE/GLC(GLY)}$ ($mg\ g^{-1}$) | Volumetric productivity ($mg\ L^{-1}\ h^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| KC3 | Glucose complex medium | 0.6 | 922 | 27.6 | 2.6 | 34.3 | 27.7 | 13.0 |
| | Glycerol complex medium | 0.6 | 246 | 16.1 | 1.7 | 15.3 | 7.38 | 3.67 |
| | | 0.3 | 332 | 27.2 | 0.78 | 15.8 | 18.8 | 4.68 |
| | | 0.15 | 241 | 37.6 | 0.64 | 14.1 | 26.8 | 3.35 |
| | | 0.3–0.15[a] | 430 | 18.3 | 0.66 | 23.7 | 28.2 | 5.06 |
| | Glycerol minimal initial medium | 0.3 | 520 | 18.0 | 0.44 | 28.9 | 29.1 | 5.53 |
| | | 0.3[b] | 190 | 15.1 | 0.98 | 12.6 | 6.87 | 2.21 |
| YL15 | Glycerol general minimal medium | 0.3[b] | 400 | 15.9 | 0.78 | 27.0 | 25.0 | 5.12 |
| | | 0.6[b] | 813 | 16.8 | 3.1 | 49.3 | 27.1 | 9.45 |
| | Glycerol optimized minimal medium (MSMopt) | 0.3[b] | 274 | 46.2 | 0.70 | 14.0 | 13.7 | 3.33 |
| | | 0.9[b] | 650 | 31.3 | 0.70 | 24.1 | 16.5 | 7.56 |
| | | 1.2[b] | 421 | 24.3 | 0.83 | 13.8 | 11.2 | 6.01 |

[a] Two stage feeding strategy, the glycerol feeding rate was controlled at 0.3 $g\ L^{-1}\ h^{-1}$ during the pre-induction stage, when the cell optical density reached a certain value (e.g. 8–10), the post-induction stage began and the glycerol feeding rate was shifted to 0.15 $g\ L^{-1}\ h^{-1}$.
[b] Minimal feeding solution containing 7% glycerol, 0.2% $MgSO_4\ 7H_2O$ and 0.1% $(NH_4)_2SO_4$ without 7% yeast extract.

### 3.1.2. Optimization of culture medium

To develop a low-cost fermentation medium for KC3 mutant, the fermentation performance of a chemically defined medium was investigated. With a tryptone and yeast extract-based initial medium and a yeast extract-based feed medium as the control medium (the two-stage feeding strategy was applied in this condition), a minimal initial medium with a complex feed medium (condition 1) and a minimal initial medium with a minimal feed medium (condition 2) were applied in the fermentation. Little negative effect on biomass production was observed in both condition 1 and 2 during the fed-batch processes. The cell growth rate in condition 2 decreased significantly, and the maximal $OD_{600}$ reached 15.1, approximately 17% lower than that of control condition and condition 1 (Fig. 3A). The ethanol accumulations in three different conditions were similar. Unexpectedly, a maximum ethanol concentration of 0.98 $g\ L^{-1}$ was obtained in condition 2, which is about 48% and 123% higher than that of control condition and condition 1, respectively (Fig. 3B).

With respect to the FAEE production and the productivity which was calculated as $Y_{FAEE/OD}$, the cells accumulated FAEE to

