Medium optimization for biotechnological production of single cell oil using *Yarrowia lipolytica* M_7 and *Candida* sp.

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Abstract

Microbial lipids have a great similarity to the lipids obtained from plants and animals. Triacylglycerol is the storage lipid in most of eukaryotic cells; this characteristic attracts a lot of attention for using these lipid sources in biodiesel production. Accumulation of neutral lipid composed of triacylglycerol and sterylster is an induced response to environmental stresses in many living organisms. In this situation lipid accumulates as intracellular lipid bodies in yeast cells. Production of microbial oil has more cost than plant's oil. In this case, reducing the cost of this process must be done by optimization of culture conditions to reach higher production yield. In this study the effect of physical parameters on lipid production of two oleaginous yeasts: *Yarrowia lipolytica* M7 and *Candida sp.* was investigated. The mentioned parameters were pH range of 4-7; centrifugation rates of 100, 150, 200 rpm; temperature of 15, 25, 35 and 45ºC and times of incubation of 24, 48, 72 and 96 h. Temperature and time of incubation had a significant effect on lipid production by these strains and optimization of them resulted in increased production of lipid from 25% to 34% in *Yarrowia lipolytica* M7.

Keywords: physical parameters, oleaginous yeasts, *Yarrowia lipolytica* M_7

Introduction

Eukaryotic cells can accumulate lipid in intracellular lipid bodies. The structure of these lipid droplets is similar in all eukaryotic cells with a hydrophobic nucleus and a phospholipid layer around it (Drucken, 2008; Mullner and Duam, 2004; Melickova et al., 2004). The similarity of the lipid accumulated in microorganisms such as molds and yeasts is very important because it can be used as the substrate for biodiesel production and many other industrial purposes. Yeasts cells that can accumulate lipid more than 20% of their biomass are called as oleaginous yeasts (Meng et al., 2009; Liu et al., 2010). Among oleaginous yeasts less than 5% of them can accumulate more than 25% of lipid (Manuel et al., 2011). Two important enzymes i.e. malic enzyme and ATP-citrate lyase are involved in lipid accumulation process. There is a great relationship between ATP-citrate lyase activity and potential of lipid accumulation in yeasts cells (Meng et al., 2009; Fidler et al., 1999; Fei et al., 2008). Lipid body formation starts at the end of exponential phase and continues during stationary phase (Raschke and Knorr, 2009). When nitrogen limited condition occurs, nicotine amid adenine dinucleotide isocitrate dehydrogenase activity reduced and affect the tricarboxilic acid cycle, change the metabolism pathway and interrupt protein synthesis, resulting in the activation of lipid accumulation process (Pan et al., 2009; Wynn and Ratledge, 2005). Beyond nitrogen limitation, phosphate limitation can also improve lipid accumulation in oleaginous microorganisms (Muniraj et al., 2013).

Oleaginous micro-organisms attract a lot of attention because of their high growth rate and ability to use different carbon sources (Economou et al., 2011; Liu et al., 2010). Also they have short life cycle and are resistant against climatic and seasonal changes, so they have good advantages over plants, being used as oil producing organisms (Li et al., 2008; Amaretti et al., 2008; Zhao et al., 2008). Substituting of microbial lipid instead of plant's oil for biodiesel production is a developing idea (Fakas et al., 2008; Karatay and Donmes, 2010).

Physical parameters such as pH, shaker rpm, time and temperature of incubation can effect on lipid production in oleaginous yeasts (Li et al., 2008). Lipid production decreases remarkably in pH 4 and 8 (Syed et al., 2006). The optimum pH is not only different for various oleaginous yeasts but

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also is different for different carbon sources (Angerbauer et al., 2008). About the temperature of incubation, oleaginous yeasts have two groups: the first group has higher lipid production in lower temperature (25-30ºC) and the second group has higher lipid yield when increasing in temperature accrues 35-45ºC. The composition of the lipid also varies in different temperatures (Saxena et al., 2009). Time of incubation and shaker rpm also effect lipid production (Leesing et al., 2011). According to this information about effective parameters on lipid yield of oleaginous yeasts, they need different culturing conditions for optimal lipid production. In this study, the effect of physical parameters such as pH, shaker rpm, temperature and time of incubation on lipid production of *Yarrowia lipolytica* M_7 and *Candida sp.* was investigated. Optimization of these physical parameters cause higher lipid production by the evaluated yeasts and have shown their potential for industrial application. Optimization is an essential step of each industrial process because it can result in higher production under economical cost. The important parameters that determine the cost of microbial oil are the substrate cost, production rate and the ultimate lipid concentration (Meester et al., 1996). For increasing the rate of production and concentration of the product, optimization of culture condition, has great importance. FTIR spectroscopy was used to confirm the composition of produced lipid and the results showed the potential of this lipid in biodiesel production.

