Conversion of Mutton Fat to Cocoa Butter Equivalent by Increasing the Unsaturated Fatty Acids at the Sn-2 Position of Triacylglycerol Through Fermentation by Yarrowia Lipolytica

Dan Xiong, Huaiyuan Zhang, Yifang Xie, Nianchu Tang, Aydin Berenjian and Yuanda Song

Center of Excellence for Functional Food and Health, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, Peoples Republic of China
School of Engineering, Faculty of Science and Engineering, The University of Waikato, Hamilton, New Zealand

Abstract: Mutton fat has a similar fatty acid profile with Cocoa Butter (CB), except that its degree of unsaturation of Triacylglycerol (TAG) at the Sn-2 position is considerably lower than CB and maybe increased by Sn-2 specific lipase to produce Cocoa Butter Equivalent (CBE), a healthy functional lipid. However, there is no commercially available Sn-2 specific lipase that can be used to convert mutton fat to CBE by improving its Unsaturated Fatty Acids (UFA) at Sn-2 position. Similar to plant, yeast fat contains higher UFAs at the Sn-2 position than animal fat. In this study, we investigated the conversion of mutton fat to CBE by fermentation of oleaginous yeast Yarrowia lipolytica which acts as a “Sn-2 specific lipase”. The yeast was able to grow on mutton fat as the sole carbon source yielding a dry cell weight of 14.11 g L⁻¹ and 33.1% lipid content after 3 days of cultivation. At optimal fermentation conditions, the degree of unsaturation of TAGs at the Sn-2 position increased from 61.5 (mutton fat) to 89.3% (cellar lipid, 72 h) while the amount of Saturated Fatty Acids (SFA) of the Total Fatty Acids (TFA) was decreased from 58.9 to 34.5%. In addition, the presence of methyl stearate as the co-substrate in the medium improved the ratio of SFAs/TFAs. It was found that fatty acid profile of the yeast fat with 24.60% palmitic acid, 31.34% stearic acid, 34.29% oleic acid, 5.57% linoleic acid and degree of unsaturation at Sn-2 position in TAGs (84.66%) resembled that of CB when the yeast was grown on mutton fat/methyl stearate (with a ratio of 60/40) as carbon source. These results suggest that biotransformation or metabolism could be directed by using mixtures of inexpensive animal fats and saturated fatty acid or methyl as co-substrates, to produce functional lipids with predetermined composition, such as CBE.

Keywords: Mutton Fat, TAGs, Sn-2 Position, Methyl Stearate, Cocoa Butter Equivalent

Introduction

Animal fats are acquirable in large quantities from the meat-packing industry (Santos et al., 2013). A main application of edible fats is as a food cooking medium, but fats have recently lost most of the market share to vegetable oils due to the fact that animal fat contains too much SFAs, which may cause health problems (Santos et al., 2013). With respect to this scenario, it is crucial to seek new applications for animal fat to enhance its value. Mutton fat is a kind of common animal fats, with fatty acids composition similar to CB. The unsaturation degree of fatty acids at the Sn-2 position of mutton fat and CB is about 60 and 90% (w/w) respectively. 87% (w/w) oleic acid is found esterified at the position Sn-2 of glycerol skeleton of CB (Papanikolaou and Aggelis, 2011). The key point for transformation of mutton fat into CBE is to increase the content of UFAs, particularly oleic acid, at the Sn-2 position. Lipase-catalyzed transesterification reaction is excluded owing to the absence of lipase with Sn-2-specificity towards triglycerides (Pfeiffer et al., 2006).
Oleaginous yeasts, which are considered as ideal candidates for this application of the synthesis of microbial substitutes of CB (Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2010), have been utilized by many investigators due to the fact that these microorganisms reserve their lipids mostly in the form of TAGs esterified in the Sn-2 position by UFAs (Padley, 1994). Papanikolaou et al. (2003) used stearin (an industrial derivative of animal fat composed of 100% free fatty acids), glycerol and glucose as co-substrates (Papanikolaou et al., 2003) or stearin and hydrolyzed rapeseed oil as co-substrates (Papanikolaou et al., 2001) for Y. lipolytica LGAM S(7)1 to synthesize a cocoa butter substitute, which contained large amount of stearic acid (from 40 to 80%, w/w). However, some research teams conducting the production of CBE by fermentation processing faced exactly the opposite problem (Moreton, 1985; Ykema et al., 1989; Gierhart, 1984; Roux et al., 1995), that is how to enhance stearic acid content, which is generally low in the oleaginous microorganisms and numerous methods have been used to achieve this target, such as inhibition of Δ9 and Δ12 desaturase (Moreton, 1985), genetic manipulation of oleaginous yeast (Ykema et al., 1989), addition of SFAs or derivative into the medium (Gierhart, 1984) and cultivation of yeasts on low oxygenation medium (Roux et al., 1995).