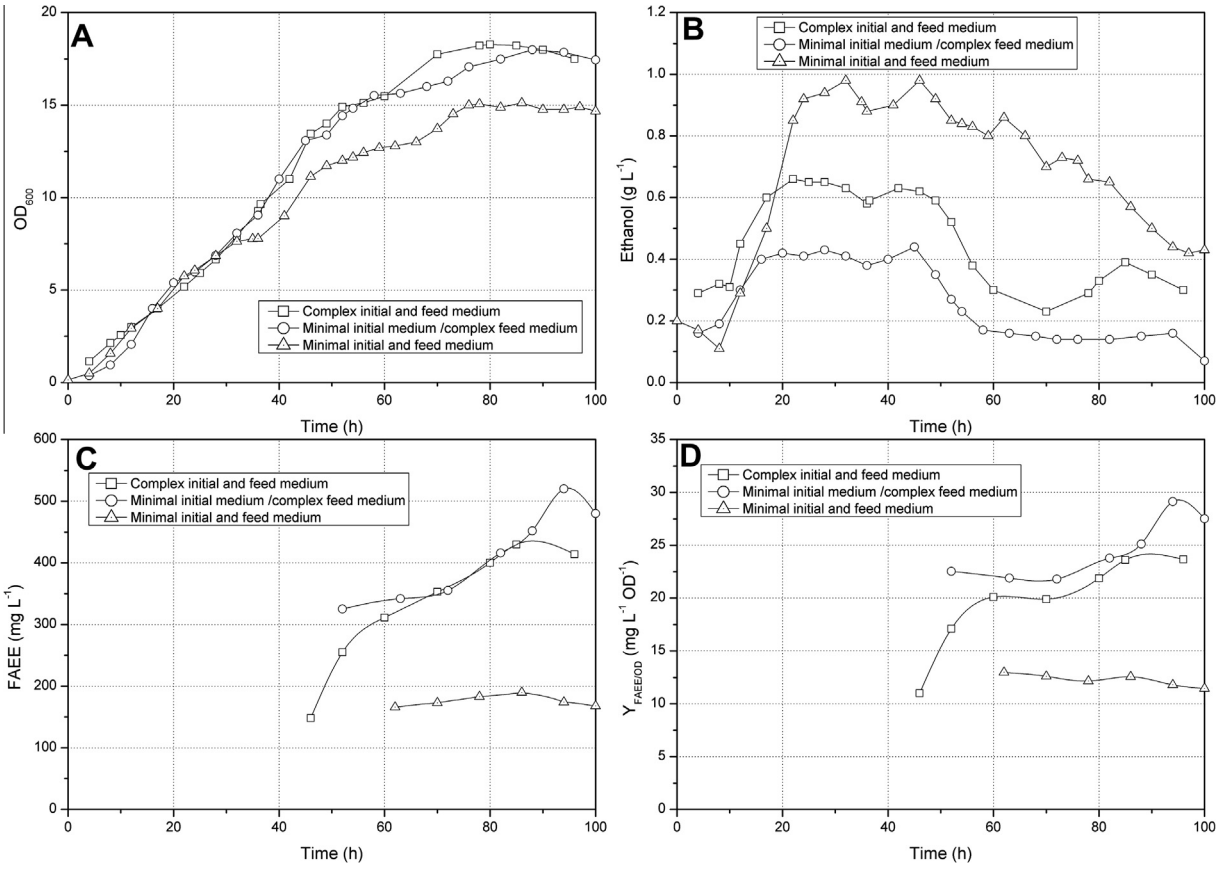


**Fig. 3.** Comparison of the fed-batch fermentation profiles for (A) cell growth, (B) ethanol accumulation, (C) FAEE production, and (D) $Y_{FAEE/OD}$ of the *E. coli* mutant strain KC3 in three different medium conditions. A two-stage glycerol feeding strategy was used in the condition of complex initial and feed medium, 0.3 $g\ L^{-1}\ h^{-1}$ glycerol feeding strategy were used in the other two conditions. Experimental conditions were the same as reported in Fig. 2.

520 mg $L^{-1}$ and $Y_{FAEE/OD}$ to 29.1 mg $L^{-1}$ $OD^{-1}$ as the maximum after 58 h induction in condition 1. FAEE content increased 21% and 174% in condition 1 compared with the control condition and condition 2, respectively. $Y_{FAEE/OD}$ in condition 2 was lower than the control condition. However, higher $Y_{FAEE/OD}$ was obtained in condition 1 than the control condition. These results indicated that nutrient accumulation might happen with complex compounds as the initial medium, which might cause changes in cell physiology and metabolism after induction and influence FAEE production subsequently. Therefore, the minimal initial medium and feed medium containing organic nitrogen might be optimal in FAEE fed-batch fermentation with glycerol as the sole carbon source. However, the chemically defined medium is required for industrial production after optimization (Fig. 3C and D).

