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Cocoa-Butter-Equivalent Production from *Yarrowia lipolytica* by Optimization of Fermentation Technology

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Abstract: In order to obtain the optimal control point for single-cell lipid production with fatty acid profile similar to cocoa butter, this study explored the effects of carbon source, nitrogen source, temperature, sterculic acid methyl ester, cobalt on cell growth and lipid accumulation of *Yarrowia lipolytica*. Glycerol and ammonium tartrate-yeast extract were chosen as carbon source and nitrogen source for production of cocoa butter equivalent. Gradual increase in temperature (20-35°C) resulted in growth reduction, while increased in lipid content per Cell Dry Weight (CDW) (10.13 to 19.7%, w/w) and Saturated Fatty Acids (SFA). Our results suggests that the optimum conditions for *Y. lipolytica* to synthesize cocoa butter equivalent was 30°C with 0.6 mg L^{-1} of $CoCl_2 \cdot 6H_2O$ or 0.03 mL L^{-1} sterculic acid methyl ester in the medium with glycerol and ammonium tartrate-yeast extract as carbon source and nitrogen source.

Keywords: Cobalt, Cocoa Butter Equivalent, Saturated Fatty Acids, Sn-2 Position, Sterculic Acid Methyl Ester, Triacylglycerols

Introduction

Cocoa butter mainly consists of Triacylglycerol (TAG) of POP, POS and SOS (P: Palmitic acid, O: Oleic acid, S: Stearic acid), commonly used in the food industries and particularly in chocolate manufactures due to its specific characteristics (Papanikolaou and Aggelis, 2011). Due to regional diversity and climate differences, the composition of saturated fatty acid in cocoa butter is a little different, from 55 to 67% (w/w). An average fatty acid composition of saturated fatty acid is as follows: Stearic acid is 32-37%, oleic acid is 30-37%, palmitic acid is 23-30% and linoleic acid is 2-4% (Lipp and Anklam, 1998; Papanikolaou and Aggelis, 2010; Ratledge, 1994).

Oleaginous yeasts are able to accumulate intracellular lipid more than 70% (w/w) of their dry matter (Papanikolaou *et al*., 2003). One common industrial applications of yeast lipid is to synthesize microbial substitutes of cocoa butter (Papanikolaou and Aggelis, 2010; Ratledge and Wynn, 2002). These microorganisms reserve their lipids mostly in the form of Triglycerides (TAGs) esterified in the Sn-2 position by unsaturated fatty acids (Ratledge, 1994). Therefore, they are considered as ideal candidates for this approach and have been utilized by many investigators. Since oleaginous yeasts can accumulate unsaturated fatty acids more than 65% (w/w) of the total lipids but cocoa butter contains saturated lipid (palmitic and stearic acid) more than 60% (w/w) (Ratledge, 1994). Hence, the main problem to solve is how to increase the level of total saturated fatty acids inside the yeast cells specially the content of C18:0 (Lipp and Anklam, 1998; Papanikolaou and Aggelis, 2010; Ratledge and Wynn, 2002). Numerous strategies have been explored to the increase of Saturated Fatty Acids (SFA), such as the genetic manipulation of oleaginous yeast (Ykema, 1989), inhibition of Δ_9 and Δ_{12} dehydrogenase (Moreton, 1985), cultivation of yeasts on low-oxygenated media (Davies *et al*., 1990) and addition

of stearic acid or derivative into the medium (Papanikolaou *et al*., 2001).

Yarrowia lipolytica can stores more than 90% of TAGs in its cells and it has the potential to be used as cell factory for the production of oils, the yeast also could be used as raw material to apply in large-scale fermentation of food industry with its safety record (Beopoulos *et al*., 2009; Groenewald *et al*., 2014). In this study, we studied the effects of carbon source, nitrogen source, temperature, sterculic acid methyl ester, cobalt on cell growth and lipid accumulation of *Yarrowia lipolytica*, in order to obtain the optimal control point for single-cell lipid production with fatty acid profile similar to cocoa butter and this study could provide theoretical basis for using the yeast as raw material to commercialized production of cocoa butter.

Materials and Methods

Strain and Growth Conditions

Strain *Y. lipolytica* CICC1778 was purchased from China Center of Industral Culture Collection (CICC). The yeast was maintained on Yeast Extract Peptone Dextrose (YPD) agar medium at 4°C prior to the experiments. Inoculum was prepared by incubation at 28°C for 24 h on YPD liquid medium. Fermentation was performed on medium containing KH_2PO_4 7 g L^{-1}, Na_2HPO_4 2 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 1.5 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.1 g L^{-1}, $FeCl_3{\cdot}6H_2O$ 0.008 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.001 g L^{-1}, $CuSO_4{\cdot}5H_2O$ 10^{-4} g L^{-1}, $MnSO_4{\cdot}5H_2O$ 10^{-4} g L^{-1}, used glycerol 50 g L^{-1} as carbon source, used 3 g L^{-1} ammonium tartrate and 1.5 g L^{-1} yeast extract as nitrogen source (the rate of C:N = 80) and the initial pH of the medium was 6.0.

