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Selection and optimization of single cell oil production from *Rodotorula* 110 using environmental waste as substrate

Marjan Enshaeieh[1*], Azadeh Abdoli[1], Iraj Nahvi [2] and Mahboobeh Madani[3]

[1]*Department of Microbiology, Falavarjan branch, Islamic Azad University, Esfahan, Iran*
[2]*Department of Biology, Esfahan University, Esfahan, Iran*
[3]*Department of Microbiology, Falavarjan branch, Islamic Azad University, Esfahan, Iran*

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Abstract

Micro-organisms such as bacteria, yeasts, molds and algae that accumulate lipid more than 20% of their biomass are called oleaginous. Microbial lipid has high similarity to the oil obtained from plants and animals. Microbial lipids are renewable sources that can be used for different purposes such as biodiesel production. Production of oil from yeasts has more advantages than that from plants. Accordingly, isolation of oleaginous yeasts with high ability of lipid production is very valuable. In this study we isolated 176 yeasts from 34 soil samples, from which 68 could produce lipid. The strains were screened by an enrichment technique in glycerol and then Sudan black B staining. After lipid extraction by Bligh and Dyer method, the best strain, *Rodotorula* 110**,** was selected. This strain proved to comprise lipid, dry biomass and lipid productivity at levels of 8.9 g/l, 15.29 g/l and 58.2% in optimized conditions, respectively. Then lipid production by the selected strain was evaluated on corn stalk and wheat straw hydrolysate as sole carbon sources. Lipid content on these media was 38.9% and 43.4%, respectively. The extracted lipid was analyzed by thin layer chromatography and FTIR spectroscopy.

Keywords**:** Oleaginous yeast**,** lipid extraction**,** Microbial lipid, FTIR spectroscopy

Introduction

Oleaginous micro-organisms such as yeasts, fungi and micro algae can accumulate high amounts of reserved lipids under appropriate cultivation condition, so their potential as lipid producing sources has attracted high attentions. Unicellular yeasts have high growth rate and can accumulate lipid in separate lipid bodies (Li et al., 2008; Drucken, 2008; Mullner and Daum, 2004; Melickova et al., 2004). They can also use low cost fermentation media such as waste material of agricultural and industrial products (Amaretti et al., 2010). Storage lipids are in the form of tri acyl glycerol (TAG) so different types of fatty acids are the main target for improving the biotechnological products. Phospholipids, sphingolipids, glycolipids, sterols and carotenoids as well as other lipid compounds are used for production of bioactive molecules used in cosmetics, nutritional and pharmaceutical products (Schorken and Kempers, 2009). Microbial oil has potential to substitute the plant oil in market. Micro-organisms that produce lipid more than 20% of their biomass are called oleaginous (Wynn and Ratledge, 2005; Amaretti et al., 2010).

The great part of microbial lipid is TAG which contains long chain fatty acids and is comparable with conventional plant oil (Kosa and Ragauskas, 2010; Pan et al., 2009). Some yeasts have evolved sophisticated metabolic pathways that allow them to grow on lipid substrates. Some of oleaginous yeasts can metabolize pentoses. This shows the ability of TAG production from lignocelluloses substrates and other low cost materials (Sabirova et al., 2010; Li et al., 2007, Zheng et al., 2012). Yeasts spp. that are known as oleaginous include *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces* (Pan et al., 2009).

Rhodotorula is a pigmented yeast that can be identified easily by distractive orange/red colonies when grow on Sabouraud's Dextrose Agar. This color is the result of pigments that yeasts produce to block certain wavelengths of light and protect them. The color of colonies is very different from cream to orange, red, pink or yellow. This yeast has high potential of lipid production and some of its strains can accumulate lipid on xylose and lignocelluloses

∗Corresponding author E-mail:
m_enshaeieh@yahoo.com

substrate as sole carbon sources. This ability is important for using these strains in industrial processes (Postgate, 1994; Dai et al., 2007).

