Fed-batch fermentation of *Yarrowia lipolytica* using defatted silkworm pupae hydrolysate: A dynamic model-based approach for high yield of lipid production

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ABSTRACT: Lipid production by *Yarrowia lipolytica* W29 in fed-batch mode was investigated by using low-cost substitutable defatted silkworm pupae hydrolysate (DSWPH) as a feedstock. Based on the optimized lipid fermentation conditions, three media (yeast extract, DSWPH, yeast extract-DSWPH as N sources) were investigated in a batch fermentation process. The DSWPH medium displayed the optimal lipid accumulation ability with a lipid yield raised by 16.13%, a ratio of unsaturated fatty acids *vs.* saturated fatty acids inproved by 0.96-fold, and a ratio of unsaturated fatty acids in total fatty acids increased to 87.23%. The mathematical equations based on experimental data provided a good description of temporal variations such as dry cell weight, glucose consumption, and product formation in lipid fermentation. The results showed that the Luedeking–Piret type equation successfully described glucose consumption and lipid accumulation in the batch culture process. A fed-batch fermentation system was designed based on the model prediction. In the lag phase, rapid biomass growth and lipid accumulation were sequentially achieved with the adjustment of temperature, pH, and dissolved oxygen. Finally, the maximum biomass and lipid productivity were 24.01 g/L and 2.76 g/L/d, respectively.
KEY WORDS: *Yarrowia lipolytica*, defatted silkworm pupae, microwave, unsaturated fatty acids, kinetics, fed-batch.

Introduction

Microbial oils, also called single cell oils (SCOs), have been considered as potential feedstock for oil sources due to relatively high unicellular growth rate and rapid lipid accumulation ability [1]. SCOs have been expected as alternative oil sources which are rarely found in plant or animal (i.e. lipids containing rare polyunsaturated fatty acids or cocoa butter equivalents). Another attractive perspective is to use SCOs as the material for the production of bio-diesel. The lipid productivity and quality of microorganism are pivotal factors in making microbial edible oil economically viable. However, industrial scale implementations are presently prohibitive due to the high cost of the process, especially the cost of the medium components. Most researchers focused on seeking low cost raw materials instead of glucose as carbon source for the heterotrophic fermentation, including raw glycerol [2], volatile fatty acids[3], cassava starch hydrolysate [4], sugar beet molasses [5], wheat straw hydrolysate [6], olive mill wastewater [7], sweet sorghum juice [8], Jerusalem artichoke [9], etc.. However, organic nitrogen sources, such as yeast extracts or peptone, are at least 5-fold more expensive than conventional carbon sources (i.e. glucose) [10]. Therefore, a cost-effective and efficient nitrogen source is critical to economically enhance lipid accumulation in microorganism. For example, corn steep liquid (CSL) is a kind of nitrogen source substitution to replace yeast extract in *Clostridium* fermentation, which enhanced butanol production by seven-fold compared to yeast extract in bottle fermentations [11].

The defatted silkworm pupae (DSWP), an agro-industry byproduct derived from silk reeling process, contains necessary nutrients for microbial growth. This unique waste biomass was considered as a new nitrogen source substitution. As we all know, silkworm pupae is the largest by-product in silk industry. In China, 650,000 tons of silkworm pupae is generated every year. Some of them were utilized to extract oil, which could be used for structural lipids formation. It's worth mentioning that the DSWP residue contains 85.2% protein. In our previous study, conversion of DSWP to microbial lipids by the oleaginous yeast *Yarrowia lipolytica* was studied, and several methods of DSWP hydrolyzation were compared. After pretreatment, DSWP proteins were converted to soluble polypeptide in the DSWP hydrolysate (DSWPH), making it easier to be used by microorganism. The prior results demonstrated the feasibility of using DSWP as an alternative nitrogen source by achieving 13.64 g L^{-1} dry cell weight (DCW) and 3.71 g L^{-1} lipid production.

Oleaginous yeasts, especially *Y. lipolytica*, have shown to be excellent SCOs producers in terms of its unique physiological characteristics and GRAS (generally recognizes as safe). It has been a model organism for lipid production and claimed having a great potential in industrial application [12]. Although these previous studies have extensively investigated and discussed the effect of fermentation conditions on lipid yield, to pursue a high productivity and low cost bio-product, it is vital to develop a suitable cultivation method to investigate and evaluate the potential growth rate and lipid production of *Y. lipolytica* by using DSWPH. Fermentation models can provide useful information for the description, prediction and evaluation of a fermentation process without complexity [13]. Inherence mechanism of microbial fermentation processes are very complex, and fermentation model could be critical for process investigation, control, and optimization in practical applications [14]. Hence, developing mathematical models of fermentation kinetics is a useful tool for fermentation system.

This study presented the application of DSWPH as organic nitrogen source for enhancing oil accumulation by *Y. lipolytica* W29. Microwave assisted enzymatic pretreatment was investigated to hydrolyze DSWP because multiple reactions and processes, including enzymatic digestion of proteins, can be accelerated under microwave irradiation [15]. To pursue the optimization of fed-batch condition, several parameters were studied to determine their effects on cell growth and lipid accumulation of *Y. lipolytica* W29. An efficient feed strategy for producing the maximum lipid and high unsaturated fatty acids (UFAs) yield was developed in fed-batch mode fermentation by investigating kinetic model and culture condition. The long-term stability of the strain cultivated on fed-batch mode was detected. The overall goal is to demonstrate the feasibility and potential of utilizing agricultural and forestry waste as nitrogen source for fermentation to produce lipid and biodiesel.

Materials and Methods

Microwave assisted enzymatic hydrolysis of DSWP

Dried silkworm pupae (Sericultural Research Institute, Chinese Academy of Agricultural Sciences) was mechanically milled to fine powder (about 50-100 in diameter) and defatted. In the enzymatic hydrolysis stage, a mixture of 5 g DSWP, 45 mL water (corresponding to a 10% (w/w) solid loading) and 3% (kg kg^{-1} DSWP) neutrase (Nantong Feiyu Biological Technology Co. Ltd., Nantong, China) was conducted at 55 °C, pH 7.0 for 30 min under microwave irradiation (CEM, MARS5, USA). During the process, neutrase was applied to degrade the protein of DSWP. After hydrolyzing, the mixture was centrifuged at 7000 rpm for 10 min to remove insoluble substance. The residual obtained was washed by pure water three times and collected together.