Materials and Methods

Preparation of inoculums

The oleaginous yeast colonies were first streaked on to YPD (Yeast Extract Peptone Dextrose agar) plates and incubated for 2 days. After that they were transferred in to 250 ml Erlenmeyer flask containing 50 ml of inoculation medium containing: glucose 15g/L, $(NH_4)_2SO_4$ 5g/L, KH_2PO_4 1g/L, $MgSO_4.7H_2O$ 0.5g/L, and yeast extract 0.5g/L and were grown at 28ºC on a shaker at 180 rpm for 2 days(Pan et al., 2009). *Yarrowia lipolytica* M_7 was isolated previously (Mirbagheri et al., 2012) (GenBank accession number, HM011048) and further studies on this strain was done by our research group. *Candid* sp. was isolated from peanut garden and its potential for lipid production was evaluated. Identification of the strain was not important in this study because the work was focused on higher lipid production by doing optimization process for industrial applications.

Preparation of production medium

5 ml of inoculums was transferred to 45 ml of nitrogen-limited medium containing: glucose 40 g/L, $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 7 g/L, NaH_2PO_4 2 g/L, $MgSO_4.7H_2O$ 1.5 g/L, yeast extract 1 g/l, $CaCl_2$ 0.15 g/L, $MnSO_4.H_2O$ 0.06 g/L, $ZnSO_4.7H_2O$ 0.02 g/L and $FeCl_3.6H_2O$ 0.15 g/L in 250 ml Erlenmeyer flask and incubated in a rotary shaker at 100 rpm, pH 4 and 28ºC for 48h (Pan et al., 2009; Papanikolaou et al., 2001; Kraisintu et al., 2010). This condition is only a trial one to evaluate the lipid production by FTIR spectroscopy. After this step one factorial method was used for evaluating physical parameters on lipid production. First of all, the pH was optimized by changing pH of the culture from 4 to 5, 6 and 7, keeping the other parameters constant and the same method was applied to optimize all other parameters. In one factorial method experiments must be done step by step and in each step only one parameter is variable. The first column of table 1 and 2 shows the variable factor in each trial conditions.

Table1. Lipid yield (g/L), lipid content (%) and biomass (g/L) of *Candida sp.* in different condition

Cultivation condition	Lipid yield (g/L)	Lipid content (%)	Biomass (g/L)
pH			
4	3.01	21.48	14.01
5	3.58	22.85	15.66
6	**3.73**	**22.90**	**16.28**
7	3.15	23.21	13.57
agitation speed (rpm)			
100	3.72	22.90	16.24
150	3.85	23.50	16.38
200	**4.15**	**24.25**	**17.11**
Temperature (°C)			
15	3.80	23.15	16.41
25	**4.25**	**25.16**	**16.89**
35	3.26	22.28	14.71
45	2.80	21.37	13.10
Time of incubation (h)			
24	4.01	24.28	16.51
48	4.35	26.18	16.61
72	4.72	28.72	16.43
96	**4.85**	**31.15**	**15.56**

Table2. Lipid yield (g/L), lipid content (%) and biomass (g/L) of *Yarrowia lipolytica* M_7.

Cultivation condition	Lipid yield (g/L)	Lipid content (%)	Biomass (g/L)
pH			
4	4.48	25.11	17.84
5	**4.78**	**25.80**	**18.52**
6	4.63	25.95	17.84
7	4.60	25.25	18.21
agitation speed(rpm)			
100	4.52	25.12	17.99
150	4.91	26.25	18.70
200	**5.25**	**27.30**	**19.23**
Temperature(°C)			
15	4.95	26.80	18.47
25	**5.59**	**28.15**	**19.40**
35	5.10	26.10	19.54
45	4.60	25.02	18.38
Time of incubation(h)			
24	5.35	29.14	18.35
48	5.68	32.50	17.67
72	**6.25**	**34.15**	**18.30**
96	5.26	31.52	16.68

Determination of lipid productivity to the dry biomass:

5ml of production cultures were harvested by centrifugation at 6000 rpm for 20 min. harvested biomass was washed twice with 5ml of distilled water and then dried at 80ºC to constant mass. The biomass was determined gravimetrically (El-Fadaly et al., 2009; Sriwongchai et al., 2013). Lipid content was determined by the following equation (Kraisintu et al., 2010).

