Original Research Paper

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*

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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 skeletonin 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 (Pfeffer *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.*, 1999), 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 (Beopoulos *et al.*, 2009; Mlíčková *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 KH_2PO_4 7, Na_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 1.5, $CaCl_2 \cdot 2H_2O$ 0.1, $FeCl_3 \cdot 6H_2O$ 0.008, $ZnSO_4 \cdot 7H_2O$ 0.001, $CuSO_4 \cdot 5H_2O$ 10^{-4} and $MnSO_4 \cdot 5H_2O$ 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 $(NH_4)_2C_4H_4O_6$ (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 also conducted in this way.

The extracted total lipids, 2-monoglyceride fraction and aliquot of the original TAGs fraction were scraped, 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 (Papanikolaou 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_0, 10, 30, 50 g L^{-1}) and $(NH_4)_2C_4H_4O_6$ (1.0, 3.0 and 9.0 g L^{-1}) on the growth and lipid production were studied in Fig. 1. When S_0 was set at 10 g L^{-1}, 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^{-1}) compared to that of other two cultures with 30 g L^{-1} mutton fat or 50 g L^{-1} mutton fat. Another observation was that, a significant amount of fat remained unconsumed (around 5 g L^{-1}) at the end of the fermentation while at higher mutton fat concentrations (30, 50 g L^{-1}), cell mass increased with the increasing amount of nitrogen source. The lipid production of the yeast with $S_0 = 30$ g L^{-1} was not much different from $S_0 = 50$ g L^{-1}, with maximum dry cell weight of 17.23 g L^{-1} and maximum lipid production of 5.39 g L^{-1} being achieved at ammonium tartrate concentration of 3 g L^{-1}. A large amount of fat (about 30 g L^{-1}) was unused in the media at the end of the fermentation when the initial concentration of mutton fat was 50 g L^{-1}. The highest lipid content (33.1%, w/w) was achieved at the initial fat concentration of 30 g L^{-1} and the ammonium concentration of 3 g L^{-1}. Considering fat dosage, biomass and lipid content, the culture with $S_0 = 30$ g L^{-1} and $(NH_4)_2C_4H_4O_6$ concentrations with 3.0 g L^{-1} 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^{-1} 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^{-1}).
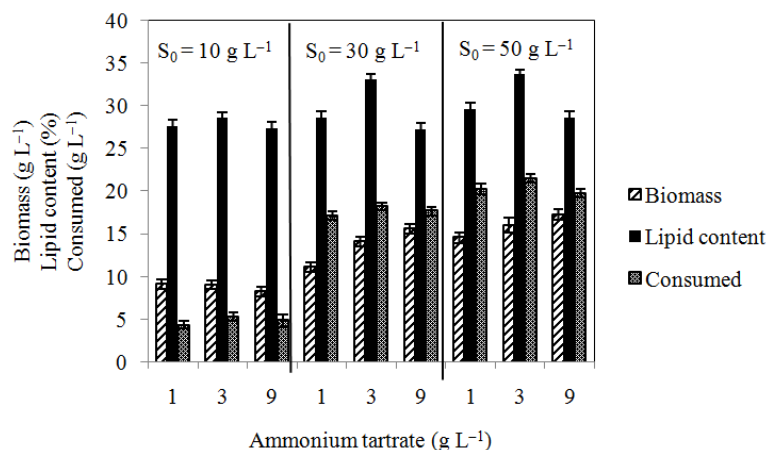
Fig. 1. The dry cell weight (g L^{-1}), lipid content per dry cell weight (%, g g^{-1}) and consumed mutton fat (g L^{-1}) of *Y. lipolytica* during growth on different initial amount of mutton fat and ammonium tartrate in 500 mL flask incubated for 72 h at 30°C and at 200 rpm
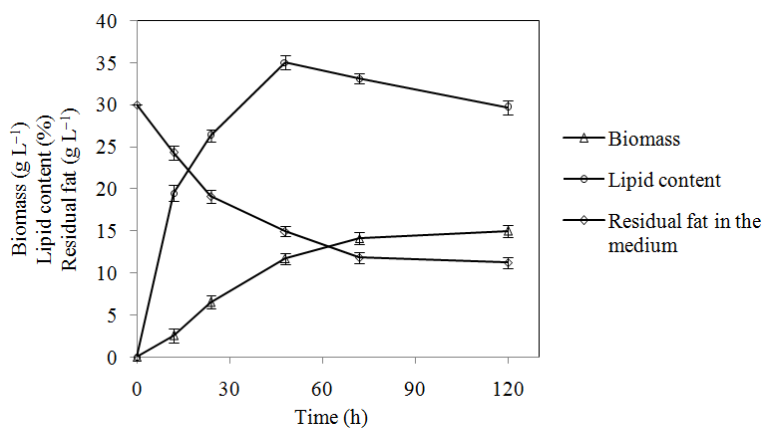

Fig. 2. The dry cell weight (g L^{-1}), lipid content per dry cell weight (%, g g^{-1}) and residual mutton fat in the medium (g L^{-1}) of *Y. lipolytica* during growth on mutton fat (30 g L^{-1}) and ammonium tartrate (3 g L^{-1}), in 500 mL flask incubated at 30°C and at 200 rpm (n = 3)

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)

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	29.9±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)

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 at the Sn-2 position was much higher than that of mutton fat and increased significantly from 75.69 to 83.85% throughout the culture time. The content of the stearic acid and palmitic acid was lower than the fat substrate. The optimal harvest time point of the yeast was 72 h with the consideration of biomass, the cellular total lipid content and fatty acids composition.

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).

