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RESEARCH PAPER

Optimization of Culture Conditions for Lipid Production by *Rhodosporidium toruloides*

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Abstract: The effects of culture conditions on the lipid production by *Rhodosporidium toruloides* Y4 were investigated using uniform design principles and single-factor experiments. The optimal medium was obtained as follows: 70 g/L glucose, 0.1 g/L $(NH_4)_2SO_4$, 0.75 g/L yeast powder, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L KH_2PO_4, sterilized at 121 °C for 15 min, and then supplemented with 1.91×10^{-6} mmol/L $ZnSO_4$, 1.50 mmol/L $CaCl_2$, 1.22×10^{-4} mmol/L $MnCl_2$, and 1.00×10^{-4} mmol/L $CuSO_4$. The optimal fermentation conditions were also developed as: 50 mL of medium (pH 6.0) in a 250-mL Erlenmeyer flask with 10 % inoculum (28-h-old) under orbital shaking at 200 r/min for 120 h at 30 °C. Under the optimized conditions, yeast accumulated lipids up to 76 % on a cellular biomass basis with biomass yield of 18.2 g/L.

Key Words: *Rhodosporidium toruloides*; microbial lipids; culture optimization; uniform design

Some microorganisms including bacteria, yeasts, molds, and algae can accumulate lipids over 20 % of their dry biomass[1]. For some oleaginous yeasts and molds, over 70 % of the cellular biomass can be lipid[2]. It has been known that microbial oil technology could be operated at various operation conditions with a much higher growth rate than that by using plants. Microbes can be genetically optimized and used to produce oils in closed manufacturing systems using cheap substrates such as crop residues, wood wastes, municipal solid waste (MSW) biomass, or even pyrolysis oils. In recent years, modern biotechnology and bioengineering developments have endorsed high microbial oil production and/or high content of rare fatty acids, such as polyunsaturated fatty acids, which promote the techno- economical feasibility of microbial oil technology to a large extent[3–5]. There has also been a better understanding of biosynthetic machinery and metabolic regulation mechanism for microbial oil accumulation[6]. As petroleum resources have been increasingly diminished, it has been realized that sustainable development requires fuels and chemicals be produced from carbohydrates through bioconversion. Therefore, cost-effective microbial oil has been suggested for biodiesel production in the near future[7]. In this article, we optimized the medium composition and fermentation conditions for lipid production using *Rhodosporidium toruloides* Y4 through single-factor and uniform-design experiments.

Uniform design is a process optimization strategy developed by Chinese mathematicians Fang KT and Wang Y. This method reduces the number of experiments when many factors are selected at different levels[8], which has been applied in the development of the flight-guided missile in China. Now, uniform design has also been used in the laboratory's experimental design[9].

1 Materials and methods

1.1 Microorganism and media

Rhodosporidium toruloides AS2.1389, originally from China General Microbiological Culture Collection Center (CGMCC), had been domesticated five times from

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lingocellulosic hydrolysate to obtain a strain *R. toruloides* Y4, which is used in this study. This strain can ferment and accumulate lipids at low pH (less than 2.0); it shows higher biomass and intracellular lipid production compared to those of the initial strain.

Yeast strains were maintained at 4 °C on yeast extract peptone dextrose (YEPD) slopes containing: glucose 20 g/L, peptone 10 g/L, yeast extract 10 g/L, and agar 20 g/L. Precultures were produced in YEPD broth (glucose 20 g/L, peptone 10 g/L, yeast extract 10 g/L as seed culture). Initial nitrogen-limited medium was composed of (g/L): glucose 70, yeast extract (nitrogen content 7 % *W*/*W*) 0.5, $(NH_4)_2SO_4$ 2.0, KH_2PO_4 1.0, $MgSO_4·7H_2O$ 0.5, with no pH adjustment unless otherwise notified. Uniform design of the medium composition was carried out as shown in Table 1 and Table 2. Single-factor experiment was carried out by adding metal ions into the medium obtained in Uniform design 1. The medium was further optimized according to Uniform design 2. All media were sterilized by autoclave at 121 °C for 15 min.

1.2 Culture conditions

Shake flask experiments were carried out in 250-mL Erlenmeyer flasks containing 50 mL nitrogen-limited medium and inoculated with 5 mL 20-h-old preculture (around 2.0×10^7–3.0×10^7 cells/mL). All cultures were incubated in an orbital shaker at 200 r/min for 96 h, unless otherwise specified.

1. 3 Analytical methods

Total lipid was extracted according to known methods[10]. In brief, wet cells in 20-mL broth culture were harvested by centrifugation, washed with distilled water, and dried at 105 °C to a constant weight to obtain biomass (g/L). Lipid content is calculated on the basis of total cellular lipid over dried biomass.

Glucose concentration was determined using a SBA-50B glucose analyzer (Shandong Academy of Sciences, China).

1.4 Experimental design

1.4.1 Uniform design 1: Based on the previous data, yeast strain Y4 needed magnesium and phosphate for lipid production. Therefore, $MgSO_4·7H_2O$ and KH_2PO_4 concentrations were fixed at 0.5 g/L and 1 g/L, respectively. As microbial oil productivity was drastically affected by carbon-to-nitrogen (C/N) molar ratio of the culture, glucose was selected as the carbon and energy source, ammonium sulfate as the inorganic nitrogen source, and yeast extract as the organic nitrogen source. Uniform design 1 involved three factors: glucose, ammonium sulfate, and yeast extract concentrations. These were selected as process parameters, whereas lipid content was selected as the response parameter (dependent parameter). The experiment design, data analysis and modeling were performed using uniform design software UD 3.0 developed by the Uniform Design Sub-society of Chinese Mathematical Society. As shown in Table 1, fifteen experiments were designed so that each factor had 15 levels.

Table 1 Uniform design matrix of the medium composition

Trial No.	Glucose/(g/L)	$(NH_4)_2SO_4$/(g/L)	Yeast extract/(g/L)
1	70	1.0	0.15
2	75	0.8	0.60
3	80	3.0	0.05
4	85	0.2	0.35
5	90	0.1	0.75
6	95	0.5	0.50
7	100	2.0	0.40
8	105	0.3	0.10
9	110	1.5	0.70
10	115	0.7	0.30
11	120	2.5	0.65
12	125	0.4	0.45
13	130	0.9	0.20
14	135	3.5	0.25
15	140	0.6	0.55

1.4.2 Uniform design 2: In contrast to the results of Uniform design 1 and Single-factor design, we found that the medium C/N ratio and the metal ion concentration were the key factors affecting lipid accumulation (*vide infra*), but the biomass was no more than 10 g/L. To further improve biomass production, $MgSO_4$, KH_2PO_4, and K_2HPO_4 were considered as additional process parameters, and experiments were carried out based on data in Table 2 from uniform design software UD 3.0.

Table 2 Uniform design matrix of inorganic salt composition

Trial No.	KH_2PO_4 / (g/L)	K_2HPO_4 / (g/L)	$MgSO_4·7H_2O$ / (g/L)
1	0.5	0.5	0.30
2	1.0	1.5	0.45
3	1.5	2.5	0.25
4	2.0	0	0.40
5	2.5	1	0.20
6	3.0	2	0.35
7	3.5	3	0.50

1.4.3 Single-factor design: Various amounts of mineral salts were supplemented into the resulting medium from Uniform design 1, to investigate the effects of metals on lipid production. All experiments were done in duplicate.

2 Results and discussion

2.1 Effect of carbon-to-nitrogen mole ratio on lipid accumulation

Fifteen trials were performed to locate the optimal medium composition as shown in Fig. 1.