Some oleaginous microorganisms can utilize various hydrophobic materials as substrate for Single Cell Oil (SCO) production, such as vegetable oils (Najjar et al., 2011), crude fish oil (Guo et al., 1997), fatty esters (Matsuo et al., 1981) and alkanes (Alvarez et al., 1997). Animal fat can be a competitive substrate for SCO production. Few researches have been implemented to evaluate this low value-added material for biotechnological applications (Ashby and Foglia, 1998). The information about the biochemical behavior of these microorganisms growing on fats is not very clear and also fat depletion from the growth media (Aggelis and Sourdis, 1997), the degradation of the storage lipids and the microbial fatty acid specificity (Papanikolaou et al., 2001), are not studied in details. Moreover, animal fat was generally added to the medium as substrate after being hydrolyzed to free fatty acids (Papanikolaou et al., 2003; Papanikolaou et al., 2001) instead of intact triglycerides owing to its lack of extracellular lipase of these microorganisms.

Species of the yeast Y. lipolytica can secrete an extracellular lipase to hydrolyze triglycerides into free fatty acid and glycerol (Fickers et al., 2005) and the internalized aliphatic chains are then disintegrated to meet needs for growth, or reserve in an unchanged or modified form (Beopoulou et al., 2009; Miléková et al., 2004).

Y. lipolytica stores mostly TAGs (>90%, w/w) and has considerable potential to be used as a cell factory for oil production applied in food industry on the basis of commercial-scale fermentation performance and an excellent safety record (Groenewald et al., 2014). In the present work, the low quality feedstock, mutton fat, was considered as intact triglycerides substrates to produce SCO and the effects of nitrogen and substrate fat, incubation time, dissolved oxygen and addition of methyl stearate on Y. lipolytica cell growth and lipid synthesis were studied. It was explored that intracellular lipid presented a profile in fatty acids similar to that of CB and can be considered as a cocoa butter substitute by adjusting culture conditions.

**Materials and Methods**

**Strain and Culture Conditions**

Y. lipolytica strain CICC1778 was collected from China Center of Industrial Culture Collection (CICC). Yeast Extract Peptone Dextrose (YPD) agar medium was used to maintain the strain at 4°C prior to all experiments. Fermentation was performed in medium containing KH₂PO₄ 7, Na₃HPO₄ 2, MgSO₄·7H₂O 1.5, CaCl₂·2H₂O 0.1, FeCl₃·6H₂O 0.008, ZnSO₄·7H₂O 0.001, CuSO₄·5H₂O 10⁻⁴ and MnSO₄·5H₂O 10⁻⁴ g L⁻¹. Mutton fat 30 g L⁻¹ was used as carbon source; ammonium tartrate 3 g L⁻¹ and yeast extract 1.5 g L⁻¹ were used as nitrogen source; C/N = 40. Initial pH was adjusted to 6.0 and the culture temperature was kept at 30°C in all experiments. All experiments were carried out in 500 mL conical flasks, unless otherwise stated, containing 100 mL of growth medium and incubated in a rotary shaker at 200 rpm.

Mutton fat for the fermentation was provided by the company of Qinghai Yutai Co Ltd (Qinghai, China). Tween 80 (1% w/w, of fat content) was employed as emulsifier. An Ultrasonic Cell Disruptor (Sonics, America) allowed the dispersion of the fat particles into the aqueous phase.

Effects of the initial concentration of mutton fat (10, 30, 50 g L⁻¹) and the initial concentration of (NH₄)₂C₂H₃O₆ (1.0, 3.0 and 9.0 g L⁻¹) on lipid production and growth of the yeast were analyzed during the experiments. Effect of dissolved oxygen and agitation (various conical flasks of 250, 500, 1000 mL and mixing speeds of 100, 200, 250 rpm), effect of addition of methyl stearate (mixtures of mutton fat/methyl stearate 100/0; 80/20; 60/40; 40/60; 20/80) on lipid production and the growth of the yeast were also investigated.

**Dry Cell Weight Determination and Lipid Extraction**

Culture samples were centrifuged (8000 rpm, 3min) and washed twice with ethanol and hexane, in order to remove extracellular fat attached to the cell surface (Papanikolaou et al., 2002). The harvested cells were lyophilized till constant weight and dry cell weight was determined gravimetrically. Cellular lipids were extracted using chloroform-methanol (2:1, v/v) and
the total lipids were derived and measured by GC-MS. Unconsumed lipids were extracted from the culture medium twice using petroleum ether and chloroform as solvents and combined with the washing fractions of the yeast cells.