Lipid accumulation occurs when one of the nutrients (usually nitrogen) is exhausted. At the same time, there are excess amount of carbon such as glucose in the medium. The cell response to exhaustion of nutrients is that they don't grow and multiply, but they continue to take up glucose from the medium. The surplus sugar is used for lipid biosynthesis. Under nitrogen limited condition the first requirement for the cells is to cease production of energy (i.e. , ATP) that is no longer needed for the synthesis of macromolecules, such as proteins and nucleic acids; because the cells do not grow and divide any more. During nitrogen limitation, oleaginous and non oleaginous yeasts continue to assimilate carbon but only oleaginous organisms metabolize it and increase the ATP/AMP ratio. These cells become larger when lipid particles grow (Meng et al., 2009; Fei et al., 2008; Raschke and Knorr, 2009; Fakas et al., 2008; Wynn and Ratlege, 2005).

The purpose of this study was isolation of oleaginous yeast with high potential of lipid production and evaluating the efficiency of lipid extraction by Bligh and Dyer method from oleaginous yeasts. Optimization of medium condition and its effect on increasing lipid production was evaluated. Analysis of produced lipid was done by FTIR spectrometry. Also the potential of lipid production by this strain on agricultural residues was evaluated.

Materials and Methods

Isolation and selection of oleaginous yeast

34 soil samples were collected to isolate yeasts. The soils were from peanut, walnuts, sunflower and almond gardens. A mass of 1 g of soil was added in to 50 ml enrichment medium which includes (g/L): glycerol 100, $(NH_4)_2SO_4$ 1, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5 and yeast extract 0.2 in a 250 ml erlenmeyer flask, then incubated at 30ºC for 96 h with shaking at 180 rpm (Pan et al., 2009).

Then 0.5 ml of this pre-cultured yeasts was added to solid medium which includes glucose (20 g/L), $(NH_4)_2SO_4$ (2 g/L), KH2PO4 (0.5 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $CaCL_2.2H_2O$ (0.1 g/L) and 2% agar. The plates were incubated at 28ºC for 48 h. After incubation, the yeast colonies were isolated for screening process (Zheng et al., 2012).

Screening for oleaginous yeasts by qualitative methods

Lipid production of isolates was evaluated by Sudan black B staining. The potential oleaginous yeast colonies were maintained on YPD slant which contains (g/L): glucose 20, yeast extract 20 and peptone 20 at 4ºC.

Determination of lipid production in isolated yeast

After qualitative analysis by Sudan Black B staining, oleaginous yeasts were cultured in nitrogen limited medium for 5 days. This medium includes glucose (40 g/L), $(NH_4)_2SO_4$ (2 g/L), KH_2PO_4 (7 g/L), NaH_2PO_4 (2 g/L), $MgSO_4.7H_2O$ (1.5 g/L) and yeast extract (1 g/L). 50 ml of this medium in 250 erlenmeyer flask was used on a shaker at 180 rpm and 28ºC. Before culturing in nitrogen limited medium, the yeast colonies were activated in inoculation medium, containing (g/L): glucose (15), $(NH_4)_2SO_4$ (5), KH_2PO_4 (1), $MgSO_4.7H_2O$ (0.5) and yeast extract (0.5) grown at 28ºC at 180 rpm for 48h (Pan et al., 2009; Kraisintu et al., 2010).

Lipid extraction was done, based on Bligh and Dyer method with few modifications (Pan et al., 2009). 50 ml of the sample was centrifuged at 5000 rpm for 10 min. After that the yeasts were washed with 50 ml of distilled water. Then 10 ml of HCl (4M) was added and incubated at 60 ºC for 3h. Then acid hydrolyzed mass was stirred with 20 ml chloroform/methanol mixture (1:1) at room temperature for 3 h. At the end centrifugation was done at 5000 rpm for 3-5 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.

The ability of using xylose and subsequently lipid production with xylose as sole carbon source was investigated. Then the best strain was cultured on the nitrogen-limited medium which includes (g/L): xylose (40), $(NH_4)_2SO_4$ (1), KH_2PO_4 (7), $MgSO_4.7H_2O$ (1.5) and yeast extract (1) grown at 28ºC and 180 rpm in shaker incubator for 72 h (Pan et al., 2009).