Effects of different carbon sources (glycerol, glucose, beef tallow, mutton tallow; 50 g L^{-1}) and nitrogen source [NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2C_4H_4O_6$, peptone, yeast extract, ammonium tartrate-yeast extract] at C/N = 80 were analyzed during the experiments. Effects of temperature (20-35°C), sterculic acid methyl ester (0-0.12 mL L^{-1}) and cobalt ($CoCl_2{\cdot}6H_2O$, 0-1.8 mg L^{-1}) with temperature being kept constant at 30°C were also studied.

Determination of Cell Dry Weight and Lipid Accumulation

The yeast samples after harvesting and washing twice with deionized water centrifuged at 8000 rpm for 5 min and then frozen at -80°C, lyophilized and the Cell Dry Weight (CDW) was determined gravimetrically. About 500 mg of dried cells was grinded and mixed with 3 mL chloroform-methanol (1:2, v/v) in a screw caped tube and whirling in the rotator for 1.5 h. The extract was washed with 1 mL chloroform and sodium chloride (0.1%, w/w). After centrifugation, chloroform phase was separated in new tube and 2 mL chloroform was added in former tube to extracted residual lipid. After washing with sodium chloride (0.1%, w/w), chloroform phase was pooled into the first extracted phase and evaporated in a stream of nitrogen. Impurities were removed by adding 1 mL diethyl ether and centrifugation. The total lipids were measured by gravimetric method.

Sn-2 Positional Analysis and Fatty Acid Profile Analysis

The method of Sn-2 positional analysis was followed by (Xiong *et al*., 2015) of our laboratory reported before. The fatty acid profile analyzed in GC-MS-QP2010 (Shimadzu, Japan) and the parameter setting was accorading to (Zhao *et al*., 2015).

Statistical Analysis

A statistical analysis of the obtained data was carried out using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). The mean values and the standard error of the mean were calculated from the data obtained from two independent experiments. The differences between the means of the test were evaluated by Student's t-test and $p<0.05$ was considered as significantly different.

Results and Discussion

Effect of Different carbon Source on Cell Growth and Lipid Accumulation in Y. lipolytica

The lipid content per CDW (%, g/g dry weight) of the cells grown on beef tallow and mutton tallow (30.09 and 33.1%) were much higher than grown on glycerol and glucose (15.19 and 17.22%, Fig. 1). It has been already reported that the pathway of lipid accumulation is different when yeast cultured on hydrophobic substances (so-called ‘ex novo’ lipid accumulation) and hydrophilic substances (so-called ‘de novo’ lipid accumulation). To meet the needs for growth, the internalized aliphatic chains are broken down, or modified form or accumulate in an unchanged and lipid accumulation enhanced when hydrophobic substances were uptake (Mlícková *et al*., 2004; Beopoulos *et al*., 2009).

Fatty acid composition of TAGs in *Y. lipolytica* with different carbon source was shown in Table 1. When glycerol was used as substrate, the content of SFA was up to 54.67% (w/w), much higher than other substrates. The Sn-2 position fatty acid composition of TAGs in *Y. lipolytica* after 3 days of cultivation with different carbon source was also shown in Table 1. The contents of oleic acid (C18:1) and total unsaturated fatty acids (UFA) at Sn-2 position were the highest (83.39% and 93.63%, respectively) when glucose was utilized as substrate. In glycerol medium, Unsaturated Fatty Acid (UFA) of Sn-2 position was declined slightly to 91.83%. Taking the high saturation of fatty acids into account, we choose glycerol as the carbon source in follow-up experiments. Glycerol has better industrialization prospect as it may be supplied from the by-products of many soap industries, as well as waste water from

biodiesel production which contains high concentration glycerol. These by-products, after proper treatment, can be used as cost-effective carbon source for yeast culture.

Effect of Different Nitrogen Source on Cell Growth and Lipid Accumulation in Y. lipolytica

Y. lipolytica was cultured on different organic and inorganic nitrogen sources. Organic nitrogen resource was considered as more beneficial to yeast cell growth and lipid accumulation. When ammonium tartrate-yeast extract was used as co-nitrogen source, the CDW (8.38 g L^{-1}) and lipid content (15.19%, w/w) were higher than that of cultured using ammonium tartrate or yeast extract as sole nitrogen source (Fig. 2). Organic nitrogen source benefited yeast growth and lipid accumulation, but different nitrogen source almost had no effect on the fatty acid profile of TAGs and the Sn-2 position in TAGs (Table 2). So ammonium tartrate-yeast extract was taken as the co-nitrogen source in next experiments.