**Sn-2 Fatty Acid and Fatty Acid Profile Analysis**

Pancreatic lipase hydrolysis was used to determine the positional distribution of fatty acids in yeast oil (IUPAC, IUPAC Official Method 2.210, 1992). The components of the mixture were separated by preparative Thin-Layer Chromatography (TLC), using Silica gel HSGF254 plates (10×20 cm; Yantai, China) applying n-hexane-diethyl ether-acetic acid (50:50:1 v/v/v) as the developing solvent. Lipid fractions were visualized by iodine vapor. Triglyceride analysis was conducted using known quantities of C19:0 added as an internal standard.

The extracted total lipids, 2-monoglyceride fraction and aliquot of the original TAGs fraction were saponified and methylated for gas chromatographic analysis. Quantification of triglyceride and fatty acid in the 2-monoglyceride fractions was conducted using known quantities of C19:0 added as an internal standard and analyzed in GC-MS-QP2010 (Shimadzu, Japan).

**Statistical Analysis**

Descriptive statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 16.0. Results were expressed as a mean ± Standard Deviation (SD). The level of significance was taken as p<0.05.

**Results and Discussion**

**Lipid Profile of Mutton Fat from Different Tissues**

Fatty acids of mutton fats were mainly composed of C16, C18 and a small amount of C14 and C17. It was shown that oleic acid (18:1) was the most abundant followed by stearic acid (18:0) and palmitic acid (16:0). Discrepancy of fatty acids was existed among mutton fats from different tissues (Table 1A).

The content of SFAs in CB is 55-67% (w/w), whereas its composition varies slightly owing to the regional variety and the climatic discrepancy. A general fatty acids composition of this fat is: Palmitic acid 23-30% w/w; stearic acid 32-37% w/w; oleic acid 30-37% w/w; linoleic acid 2-4% w/w (Papanikolau and Aggelis, 2010; Padley, 1994; Lipp and Anklam, 1998).

It was noted that fatty acids composition of the fat in the tissues of waist and intestines was very close to that of CB. So, mutton fat, particularly the fat in the tissues of intestines was a perfect substrate to promote accumulation of a cocoa-butter-like lipid by *Y. lipolytica*. Also, C18:1 existed at the Sn-2 position of the mutton fat TAGs, is only about 50% (Table 1B), which is significantly lower than that of CB of which C18:1 content at Sn-2 position reaches up to 87%. This proves the fact that proper biotransformation of the mutton fat is required to convert this low value-added fat to CBE.

**Effect of Fat and Nitrogen Concentration on Fermentation**

Our preliminary experiment showed that mutton fat can be used as the sole carbon source for the growth of *Y. lipolytica* and the accumulated fat had a similar unsaturation degree of Sn-2 fatty acids to cocoa butter (data not shown). To optimize the fermentation process, the effect of initial concentration of mutton fat (S<sub>0</sub>, 10, 30, 50 g L<sup>-1</sup>) and (NH<sub>4</sub>)<sub>2</sub>C<sub>2</sub>H<sub>3</sub>O<sub>2</sub> (1.0, 3.0 and 9.0 g L<sup>-1</sup>) on the growth and lipid production were studied in Fig. 1. When S<sub>0</sub> was set at 10 g L<sup>-1</sup>, relative low cell mass, but high lipids content were observed despite the extracellular nitrogen availability of the media. It resulted in lower amount of total cellular lipid was being produced (2.25 g L<sup>-1</sup>) compared to that of other two cultures with 30 g L<sup>-1</sup> mutton fat or 50 g L<sup>-1</sup> mutton fat. Another observation was that, a significant amount of fat remained unconsumed (around 5 g L<sup>-1</sup>) at the end of the fermentation while at higher mutton fat concentrations (30, 50 g L<sup>-1</sup>), cell mass increased with the increasing amount of nitrogen source. The lipid production of the yeast with S<sub>0</sub> = 50 g L<sup>-1</sup> was not much different from S<sub>0</sub> = 50 g L<sup>-1</sup>, with maximum dry cell weight of 17.23 g L<sup>-1</sup> and maximum lipid production of 5.39 g L<sup>-1</sup> being achieved at ammonium tartrate concentration of 3 g L<sup>-1</sup>. A large amount of fat (about 30 g L<sup>-1</sup>) was unused in the media at the end of the fermentation when the initial concentration of mutton fat was 50 g L<sup>-1</sup>. The highest lipid content (33.1%, w/w) was achieved at the initial fat concentration of 30 g L<sup>-1</sup> and the ammonium concentration of 3 g L<sup>-1</sup>. Considering fat dosage, biomass and lipid content, the culture with S<sub>0</sub> = 30 g L<sup>-1</sup> and (NH<sub>4</sub>)<sub>2</sub>C<sub>2</sub>H<sub>3</sub>O<sub>2</sub> concentrations with 3.0 g L<sup>-1</sup> was chosen as the optimum for the rest of the experiments.