Determination of single cell oil productivity (lipid content)

Lipid content in each trial condition was determined by the following equation (Kraisintu et al., 2010):

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

Analysis of single cell oil production by FTIR spectroscopy

Lipid production in oleaginous yeast was verified by sudan black staining at first, further confirmation of certain oil compounds was detected by FTIR (Fourier Transform Infrared) spectroscopy using JASCO FT/IR-6300, Japan device. The range of spectrum, analyzed by device, was set from 400 cm^{-1} to 4000 cm^{-1}. Triolein (bought from sigma Aldrich) was used as control sample for comparing with the produced single cell oil.

Effects of nutrient composition, pH, rpm, incubation time and temperature on lipid production

Effects of nutrient composition such as glucose and ammonium sulfate concentration and physical parameters such as pH, rpm, temperature and time of incubation on lipid production were evaluated. Glucose concentration was varied at 35, 55, 75, 95, 115g/L. Effect of combined organic nitrogen sources (yeast extract and peptone at 1 g/L) and inorganic compounds (ammonium sulfate and ammonium chloride at 1g/L) on lipid production were investigated. Also lipid production was evaluated under different ammonium sulfate concentration such as 0.5, 1, and 1.5 g/L. Other factors were variable; pH rates of 5, 5.5, 6, 6.5; temperatures of 25ºC and 35ºC; rpm of 150 and 200; and times of incubation of 24 h, 48 h, 72 h and 96 h (Kraisintu et al., 2010).

Lipid production using wheat bran and corn stalk as sole carbon sources

Before using wheat bran and corn stalk, they must be prepared by acid hydrolysis. For this purpose the materials were ground, then hydrolyzed by using sulfuric acid (5%). This digestion was performed at a solid:liquid ratio of 1:8, upon completion of the process they were autoclaved at 110°C for 25 min. Then the suspension was centrifuged to remove unhydrolyzed residues (Dai et al., 2007). 10 ml of this suspension was brought to 45 ml with sterile water. After adjusting the pH at 6, the other components (the same as nitrogen-limited medium) were added. 5 ml of the inoculation was added and incubated at the optimum condition.

Results

Isolation of oleaginous yeasts

174 yeasts were isolated, from which 68 were oleaginous according to our first qualitative analyses by Sudan black B staining. The yeast lipid bodies were obvious as black droplets inside the oleaginous yeast cells under optical microscope. Some of the strains had multi-lipid bodies. These strains were selected for lipid extraction after cultivation in nitrogen limited medium. The result of lipid extraction, dry biomass, lipid productivity, and the ability of xylose assimilation were shown in table 1.

As the results show the strain *Rodotorula* 110 (Yr_2) produced the highest amount of lipid. Also this strain could produce lipid in a medium with xylose as a sole carbon source. Yr_2 produced 6.17 g/L lipid per 17.82 g/L dry biomass and its lipid productivity was 34.62%. So this strain was selected for further investigation.

Effect of nitrogen source on lipid production in yeast

In the nitrogen limited condition the excess amount of carbon in the medium is used to produce lipid bodies by oleaginous yeasts. The effects of organic nitrogen such as yeast extract and peptone and inorganic nitrogen such as ammonium sulfate and ammonium chloride were determined in the nitrogen limited medium with 40 g/L glucose. Results showed that lipid production by this strain reached to its highest level at the presence of yeast extract and ammonium sulfate, although there was no significant difference for production examine between the evaluated nitrogen sources (table 2).

Effect of glucose and xylose as carbon sources on lipid production in yeast

Production of lipid in the medium with glucose as a sole carbon source was more efficient. Therefore glucose was used as carbon source for evaluating the yeast capacity for lipid production under different conditions. Lipid production on xylose with *Rhodotorula* 110, was 5.15 g/L in this study. Xylose is one of the main sugars in the hydrolysis of Lignocellulosic materials and environmental wastes, so oleaginous yeasts with capacity of using it are valuable from economical point of view.

