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Single Cell Oil (SCO) Production by *Rhodotorula mucilaginosa* and Its Environmental Benefits

M. Enshaeieh[1*], A. Abdoli [1], and M. Madani[2]

ABSTRACT

Microbial oil has high similarity to the oil obtained from plants and animals. They are renewable sources that can be used for different purposes such as production of biofuels. Biofuels are renewable, biodegradable, and nontoxic, which makes them highly environmentally friendly. Producing oil from yeasts has more advantages than that from plants. Accordingly, isolation of oleaginous yeasts with high ability of lipid production is highly valuable. A total of 138 yeasts were isolated for the purpose of this study. From this amount, 35 were capable of producing lipid. After extracting lipid, the best strain was selected and, by using PCR method, identified as *Rhodotorula mucilaginosa*. Optimization was done using the design of experiments; Qualitek-4 (W32b) software was used for analyzing the experimental data. According to the results, *Rhodotorula mucilaginosa* proved to comprise lipid, dry biomass, and lipid productivity at levels of 10.97 g L $^{-1}$, 18.84 g L^{-}1, and 58.2% in optimized conditions, respectively. Lipid content on corn stalk and wheat straw hydrolysate was 36.9 and 41.8%, respectively. The extracted lipid was analyzed by FTIR spectroscopy and gas chromatography-mass spectrometry (GC-MS). The study showed high potential of lipid production in *Rhodotorula mucilaginosa* and also high efficiency of using Taguchi design in optimization of medium condition; therefore, by using this method, the optimization process can be done as best as possible. The economic values of microbial lipid production become more favorable when waste materials with zero or negative economic value are utilized as carbon source. Using bioprocesses such as microbial lipid production from waste materials, the problem of shortage of energy resources, and also air pollutions caused by fossil fuels, could be eliminated.

Keywords: Air pollution**,** Biofuels**,** Microbial lipid.

INTRODUCTION

Under appropriate cultivation methods, oleaginous microorganisms can accumulate high amounts of lipids. Therefore, their potential for application as lipid producing sources has attracted a lot of attention. The similarity of the lipid accumulated in microorganisms such as mold and yeasts to the oil obtained from plants is very important because it can be used as a substrate in biodiesel production as well as in many other industrial activities. Application of microorganisms in microbial oil production and subsequently in biodiesel production is valuable from economical point of view considering that they can be derived from such low cost sources as corn stalk, rice straw, and even from forestry residues (Mullner and Daum, 2004; Melickova *et al*., 2004; Li *et al*., 2008; Drucken, 2008; Zhu *et al*., 2008). Oleaginous yeasts attract a lot of attention because of their high growth rate and their ability to use different carbon sources. They can also utilize such low cost fermentation

[1] Young Researchers and Elite Club, Falavarjan Branch, Islamic Azad University, Isfahan, Islamic Republic of Iran.

[*] Corresponding author, email: m_enshaeieh@yahoo.com

[2] Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Islamic Republic of Iran.

media as waste materials from agricultural and industrial products (Amaretti *et al*., 2010, Benjamas and Louhasakul, 2013). Storage lipids are in the form of tri acyl glycerol (TAG). Therefore, different types of fatty acids are the main objectives for improving the biotechnological products (Schorken and Kempers, 2009). Microbial oil has a potential to substitute the plant oil in the market. Microorganisms 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 to conventional plant oil (Pan *et al*., 2009; Kosa and Ragauskas, 2010; Fei *et al*., 2010). Some oleaginous yeasts can metabolize pentoses. This shows the ability of TAG production from lignocelluloses substrates and other low cost materials (Li *et al*., 2007; Sabirova *et al*., 2010; Zheng *et al*., 2012).The advantages of microbial lipids include the short life cycle of microorganisms, less labor requirement, little affection by season and climate and also the relevant ease in scaling them up (Syed *et al*., 2006; Li *et al*., 2008; Leesing and Baojungharn, 2011).

Strains of *Rhodotorula* are pigmented yeasts that have high potential of lipid production and some of their strains can accumulate lipid on xylose and lignocellulosic substrate as sole carbon sources. This ability is important in using these strains in industrial processes (Postgate, 1994; Dai *et al*., 2007).

The structure of lipid droplets in all eukaryotic cells is similar to that of hydrophobic nucleus and the phospholipid layer around it. Lipid accumulation occurs when one of the nutrients (usually nitrogen) is exhausted. At the same time, there are excess amounts of carbon sources such as glucose in the medium. The cell's response to exhaustion of nutrients is that they cease to grow or 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 energy production i.e. ATP, as it is no longer required for the synthesis of macromolecules such as proteins and nucleic acids. This is because the cells have stopped to grow or divide. 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 (Wynn and Ratlege, 2005; Fei *et al*., 2008; Fakas *et al*., 2008; Meng *et al*., 2009; Raschke and Knorr, 2009). In addition to nitrogen limitation, phosphate limitation can also improve lipid accumulation in oleaginous microorganisms too (Muniraj *et al*., 2013).