Detection of metals concentration from DSWP by ICP-MS

All reagents were made in water purified (>18.2 ΩWcm^{-1}) using a Milli-Q (MQ) system (Millipore Corp., Billerica, USA) (Millipore). Optima grade nitric acid (Fisher Scientific, St. Louis, USA), and Fluka trace select grade hydrogen peroxide (Sigma-Aldrich, St. Louis, USA) were used in all sample preparation and dilution steps. The stock solutions (1g L^{-1}) were obtained by dissolving appropriate amounts of corresponding salts in 1% (v v^{-1}) diluted HNO_3 and diluted to 100 mL high purity deionized water, respectively. The microwave digestion procedure for DSWP samples and operating parameters of ICP-MS were shown on Supporting Materials [16].

The DSWP and its residual were dried at 60 °C in vacuum overnight. The dried specimens were homogenized and then weighed precisely. The sample was digested with concentrated HNO_3 in the microwave oven. Samples were analyzed using an ICP-MS (Thermo X Series2, Thermo Scientific, USA). Standard solutions (E. Merck, Darmstadt, Germany) were used to determine elemental concentrations.

Culture and medium

Y. lipolytica W29 (the American Type Culture Collection center, ATCC) was maintained at 4 °C. It was kept on YEPD agar plates (%, yeast extract 1, peptone 2, glucose 2, and agar 1.5). Every month, single colonies were transferred to a fresh plate, incubated for 4 days, and then maintained under refrigeration condition.

The seed medium contained (per liter): 20 g glucose, 10 g yeast extract, and 10 g tryptone. The initial pH was nature (about 6.5~7.0). Baffled hybrid flasks (250 mL) containing 50 mL of culture medium were used for cultivation with rotary shaking (200 rpm) at 28 °C for 24 h. The growth medium using yeast extract as N source contained (per liter): 40.0 g glucose, 2.0 g ammonium tartrate, 1.5 g yeast extract, 7.0 g KH_2PO_4, 2.0 g Na_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$. The growth medium using DSWPH contained (per liter): 40.0 g glucose, 2.0 g ammonium tartrate, 30 mL DSWPH, 7.0 g KH_2PO_4, 2.0 g Na_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$.

Parameters optimization for fed-batch fermentation

Five factors including glucose concentration, inoculum dose, pH, temperature, and dissolved oxygen (DO) on lipid accumulation and distribution were investigated in batch experiments. The seed culture was inoculated at 5 % (by volume) into the 3 L bioreactor (BioFlo/CelliGen 115, New Brunswick Scientific, USA) containing 2 L initial growth medium. The inoculated flasks were kept on a rotary shaker at 28 °C, 200 rpm for 72 h.

Kinetics of the growth and lipid production

The kinetic models for lipid batch fermentation including variables of biomass (X, DCW, g L^{-1}), substrate (S, glycerol, g L^{-1}), and product (P, Lipid, g L^{-1}) were established. Microbial growth, lipid accumulation, and sugar utilization of *Y. lipolytica* W29 in batch fermentation process utilizing DSWPH, yeast extract, or the mixture were modeled according to mathematical models based on the Logistic and Luedekinge-Piret equations [17]. Values of the kinetic parameters and model simulations, were calculated using the methods described by Sattur [18] for the speculation of the fermentation data.

Experimental data from batch fermentation in 3.0 L bioreactor was utilized to simulate the kinetic parameters in the proposed set of model equations [19]. Nonlinear curve was fitted and kinetic parameters were estimated. The fed-batch was conducted under the estimation of the model that established.

The experiments were carried out in three replicates and results were presented as the average with standard deviation (SD) represented by error bars in graphs. Analysis of variance (ANOVA) procedure followed by Duncan's test was applied to determine the significant difference (p<0.05) between treatment means [20].

Batch and fed-batch fermentation

The inoculum used was obtained from a two-stage progressive scale-up from 1 mL to 50 mL and finally to 2 L. First one tube of cells was inoculated into 50 mL seed medium in a 250 mL flask and incubated at 28 °C, 180 rpm on a reciprocal shaker until the optical density (OD) at 600 nm of seed culture reached 1.2±0.1. Then the seed broth was inoculated into the 3 L bioreactor containing 2.0 L initial growth medium. The inoculated flasks with culture medium were kept on a rotary shaker at 28 °C, 200 rpm for 72 h.

After seed preparation, 5% of seed broth (400 mL) was transferred into a 3 L bioreactor containing 1.6 L initial growth medium pumped into the bioreactor. After 24 h of fermentation at the lipid accumulation stage, the stock solution containing growth medium supplemented with 160 g L^{-1} glucose and 40 mL L^{-1} DSWPH was added to maintain the concentrations of C and N at desired levels. The medium volume was maintained at 2 L.

The bioreactor was operated isothermally at 28±1 °C with a stirring rate of 200 rpm and an aeration rate of 1.5 vvm. The pH of the medium was adjusted to 5.5 before sterilization. The DO was natural at the beginning but kept at 30% in the lipid accumulation stage. The pH-value was automatically controlled by adding 2 M H_2SO_4 and 2 M NaOH solutions during fermentation. Foam was controlled by the addition of 25 % organ silicon. Samples were taken at intervals for determination of DCW, glucose concentration and lipid concentration.

Analytical methods

DCW was determined by transferring 5 mL cell suspension to a pre-weighted centrifuge tube followed by centrifugation at 8000 × g for 5 min. The cell pellet was washed twice with distillated water and freeze-dried until constant (24 h). Glucose was determined by an SBA-40D biosensor analyzer (Institute of Biology of Shandong Province Academy of Sciences, Shandong, China). Before detection the supernatant containing residual glucose was diluted into a proper concentration [21].