**Effect of Fermentation Time on Fat Assimilation and Conversion**

The growth rate of *Y. lipolytica* on mutton fat was shown in Fig. 2. During the first 72 h of incubation, large amount of substrate was highly consumed leading to significant cell growth and lipid accumulation. Furthermore, the lipid content reached the maximum value of 35.02% (w/w) at 48 h and lipid yield reached the maximum value of 4.67 g L<sup>-1</sup> at 72 h. From 72 to 120 h of incubation, the extracellular fat was utilized very slowly and the biomass remained stable. Degradation of the cellular lipids occurred as suggested by the decrease of cellular lipids, though a relatively significant amount of fat remained unconsumed (at 120 h, unconsumed fat was about 11 g L<sup>-1</sup>).
Fig. 1. The dry cell weight (g L$^{-1}$), lipid content per dry cell weight (%, g g$^{-1}$) and consumed mutton fat (g L$^{-1}$) of *Y. lipolytica* during growth on different initial amount of mutton fat and ammonium tartrate in 500 mL flask incubated for 72 h at 30°C and at 200 rpm.

Fig. 2. The dry cell weight (g L$^{-1}$), lipid content per dry cell weight (%, g g$^{-1}$) and residual mutton fat in the medium (g L$^{-1}$) of *Y. lipolytica* during growth on mutton fat (30 g L$^{-1}$) and ammonium tartrate (3 g L$^{-1}$), in 500 mL flask incubated at 30°C and at 200 rpm (n = 3).

Table 1. A. Total fatty acids composition (%, w/w) of different tissues of mutton fat, B. The Sn-2 position fatty acids composition (%, w/w) of TAGs of different tissues of mutton fat

|          | Waist | Belly | Tail | Intestines | Suet |
|----------|-------|-------|------|------------|------|
| A C14:0  | 4.74±0.39 | 5.99±0.55 | 3.02±0.12 | 3.89±0.24 | 4.29±0.32 |
| C16:0    | 25.84±2.03 | 28.52±0.42 | 22.64±0.54 | 25.63±1.23 | 22.29±0.23 |
| C16:1    | 1.29±0.24 | 1.54±0.23 | 1.45±0.18 | 1.26±0.53 | 1.78±0.32 |
| C17:0    | 1.53±0.41 | 1.43±0.57 | 1.37±0.65 | 1.59±0.32 | 1.91±0.35 |
| C18:0    | 28.83±1.13 | 21.71±0.21 | 26.89±0.15 | 27.79±0.61 | 23.06±1.33 |
| C18:1    | 33.49±0.52 | 36.16±0.56 | 40.56±2.12 | 35.96±0.37 | 43.47±0.56 |
| C18:2    | 2.83±0.31 | 4.01±0.43 | 3.47±0.19 | 3.39±0.98 | 2.34±0.67 |
| C18:3    | 1.45±0.43 | 0.64±0.65 | 0.60±0.71 | 0.49±0.24 | 0.86±0.56 |
| B C14:0  | 9.78±2.14 | 7.59±0.38 | 4.42±0.53 | 4.68±0.54 | 6.23±0.31 |
| C16:0    | 18.77±0.32 | 17.18±0.85 | 16.97±1.76 | 16.93±2.08 | 17.78±0.86 |
| C16:1    | 1.54±0.41 | 1.90±0.44 | 1.77±0.19 | 1.60±0.11 | 2.90±0.03 |
| C18:0    | 17.26±0.19 | 13.94±0.53 | 14.78±2.01 | 15.94±0.41 | 14.63±0.17 |
| C18:1    | 44.44±1.34 | 52.22±0.23 | 55.63±0.49 | 53.34±0.46 | 50.22±1.92 |
| C18:2    | 7.37±0.75 | 6.45±0.32 | 5.78±0.66 | 6.52±1.21 | 7.54±0.42 |

Data expressed as mean of three data points (n = 3)
The fatty acids composition of cellular TAGs showed that, throughout the culture, oleic acid content was much higher than that of the substrate and increased significantly from 39.73 to 65.47% with the progress of the fermentation. The percentage of the stearic acid was lower compared with the fat substrate and decreased from 17.25 to 7.06% (Table 2A). The amount of palmitic acid did not change significantly as compared to the substrate (25.63%), except for the last sampling point (120 h, 15.56%). The amount of linoleic acid almost kept constant throughout the fermentation, but was higher than that of mutton fat.