Glucose represents the most abundant carbon source available for the production of chemicals and fuels via microbial fermentations. The fermentation variables with glycerol as the carbon source in diverse feeding strategies were compared with the optimal results of our previous work with glucose as the carbon source in Table 1. For fed-batch fermentations in complex medium, the maximum titers of FAEE production, volumetric productivity and $Y_{FAEE/OD}$ obtained by feeding glycerol decreased 53%, 61%, and 31% respectively compared to those obtained by feeding glucose. When the yields $Y_{FAEE/Gly(Glc)}$ were calculated as FAEE production of per gram glycerol (or glucose), feeding of glycerol resulted in 2% higher yield compared to feeding of glucose. About a twofold increase of FAEE production in the minimal medium with glycerol as the sole carbon source was obtained compared to that in the complex medium, however, the FAEE production was still 12% lower than that with glucose as the sole carbon source.

The comparison of the variables indicated that the overall results obtained with glucose as carbon source were superior to that obtained with glycerol. However, glycerol was much competitive considering that the low-priced minimal medium can be applied.

### 3.2. Genetic modifications of FAEE biosynthetic pathway to improve production of FAEEs

Free fatty acids (FFAs) must be activated to fatty acyl-CoA by acyl-CoA synthetase (ACS, EC 6.2.1.3) prior to the synthesis of FAEE. Overexpression of ACS may improve production of FAEEs, so *fadD* gene from *E. coli* DH5α and *FAA2* gene from *S. cerevisiae* INVSc1 were overexpressed in YL14 and YL16 mutant, respectively. However, the FAEE productivity in YL14 or YL16 mutant had not been improved significantly in chemically defined medium with glycerol as the sole carbon source (data not shown).

It seems that the relatively low activity of WS/DGAT is the bottleneck in *de novo* FAEE production. An *E. coli* mutant overexpressing two copies of *atfA* gene and other elements for *de novo* FAEE biosynthesis (YL15) was constructed. In the YL15 strain, FAEE production was significantly improved and FFA accumulation dropped remarkably compared with KC3 strain. When cultivated in the chemically defined medium with glycerol as the sole carbon source in shake flasks, the FAEE production in YL15 mutant was found 70% higher than that of KC3 strain, up to 22.9 mg $L^{-1}$ $OD_{600}^{-1}$.

Fed-batch fermentations in minimal medium with glycerol as sole carbon source were employed to compare the capability of KC3 and YL15 strain at the same feeding rate of 0.3 g $L^{-1}$ $h^{-1}$. The complete profiles for KC3 and YL15 strain during the fed-batch fermentation were shown in Table 1. The YL15 strain accumulated 400 mg $L^{-1}$ FAEE as the maximum, which translates into more than twice the value of $Y_{FAEE/OD}$ compared with the reference strain KC3, since slight difference of cell growth occurred. With respect to the ethanol accumulation during the fermentation, very low level of