The DH, which was defined as the percentage of the ratio of the number of peptide bonds cleaved (h) to the total number of bonds per unit weight ($h_{tot.}$), was determined using the ninhydrin reaction and calculated with the following equation. The $h_{tot.}$ of silkworm pupae protein was 7.8 mmol g^{-1}.

$$\text{Degree of hydrolysis (DH)} = \frac{\text{Number of peptide bonds cleaved } (h)}{\text{Total number of peptide bonds } (h_{tot.})} \times 100\ \% \quad (1)$$

A two-step methylation process was applied for the determination of fatty acid (FA) composition in *Y. lipolytica* W29 [22]. The amount of fatty acid methyl esters (FAMEs) in the samples was determined by the gas chromatography (GC) equipped with a flame ionization detector (FID) (Model 6820 GC system, Agilent Technologies, USA) and a fused silica capillary column HP-Innowax (Thickness 0.25 lm, I.D. 0.25 mm, Length 30 m, Agilent Technologies, USA). The oven was initially set at 80 °C for 1 min, heated to 200 °C at a rate of 15 °C min^{-1} and then to 250 °C at a rate of 2 °C min^{-1} and maintained for 6 min. Highly pure N_2 (99.99 % purity) was the carrier gas (1.0 mL/min), and the stigma pressure was 0.07 Mpa. The split ratio was 50:1. All samples were assayed in triplicate. Based on these response factors, the FAs in samples were quantified by comparing their peak area with the internal standard (C 17:0) and corrections were applied based on their response factors.

Total lipid extractions were obtained from 100 mg samples. The freeze-dried yeast cells were suspended in a 2:1 chloroform/methanol solution by glass beads for 20 min. The organic phase was collected and washed with pure water before being dried at 60 °C and weighed to quantify lipid production [23].

Results and discussion

Characteristics of DSWPH

The DSWP used in the study contained 85.2% (w w^{-1}) protein and 3.4% oil. The protein was converted into fermentable micro-molecular substrates after the microwave-assisted enzymatic hydrolysis, and the final concentration of total N is about 5.9 g L^{-1}. Considering that the total N in yeast extract is 10.0-12.5 (wt %), per gram of yeast extract should be replaced by ca. 20 mL hydrolysate with a N content ranged in 5-6.25 mg mL^{-1}.

Table 1 shows slightly difference in metal contents of the DSWP when using different pretreatment. The microwave showed a slight increase of the heavy ions solubilizing, such as Cu, Fe, and Zn. After microwave assisted enzymatic hydrolysis, only 15.03% biomass was left. However, the value was 40.53% when DSWP went through enzymatic hydrolysis. According to the weight loss of biomass and metal content before and after the pretreatment, the metal concentration in hydrolysate could be calculated. In the medium using DSWPH as N source, the metals concentration were 0.03 Cu, 0.31 Zn, 0.11 Fe, 0.01 Mn, 14.00 Mg, 33.63 K, 3.26 Ca (mg L^{-1}) after enzymatic hydrolysis while the metals concentration were 0.04 Cu, 0.48 Zn, 0.18 Fe, 0.02 Mn, 18.96 Mg, 50.37 K 5.06 Ca (mg L^{-1}) after microwave assisted enzymatic hydrolysis.

The metabolic pathways for de-novo lipid synthesis in *Y. lipolytica* are normally made of tricarboxylic acid (TCA) cycle and Kennedy pathway [24]. Acyl-CoA is the precursor to form phosphatidic acid (PA) [25]. In addition, the ATP citratelyase (ACL)-mediated cleavage of citrate *via* the citrate shuttle yielding acetyl-CoA and oxaloacetate (OAA). Then, acetyl-CoA was transported from the mitochondria to the cytosol [26]. The key enzyme of lipid accumulation in oleaginous microorganisms, ATP-citrate lyase (ACL), is metal ion-dependent enzyme [27]. Likely, the Fe^{3+}, Cu^{2+}, and the other metals also could enhance lipid accumulation [28, 29].

Table 1 Analytical results of metals in different DSWP samples.

Metal	Control [a]	EH [b]	MWEH [c]
	(μg/g)	(μg/g)	(μg/g)
^{65}Cu	14.64±0.19	11.86±0.21	11.46±0.13
^{66}Zn	172.45±3.03	183.16±3.44	199.75±4.35
^{56}Fe	65.71±0.51	73.56±2.03	78.25±0.80
^{55}Mn	10.38±0.11	14.01±0.11	19.36±0.42
^{24}Mg	6031.31±48.51	4369.23±62.53	3635.89±72.38
^{39}K	16741.72±72.38	15332.52±45.22	13835.84±30.01
^{40}Ca	1774.40±12.18	1812.81±18.21	1927.78±17.71
^{60}Ni	0.33±0.01	0.35±0.01	0.37±0.01

[a] Condition: the material was just defatted, dried and washed by ultrapure water. And the weight loss was at 12.36%.
[b] Condition: 5 g of DSWP were mixed with 45 mL water. The neutrase was autoclaved at 55 °C, pH 7.0 for 30 min. EH: Enzymatic hydrolysis. And the weight loss was at 59.14%.
[c] Condition: 5 g of DSWP were mixed with 45 mL water. The neutrase was autoclaved at 55 °C, pH 7.0 for 30 min in polypropylene tubes of the microwave accelerated reaction system. MWEH: Microwave-enzymatic hydrolysis. And the weight loss was at 84.97%.

Comparison of different nitrogen source for yeast culture

Fig. 1 shows that biomass, total lipid and residual glucose time profiles of *Y. lipolytica* when using different organic nitrogen source. The results indicated that DSWPH accelerated the degradation of lipid. For medium with DSWPH, the DCW appears at the highest level in the stationary phase. Meanwhile, this condition achieved the highest lipid production. However, the medium with DSWPH holds the longer lag phase than the other. Accordingly, at the lag phase, the yeast grew on the medium with yeast extract has the highest glucose consumption rate. This is because the organic nitrogen source applied in seed medium was yeast extract, when the strain was transferred to a new medium, it needed time to adjust to the growing environment. However, the DSWPH contains about 18 kinds of amino acids. As a type of high quality nitrogen source and virtual growth factor, amino acids have significant effect on cell growth and lipid accumulation. Besides, there were small amounts of oils remained in the DSWPH. When the glucose concentration was at low level, the enzyme activity of the lipase was activated. The residual oil was digested and converted into the nutrition for cell growth. Fig. 1 also shows that the yeast cultivated on the medium with DSWPH grown faster than the other two media.