Fatty acids composition at the Sn-2 position of cellular TAGs was shown in Table 2B. Oleic acid percentage significantly increased with the increase of mixing speed, whereas stearic acid percentage at the speed of 200 and 250 rpm was much lower than 100 rpm (Table 3A). Fatty acids composition at the Sn-2 position of cellular TAGs was shown at Table 3B. Oleic acid percentage and the amount of UFAs at the Sn-2 position increased with the increase of mixing speed. Higher dissolved oxygen has positive effect on cell growth and influences the fatty acid profile of *Y. lipolytica*. It has been reported that UFAs formation in yeast and fungi were an oxygenation process, which strictly required oxygen molecules for the desaturation process and the presence of oxygen activated transcriptional expression of desaturase genes (Ruenwai et al., 2010). Significant amounts of medium-and long-chain SFAs were produced when the yeast cells were cultivated under anaerobic conditions (Jeemnor et al., 2006). It is feasible to increase the total SFAs without decreasing the biomass significantly by reducing dissolved oxygen properly.

**Effect of Dissolved Oxygen and Agitation on the Conversion of Mutton Fat**

Agitation can affect the oxygen diffusion, thus impacting the mass transfer and the aerobic metabolite production of microorganisms (Yadav et al., 2014). The effect of dissolved oxygen and the agitation (100, 200, 250 rpm) on yeast growth and lipid production were examined at different volumes (250, 500 and 1000 mL). Greater biomass production was observed with increasing mixing speeds and greater volume of the flask. The result showed that biomass reached the maximum value of 22.46 g L$^{-1}$ in 1000 mL flask with agitation at 250 rpm. Nonetheless, the lipid content of the yeast grown in 250 and 500 mL flasks was higher when agitated at 200 rpm than other speeds (Fig. 3).

**Addition of Methyl Stearate as Co-substrate for Y. lipolytica to Produce CBE**

Although mutton fat can be converted to TAGs with UFAs (especially oleic acid) at the Sn-2 position close to CB, it’s SFAs (especially stearic acid) is much lower than CB and it contains more linoleic acid than CB. Hence methyl stearate was used as the co-substrate of mutton fat to improve the fatty acid profile of lipid in *Y. lipolytica* cell. Addition of methyl stearate (less than 40%) increased the cells growth rate even though lipid accumulation was inhibited with the addition of methyl stearate in a dose dependent manner (Fig. 4).

### Table 2. A, B. Storage lipid composition of *Y. lipolytica* during growth on mutton fat (30 g L$^{-1}$) and ammonium tartrate (3 g L$^{-1}$), in 500 mL flask at 30°C and at 200 rpm. A. Fatty acids composition (%), w/w) of TAGs, B. The Sn-2 position fatty acids composition (%), w/w) of TAGs

| Time (h) | C16:0 | C18:0 | C18:1 | C18:2 | SFA (%) |
|---------|-------|-------|-------|-------|---------|
| **A**   |       |       |       |       |         |
| 12      | 28.12±1.03 | 17.25±1.25 | 39.73±0.79 | 11.51±0.45 | 45.38±2.73 |
| 24      | 25.58±0.98 | 16.93±0.54 | 40.85±2.67 | 12.84±0.23 | 42.51±3.01 |
| 48      | 20.96±0.24 | 8.94±0.67 | 50.57±0.31 | 15.95±0.32 | 29.5±0.55 |
| 72      | 23.17±0.23 | 11.36±0.68 | 51.67±1.02 | 9.36±0.33 | 34.53±1.33 |
| 120     | 15.56±0.34 | 7.06±0.86 | 65.47±1.75 | 7.95±0.98 | 22.62±0.47 |
| **B**   |       |       |       |       | UFA (%) |
| 12      | 4.80±0.41 | 4.68±0.65 | 75.69±0.68 | 11.98±0.31 | 87.67±1.36 |
| 24      | 3.43±0.52 | 3.66±0.52 | 78.98±1.38 | 11.48±0.98 | 90.46±1.95 |
| 48      | 2.21±0.51 | 1.88±0.42 | 80.67±0.99 | 12.48±0.44 | 93.15±0.74 |
| 72      | 2.56±0.58 | 3.80±0.16 | 80.33±0.14 | 8.95±0.59 | 89.28±0.63 |
| 120     | 1.95±0.49 | 2.28±0.41 | 83.85±0.64 | 9.56±0.43 | 93.41±2.98 |

Data expressed as mean of three data points (n = 3); other non-presented fatty acids are mainly palmitoleic acid-C16:1 (3%), w/w) and heptadecanoic acid-C17:0 (2%, w/w)
Fig. 3. The dry cell weight (g L\(^{-1}\)) and lipid content per dry cell weight (\%, g g\(^{-1}\)) of \textit{Y. lipolytica} during growth on mutton fat (30 g L\(^{-1}\)) and ammonium tartrate (3 g L\(^{-1}\)), in 500 mL flask for 72 h at 30\(^\circ\)C and at different rpm (n = 3).