Lipid content = SCO Weight (g/L) / Cell dry weight (g/L) × 100

Single cell oil extraction

Extraction of lipid was carried out according to Bligh and Dyer with modification (Pan et al., 2009). 40 ml of sample was centrifuged at 6000 rpm for 10 min. After that the yeasts were washed with 40 ml of distilled water. This step was repeated, and then 8 ml of 4 M HCl was added in to the biomass and incubated at 70ºC for 2 h. Then acid hydrolyzed mass was stirred with 16 ml chloroform/methanol mixture (1:1) at room temperature for 3 h. At the end centrifugation was done at 5000 rpm for 5 min at room temperature to separate the aqueous upper phase and organic lower phases. Then the lower phase containing lipid was recovered with Pasteur pipette and evaporated in the vacuum. After that the dry lipid was weighed.

Evaluating of physical parameters

The effect of pH varying from 4 to 7 on lipid production was investigated. The culture was prepared same as the production medium, mentioned in previous sections (in each trial condition only one factor was variable). For example at first, pH is the variable parameter and the others are constant; after optimizing this parameter, the other parameters were also optimized repeating the same procedure. After the optimization of pH, the agitation speed of 100, 150 and 200 rpm was evaluated. Cultivation temperature was varied from 15°C to 25°C and 35°C and also 45°C. At the end, the time of incubation at 24, 48, 72 and 96h was varied to evaluate lipid production at each time.

Single cell oil analysis by FTIR spectroscopy:

One of the techniques that can be used to confirm the composition of a product is FTIR spectroscopy. The basic of this method is creating peaks in a special spectrum based on cm^{-1} unit, so each chemical group has a specific peak at a certain point in determined spectrum. Confirmation of certain oil compounds was determined by FTIR spectroscopy using JASCO FT/IR-6300, Japan device. The range of spectrum analyzed by the device was set from 400cm^{-1} to 4000 cm^{-1}. Triolein was used as control sample for comparing with produced single cell oil.

Results

Table 1 and 2 show the results of lipid extraction for *Candida sp.* and *Yarrowia lipolytica* M_7, respectively. The results showed that lipid content of *Candida sp.* reached from 21.48% to 31.15% by optimizing physical condition. About *Yarrowia lipolytica* M_7 lipid content reached from 25.11% to 34.15%. Among physical parameters, temperature and time of incubation have more effect on growth and lipid content. Optimization of physical parameters as well as chemical factors can increase the lipid yield in oleaginous yeasts.

FTIR spectroscopy results

Microbial lipid graphs have been shown in figure 1. Comparison of two graphs shows significant similarity between extracted oil from oleaginous yeasts and the standard (triolein). Significant peaks were between 1670 to 1820 cm^{-1}, confirmed presentation of carbonyl groups. The peaks between 2850 to 2929 cm^{-1} show methyl groups. All of the

peaks in mentioned points showed that produced oil can be converted to biodiesel (Elumalai et al., 2011; European Standard EN 14078; Lin-Vien et al., 1991). Analyzing and confirming of biodiesel compound as an identification method is in number EN 14078 European standard (European Standard EN 14078). The X axis shows wave number which was set between 400 to 4000 (cm^{-1}) and the Y axis shows the percentage of different chemical groups in the evaluating material.