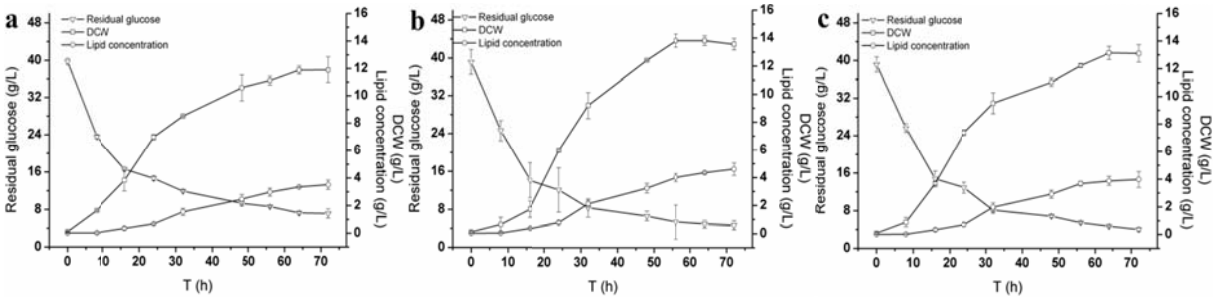

Fig. 1. Comparisons among yeast extract, DSWPH, yeast extract-DSWPH as organic nitrogen sources for cell growth and lipid production in batch cultures by *Y. lipolytica* W29. (a. yeast-extract; b. DSWPH; c. yeast extract-DSWPH.)

At the end of exponential growth phase, the strain began to accumulate large amounts of lipids. The cells were observed by a Leica microscope (GmbH, Göttingen, Germany) at 1000× (Fig. 2). During the whole growth phase, the yeast went through about two stages. At the proliferation stage, most of the cells were shown to be short true mycelia and pseudo-mycelia. While at the lipogenic phase, the yeast-like cells were predominant. The size of the cells, only utilize DSWPH as the nitrogen source, were larger than the other. There were lipid droplets being observed. It indicated that the whole cell has the ability to accumulate lipid, which was consistent with the growth curve at Fig.1. Comparing the Figs. 2a and 2c, the cells cultured on the media with yeast extract still at the growth stage after 2 days cultivation since the morphology of *Y. lipolytica* was still shown as short true mycelia and pseudo-mycelia. After cultivated for 2 days, only the strain cultivated on the medium with DSWPH showed the comprehensive ability of lipid accumulation (Fig. 2b). In the medium with yeast extract-DSWPH, most of the cells look like typical yeast. Lipid storage stage might have almost been done. As growth proceeded only yeast cells appeared in the culture, while the different percentage of lipid accumulated in the various growth phases was notably reflected on the cell size. Lipogenic phase as significant quantities of lipid were stored in the cells, which could have been effected by using the medium.

Table 2 shows FAs composition of the various lipid fractions when the strain grown on three kinds of media. C18:1 proportions increased to 62% and 67% in both media containing DSWPH. Oleic acid (C18:1) was the predominant FA regardless the medium used. Palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and linoleic (C18:2) acids were also found in significant quantities in the lipid structures, which are different with the

previous investigation [30]. The media containing half or whole DSWPH both contained reduced percentages of saturated fatty acids (SFAs). In other word, the percentages of unsaturated fatty acids (UFAs) have raised by changing the culture medium. Interestingly, only the strain utilizing DSWPH has the ability of accumulating slight amount of α-linolenic acid (C18:3). Obviously, the fractions in hydrolysate have promoted the desaturation reaction and UFAs accumulation. The cells grew on yeast extract reached UFAs/SFAs ratio at 3.48 while DSWPH reached at 6.81. The value has increased by about 0.96-fold. The result was verified with the assumption growth factors, like ions, free amino acids, and the other components, are conducive to the cell growth and UFAs accumulation. Moreover, the FA composition was quite similar to that of vegetable oil [31].

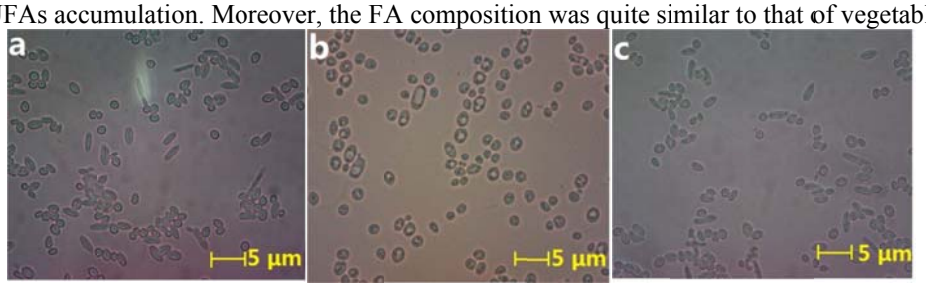

Fig. 2. Morphologies of the *Y. lipolytica* W29 grown on media made of different organic nitrogen sources. The strain was cultivated on (a) medium containing yeast extract; (b) the strain was cultivated on medium containing DSWPH; (c) the strain was cultivated on medium mixed yeast extract with DSWPH at the same nitrogen concentration. The image was captured at the end of 2 d cultivation. Conditions: initial glucose concentration 40 g/L; DO 30%; pH 5.5; incubation temperature 28 °C.

Table 2 Effects of nitrogen source on lipid profile and UFAs/SFAs of *Y. lipolytica* W29 in the batch fermentation.

Fatty acid (relative %, w/w)	Nitrogen source		
	Yeast extract	Yeast extract and DSWPH	DSWPH
C16:0	[a] 12.51±0.86	[b] 11.12±0.35	[b] 10.68±0.23
C16:1	[a] 10.45±0.37	[b] 8.96±0.09	[b] 8.76±0.35
C18:0	[a] 9.82±0.46	[b] 5.82±0.33	[c] 2.12±0.03
C18:1	[c] 51.74±1.06	[b] 62.42±0.42	[a] 67.30±0.96
C18:2	[a] 16.48±0.75	[b] 10.69±0.41	[c] 7.68±0.42
C18:3	[c] 0.00±0.00	[b] 1.79±0.31	[a] 3.46±0.02
UFAs/SFAs	3.48	5.05	6.81
UFAs/TFAs	78.67	83.86	87.20

[a, b, c] The mean values in the same row for *Y. lipolytica* oil TFAs culturing on different media are significantly different ($p < 0.05$). UFAs: unsaturated fatty acids; SFAs: saturated fatty acids; TFAs: total fatty acids. For the yeast *Y. lipolytica* W29, main UFAs are C16:1, C18:1, C18:2 and C18:3, main UFAs are C16:0 and C18:0.