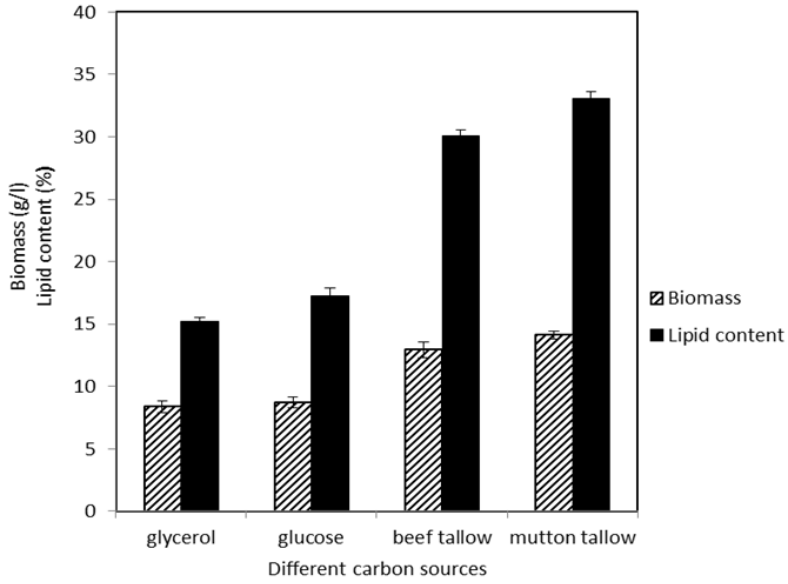

Fig. 1. The cell dry weight (g/L) and lipid content per cell dry weight (%, g/g) of *Y. lipolytica* after 3 days of cultivation for different carbon source (n = 3)

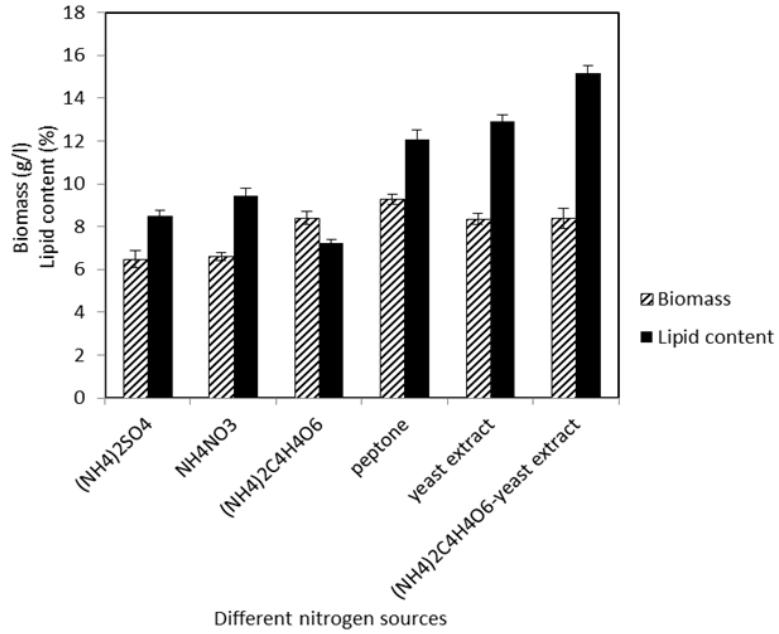

Fig. 2. The cell dry weight (g/L) and lipid content per cell dry weight (%, g/g) of *Y. lipolytica* after 3 days of cultivation for different nitrogen source (n = 3)

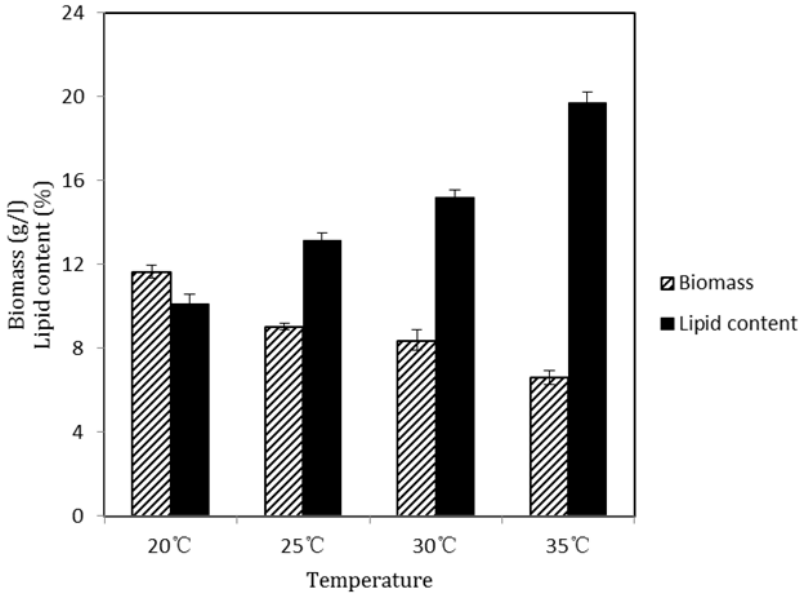

Fig. 3. The cell dry weight (g/L) and lipid content per cell dry weight (%, g/g) of *Y. lipolytica* after 3 days of cultivation for different temperature (n = 3)