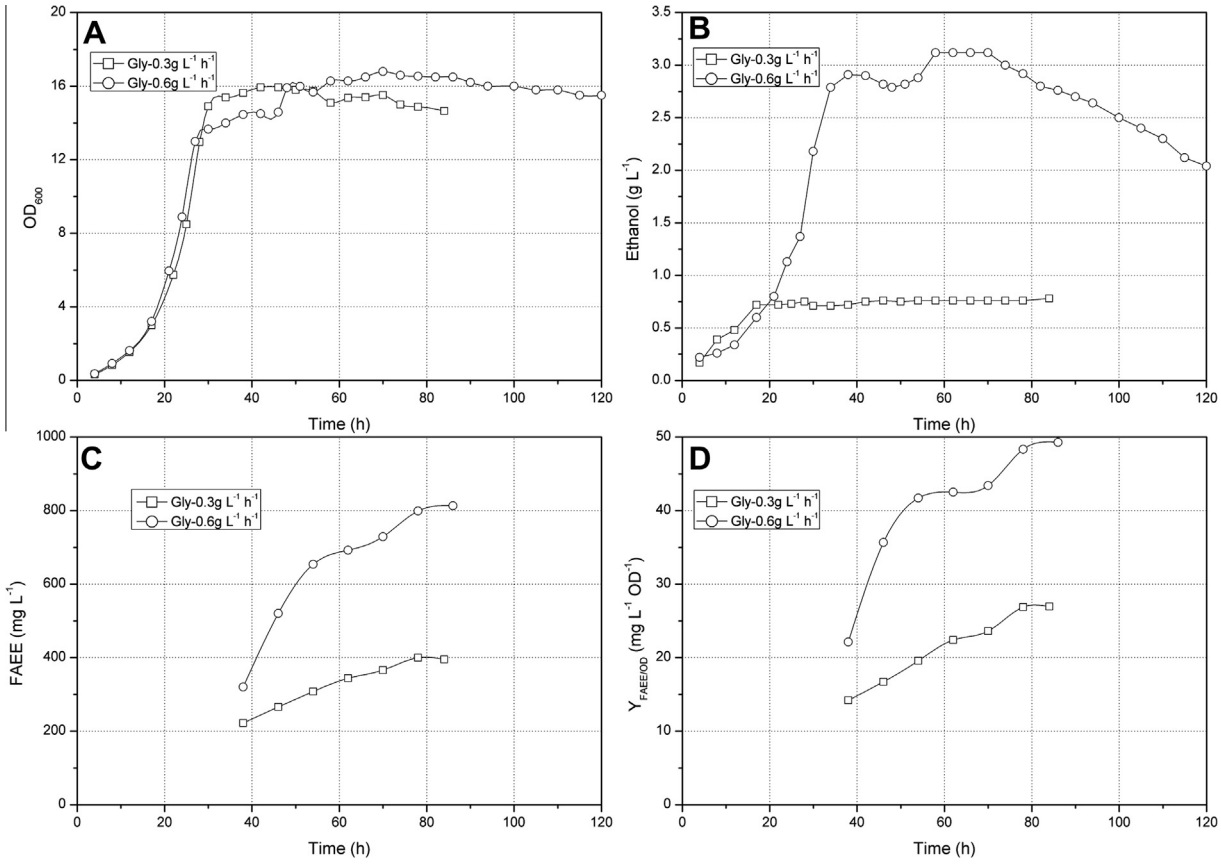


**Fig. 4.** Comparison of the fed-batch fermentation profiles for (A) cell growth, (B) ethanol accumulation, (C) FAEE production, and (D) $Y_{FAEE/OD}$ of the *E. coli* mutant strain YL15 with two glycerol feeding rates in the general minimal medium. Experimental conditions were the same as reported in Fig. 2.

ethanol in either KC3 or YL15 strain after about 20 h cultivation. Therefore, the performance of YL15 strain was superior to KC3 strain under the culture condition using glycerol as sole carbon source in the minimal medium.

### 3.3. Production of FAEEs in the mutant E. coli strain YL15 under the optimized fed-batch fermentation

Feeding strategies were optimized to further improve FAEE production because it was of great importance in fed-batch fermentation. The YL15 strain exhibited similar growth characteristics during the fermentation process at two different feeding rates (0.3 and 0.6 g $L^{-1}$ $h^{-1}$), and the maximum $OD_{600}$ value at feeding rate of 0.3 and 0.6 g $L^{-1}$ $h^{-1}$ were 15.9 and 16.8, respectively. The minimal medium was fed with glycerol at an $OD_{600}$ of ~12, and no further increase in biomass could be observed (Fig. 4A).

Ethanol accumulation varied remarkably at different glycerol feeding rates. When the feeding rate increased to 0.6 g $L^{-1}$ $h^{-1}$, ethanol accumulated exponentially to 2.8 g $L^{-1}$ from the beginning to 34 h of fermentation process, and maintained this comparatively high value till 70 h, then decreased slowly to 2.0 g $L^{-1}$ till the end of the cultivation. The maximum accumulation of ethanol reached 3.12 g $L^{-1}$ at the feeding rate of 0.6 g $L^{-1}$ $h^{-1}$, which increased more than 3 times in comparison to the rate of 0.3 g $L^{-1}$ $h^{-1}$ (Fig. 4B).

FAEE production and $Y_{FAEE/OD}$ reached 813 mg $L^{-1}$ and 49.3 mg $L^{-1}$ $OD^{-1}$ respectively at the feeding rate of 0.6 g $L^{-1}$ $h^{-1}$, which both doubled that obtained at 0.3 g $L^{-1}$ $h^{-1}$ since the cell growth were such similar at the two feeding rates. A twofold glycerol feeding rate resulted in about a fourfold increase in ethanol and a twofold increase in FAEE production rather than higher biomass in fed-batch fermentation of YL15 strain (Fig. 4C and D).