Interestingly, the maximum yield of cellular total lipid (4.67 g L^{-1}) with 14.11 g L^{-1} cell biomass was achieved in the 500 mL flask at 200 rpm. Mixing speed also showed significant effect on the fatty acid profile of cellular lipid. SFAs significantly decreased as the mixing speed increased. 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 the 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 (Jeennor *et al.*, 2006). It is feasible to increase the total SFAs without decreasing the biomass significantly by reducing dissolved oxygen properly.

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).
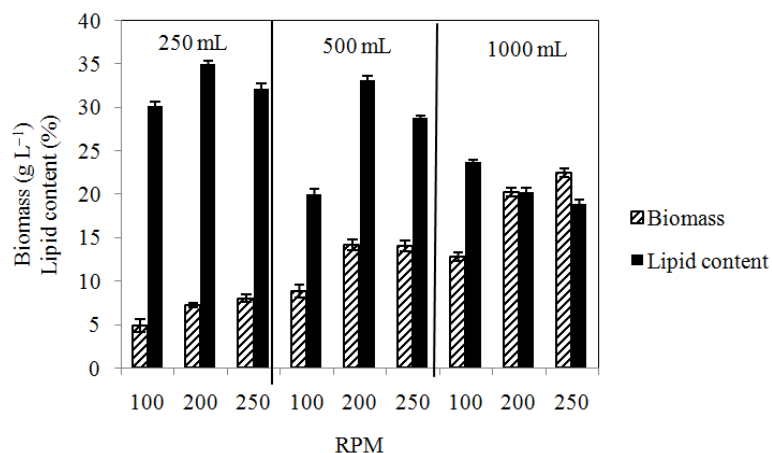
Fig. 3. The dry cell weight (g L^{-1}) and lipid content per dry cell weight (%, g g^{-1}) of *Y. lipolytica* during growth on mutton fat (30 g L^{-1}) and ammonium tartrate (3 g L^{-1}), in 500 mL flask for 72 h at 30°C and at different rpm (n = 3)
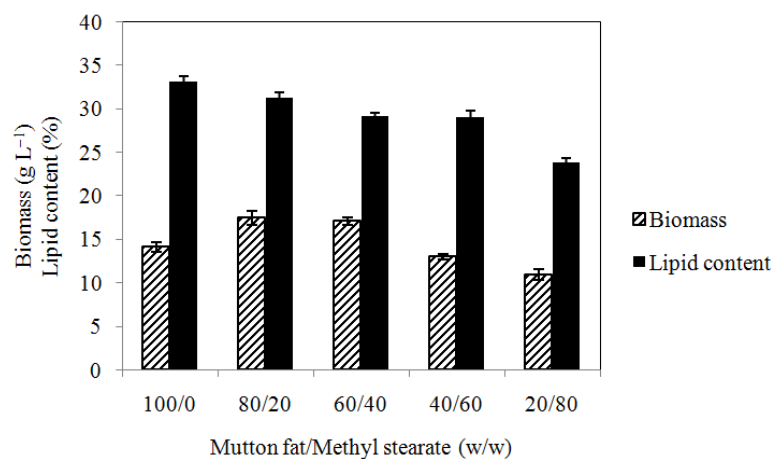

Fig. 4. The dry cell weight (g L^{-1}) and lipid content per dry cell weight (%, g g^{-1}) of *Y. lipolytica* during growth on mixtures of mutton fat/methyl stearate 100/0; 80/20; 60/40; 40/60; 20/80 (30 g L^{-1}) and ammonium tartrate (3 g L^{-1}), in 500 mL flask for 72 h at 30°C

Table 3. 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 for 72 h at 30°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	54.078±2.56	10.37±0.97	30.57±1.47
B					
					UFA (%)
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.
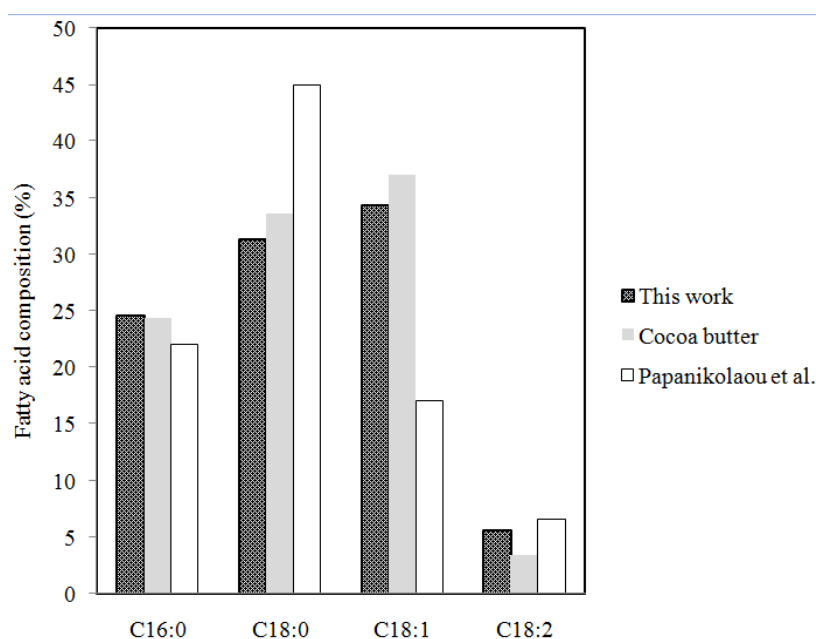

Fig. 5. Fatty acids composition (%, w/w) (mean value) of TAGs of CBE from this work (on mixtures of mutton fat/methyl stearate 60/40 after 72 h of cultivation) and published work (on 25 g L^{-1} glucose and 7 g L^{-1} stearin after 120 h of cultivation) compared to cocoa butter

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	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					
					UFA (%)
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

Funding Information

Author's Contributions

Dan Xiong: Participated in all experiments and aquisition 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.

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