Effects of culture conditions on cell growth and lipid accumulation

Fig.3a shows that the DCW and lipid content climbed to the highest level of 13.62 g L^{-1} and 0.28 g g^{-1}, respectively, when the initial glucose concentration was 60 g L^{-1}.

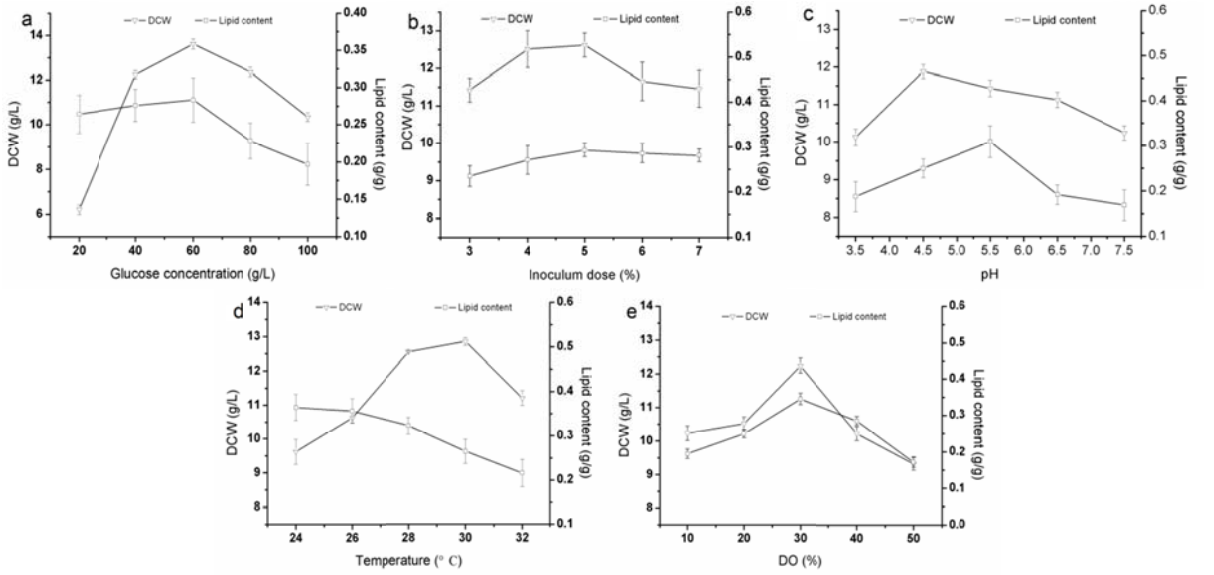

Fig. 3. Growth and lipid accumulation of *Y. lipolytica* W29 under different cultivation conditions by batch culture. (a) glucose concentration; (b) inoculum dose; (c) pH; (d) temperature; (e) DO. Reaction conditions: (a) the inoculum was 5%, temperature was 28 °C, pH and DO was nature; (b) the glucose concentration was 60 g/L, temperature was 28 °C, pH and DO was nature; (c) the glucose concentration was 60 g/L, inoculum dose was 5%, culture temperature was 28 °C.

A significant increase of DCW was observed at the glucose concentration increased from 20 g L^{-1} to 40 g L^{-1}. The effect of carbon source for microbial growth metabolism mainly provided cells carbon frame, energy for the cell life activities, and carbon frame of synthetic products. When the glucose concentration was 20 g L^{-1}, the strain has inadequate carbon for cell proliferation. Accordingly, the lipid content was still at the low level which was confirmed with the fact that lower C/N went against lipid accumulation [32]. However, the cell did not grow very well when the glucose above 60 g L^{-1}. The thick fermentation broth and toxic substance after the sterilization inhibited the mass transfer and cell growth.

Fig. 3b shows that DCW and Lipid content were highest when the inoculum dose was 5%. Lipid production did not show significant difference due to the compensation between lipid content and ultimate DCW. Higher inoculum dose might rapidly decrease the lag phase, however, the initial high nutrient conditions might inhibit the activity and become the barrier for the yeast to accumulate lipid.

The initial pH was critical for yeast growth and had a direct impact on the kinetics and yields [33]. From Fig. 3c, when pH was 4.5, DCW runs to the highest. When pH continued to increase, DCW decreased gradually. However, lipid content reached its highest value when pH was 5.5, then the lipid content decreased rapidly as pH increased from 5.5 to neutral.

As Fig. 3d shown, although the lower temperature led to the higher lipid content, the lower temperature increased the production cost and slowed down the cell growth. This indicated that the desaturase activity was reduced with the increased temperatures [34]. Therefore, 28 °C would be the best culture temperature.

DO value in the media directly affects the accumulated lipid content [35]. According to Fig. 3e, the DO value maintained at 30 % at stable phase showed a well growth of cell and accumulated the highest level of lipid. The oleaginous yeast possessed a PUFA synthase complex mode which required abundant oxygen. When the oxygen was deficient, the synthesis of TAG will be inhibited. Based on the above results, the optimized glucose concentration (60 g L^{-1}), inoculum dose (5 %), pH (5.5), temperature (28 °C), and DO (30 %) were verified.

Effects of culture conditions on the FA composition of yeast oil

To study the effect of culture condition on lipid compositions, the total cellular lipid content was determined in harvested biomass. The distribution of intracellular FAs depended on the nitrogen source and culture conditions. Fig. 4 showed that the C18:1 was the predominate fatty acid in the total lipid. The content of C18:1 maintained from 50 % to 65 %. Fig. 4a showed that the glucose concentration had slightly influence on C18:2 and C18:3 and other FAs. The improved glucose concentration to 60 g L^{-1} led to the higher C/N ratio which could make the content of UFAs at slightly higher level. But when the glucose concentration higher than 60 g L^{-1}, the growth rate will be reduced which also inhibit UFAs accumulation. In addition, the high level of nitrogen source (with a low C/N ratio) enhanced the biomass growth, but impeded the lipids accumulation [36].

