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Bioconversion of different carbon sources into microbial oil and biodiesel using oleaginous yeasts

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ABSTRACT

Micro-organisms such as bacteria, yeasts, molds and algae that store lipid more than 20% of their biomass are called Oleaginous. Microbial lipid has a lot of similarity to the oil obtained from plants and animals and this similarity is valuable because it can be used for biodiesel production with many environmental benefits. Production of microbial oil is valuable when the cost of production is decreased by optimization of cultivation condition and using low cost substrate. In this study the effect of carbon and nitrogen sources on lipid production was investigated. These parameters have the most important effect on lipid production in many oleaginous types of yeast. Among the carbon sources which were evaluated, glucose leads to higher lipid content but from economical point of view rice bran was the best carbon source. Between nitrogen sources, yeast extract and ammonium sulfate exhibited higher lipid content. *Rhodotorulla* 110 had lipid content of 40%, 36% and 30% in glucose, xylose and bran as carbon source respectively with yeast extract and ammonium sulfate as nitrogen sources.

Key words: Oleaginous yeast, Carbon source, Nitrogen source, Rhodotorulla 110, Microbial lipid

1. INTRODUCTION

Application of microbial lipid as superseding source of lipid has attracted attention from the first years of twentieth century (1). Another important characteristic of microbial lipid is its application in biodiesel production. Application of micro-organisms with ability of using some of low cost sources such as corn stalk, rice straw and other plant's and forestry residues for microbial oil production and ultimately biodiesel production is valuable from economical point of view (2, 3). Lipid production from oleaginous micro-organisms in a medium rich in carbon source occurs in a two stage process: in the first stage cell grows and increase their number. This step goes to the end by eliminating some nutrition except carbon. During the second stage the excess amount of carbon accumulate in the form of intracellular lipid bodies (4). Yeast geniuses applicable for lipid production are *Yarrowia*, *Candida, Rhodotorulla, Rhodosporidium, Cryptococcus*, *Trichosporon and Lipomyces*. Oleaginous strains can accumulate different type of lipids because according to the medium composition they can substitute their fatty acids in triglyceride and change their lipid composition (5). These geniuses can utilize different carbon sources or fats in the medium so they can change their lipid composition. Oleaginous yeasts have some advantages to the other microbial sources. For example the time that is needed for them to become two fold is less than one hour and also cultivation of them is easier than algae. According to variety of micro-organisms and cultivation condition, oleaginous yeasts are good sources for triglycerides, surfactants and poly unsaturated fatty acids (6). During nitrogen limiting stage oleaginous and non-oleaginous strains continue carbon assimilating but only in oleaginous organisms ATP/AMP ratio increases and lipid accumulation occur. The size of these cells increase as the lipid particles will become larger (7, 8). In non-oleaginous yeasts the excess amount of lipid can turn to poly saccharides or they stay without any utilization but in oleaginous yeast this amount of carbon change to lipid and accumulate in the form of TAG in lipid bodies of the cells

(4, 9). The important parameters that determine the cost of microbial oil are the cost of substrate, production rate and the ultimate lipid concentration (9). For increasing the rate of production and concentration of the product, optimization of cultivation condition, has great importance. The present study was done to optimize native oleaginous yeast in carbon and nitrogen sources. Different carbon sources such as glucose, xylose, glycerol, rice bran and also different nitrogen sources such as yeast extract, peptone and urea as organic sources and ammonium sulfate & ammonium chloride as mineral sources were evaluated for lipid production in oleaginous yeast *Rhodotorulla* 110 and *candida* 14.

2. MATERIALS AND METHODS

2.1. Yeast strains

Yeast strains used in this investigation were *candida* 14 and *Rhodotorulla* 110 that were isolated from soil samples and their lipid production was evaluated in different condition.

2.2. Preparation of inoculums

The oleaginous yeast colonies were first grown on YPD plates and incubated for 2 days. After that they were transferred in to 250 ml Erlenmeyer flask that contains 50 ml of inoculation medium containing (g per liter) Glucose 15, $(NH_4)_2SO_4$ 5, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5, and yeast extract 0.5 and in a shaker at 180 rpm for 2 days at 28ºC.