Table 1. Fatty acid composition (%, w/w) of TAGs and the Sn-2 position fatty acid composition (%, w/w) of TAGs in *Y. lipolytica* cellular lipids after 3 days of cultivation for different carbon source

TAGs	Carbon source	C16:0	C18:0	C18:1	C18:2	SFA (%)
	Glycerol	22.78±1.01	31.90±0.97	32.94±0.18	8.04±0.27	54.67±1.97
	Glucose	17.11±0.83	15.18±2.03	56.30±0.59	6.6±0.56	32.89±2.89
	Beef tallow	25.57±1.38	6.38±0.33	55.94±2.03	8.03±0.78	31.95±0.99
	Mutton tallow	23.17±0.23	11.36±0.68	51.67±1.02	9.36±0.33	34.53±1.33
Sn-2 position	Carbon source	C16:0	C18:0	C18:1	C18:2	UFA (%)
	Glycerol	2.24±0.70	2.85±0.02	73.32±3.26	18.52±2.94	91.83±0.32
	Glucose	1.39±0.25	1.43±0.18	83.39±1.04	10.24±0.45	93.63±1.10
	Beef tallow	2.93±0.33	1.87±0.09	79.62±1.43	12.43±024	92.05±1.04
	Mutton tallow	2.56±0.58	3.8±0.16	80.33±0.14	8.95±0.59	89.28±0.63

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)

Table 2. Fatty acid composition (%, w/w) of TAGs and the Sn-2 position fatty acid composition (%, w/w) of TAGs in *Y. lipolytica* cellular lipids after 3 days of cultivation for different nitrogen source

TAGs	nitrogen source	C16:0	C18:0	C18:1	C18:2	SFA (%)
	1	22.35±0.29	30.65±1.12	32.57±0.41	9.38±0.35	53.00±1.35
	2	22.89±0.55	31.62±1.41	31.56±1.01	8.75±0.15	54.51±0.86
	3	20.36±0.81	29.53±0.29	33.65±1.32	11.36±0.24	49.89±2.01
	4	21.6±0.38	31.65±0.27	29.87±0.47	11.62±0.39	53.25±0.92
	5	19.32±0.33	28.77±0.67	36.72±2.03	10.32±0.16	48.09±0.87
	6	22.78±1.01	31.90±0.97	32.94±0.18	8.04±0.27	54.67±1.97
Sn-2 position	nitrogen source	C16:0	C18:0	C18:1	C18:2	UFA (%)
	1	3.21±0.06	3.56±015	70.58±0.25	20.62±0.32	91.20±0.56
	2	4.01±0.19	3.16±0.34	73.02±0.45	17.53±0.22	90.55±0.78
	3	2.77±0.32	2.05±0.31	71.89±0.14	21.17±0.24	93.06±0.21
	4	3.18±0.31	2.98±0.12	68.65±0.21	22.71±0.11	91.36±0.55
	5	1.85±0.21	1.92±0.32	72.32±0.11	21.89±1.32	94.21±0.76
	6	2.24±0.70	2.85±0.02	73.32±3.26	18.52±2.94	91.83±0.32

1: $(NH_4)_2SO_4$, 2: NH_4NO_3, 3: $(NH_4)_2C_4H_4O_6$, 4: peptone, 5: yeast extract, 6: ammonium tartrate-yeast extract. 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)

Table 3. Fatty acid composition (%, w/w) of TAGs and the Sn-2 position fatty acid composition (%, w/w) of TAGs in *Y. lipolytica* cellular lipids after 3 days of cultivation for different temperatures

TAGs	Temperature (°C)	C16:0	C18:0	C18:1	C18:2	SFA (%)
	20	24.89±0.02	21.15±0.41	35.16±1.29	11.96±1.48	46.04±0.76
	25	21.36±0.23	23.14±0.09	32.77±0.83	15.20±0.76	44.50±0.18
	30	22.78±1.01	31.90±0.97	32.94±0.18	8.04±0.27	54.67±1.97
	35	13.57±1.29	45.60±1.17	30.81±1.63	2.73±0.09	59.17±3.00
Sn-2 position	Temperature (°C)	C16:0	C18:0	C18:1	C18:2	UFA (%)
	20	2.64±0.58	3.17±0.86	64.22±1.32	27.47±1.52	91.69±0.20
	25	2.04±0.03	3.77±0.77	68.25±1.03	20.57±1.14	88.81±2.17
	30	2.24±0.70	2.85±0.02	73.32±3.26	18.52±2.94	91.83±0.32
	35	1.11±0.05	1.68±0.72	70.74±0.27	19.97±0.14	90.71±0.13

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)

Effect of Temperature on Cell Growth and Lipid Accumulation in Y. lipolytica

The lipid content per CDW of *Y. lipolytica* at different temperatures was shown in Fig. 3. With the decrease of growth temperature from 35 to 20°C, the CDW of *Y. lipolytica* was increased from 6.59 to 11.65 (g L^{-1}), but resulted in sharp decline of lipid content (19.70 to 10.13%). There was a strong negative correlation between CDW and temperatures in our study and also in accordance with the results of (Karasu-Yalcin *et al*., 2009). The lipid content showed positive correlation with temperatures but the difference between 25 and 30°C was not significant ($p<0.05$). It has been reported that different fermentation temperature can influence the growth of *Y. lipolytica* and led to the formation of different product (Karasu-Yalcin *et al*., 2009). According to our results, the optimal cultivation temperature for *Y. lipolytica* growth was 20°C and for lipid was 35°C.