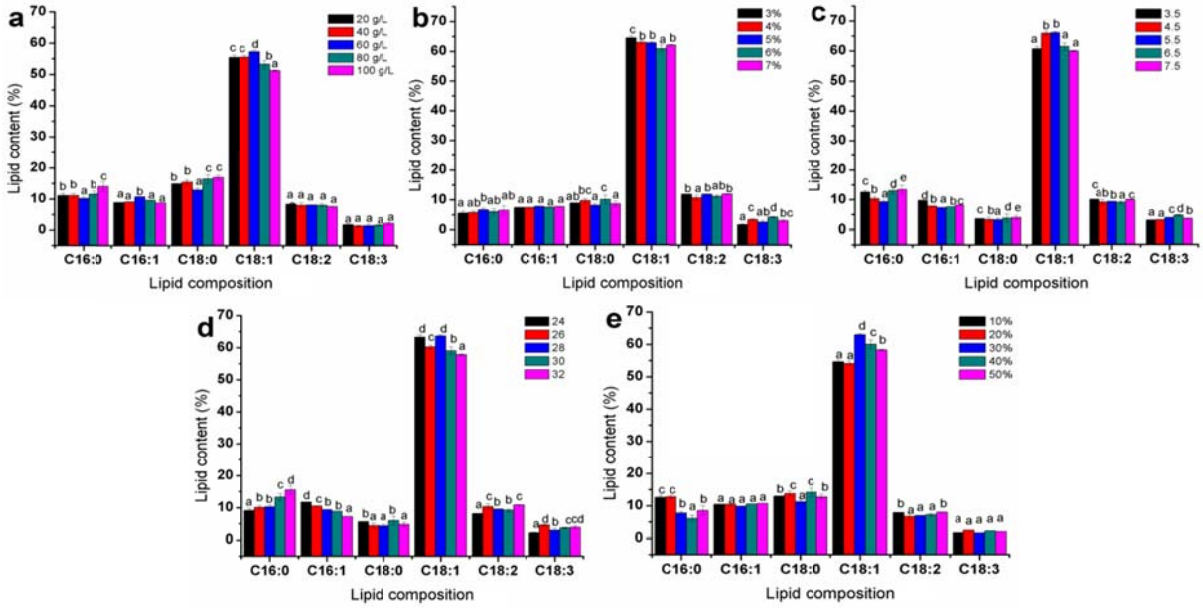

Fig. 4. Lipid profiles of *Y. lipolytica* W29 cultivated on DSWPH in batch culture. (a) glucose concentration; (b) inoculum dose; (c) pH; (d) temperature; (e) DO. Reaction conditions: (a) the inoculum was 5%, temperature was 28 °C, pH and DO was nature; (b) the glucose concentration 60 g/L, temperature 28 °C, pH and DO was nature; (c) the glucose concentration 60 g/L, inoculum dose 5%, culture temperature 28 °C; (d) the glucose concentration 60 g/L, inoculum dose 5%, pH 5.5, DO was nature; (e) the glucose concentration 60 g/L, inoculum dose 5%, pH 5.5, culture temperature 28 °C.

From Fig. 4b, the inoculum dose has a little effect on lipid distribution. The changed inoculum dose led to the significant variation of each fatty acid. Obviously, the pH, temperature, and DO have prominently effects on the cell growth and lipid profiles. From the results on Fig. 4c, pH maintained at 4.5, the UFAs achieved the

highest level. It was obvious that the content of C18:0 decreased sharply under the controlled pH value. When the pH was set at 4.5, ratio of UFAs was high but cells not grew very well. Similarly, the lipid profile and cell growth were also not consistent as temperature changed.

Temperature is one of the main factors that influence the FAs produced by yeasts. When the yeast grown at a low temperature, the ratio of UFAs/SFAs will be increased, as well as membrane fluidity. Previously, the proportion of UFAs in *Y. lipolytica* was found to be higher at lower temperatures [37]. Total fatty acids (TFAs) profiles have shown an apparent increase at the lower temperature with a less significant decrease of UFAs level. The content of C18:1 and the other UFAs were improved on the highest level when the DO raise up to 30%.

Kinetic model for lipid fermentation

The fermentation cycle was 72 h when cells grow on the medium made by DSWPH. Fig. 1 showed the first 16 h was the lag phase. At this stage, yeasts grow slowly and consumed nutrition for cell growth. Therefore, it did not accumulate lipid at the first 16 h. From 16 h to 56 h, the exponential phase, abundant cells were collected and glucose was utilized at the short time. At 24 h, the cells start accumulating the lipid. Clearly, after 24 h cultivation entered the stage of lipid formation. During this stage, the longer cultivation time, the more lipid yeast produced. After 56 h, the fermentation entered the stable stage. The DCW almost had no change and maintained at 13.81 g L^{-1}, but lipid concentration still rise. At the end of 72 h cultivation, the lipid concentration and DCW were both retained at the stable value.

Because of the "S" like shape of the growth curve, Logistics equation was applied to fit the strain growth model. The results well reflected the cell growth when *Y. lipolytica* accumulated the lipid (Fig. 5a). Equations used to model growth of *Y. lipolytica* W29 was shown in Table 3. The experimental values fitted with nonlinear formula, and the relationship between *X* and t was shown as:

Table 3 Equations used to model growth of *Y. lipolytica* W29 on glucose and DSWPH.