2.3. Preparation of flask culture

5 ml of inoculum was transferred to 45 ml of nitrogen-limited medium which contains (g per liter): Glucose (or other carbon sources) 40, $(NH_4)_2SO_4$ 1, KH_2PO_4 7, NaH_2PO_4 2, $MgSO_4.7H_2O$ 1.5, yeast extract 1, $CaCl_2$ 0.15, $MnSO_4.H_2O$ 0.06, $ZnSO_4.7H_2O$ 0.02 g/L and $FeCl_3.6H_2O$ 0.15 g/L in 250 ml Erlenmeyer flask and incubated in shaker at 180 rpm and 28ºC for 3 days (10-12).

2.4. Qualitative analysis by Sudan black staining

Sudan Black staining was used as a quick method for analysis of lipid production in the yeasts. Black lipid bodies can be observed with optical microscope.

2.5. Determination of yeast Dry mass

Portions of 5ml of fermented 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 (13).

2.6. Single cell oil extraction

Extraction of lipid was carried out according to Bligh & Dyer with modification. 40 ml of sample was centrifuged at 6000 rpm for 10 min. After that the yeasts was washed with 40ml of distilled water for two times. Then 8ml of 4M HCl was added and incubated at 60ºC for 2h. Then acid hydrolyzed mass was stirred with 16ml chloroform/ methanol mixture (1:1) at room temperature for 3h. Separation of the aqueous upper phase and organic lower phases was done by centrifugation at 5000rpm for 5min at room temperature. Then the lower phase containing lipid was recovered with Pasteur pipette and evaporated in the vacuum. After that the dry lipid was weighed.

2.7. Effect of nitrogen source on lipid production

Organic nitrogen sources used in this study were yeast extract, peptone and urea (1g/L) and mineral sources were ammonium sulfate and ammonium chloride (1g/L).

2.8. Effect of carbon source on lipid production

Different carbon sources such as glucose (75 g/L), xylose (75g/L), glycerol (75g/L) and rice bran (10 mL of hydrolyzed mass in 40 mL of distilled water) were used. After that we evaluate glucose concentration on lipid production. For using rice bran as carbon source it must be hydrolyzed by HCl 5% and incubated at 110ºC for 20 min before use.

2.9. Analysis of extracted lipid by TLC

Silica gel plates were used with lipid standards such as Triolein as reference substance for Triacylglycerol. The solvent was n-hexane-diethyl ether-acetic acid (90:10:2). The bands were observed after staining the TLC plate by Iodine vapor (14).

2.10. Lipid analysis by FTIR spectroscopy

Confirmation of certain oil compounds was determined by FTIR spectroscopy using JASCO FT/IR-6300, Japan device. The range of spectrum analyzed by device was set from 400cm^{-1} to 4000 cm^{-1}. Triolein (bought from sigma Aldrich) was used as control sample for comparing with produced single cell oil (15).

2.11. Biodiesel production

Extracted oil was used for Tran's esterification using methanol with molar ratio of 30:1, at 55°C, 150 rpm, and 5.5h as reaction time and based on amount of oil weight 80% sulfuric acid was used as catalyst (16, 17). After that the upper layer contains biodiesel separated by petroleum ether. Fatty acid methyl esters were analyzed by GC-MS (HP5890, serieII gas chromatography, HP 5972 mass selective detector).

3. RESULTS AND DISCUSSION

3.1. Lipid production in nitrogen limited medium before optimization

Lipid yield (g/L), lipid content (%) and dry biomass (g/L) of the evaluating strains were shown in Table 1.

Table 1. The lipid yield, lipid content and dry biomass of two strains in 40 g/L glucose containing medium.

Yeast strain	*Lipid yield(g/L)*	*Lipid content*	*Dry biomass(g/L)*
Rhodotorulla 110	6.17	34.62	17.82
Candida 14	4.95	27.04	18.30

3.2. Lipid production in different nitrogen sources

The results of different nitrogen sources were shown in Table 2 for *Rhodotorulla* 110 and in Table 3 for candida 14. According to the results yeast extract and ammonium sulfate was selected for further investigations.

Table 2. The lipid yield, lipid content and dry biomass of *Rhodotorulla* 110 in different nitrogen sources.