Fig. 4. The dry cell weight (g L\(^{-1}\)) and lipid content per dry cell weight (\%, g g\(^{-1}\)) of \textit{Y. lipolytica} during growth on mixtures of mutton fat/methyl stearate 100/0; 80/20; 60/40; 40/60; 20/80 (30 g L\(^{-1}\)) and ammonium tartrate (3 g L\(^{-1}\)), in 500 mL flask for 72 h at 30\(^\circ\)C.

Table 3. A, B. Storage lipid composition of \textit{Y. lipolytica} during growth on mutton fat (30 g L\(^{-1}\)) and ammonium tartrate (3 g L\(^{-1}\)), in 500 mL flask for 72 h at 30\(^\circ\)C and at different rpm, A. Fatty acids composition (\%, w/w) of TAGs, B. The Sn-2 position fatty acids composition (\%, w/w) of TAGs

| RPM | C16:0   | C18:0   | C18:1   | C18:2   | SFA (%) |
|-----|---------|---------|---------|---------|---------|
| A   |         |         |         |         |         |
| 100 | 19.87±0.81 | 31.57±2.49 | 34.28±0.57 | 10.04±0.65 | 51.44±2.43 |
| 200 | 23.17±0.23 | 11.36±0.68 | 51.67±1.02 | 9.36±0.33  | 34.53±1.33  |
| 250 | 14.68±0.72 | 15.88±0.74 | 54.07±2.56 | 10.37±0.97 | 30.57±1.47  |
| B   |         |         |         |         |         |
| 100 | 4.02±0.42  | 8.77±0.87  | 70.65±1.97 | 12.54±0.76 | 83.19±3.06  |
| 200 | 2.56±0.58  | 3.80±0.16  | 80.33±0.14 | 8.95±0.59  | 89.28±0.63  |
| 250 | 2.02±0.14  | 2.43±0.34  | 83.51±0.51 | 8.59±0.68  | 92.10±1.35  |

Data expressed as mean of three data points (n = 3); other non-presented fatty acids are mainly palmitoleic acid-C16:1 (3\%, w/w) and heptadecanoic acid-C17:0 (2\%, w/w).

In all of the cultures, the cellular lipids contained significant amounts of palmitic, stearic, oleic and linoleic acid. It seems that when methyl stearate was used as the co-substrate, lipid produced in the yeast contained more SFAs than that when mutton fat was used as the only carbon source. Moreover, the ratio of
SFAs was higher when more methyl stearate was added. Indeed, when mutton fat/methyl stearate ratio was 20/80, the stearic acid concentration was multiplied by five times compared with the cases in which fat was used as sole carbon source (Table 4A). It was noted that, when mutton fat/methyl stearate ratio was 60/40, lipid profiles of TAGs with 24.60% palmitic acid, 31.34% stearic acid, 34.29% oleic acid, 5.57% linoleic acid and degree of unsaturation of Sn-2 position in TAGs (84.66%) resembled that of CB.

Fatty acids composition at the Sn-2 position of cellular TAGs was shown in Table 4B. The oleic acid percentage of the Sn-2 position decreased as the amount of methyl stearate increased. The addition of methyl stearate showed significant influence on fatty acid profile of cellular lipid. As a consequence, the fatty acids composition of the substrate was very important to the fatty acids composition of the storage lipid in the yeast cell. The yeast intended to store the cellular lipids with fatty acids which existed largely in the growth medium.

Figure 5 showed the comparison of major CBE fatty acids from this study and published work by Papanikolaou et al. (2003) utilizing glucose and stearin as the co-substrates compared to CB.

Table 4. A, B. Storage lipid composition of Y. lipolytica during growth on mixtures of mutton fat/methyl stearate 100/0; 80/20; 60/40; 40/60; 20/80 (30 g L⁻¹) and ammonium tartrate (3 g L⁻¹), in 500 mL flask for 72 h at 30°C and at 200 rpm, A. Fatty acids composition (% w/w) of TAGs, B. The Sn-2 position fatty acids composition (% w/w) of TAGs