Effect of glucose and ammonium sulfate concentration on lipid production

Table 2 shows that when glucose concentration increased from 35 g/L to 55 g/L and 75 g/L, the lipid production was also raised; but increasing of glucose concentration to 95 g/L and 115 g/L had reverse effect on lipid production because optimum concentration of glucose depends on the yeast strain and each strain has a different capacity for tolerating osmotic effect of sugar in the medium.

About ammonium salt the second concentration (1g/L) was the best one for lipid production. It has

been shown that nitrogen is necessary for growing but the limited condition is also important for lipid production.

Effects of physical parameters

The best temperature for lipid production by this strain was 25ºC and had significant effect on lipid production. Time of incubation had significant effect on lipid production too, but when agitation rates increased the lipid production decreased slightly. Lipid production after 24 h, 48 h and 72 h was 4.6 g/L, 6.03 g/L 8.85 g/L, respectively ; but after 96 h this production decreased to 7.95 g/l. The best pH for lipid production was 6.5 and lipid production at this pH with other optimized factors was 8.9 g/L.

Lipid production using agricultural residues

The results of lipid production in these media are shown in table 3. The results obtained in our study were excellent and showed high potential of lipid production by this yeast strain (Yr2).

FTIR spectroscopy analysis of lipid products

Microbial lipid graphs obtained from the FTIR analysis are shown in figure 1. Comparison of two graphs shows the highest similarity between extracted oil from oleaginous yeast with the standard oil of triolein. Significant peaks were created between 1670 to 1820 cm^{-1}, confirming presence of carbonyl groups. There are peaks between 2850 to 2929 cm^{-1} that show presence of methyl groups. All of the mentioned peaks confirm that the produced oil can be converted to biodiesel potentially (Elumalai et al., 2011; Lin-Vien,1991). Biodiesel compounds are analyzed based on the European standard of EN 14078 (European Standard EN 14078). FTIR has also been used for analyzing and confirmation of biodiesel based on the methy and ethyl ester of long chain fatty acids in products from *Chloralla vulgaris* and *Senedesmis* sp. (Elumalai et al., 2011).

Table 1. Results of lipid extraction, dry biomass, lipid productivity and Xylose assimilation for oleaginous colonies

strain	Lipid yield (g/L)	Dry biomass(g/L)	Lipid productivity (%)	Xylose assimilation
Y6	4.45	14.21	31.35	w
Yc1	2.13	8.61	24.78	++
Yb1	2.87	11.19	25.67	+
Yb2	2.19	9.18	23.88	++
Yd1	1.97	8.90	22.13	-
Yg1	2.06	9.17	22.53	++
Yq2	2.36	9.78	24.18	++
Ya2	2.35	10.06	23.35	-
Yr2	6.17	17.82	34.62	+
YK	2.16	8.08	26.78	+
YL2	1.94	8.16	23.81	-
Yq3	2.29	10.18	22.45	++
Yq1	2.36	10.02	23.57	+
Yt4	6.04	18.25	33.09	-
Yv2	3.86	13.87	27.88	+
Yh1	1.71	7.53	22.68	+
Yv1	1.92	8.37	22.92	+
Yu2	2.03	8.00	25.38	+
Yv3	3.04	10.72	28.37	+
yL3	3.67	14.22	25.85	++
Am1	3.95	16.91	23.39	-
An	2.91	11.80	24.33	+
Yu1	3.18	12.02	26.50	++
Ab1	4.97	15.47	32.15	+
Ak1	3.99	16.09	24.82	+
Yy1	1.95	8.83	22.17	++
Aa1	5.35	17.98	29.77	++
Ab2	5.09	15.15	33.62	-
Ad1	3.33	12.74	26.18	-
Ag1	4.88	19.93	24.48	+
Ai1	3.90	16.18	24.15	+
Aj	3.14	13.12	23.92	+
Ao1	4.13	15.08	27.38	w
Ao2	4.07	16.35	24.89	++
Ao3	3.30	14.04	23.55	++
Ap1	4.15	15.88	26.13	++