Item	Kinetic equation
Microbial growth rate (Eq.2)	$\frac{dX}{dt} = \mu_m X(1-\frac{X}{X_m})$
Lipid production rate (Eq.3)	$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X$
Glucose consumption rate (Eq.4)	$-\frac{dS}{dt} = \frac{dX}{dt}\frac{1}{Y_{X/S}} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + mX$

X: non lipid biomass concentration (g/L) at time t (h), X_0=0.106; μ_m: maximum specific growth rate (1/h), 0.191; X_m: maximum carrying capacity (g/L), 13.821; P: lipid concentration (g/L) at time t (h); α: growth-associated lipid production parameter (g/g), 0.141; β: non growth-associated lipid production parameter (g/g/h), 0.004; S: residual glucose concentration (g/L) at time t (h), S_0=39.183; $Y_{X/S}$: non lipid biomass yield coefficient (g/g), 0.345; $Y_{P/S}$: lipid yield coefficients, respectively (g/g), 0.118; m: maintenance constant, 0.010.

$$X = \frac{1.464e^{0.191t}}{13.715 + 0.106e^{0.191t}} \quad (5)$$

Here, X_0=0.106 g/L, X_m=13.821 g/L, μ_m=0.191 h^{-1}. From Fig.5a, the simulated dates were close to the actual values. This maximal growth rate was very similar to those reported for *Cryptococcus curvatus* grown on acetate (0.19 h^{-1}) [33] and *Y lipolytica* grown on glucose (0.27 h^{-1}) [38]. In addition, the R^2=0.997 verified that the model of confirmatory factor analysis fitted well.

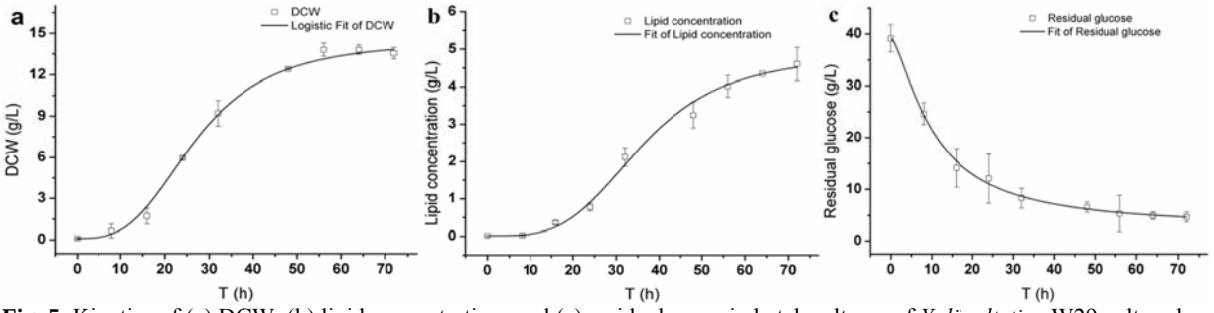

Fig. 5. Kinetics of (a) DCW, (b) lipid concentration, and (c) residual sugar in batch cultures of *Y. lipoltytica* W29 cultured on DSWPH medium with time-corrected Luedeking-Piret models. Culture conditions: initial glucose concentration 40 g/L; DO was 30% of saturation; pH 5.5; incubation temperature 28 °C.

Fig.1 shows a delay of lipids accumulation was found compared to the cell growth. Hence, the Luedeking-Piret equation was adopted to analyze the relationship between cell growth and lipid accumulation (Eq.3). Combining the integrated form of Eq.3 with Eq.2 and Eq.5, the model of lipid production were established. Considered the first 8 h, rare lipid was produced. The production of lipid was described as Eq.6. At the first 8 h, cell growth and lipid accumulation were ignored (Fig. 5b).

$$P = \alpha \frac{X_0 X_m e^{\mu m l}}{X_m - X_0 + X_0 e^{\mu m l}} + \beta \frac{X_m}{\mu_m} \ln\left(\frac{X_m - X_0 + X_0 e^{\mu m l}}{X_m}\right) \quad (6)$$

The experimental values fitted the nonlinear formula, the relationship between P and t was shown as:

$$P = \frac{0.208 e^{0.191t}}{13.715 + 0.106 e^{0.191t}} + 0.289 \ln\left(\frac{13.715 + 0.106 e^{0.191t}}{13.821}\right) \quad (7)$$

Based on the model, the value of α *and* β were calculated (α=0.141, β=0.004). From Fig.5a, the data from simulation were corresponding with the actual values. In addition, the R^2=0.996 verified that the model of confirmatory factor analysis fitted well.

During the fermentation stage, the glucose was mainly consumed in cell growth (Fig. 5c). Here we have integrated formula on the relation between S and t, which was based on the Eq.4 and Eq.6.

$$S = S_0 - \left(\frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}}\right)\left(\frac{X_0 X_m e^{\mu m l}}{X_m - X_0 + X_0 e^{\mu m l}}\right) - \frac{X_m}{\mu_m}\left(\frac{\beta}{Y_{P/S}} + \mathrm{m}\right) \ln \frac{X_m - X_0 + X_0 e^{\mu m l}}{X_m} \quad (8)$$

The experimental values were fitted with nonlinear formula, the relationship between P and t was shown as:

$$S = 39.183 - \frac{5.999 e^{0.191t}}{13.715 + 0.106 e^{0.191t}} - 2.566 \ln \frac{13.715 + 0.106 e^{0.191t}}{13.821} \quad (9)$$

On the Eq.9, S_0=39.183 g/L, $Y_{x/s}$=0.345 g/g, $Y_{p/s}$=0.118 g/g, m=0.01. The model described on Fig. 5 fit the experimental glucose consumption data well with a high R^2 of 0.996.

Model application in fed-batch cultivation

The kinetic parameters obtained *via* the batch experiments were used to simulate different feeding policies, which provide an insight on the operational protocol that may be implemented to obtain the high lipid production. If lipid production is able to be controlled, regulation of the environmental variables is required to maximize the stability of the metabolic state. This stability can be achieved through the precise control of culture conditions.

In fed-batch culture, feeding flows are monitored to control the specific growth rate for lipid accumulation of *Y. lipolytica*. Fig. 6 showed the fed-batch fermentation kinetics with concentrated DSWPH as the substrate. From 24 h to 27 h, stock solution (160 g L^{-1} glucose and 40 mL L^{-1} DSWPH) was added to the culture medium and the final glucose concentration was around 74.20 g L^{-1}. Dilution rate was 0.13 h^{-1} below the μ_m, full working volume was 2.0 L, and the added solution volume was 0.4 L. According to the the yeast cell growth, lipid accumulation, and lipid distribution, the feeding strategy was preliminary determined. At the first stage, the curve was similar with the batch culture curve discussed before. At 24 h, the residual glucose was 12.91 g L^{-1} and cell growth entered exponential phase. The yeast has the sign of lipid accumulation, though barely lipid was accumulated. After adding the feeding medium, a large number of cells were generated.