Based on these results, the optimized minimal medium MSMopt was employed as initial medium in order to enhance the cell growth and further improve the FAEE production (Berger et al., 2011). As shown in Fig. 5, use of the MSMopt medium led to a remarkable enhancement of biomass formation, which yielded 50% increase of the $OD_{600}$ to 24.3 compared to the former minimal medium at the same feeding rate of 0.3 g $L^{-1}$ $h^{-1}$. However, higher biomass did not result in higher FAEE production as expected, only 274 mg $L^{-1}$ FAEE was obtained under this condition.

The glycerol feeding strategy was investigated in the fed-batch fermentation of YL15 strain with the MSMopt medium. The growth curves measured by $OD_{600}$ and the ethanol accumulation levels displayed a high reproducibility of process control until the glycerol feeding rate changed when the $OD_{600}$ were detected to ~14. The $OD_{600}$ increased faster when the glycerol feeding rate shifted from 0.3 g $L^{-1}$ $h^{-1}$ to 1.2 g $L^{-1}$ $h^{-1}$. The highest $OD_{600}$ reached 46.2 at the feeding rate of 1.2 g $L^{-1}$ $h^{-1}$, which was 48% and 90% higher than that at feeding rate of 0.9 and 0.3 g $L^{-1}$ $h^{-1}$, respectively. For ethanol accumulation, different glycerol feeding rates resulted in different profiles during the feeding process. The maximum titers of 0.7 g $L^{-1}$ ethanol were obtained and maintained 26 h at the feeding rate of 0.3 g $L^{-1}$ $h^{-1}$, whereafter the ethanol titers decreased quickly to 0.17 g $L^{-1}$. While the ethanol titers kept at 0.7 g $L^{-1}$ till the end of fermentation at the feeding rate of 0.9 g $L^{-1}$ $h^{-1}$. At the rate of 1.2 g $L^{-1}$ $h^{-1}$, ethanol accumulated to 0.83 g $L^{-1}$ as the maximum before decreased to 0.4 g $L^{-1}$ at the end of cultivation. The maximum FAEE production and $Y_{FAEE/OD}$ reached 650 mg $L^{-1}$ and 24.1 mg $L^{-1}$ $OD^{-1}$ respectively at the rate of 0.9 g $L^{-1}$ $h^{-1}$, which meant respective 20% and 51% decrease compared to the former minimal medium at the rate of 0.6 g $L^{-1}$ - $h^{-1}$. The same level of $Y_{FAEE/OD}$ was obtained at the rate of 0.3 or