| Mutton fat/Methyl stearate (w/w) | C16:0 | C18:0 | C18:1 | C18:2 | UFA (%) | SFA (%) |
|----------------------------------|-------|-------|-------|-------|---------|---------|
| A                                |       |       |       |       |         |         |
| 100/0                            | 23.17±0.23 | 11.36±0.68 | 51.67±1.02 | 9.36±0.33 | 34.53±1.33 |
| 80/20                            | 20.19±1.03 | 18.24±0.71 | 49.11±0.21 | 7.77±0.52 | 38.43±0.65 |
| 60/40                            | 24.60±0.58 | 31.34±0.31 | 34.29±0.43 | 5.57±0.65 | 55.94±0.98 |
| 40/60                            | 22.44±0.31 | 34.62±0.67 | 33.18±1.45 | 5.19±0.46 | 57.06±2.41 |
| 20/80                            | 13.06±0.32 | 56.48±0.46 | 23.32±0.65 | 3.42±0.13 | 69.54±0.68 |
| B                                |       |       |       |       |         |         |
| 100/0                            | 2.56±0.58  | 3.80±0.16  | 80.33±0.14  | 8.95±0.59  | 89.28±0.63  |
| 80/20                            | 4.38±0.65  | 6.22±0.34  | 79.79±0.57  | 6.32±0.87  | 86.11±0.46  |
| 60/40                            | 4.90±0.65  | 6.83±0.55  | 74.23±1.14  | 10.43±0.65 | 84.66±0.87  |
| 40/60                            | 6.98±0.49  | 8.89±0.43  | 70.43±1.72  | 10.13±0.15 | 80.56±1.06  |
| 20/80                            | 10.39±0.15 | 13.43±0.54 | 65.39±0.87  | 7.34±1.08  | 72.73±0.87  |

Data expressed as mean of three data points (n = 3); other non-presented fatty acids are mainly palmitoleic acid-C16:1 (3%, w/w) and heptadecanoic acid-C17:0 (2%, w/w)
The composition of the major fatty acids from Papanikolaou et al. (2003) was: 22% palmitic acid, 45% stearic acid, 17% oleic acid and 6.5% linoleic acid. Moreover, lipid synthesis was low (7%, w/w). The content of total SFAs of the cellular lipid was 67% (w/w), close to CB, which contains 62-70% (w/w) of SFAs, whereas its profile was slightly different from CB due to the high content of stearic acid and low content of oleic acid. The fatty acids composition at the Sn-2 position of TAGs in the work by Papanikolaou et al. (2003) was not given. In this study, the fatty acids composition of the CBE is very close to that of CB and the lipid production was much higher when mutton fat and methyl stearate was used as the co-substrate compared to Papanikolaou et al. (2003).

The results of the present study indicate that there is a high potential to use the cheap animal fat to produce CBE thorough the fermentation. Microbial metabolism can be directed by using mixtures of inexpensive animal fats and saturated fatty acid or its ester as the co-substrates, to accumulate functional lipids with predetermined composition.

Conclusion
The fatty acid profile of mutton fat is similar to CB, but with a much lower degree of unsaturation of at the Sn-2 fatty acids of the fat. When mutton fat was fermented by the oleaginous yeast Y. lipolytica, its unsaturated fatty acid content at the Sn-2 position increased, close to that of CB. However, the fatty acid profile of the converted fat changed significantly. It contained as low as 34.5% SFAs, while the mutton fat contained 58.9% SFAs. By optimizing the fermentation process conditions and addition of methyl stearate as the carbon source, with an optimal ratio of 60/40 (mutton fat/methyl stearate), the accumulated fat in the yeast had a fatty acid profile and its Sn-2 fatty acid unsaturation degree close to that of CB. Therefore, it is feasible to convert mutton fat to CBE by the fermentation process using Y. lipolytica.

Acknowledgement
The work was supported by the National Natural Science Foundation of China (31271812), the National High Technology Research and Development Program of China (863 Program 2012AA022105C) and Qinghai Yutai Co Ltd (Qinghai, China) are acknowledged for the funding support of the project.

Author’s Contributions
Dan Xiong: Participated in all experiments and acquisition of data, analyzed all the data and contributed to the writing of the manuscript.
Huaiyuan Zhang: Coordinated the partial work and participated in interpretation of data.
Yifang Xie: Participated in partial work and acquisition of data.
Nianchu Tang: Provided technical guidance and participated in interpretation of data.
Aydin Berenjian: Coordinated the writing and revising of the manuscript.
Yuanda Song: Designed the research plan and organized the study.

Ethics
The article is original and has not been formally published in any other peer-reviewed journal and does not infringe any existing copyright and any other third party rights.