Fatty acid composition of TAGs in *Y. lipolytica* at different temperatures was shown at Table 3. In *Y. lipolytica* cells, more than 95% of fatty acids were composed of C16 and C18, which is similar to that of cocoa butter, with C16 and C18 up to near 100%. Stearic acid (18:0) mainly contributed to the saturability, increased from 21.15% at 20°C to 45.60% at 35°C. Oleic acid (C18:1) showed no significant change and linoleic acid (C18:2) significantly ($p<0.01$) decrease from 11.96 to 2.73%. It was also observed that the content of saturated fatty acid in TAGs was 54.67% at 30°C and 59.17% at 35°C, this result suggested that it could be used as Cocoa Butter Equivalent (CBE). Moreover, contents of stearic acid, oleic acid, palmitic acid and linoleic acid were 31.90, 32.94, 22.78 and 8.04% at 30°C respectively and this fatty acid profile was very similar to cocoa butter (Fig. 4). Temperature adjustment could apply to somehow increase in SFA content and with the increase of cultivation temperature, the ratio of sum of saturated fatty acids was also increased. Wu *et al*. (2010) reported that shift in the incubation temperature of *Cryptococcus curvatus* cultured on N-acetyl-glucosamine from 30 to 22°C, the content of cellular SFAs was significantly increased from 44 to 54% (w/w), while with the decrease of the temperature, it has no effect on the biomass and the product of Single Cell Oil (SCO). *Rhodotorula glutinis* produced twice unsaturated lipids at 15°C as much as that at 30°C (Beopoulos *et al*., 2009). This difference may be caused by diversities of desaturase activity and stability in different yeasts.

The temperature can influence the lipid profile of the oleaginous mould (Kendrick and Ratledge, 1992). With the decrease of the culture temperature, the degree of saturation of fatty acid was generally decreased, the culture temperature influenced the fatty acid composition of *Y. lipolytica*. At low temperatures, the desaturase stability was increased and other enzymes were not increased in stability, led to the different formation of lipid profile and low temperatures led to the decrease of cellular activity and metabolism, it is not conducive to the synthesis of lipids (Groenewald *et al*., 2014). So with the change of incubation temperature, it maybe increase the SFA content of lipids produced and then synthesized intracellular lipids similar to cocoa butter and could be considered to be used as cocoa butter equivalent.

The Sn-2 position fatty acid composition analysis of TAGs in *Y. lipolytica* at different cultivation temperature (Table 3) showed that the content of oleic acid (18:1) was the most and followed by linoleic acid (18:2). These two fatty acids composition summed more than 90% in the total fatty acids. Palmitic acid (16:0) and stearic acid (18:0) added up to about 6%. The degree of unsaturation fatty acids at Sn-2 position was similar to that of cocoa butter (oleic acid 87%, stearic acid 3%, linoleic acid 3% and palmitic acid 3%). Particularly, at 30°C, oleic acid (18:1) was highest 73.32%, closest to cocoa butter (Fig. 5). Among all factors on biochemical behavior of *Y. lipolytica*, temperature is a very sensitive and easy-control factor for growth and metabolic activities (Beopoulos *et al*., 2009; Ke *et al*., 2010).

Effect of Sterculic Acid Methyl Ester on Cell Growth and Lipid Accumulation in Y. lipolytica

As a specific inhibitor of the Δ_9-desaturase, sterculic acid methyl ester was added to the culture medium from

0 to 0.12 mL L^{-1}. This led to a slight decrease of CDW and lipid content (Fig. 6), but a drastic increase of saturation level of fatty acids (Table 4). This increase of saturation was mainly because of increase in C18:0 level (31.90 to 55.98%). On the contrary, there was a radical decrease of C18:1, from 32.94 to 14.63%. When added 0.03 mL L^{-1} sterculic acid methyl ester to the culture medium, the fatty acid profile of TAGs [palmitic acid (22.49%), stearic acid (34.52%), oleic acid (31.73%) and linoleic acid (6.20%)] was further improved and greatly similar to cocoa butter, compared with the fatty acid profile [palmitic acid (22.78%), stearic acid (31.90%), oleic acid (32.94%) and linoleic acid (8.04%)] of the yeast grown at 30°C without the inhibitor (Fig. 4). Sterculic acid methyl ester also effected the Sn-2 position fatty acid composition of TAGs (Table 4). Both of the contents of C16:0 and C18:0 were increased with opposite trend of C18:1 content. The degree of unsaturation of Sn-2 position fatty acids declined with the increase of additive amount of sterculic acid methyl ester.