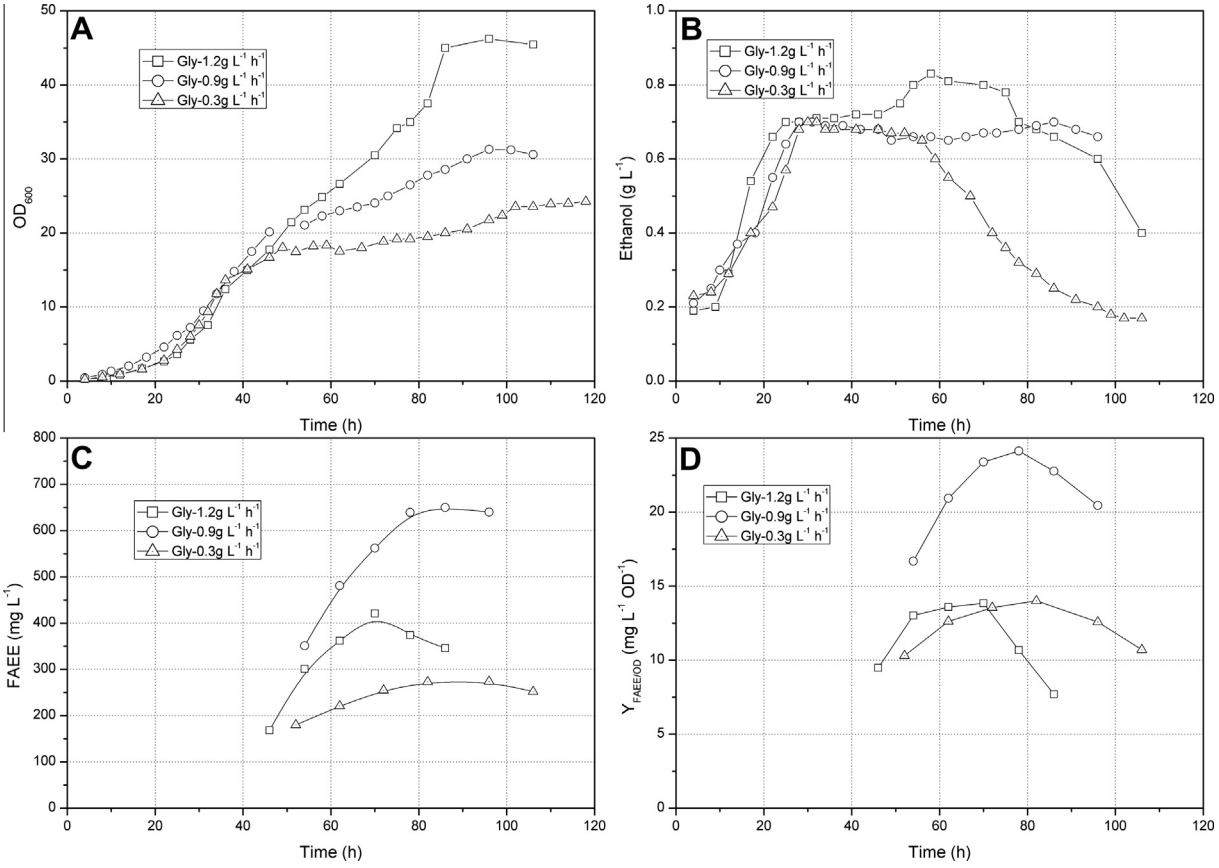


**Fig. 5.** Comparison of the fed-batch fermentation profiles for (A) cell growth, (B) ethanol accumulation, (C) FAEE production, and (D) $Y_{FAEE/OD}$ of the *E. coli* mutant strain YL15 in the optimized minimal medium (MSMopt) with three glycerol feeding rates. Experimental conditions were the same as reported in Fig. 2.

1.2 g $L^{-1}$ $h^{-1}$ (the maximum was about 14.0 mg $L^{-1}$ $OD^{-1}$), because the FAEE production was proportionate to the biomass formation (Fig. 5).

It was indicated that the use of minimal medium could be effective in FAEE fermentation. The MSMopt medium was in favor of enhancing the biomass formation, while the former minimal medium was conductive to the FAEE synthesis with appropriate glycerol feeding rates in fed-batch fermentation.

## 4. Conclusion

This work demonstrated the feasibility of converting glycerol to FAEE. FAEE production with glycerol compared well with glucose as the carbon source by applying of a two-stage feeding strategy. Metabolic engineering strategies by overexpressing two copies of *atfA* gene contributed to the efficient production of FAEE. Finally, 813 mg $L^{-1}$ of FAEE was obtained as the maximum by using glycerol as sole carbon source in optimized minimal medium in the fed-batch fermentation. Microbial recycling of glycerol to biodiesel could make full utilization of byproduct in biodiesel industry and contribute to its competitiveness.

## Competing interests

The authors declare that they have no competing interests.

## Acknowledgement

This work was supported by Grants from the "100-Talent Program of the Chinese Academy of Sciences" foundation (Grant O91001110A).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.09.073.

## References

Almeida, J.R., Favaro, L.C., Quirino, B.F., 2012. Biodiesel biorefinery: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. Biotechnol. Biofuels 5 (1), 48.

Anand, P., Saxena, R.K., 2012. A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from *Citrobacter freundii*. Nat. Biotechnol. 29 (2), 199–205.

Berger, C., Montag, C., Berndt, S., Huster, D., 2011. Optimization of *Escherichia coli* cultivation methods for high yield neuropeptide Y receptor type 2 production. Protein Expression Purif. 76 (1), 25–35.

Chatzifragkou, A., Papanikolaou, S., Dietz, D., Doulgeraki, A.I., Nychas, G.J., Zeng, A.P., 2011. Production of 1,3-propanediol by *Clostridium butyricum* growing on biodiesel-derived crude glycerol through a non-sterilized fermentation process. Appl. Microbiol. Biotechnol. 91, 101–112.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25 (3), 294–306.