Ap2	4.07	16.15	25.25	++
Ap3	3.88	16.32	23.81	+
Aq1	4.44	17.65	25.16	+
Aw3	5.79	17.45	33.18	+
As1	4.09	16.44	24.87	+
Aa4	5.13	18.98	27.05	+
Ad2	5.03	15.95	31.54	w
Ai2	4.76	19.16	24.88	++
Ak2	3.67	14.56	25.22	++
AL2	3.89	15.46	25.17	+
Am2	4.15	16.07	25.85	++
Aq2	3.87	15.54	24.95	++
Aq3	3.45	14.03	24.63	++
Ar2	4.24	16.13	26.31	++
At1	3.94	16.80	23.45	++
Ay1	4.98	16.89	29.50	w
Az1	3.76	15.92	23.67	++
Az2	3.23	13.38	24.13	++
Bc2	3.77	15.86	23.78	+
Bd1	3.56	16.09	22.15	+
Bm	4.87	17.21	28.31	w
Bn	4.17	19.67	24.27	++
Be1	4.18	17.53	23.84	+
Bd2	3.25	9.79	33.20	++
Bc4	3.00	10.60	28.29	++
Bc1	3.57	12.83	27.82	-
Bb1	2.78	12.28	22.64	+
Ba1	4.41	17.28	25.52	w
Av2	4.12	16.54	24.90	+
Av1	3.92	16.21	24.18	++
Y29	3.92	16.03	24.45	+
At2	2.83	12.45	22.73	+

w: weak assimilation, +: positive (strains with ability of xylose assimilation), ++: high assimilation of xylose, - : negative (strains that could not grow on xylose as sole carbon source).

Table 2. Results of Yr2 cultivated in nitrogen limited medium at different conditions.

condition	**Lipid production (g/l)**	**Biomass (g/L)**	**lipid productivity(%)**
Nitrogen source			
Yeast extract and $(NH_4)_2SO_4$	6.29	17.57	35.78
Yeast extract and NH_4Cl	6.15	17.9	34.35
Peptone and $(NH_4)_2SO_4$	6.11	17.68	34.37
Peptone and NH_4Cl	6.18	17.88	34.55
Carbone source			
Glucose	6.3	17.45	36.1
Xylose	5.15	17.16	30
Glucose concentration(g/l)			
35	4.35	12.42	35
55	6.52	16.21	40.2
75	7.13	12.7	56.1
95	5.84	10.97	53.2
115	5.71	11.42	50
$(NH_4)_2SO_4$ (g/l)			
0.5	6.9	12.36	55.8
1	7.2	12.78	56.3
1.5	6.7	12.4	54
Temperature			
25ºC	7.23	12.86	56.2
35ºC	6.35	11.73	54.1
rpm			
150	7.34	13.04	56.28
200	6.83	12.64	54
Time of incubation			
24h	4.6	9.58	48
48h	6.03	11.59	52
72h	8.85	15.39	57.5
96h	7.95	14.14	56.2

pH			
5	8.83	15.49	57
5.5	8.35	14.54	57.4
6	8.58	14.84	57.8
6.5	8.9	15.29	58.2

Table 3. Lipid production on agricultural residues as sole carbon sources.

condition	**Lipid production (g/l)**	**Biomass (g/L)**	**lipid productivity (%)**
wheat bran	6.50	14.97	43.4
Corn stalk	5.84	15.01	38.9