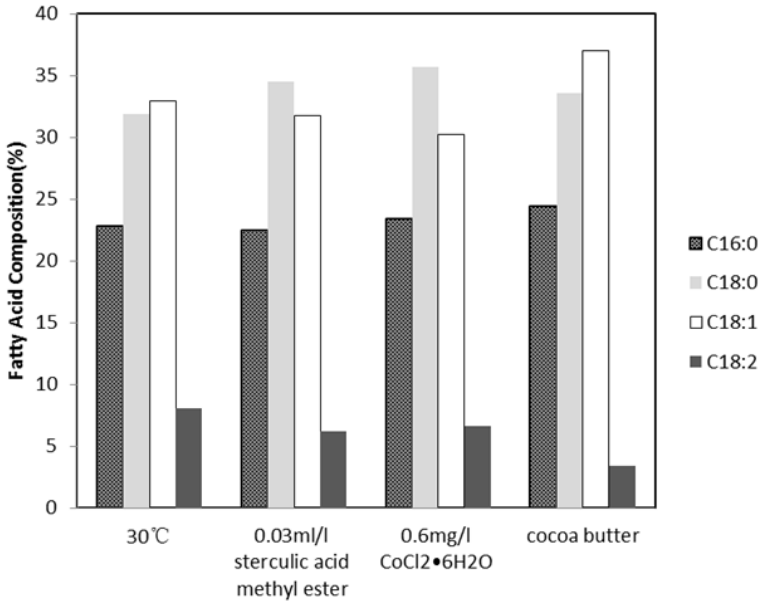

Fig. 4. Fatty acid composition (%, w/w) of cocoa butter and TAGs of cellular lipids in *Y. lipolytica* for different cultivation conditions

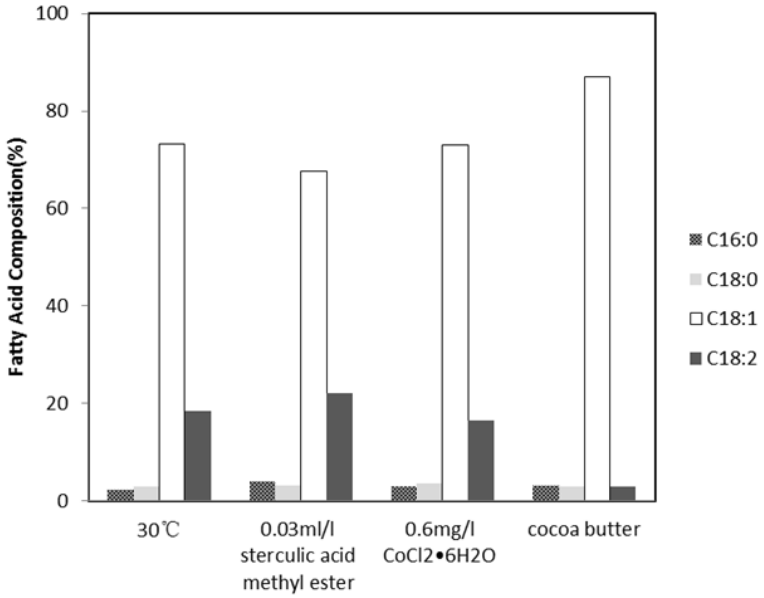

Fig. 5. The Sn-2 position fatty acid composition (%, w/w) of cocoa butter and TAGs of cellular lipids in *Y. lipolytica* for different cultivation conditions

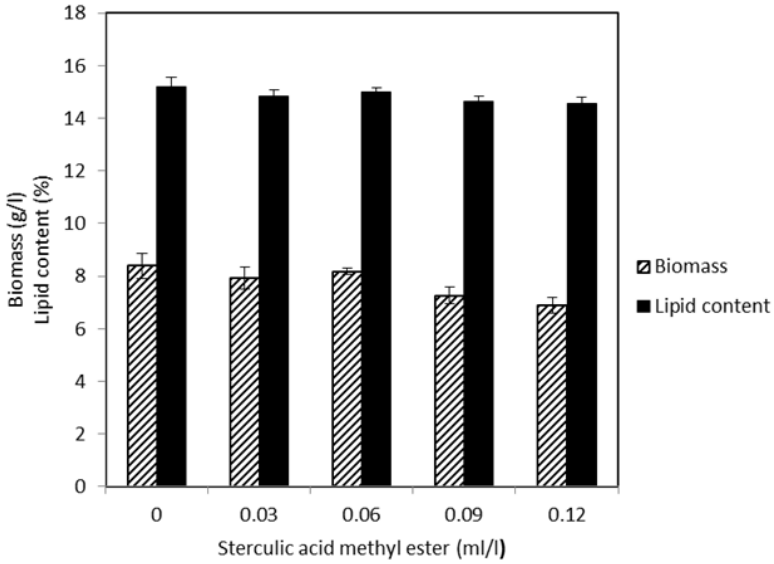

Fig. 6. The cell dry weight (g/L) and lipid content per cell dry weight (%, g/g) of *Y. lipolytica* after 3 days of cultivation for different additive amount of sterculic acid methyl ester (n = 3)