Nitrogen source	*Lipid yield*	*Lipid content*	*Dry biomass*
Yeast extract & $(NH_4)_2SO_4$	6.39	35.79	17.85
Yeast extract & NH_4Cl	6.05	34.45	17.56
Peptone & $(NH_4)_2SO_4$	6.00	34.56	17.36
peptone & NH_4Cl	6.11	34.38	17.77
Urea & $(NH_4)_2SO_4$	5.95	32.16	18.50
Urea & NH_4Cl	5.88	32.06	18.34

Table 3. The lipid yield, lipid content and dry biomass of Candida 114 in different nitrogen sources

Nitrogen source	*Lipid yield*	*Lipid content*	*Dry biomass*
Yeast extract & $(NH_4)_2SO_4$	5.38	30.39	17.70
Yeast extract & NH_4Cl	5.09	29.78	17.09
Peptone & $(NH_4)_2SO_4$	5.00	30.06	16.63
peptone & NH_4Cl	5.13	28.85	17.78
Urea & $(NH_4)_2SO_4$	5.05	27.80	18.16
Urea & NH_4Cl	5.11	27.59	18.52

3.3. Effect of carbon source

Different carbon sources were evaluated for these strains. The results of this evaluation were shown in Table 4 and Table 5 for the two strains.

Table 4. The lipid yield, lipid content and dry biomass of *Rhodotorulla* 110 in different carbon sources

Rhodotorulla 110	*Lipid yield (g/L)*	*Lipid content (%)*	*Dry biomass (g/L)*
Glucose	7.71	40	19.27
Xylose	7.04	36.1	19.50
Glycerol	3.81	25	15.24
Rice bran	5.45	30	18.16

Table 5. The lipid yield, lipid content and dry biomass of Candida 14 in different carbon sources

Candida 14	*Lipid yield (g/L)*	*Lipid content (%)*	*Dry biomass (g/L)*
Glucose	5.23	33	15.84
Xylose	4.80	25	19.20
Glycerol	3.23	22	14.68
Rice bran	4.25	24	17.70

3.4. FTIR spectroscopy results

FTIR graphs were shown in Figure 1. Comparison of graphs shows high similarity between extracted oil from oleaginous yeasts and the standard (Triolein). Significant peaks were created between 1670 to 1820 cm^{-1}, demonstrated presentation of carbonyl groups. Between 2850 to 2929 cm^{-1} was peaks that show methyl groups. All of the peaks in mentioned points confirmed that extracted oil can be converted to biodiesel (15, 18). FTIR was used for analyzing & confirming of biodiesel production from Chloralla vulgaris & Senedesmis sp (15).