References
Aggelis, G. and J. Sourdis, 1997. Prediction of lipid accumulation-degradation in oleaginous micro-organisms growing on vegetable oils. Antonie Van Leeuwenhoek, 72: 159-165. DOI: 10.1023/a:1000364402110
Alvarez, H.M., R. Kalscheuer and A. Steinbüchel, 1997. Accumulation of storage lipids in species of Rhodococcus and Nocardia and effect of inhibitors and polyethylene glycol. Lipid/Fett, 99: 239-246. DOI: 10.1002/lipi.19970990704
Ashby, R.D. and T.A. Foglia, 1998. Poly(hydroxyalkanoate) biosynthesis from triglyceride substrates. Applied Microbiol. Biotechnol., 49: 431-437. DOI: 10.1007/s002530051194
Beopoulos, A., J. Cescut, R. Haddouche, J. Uribelarrea and J. Molina et al., 2009. Yarrowia lipolytica as a model for bio-oil production. Progress Lipid Res., 48: 375-387. DOI: 10.1016/j.plipres.2009.08.005
Fickers, P., P. H. Benetti, Y. Waché, A. Marty and S. Mauersberger et al., 2005. Hydrophobic substrate utilisation by the yeast Yarrowia lipolytica and its potential applications. FEMS Yeast Res., 5: 527-543. DOI: 10.1016/j.femsyr.2004.09.004
Gierhart, D.I., 1984. Multistage process for the preparation of fats and oils. US Patent 4485, 172.
Groenewald, M., T. Boekhout, C. Neveuglise, C. Gaillardin and P.W. van Dijck et al., 2014. Yarrowia lipolytica: Safety assessment of an oleaginous yeast with a great industrial potential. Crit. Rev. Microbiol., 40: 187-206. DOI: 10.3109/1040841X.2013.770386

Guo, X., T. Tomonaga, Y. Yanagihara and Y. Ota, 1999. Screening for yeasts incorporating the exogenous eicosapentaenoic and docosahexaenoic acids from crude fish oil. J. Biosci. Bioeng., 87: 184-188. DOI: 10.1016/S1389-1723(99)89010-6

IUPAC, IUPAC Official Method 2.210, 1992.

Jeennor, S., K. Laoteng, M. Tanticharoen and S. Papanikolaou, 2010. Lipids of oleaginous yeasts. Part II: Technology and potential applications. Eur. J. Lipid Sci. Technol., 113: 1052-1073. DOI: 10.1002/ejlt.201100015

Papanikolaou, S., I. Chevalot, M. Komaitis, G. Aggelis and I. Marc, 2001. Kinetic profile of the cellular lipid composition in an oleaginous Yarrowia lipolytica capable of producing a cocoa-butter substitute from industrial fats. Antonie Van Leeuwenhoek, 80: 215-224. DOI: 10.1023/a:1010383211405

Papanikolaou, S., L. Muniglia, I. Chevalot, G. Aggelis and I. Marc, 2003. Accumulation of a cocoa-butter-like lipid by Yarrowia lipolytica cultivated on agro-industrial residues. Current Microbiol., 46: 124-130. PMID: 12520368

Papanikolaou, S., I. Chevalot, M. Komaitis, I. Marc and G. Aggelis, 2002. Single cell oil production by Yarrowia lipolytica growing on an industrial derivative of animal fat in batch cultures. Applied Microbiol. Biotechnol., 58: 308-312. PMID: 11935181

Pfeiffer, J., S. Richter, J. Nieveler, C.E. Hansen and R. Rhid et al., 2006. High yield expression of Lipase A from Candida antarctica in the methylotrophic yeast Pichia pastoris and its purification and characterisation. Applied Microbiol. Biotechnol., 72: 931-938. PMID: 16575565

Ratledge, C. and J.P. Wynn, 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Applied Microbiol., 51: 1-51. PMID: 12236054

Roux, M.P., J.L.F. Kock, J.C. Du Preez and A. Botha, 1995. The influence of dissolved oxygen tension on the production of cocoa butter equivalents and gamma-linolenic acid by Mucor circinelloides. Syst. Applied Microbiol., 18: 329-334. DOI: 10.1016/S0723-2020(11)80423-6

Ruenvai, R., S. Cheevadhanarak, S. Rachdawong, M. Tanticharoen and K. Laoteng, 2010. Oxygen-induced expression of Delta(6)-, Delta(9)- and Delta(12)-desaturase genes modulates fatty acid composition in Mucor rouxii. Applied Microbiol. Biotechnol., 86: 327-334. PMID: 20187298

Santos, D.K.F., R.D. Rufino, J.M. Luna, V.A. Santos and A.A. Salgueiro et al., 2013. Synthesis and evaluation of biosurfactant produced by Candida lipolytica using animal fat and corn steep liquor. J. Petroleum Sci. Eng., 105: 43-50. DOI: 10.1016/j.petrol.2013.03.028

Yadav, K., D. Rahi and S. Soni, 2014. An indigenous hyperproductive species of aureobasidium pullulans RYL-F-10: Influence of Fermentation Conditions on Exopolysaccharide (EPS) production. Applied Biochem. Biotechnol., 172: 1898-1908. PMID: 24293276

Ykema, A., E. Verbree, H. Nijkamp and H. Smit, 1989. Isolation and characterization of fatty acid auxotrophs from the oleaginous yeast Apiotrichum curvatum. Applied Microbiol. Biotechnol., 32: 76-84. DOI: 10.1007/BF00164826