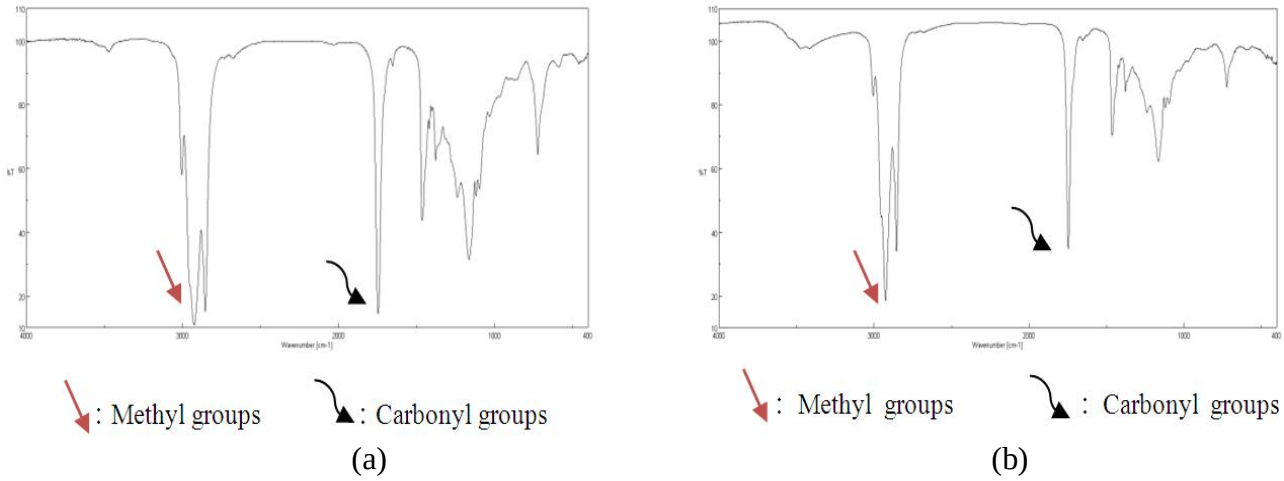

Figure 1. FTIR analytical graphs performed on the standard oil triolein (a) and the oil product from *Rhodotorula* 110 (b).

Discussion

SCO production from lignocellulosic materials, containing xylose, was carried out by some investigators (Zhao, 2005; Papanikolaou, 2008; Meester et al., 1996). Yeast strains that can use xylose in lignocellulosic hydrolysate have potential of industrial application (Dai et al., 2007). Evans and Ratledge (1984) evaluated lipid production in *Candida curvata* and its lipid content was 50% on xylose. Li et al. (2005) reported that lipid production in *Rhodosporidium toroloides* AS2.1389 was 10.6 g/L when using 100 g xylose. Pan et al (2009) isolated oleaginous yeasts with assimilating capacity of xylose and the best yeast strain could produce 5.8 g/L lipid while using 40 g/L xylose. Lignocellulosic materials are a good substrate for microbial oil production because of being low cost and also being abundance (Zheng et al., 2012; Khot et al., 2012). Lipid production on xylose with *Rhodotorula* 110, was 5.15 g/L in this study and with optimization of cultivation condition higher lipid production can be obtained.

About the effect of carbon source on lipid production, Ratledge (2002) reported that glucose was the most important factor on lipid production in oleaginous yeasts. Syed et al (2006) reported that increasing of glucose concentration to very high degrees had inverse effect on lipid production because of increasing osmotic potential of the medium; the same effect of glucose concentration on lipid production was obtained in our study.

About using environmental residues as sole carbon sources for lipid production in yeasts, Dai et al. (2007) used corn stalk and rice straw as sole carbon sources using *Rhodotorula glutinis*. The results for lipid content were 11.78% and 5.74% on corn stalk and rice straw, respectively. The yeast strain in our study had high potential of lipid production on lignocellulosic materials as sole carbon sources. The lipid productivity of this strain was 43.4% and 38.9% on wheat bran and corn stalk, respectively.

The results showed that this strain (*Rhodotorula* 110) is a high lipid producing yeast with potential of industrial applications. Its lipid yield reached to 8.9 g/L with lipid content of 58.2% after 72 h at 25ºC when cultivated in nitrogen limited medium. This medium included: 75 g/L glucose, 1 g/L ammonium sulfate and 1 g/L yeast extract with pH adjusted on 6.5 in shaking flask at 150 rpm. It could assimilate xylose and produce lipid on

lignocellulosic hydrolysate as sole carbon source. This ability shows the potential of this native strain for industrial lipid production.

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