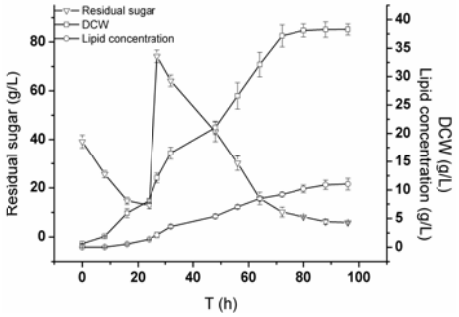

Fig. 6. Time course of fed-batch fermentation on residual glucose, DCW, and lipid concentration of the *Y. lipoltytica* W29 cultivated in the 3.0 L fermentor with a 2.0 L DSWPH medium. The feeding medium was containing 160g/L glucose and 40 mL/L DSWPH. The feeding time was began at 24 h and end at 27 h.

At the second stage, from 27 h to 36 h, the curve of DCW climbed quickly. Meanwhile, the cells start accumulating lipid stably. During the whole stage, after 24 h, DO and pH were maintained at 30 % and 4.5, respectively. The temperature changed to 24 °C just after 72 h cultivation. Finally, the DCW of 38.3 g L^{-1} and lipid concentration of 11.03 g L^{-1} were achieved after 96 h. Compared to the batch culture, the culture time extended 24 h, and the DCW and lipid concentration increased.

The results of this experiment were compared to other reported studies in Table 4. Volatile fattyacids fats [3] was used and higher lipid content were achieved with 43%, biomass obtained (3.5 g L^{-1}) was lower than that in this study (38.3 g L^{-1}). Because oleaginous microorganism has an ability to transform organic acids into acetyl-CoA, which is a central intermediate in the lipid biosynthesis [39]. It was also noted that despite continuous process achieved higher volumetric lipid productivity, but fermentation time were too long (168 h and 450 h) [40, 41]. The results also indicate the fed-batch is a more effective method than batch to investigate lipid production of *Y. lipolytica*. Thus, the lipid productivity obtained in this study is encouraging, and presented a significant improvement than before. Further research, including the expression and regulation of key enzymes, is necessary to determine the specific mechanism of DSWPH in enhancing biomass of *Y. lipolytica*.

Table 4 Applications of *Y. lipolytica* W29 use different waste sources as inexpensive alternative substrates.

Y. lipolytica	Medium	Mode culture	DCW (g/L)	Lipid content (%, w/w)	Fermentation time (h)	Lipid productivity (mg/L/d)	Refs
JMY4086	Molasses and crude glycerol	Continuous	60	31	450	430	[41][a]
LGAM S(7)1	Industrial glycerol	Continuous	8.14	43	168	120	[40][b]
MUCL 28849	Volatile fatty acids	Two-stage fed-batch	31	40	60	206	[3][c]
W29	Glucose and olive mill wastewater-based media	Flask	6.8	27.9	72	26	[7]
-	Non-detoxified liquid wheat straw hydrolysate	Flask	7.8	4.6	144	2	[6]
W29	Glucose and DSWPH	Batch	13.9	34.6	72	67	[42]
W29	Glucose and DSWPH	Fed-batch	38.3	28.8	96	115	This study

[a] The strain was cultured for 450 h in a chemostat containing a nitrogen-limited medium (dilution rate of 0.01 h^{-1}, 250 g/L crude glycerol), and volumetric lipid productivity was 0.43 g/L/h and biomass yield was 60 g CDW/L.
[b] This was produced in highly aeracted continuous. Lipid production was favoured at low specific dilution rate whilst fat-free material yield increased over the whole range of specific dilution rate.
[c] The strain was initially grown on glucose or glycerol as carbon source, then sequential additions of acetic acid under nitrogen limiting conditions were performed after glucose or glycerol exhaustion.

Conclusions

This work showed that the lipid content and the fatty acid profile of *Y. lipolytica* W29 can be manipulated by changing the growth conditions. Based on the ANOVA test, the pH, DO, and temperature have significant effect on lipid accumulation and distribution. In the fed-batch, culture conditions were optimized for lipid accumulation with 38.3 g L^{-1} DCW, 11.03 g L^{-1} lipid, and 87.23% ratio of UFAs in TFAs achieved after 96 h.

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The authors have declared no conflicts of interest.

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Electronic Supplementary Material

Table S1 The microwave digestion procedure for the determined silkworm pupa samples.

Step	Power (W)	Temperature (°C)	Heating time (min)	Holding time (min)
1	800	120	2	2
2	800	160	8	8
3	800	185	10	10
4	800	190	10	20

Table S2 The optimized operating parameters of ICP-MS.

Parameter	Set value
RF power	1250W
Outer gas flow rate	13.0 $Lmin^{-1}$
Intermediate gas flow rate	0.8 $Lmin^{-1}$
Nebulizer gas flow rate	0.93 $Lmin^{-1}$
Oxide percentage	<3
Focus voltage	11.4 V
Voltage of the detector	3000 V
Repeated sampling times	3

Table S3 Linearity ranges standard curve equations and correlation coefficients of measured metal elements.

Metal element	Linearity range (μg/L)	Standard curve equation	Correlation coefficient
^{65}Cu	$0.16\sim10^2$	$y = 314x + 5834$	0. 9997
^{66}Zn	$19.13\sim10^5$	$y = 549x + 34218$	0. 9999
^{56}Fe	$61.03\sim10^5$	$y = 2329x + 69045$	0. 9999
^{55}Mn	$0.26\sim10^2$	$y = 25135x + 6332$	0. 9999
^{24}Mg	$66.41\sim10^5$	$y = 502x + 30172$	0.9998
^{39}K	$61.07\sim10^5$	$y = 1056x + 102906$	0.9997
^{40}Ca	$12.03\sim10^5$	$y = 1546x + 2756212$	0.9997
^{60}Ni	$0.29\sim10^2$	$y = 2046x + 4123$	0.9998