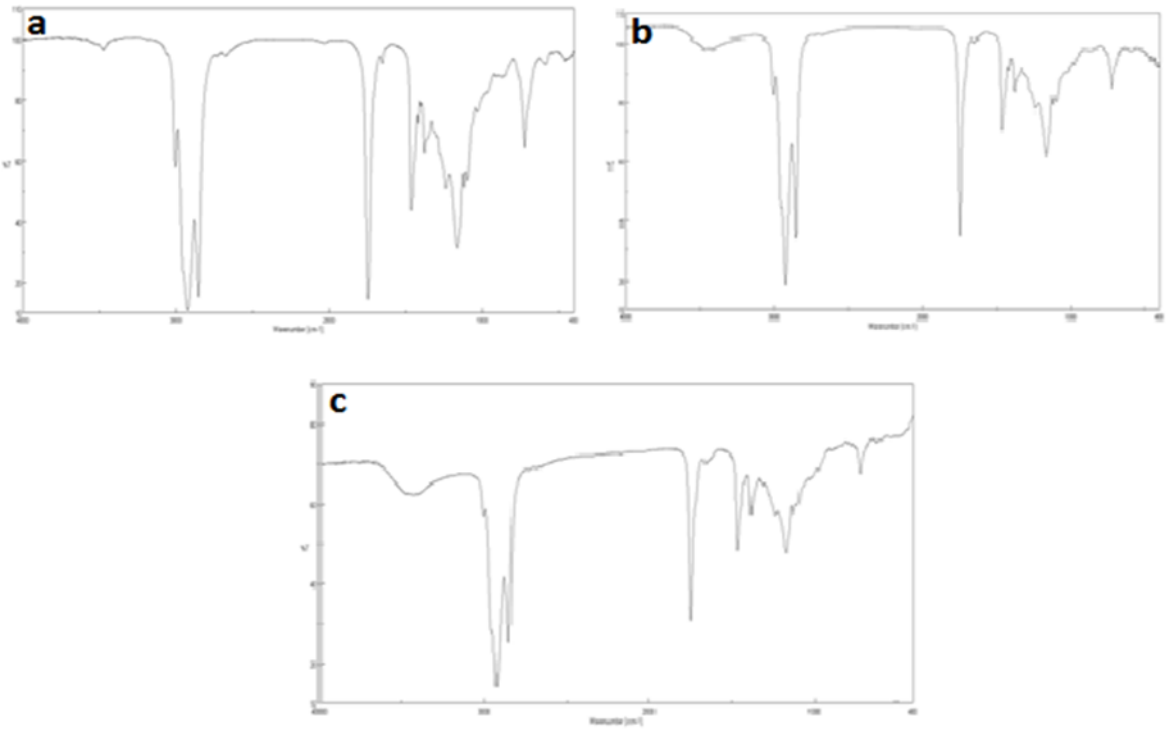

Figure 1. a): FTIR graph of Triolein standard; b): FTIR graph of produced SCO by yeast Rhodotorulla 110 c): FTIR graph of produced SCO by yeast Candida114

3.5. Biodiesel production

The yield of biodiesel production was 79% about *Rhodotorulla* 110 and the result of GC-MS show similarities with biodiesel produced from vegetable oil. Composition of biodiesel was as follows: all of them are as methyl esters: Palmitic acid 18.51%, Oleic acid 67.29%, Myristic acid 1.11%, Stearic acid 1.25%, Linoleic acid 4.76% and very low concentration of other fatty acid methyl esters. These results suggested that we can use agricultural residues for lipid production by oleaginous yeast that has ability to use them. By using such material as substrate for lipid production we can decrease the cost of production too much. Other agricultural and forestry residues also have this potential. Dai et al (2) exhibited the potential of conversion of agricultural and forestry residues to microbial lipid. They use *Rhodotorulla glutinis* as oleaginous yeast and reach to 49.25% lipid content. Jacob & Krishnamurthy (19) reported that *Rhodotorulla glutinis* had better biomass and lipid production on glucose. Sutari et al (20) reported similar results about *Lipomyces starkey*. Vijayakumar et al (21) evaluated lipid production of *Rhodotorulla glutinis* in different carbon sources. Between the sources, glucose caused lipid yield and lipid content of 2.43 g/L and 23.78 %. Syed et al (22) investigated lipid production in different fungal species in various carbon sources such as glucose, sucrose, lactose, soluble starch, and revealed that glucose was the best carbon source but besides this good effect of glucose the process must be economical. Most of investigations on lipid production focus on glucose as carbon source (23) but the high cost of this substrate persuades much effort to find low cost carbon sources and also oleaginous yeasts with potential of lipid production by utilizing these sources. In this way pectin & lactose was used by Papa Nikolaou (24), Wastes was investigated by Fakas & Xue (25) and glycerol as alternative carbon source was used by Easterling & Fakas (26) and Makri (27). Glucose is the best source for evaluating lipid production of micro-organism and it is used to compare the performance of micro-organisms to other carbon sources. About glycerol it is a byproduct of many industries, so glycerol as industrial and also rice bran as agricultural residues are two carbon sources that have potential for lipid production with optimization of other cultivation conditions for higher lipid production. About nitrogen source Huang et al (28) reported that organic nitrogen sources are good for lipid production and mineral nitrogen sources have good effect on biomass yield. Li et al (29), Yong- Hong et al (30) and papa Nikolaou & Aggelis (31) reported that ammonium sulfate was preferred for lipid production. Xue et al (25) investigated lipid

production in *Rhodotorulla glutinis* with 20% lipid content in the presence of ammonium sulfate as nitrogen source. Yeast extract & ammonium sulfate was the best nitrogen sources in this study. Lipid yield of these evaluated strains show their potential for industrial use not too far in the future.

4. CONCLUSION

The results of this investigation showed that native strains such as *Rhodotorulla* 110 has high potential for industrial use and biodiesel production. The effect of a number of different factors was investigated in this investigation. It was shown that carbon source is an important parameter which has obvious effect on lipid production in oleaginous yeasts. Among the carbon sources glucose lead to higher lipid content but from economical view rice bran was better. Between nitrogen sources yeast extract and ammonium sulfate exhibited higher lipid production. *Rhodotorulla* 110 had lipid content of 40%, 36% and 30% in glucose, xylose and bran as carbon source respectively with yeast extract and ammonium sulfate as nitrogen sources. This amount of lipid production shows the importance of these oleaginous yeasts in industrial applications such as biodiesel production.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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