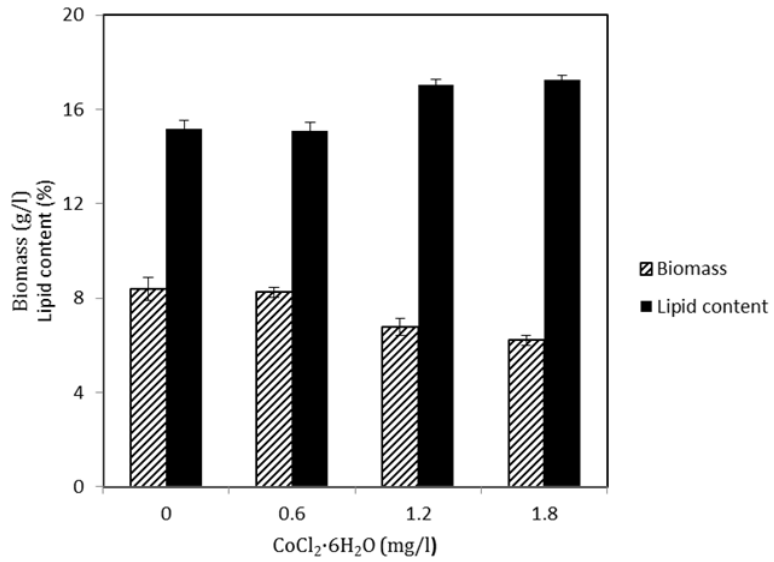

Fig. 7. The dry cell weight (g/L) and lipid content per dry cell weight (%, g/g) of *Y. lipolytica* after 3 days of cultivation for different additive amount of cobalt (n = 3)

Sterculic acidis is an inhibitor of the stearoyl-CoA desaturase, when added it to the medium, it could inhibit the saturated fatty acids synthesis of more than 16 carbon atoms and of odd-numbered fatty acids (Jeffcoat and Pollard, 1977). There was an almost complete growth suppression of *Rhodococcus opacus* PD630 when added 0.25 mM sterculic acid in the medium and the total fatty acid content was decreased to 46.7% (w/w), while the content of total fatty acid was 76% when did not added sterculic acid in the medium (Wältermann and Steinbüchel, 2000). The ratio of stearic to oleic acid showed a dose response effect, with an increase in cyclopropene fatty acid dose, stearic acid content also increased with a corresponding reduction in oleic acid when subjected to *Candida sp.* 107, *Rhodosporidium toruloides* and *Trichosporon cutaneum* for 4 days (Moreton, 1985). It has been reported that the inhibition of sterculic acid is irreversible and it occurs the reaction of cyclopropene ring with a conserved cysteine residue in the active center of the desaturase, although the mechanism of the inhibition is still not clear (Jeffcoat and Pollard, 1977).

Table 4. Fatty acid composition (%, w/w) of TAGs and the Sn-2 position fatty acid composition (%, w/w) of TAGs in *Y. lipolytica* cellular lipids after 3 days of cultivation for different additive amount of sterculic acid methyl ester

TAGs	Sterculic acid methyl ester (ml/L)	C16:0	C18:0	C18:1	C18:2	SFA (%)
	0	22.78±1.01	31.90±0.97	32.94±0.18	8.04±0.27	54.67±1.97
	0.03	22.49±0.14	34.52±0.11	31.73±2.13	6.20±0.23	57.01±0.97
	0.06	22.81±0.57	37.10±0.15	27.51±0.25	7.68±0.46	59.91±0.54
	0.09	18.94±0.12	45.15±0.31	20.30±0.13	9.46±0.56	64.09±0.29
	0.12	13.21±0.32	55.98±0.67	14.63±0.47	9.92±0.19	69.19±1.51
Sn-2 position	Sterculic acid methyl ester (ml/L)	C16:0	C18:0	C18:1	C18:2	UFA (%)
	0	2.24±0.70	2.85±0.02	73.32±3.26	18.52±2.94	91.83±0.32
	0.03	3.95±0.19	3.22±0.64	67.61±0.31	22.02±0.75	89.63±0.77
	0.06	4.42±0.35	4.15±1.01	64.18±0.38	23.98±0.15	88.16±0.13
	0.09	2.58±0.24	8.04±0.39	64.01±0.82	22.36±0.61	86.37±1.22
	0.12	5.51±0.58	7.50±0.66	60.37±0.17	23.27±0.34	83.64±0.56

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)

Table 5. Fatty acid composition (%, w/w) of TAGs and the Sn-2 position fatty acid composition (%, w/w) of TAGs in *Y. lipolytica* cellular lipids after 3 days of cultivation for different additive amount of cobalt