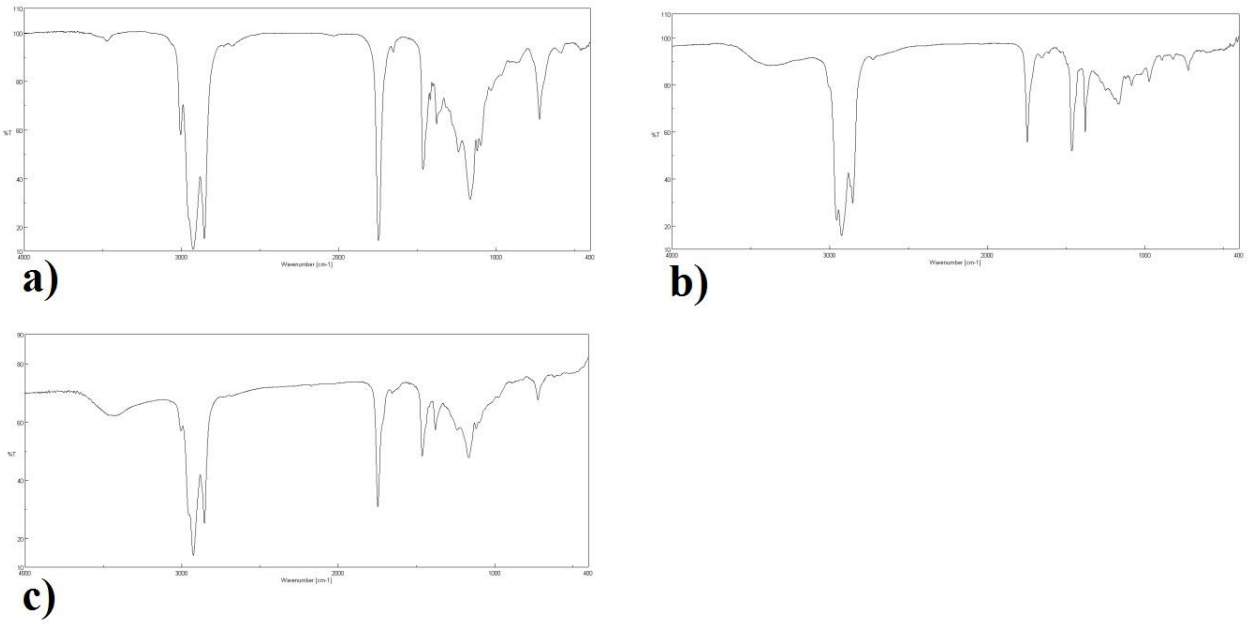

Figure 1: FTIR graphs, (a) FTIR graph of triolein standard, (b) FTIR graph of produced SCO by yeast *Yarrowia lipolytica* M7 (c) FTIR graph of produced SCO by yeast *Candida sp*.

Discussion

Oleaginous yeasts accumulate triacylglycerol rich in unsaturated fatty acids. These lipophylic microbial compounds, because of their special characteristics, are considered from industrial point of view. The first step for application of these oleaginous yeasts in industrial processes is optimization of culture condition. This step cause less time consuming and also less cost is required, so it become valuable from economical point of view. In previous study it was shown that optimization of chemical parameters such as carbon and nitrogen sources, carbon concentration and ammonium concentration can effect on lipid yield in oleaginous yeasts as well as physical parameters (Enshaeieh et al., 2012a). Also the effect of different carbon sources such as glucose, xylose, glycerol and rice bran and different nitrogen sources such as yeast extract, peptone, urea, (NH4)2SO4 and NH4Cl was investigated on lipid production in another study (Enshaeieh et al., 2012 b). Now in this study the optimization process was done by focusing on only the physical parameters.

Leesing et al., in 2011 evaluated lipid production in *Torulaspora globosa* YU5/2 and reported that lipid production decreased after 8 days incubation. Time of incubation for higher lipid production is different among various yeasts. In *Yarrowia sp.* increasing of incubation time, decrease lipid content because they consume the stored lipid after 80 h (papanikolao et al., 2001). Table 2 shows decreasing of about 1 g/L in lipid production of *Yarrowia lipolytica* M7 after increasing of incubation time from 72 h to 96 h. Results showed that by increasing rpm of agitation rate the oxygen that dissolve in the medium become higher and it increase growth and lipid content as metabolisms energy and synthesize of lipid components need oxygen. Accordingly, Oxygen content of the medium has positive relation with lipid accumulation (Liang et al., 2006; Yan et al., 2003; Yi et al., 2006; Tan and Gill, 1985; Choi et al., 1982).

pH of the medium has effect on lipid production by micro-organisms. The influence of pH on lipid production of *Rhodosporidium toruloides* DMKU3-TK16 was investigated by Karisintu et al., in 2010. They found that pH rate of 5.5 was the best one and

lipid production of this strain reach to 9.26 g/L after optimizing other parameters. Angerbauer et al., (2008) reported that in pH rate of 5 the lipid content of lipomyces starky was highest. Acidic and basic condition can affect yeast metabolism because it influence on the enzymes that are evolved in this process and also effect on other components of the yeast cell. For example Johson et al., in 1992 reported that a decrease in ergosterol content of the cell membrane happen as pH increases, so changing in cellular composition with pH seems important for the lipid production in oleaginous yeasts. Limited information about the reason of why pH influences on lipid production is available. The related studies just focus on the effect of pH on optimizing process and did not evaluate the molecular reasons. pH rates of 5-6 is better for higher lipid production in oleaginous yeasts(syed et al., 2006).

El-fadaly et al. in 2009 investigated the effect of incubation time, agitation speed, temperature and pH on lipid production of *Cryptococcus curvatus* NRRLY-1511. The optimized amount of these parameters were 72h, 28ºC, 200 rpm and pH of 5.5, respectively. Syed et al. (2006) showed lipid production decrease in pH rates of 4 and 8. pH of the medium can affect the ability of lipid production and the optimized pH for yeasts cell is between 5 to 6 (Zhu et al., 2008; Easterling et al., 2009; Li et al., 2005).

The results of this investigation showed that by optimization of physical parameters, increase in the lipid production can be done in oleaginous yeasts. By optimizing these factors, the process become more economical than usual and the lipid content of the yeasts become higher. In this investigation lipid content in *Candida sp.* and *Yarrowia lipolytica* M_7 were increased approximately about 10% after optimization. So by optimizing physical parameters higher lipid production and less cost of the process can be obtained.

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