Dharmadi, Y., Murark, A., Gonzalez, R., 2006. Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol. Bioeng. 94 (5), 821–829.

Du, W., Liu, D.H., 2012. Biodiesel from conventional feedstocks. Adv. Biochem. Eng. Biotechnol. 128, 53–68.

Duan, Y., Zhu, Z., Cai, K., Tan, X., Lu, X., 2011. De novo biosynthesis of biodiesel by *Escherichia coli* in optimized fed-batch cultivation. PLoS One 6 (5), e20265.

Durnin, G., Clomburg, J., Yeates, Z., Alvarez, P.J.J., Zygourakis, K., Campbell, P., Gonzalez, R., 2009. Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnol. Bioeng. 103, 148–161.

Fang, S., Li, J., Liu, L., Du, G., Chen, J., 2011. Overproduction of alkaline polygalacturonate lyase in recombinant *Escherichia coli* by a two-stage glycerol feeding approach. Bioresour. Technol. 102, 10671–10678.

Gungormusler, M., Gonen, C., Azbar, N., 2011. Continuous production of 1,3-propanediol using raw glycerol with immobilized *Clostridium beijerinckii* NRRL B-593 in comparison to suspended culture. Bioprocess Biosyst. Eng. 34, 727–733.

Hansen, A.C., Zhang, Q., Lyne, P.W., 2005. Ethanol–diesel fuel blends – a review. Bioresour. Technol. 96 (3), 277–285.

Hoydonckx, H.E., De Vos, D.E., Chavan, S.A., Jacobs, P.A., 2004. Esterification and transesterification of renewable chemicals. Top. Catal. 27 (1–4), 83–96.

Kalscheuer, R., Stolting, T., Steinbuchel, A., 2006. Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152 (Pt 9), 2529–2536.

Ma, F., Hanna, M.A., 1999. Biodiesel production: a review. Bioresour. Technol. 70, 1–15.

Norsyahida, A., Rahmah, N., Ahmad, R.M., 2009. Effects of feeding and induction strategy on the production of BmR1 antigen in recombinant *E. coli*. Lett. Appl. Microbiol. 49 (5), 544–550.

Posada, J.A., Cardona, C.A., 2010. Design and analysis of fuel ethanol production from raw glycerol. Energy 35, 5286–5293.

Posada, J.A., Rincon, L.E., Cardona, C.A., 2012. Design and analysis of biorefineries based on raw glycerol: addressing the glycerol problem. Bioresour. Technol. 111, 282–293.

Ratledge, C., Cohen, Z., 2008. Microbial and algal oils: do they have a future for biodiesel or as commodity oils? Lipid Technol. 20, 155–160.

Rottig, A., Wenning, L., Broker, D., Steinbuchel, A., 2010. Fatty acid alkyl esters: perspectives for production of alternative biofuels. Appl. Microbiol. Biotechnol. 85 (6), 1713–1733.

Schubert, C., 2006. Can biofuels finally take center stage? Nat. Biotechnol. 24 (7), 777–784.

Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S.B., Keasling, J.D., 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463 (7280), 559–562.

Tarabet, L., Loubar, K., Lounici, M.S., Hanchi, S., Tazerout, M., 2012. Eucalyptus biodiesel as an alternative to diesel fuel: preparation and tests on di diesel engine. J. Biomed. Biotechnol. 2012, 235485.

Thanh le, T., Okitsu, K., Sadanaga, Y., Takenaka, N., Maeda, Y., Bandow, H., 2010. A two-step continuous ultrasound assisted production of biodiesel fuel from waste cooking oils: a practical and economical approach to produce high quality biodiesel fuel. Bioresour. Technol. 101 (14), 5394–5401.

Wijffels, R.H., Barbosa, M.J., 2010. An outlook on microalgal biofuels. Science 329 (5993), 796–799.

Yang, F., Hanna, M.A., Sun, R., 2012. Value-added uses for crude glycerol – a byproduct of biodiesel production. Biotechnol. Biofuels 5, 13.

Yazdani, S.S., Gonzalez, R., 2008. Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. Metab. Eng. 10, 340–351.

Yousuf, A., 2012. Biodiesel from lignocellulosic biomass – prospects and challenges. Waste Manage. 32, 2061–2067.