TAGs	$CoCl_2·6H_2O$ (mg/L)	C16:0	C18:0	C18:1	C18:2	SFA (%)
	0	22.78±1.01	31.90±0.97	32.94±0.18	8.04±0.27	54.67±1.97
	0.6	23.38±0.14	35.65±0.19	30.19±0.31	6.65±0.16	59.03±0.34
	1.2	21.24±0.16	39.2±0.39	25.98±0.11	7.67±0.34	60.44±0.17
	1.8	18.24±0.18	45.68±0.51	22.38±0.44	7.77±0.67	63.92±0.86
Sn-2 position	$CoCl_2·6H_2O$ (mg/L)	C16:0	C18:0	C18:1	C18:2	UFA (%)
	0	2.24±0.70	2.85±0.02	73.32±3.26	18.52±2.94	91.83±0.32
	0.6	2.89±0.10	3.56±0.52	73.11±0.55	16.56±0.23	89.67±0.77
	1.2	3.53±0.36	5.10±0.12	65.74±0.39	22.37±0.21	88.11±0.44
	1.8	1.97±0.29	6.99±0.26	63.96±0.13	23.66±0.41	87.62±0.49

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)

Effect of Cobalt on Cell Growth and Lipid Accumulation in Y. lipolytica

Cobalt had an obvious reductive effect on *Y. lipolytica* growth but led to a slight increase of lipid content when the amount of $CoCl_2·6H_2O$ increased to 1.2 or 1.8 mg L^{-1} (Fig. 7). The effect of cobalt on fatty acid composition had a common with that of sterculic acid methyl ester (Table 5). There was a drastic increase of saturation levels mainly because of increase in C18:0 (31.90 to 45.68%) and decrease in C18:1 (32.94 to 22.38%) level. Cobalt had no significant effect on C16:0 and C18:2. It was also observed that contents of palmitic acid (23.38%), stearic acid (35.65%), oleic acid (30.19%) and linoleic acid (6.65%) were improved and greatly close to the fatty acid composition of cocoa butter by addition of 0.6 mg L^{-1} $CoCl_2·6H_2O$ to the culture medium at 30°C (Fig. 4). Cobalt has similar kind of effect on Sn-2 position of TAGs as sterculic acid methyl ester (Table 5).

Recently, it has been reported that cobalt could reduce the Δ_9-desaturase indices in bovine milk when supplied in large amounts, it may be influenced the Δ_9-desaturase activity (Shingfield *et al*., 2008; Taugbøl *et al*., 2010). The result of Frutos *et al*. (2014) has reported that, consistent with an inhibition of the activity of stearoyl-coenzyme A desaturase, cobalt could modify milk fat composition in cattle. The reduction of Δ_9-desaturase obtained by Co-treatment could negatively affect the synthesis of the enzyme and its activity (Karlengen *et al*., 2010). Little available information in biochemistry of lipid desaturation has been reported and our results also suggested the possibility of Δ_9-desatrase activity of cobalt on *Y. lipolytica*.

Conclusion

The ability of microorganisms to produce natural substance and convert them into reserve lipids is now becoming great attention of lipid biotechnology. Oleaginous yeast *Y. lipolytica*, is also capable of producing large amount of lipids when cultivated on different conditions. Glycerol and ammonium tartrate-yeast extract were chosen as carbon source and nitrogen source for TAGs profile of intracellular lipid analogous to cocoa butter. An increased of temperature from 20 to 35°C halved the CDW of *Y. lipolytica* (from 11.65 to 6.59 g L^{-1}) while increase of the lipid content per CDW

(10.13 to 19.7%, w/w). Degree of saturation of TAGs increased with the increase of temperature. Particularly at 30°C, the composition of palmitic acid (22.78%), stearic acid (31.90%), oleic acid (32.94%), linoleic acid (8.04%) and the degree of unsaturation at Sn-2 position (90%) resembled cocoa-butter.

Sterculic acid methyl ester and cobalt have inhibition effects on unsaturated fatty acid with lipid composition similar to cocoa butter. Conclusively, the optimum conditions for *Y. lipolytica* to synthesize cocoa butter equivalent was 30°C with 0.6 mg L^{-1} of $CoCl_2 \cdot 6H_2O$ or 0.03 mL L^{-1} sterculic acid methyl ester in the medium of glycerol and ammonium tartrate-yeast extract as carbon source and nitrogen source. This study provides theoretical base for industrial applications of yeast lipid to synthesize microbial substitutes of cocoa butter.

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Author's Contributions

Hongyin Zhang and Yuanda Song: Designed the experiments.

Lina Zhao, Bo Li and Dan Xiong: Performed the experiments.

Huaiyuan Zhang, Xin Tang and Shengru Yang: Contributed to reagents, material and analysis tools.

Lina Zhao and Bo Li: Wrote the manuscript.

Ethics

The authors declare that they have no conflict of interest. All authors have read and approved the manuscript and no ethical issues involved.

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