The aim of this study was isolation and identification of the oleaginous yeasts with high potential of lipid production and optimization of medium condition and the evaluation of its effect on increasing lipid production. This study also aimed to investigate the potential for lipid production by this particular strain on agricultural residues.

MATERIALS AND METHODS

Isolation, Selection, and Screening of Oleaginous Yeast

Twenty eight soil samples from fields of peanuts, walnuts, almonds, and sunflowers were collected to isolate the yeasts. A mass of 1g of soil was added into 50 mL enrichment medium, which included (g L^{-1}): glycerol, 100; $(NH_4)_2SO_4$, 1; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; and yeast extract, 0.2 g L^{-1} in a 250 mL Erlenmeyer flask, then incubated at 30ºC for 96 hours while shaking at 180 rpm (Pan *et al*., 2009).

Then, 0.5 ml of this pre-cultured yeast was added to solid medium which included (g L^{-1}): glucose, 20; $(NH_4)_2SO_4$, 2; KH2PO4, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; and 2% agar. The plates were incubated at 28ºC for 48 hours. After incubation, the yeast colonies were isolated for screening process (Zheng *et al*., 2012). Lipid production was evaluated by Sudan Black B staining. The potential oleaginous yeast colonies were maintained on YPD slant, which contained (g L^{-1}): glucose,

20; yeast extract, 20; and peptone, 20 g L^{-1}, 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 included (g L^{-1}): glucose, 40; $(NH_4)_2SO_4$, 2; KH_2PO_4, 7; NaH_2PO_4, 2; $MgSO_4.7H_2O$, 1.5; and yeast extract; 1 g L^{-1}. Fifty mL of this medium in 250 Erlenmeyer flask was used on a shaker at 180 rpm and 28ºC. Before culturing it in nitrogen limited medium, the yeast colonies were activated in inoculation medium containing (g L^{-1}): glucose, 15; $(NH_4)_2SO_4$, 5; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; and yeast extract, 0.5 g L^{-1} grown at 28ºC at 180 rpm for 48 hours (Pan *et al*., 2009; Kraisintu *et al*., 2010). Lipid extraction was carried out based on Bligh and Dyer method with few modifications (Pan *et al*., 2009). A sample of 50 mL was centrifuged at 5,000 rpm for 10 minutes. After that, the yeasts were washed with 50 mL of distilled water. Then, 10 mL of 4M HCl was added and incubated at 60ºC for 3 hours. Later, the acid hydrolyzed mass was stirred with 20 mL chloroform/methanol mixture (1:1) at room temperature for 3 hours. At the end, centrifugation was done at 5,000 rpm for 3-5 minutes 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 this procedure 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 included (g L^{-1}): xylose, 40; $(NH_4)_2SO_4$, 1; KH_2PO_4, 7; $MgSO_4.7H_2O$, 1.5; and 1 g L^{-1} yeast extract grown at 28 ºC and 180 rpm in shaker incubator for 72 hours (Pan *et al*., 2009).

Determination of SCO Productivity, Growth Yield Efficiency, and SCO Yield Efficiency

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

SCO productivity (Lipid content)= SCO Weight (g L^{-1})/Cell dry weight (g L^{-1})×100

The supernatant was analyzed for glucose consumption by dinitrosalicylic acid (DNS) solution. Growth yield efficiency= Cell dry weight (g L^{-1})/Sugar consumed×100

SCO yield efficiency= SCO Weight (g L^{-1})/Sugar consumed×100

Determination of Yeast Dry Mass Portions

of 5 mL cultures were harvested by centrifugation at 6,000 rpm for 20 minutes. Harvested biomass was washed twice with 5 mL of distilled water and then dried at 80ºC to create a constant mass. The biomass was determined gravimetrically (Vijayakumar *et al*., 2010; Sriwongchai *et al*., 2013).

Analysis of SCO Production by FTIR Spectroscopy

Lipid production in oleaginous yeast was first verified using Sudan Black B staining, 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 was set from 400 to 4000 cm^{-1}. Triolein (bought from sigma Aldrich) was used as the control sample for comparing it with the produced single cell oil (Elumalai *et al*., 2011; Enshaeieh *et al*., 2013a).

Analysis of SCO Production by Gas Chromatography-Mass Spectrometry

(GC-MS)After analysis of the extracted oil by FTIR spectrometry, GC-MS was done for

further confirmation. FTIR spectrometry is a standard method for confirming that the extracted materials from the yeasts have neutral lipid composition (especially tri acyl glycerol), but further analysis by GC-MS can show that the obtained oil has similar structure to the plants oil and can be converted to biodiesel or be used for other industrial applications. The obtained oil was used for trans-esterification using methanol with molar ratio of 30:1, at 55°C, 180 rpm, and 5.5 hours as reaction time. Based on the amount of oil weight, 80% sulfuric acid was used as catalyst (Liu *et al*., 2004; Wu *et al*., 2006). After that, the upper layer containing biodiesel was separated by petroleum ether. Fatty acid methyl esters were analyzed by GC-MS (HP5890, serieII gas chromatography, HP 5972 mass selective detector) (Enshaeieh *et al*., 2012a).

Medium Optimization by Taguchi Design

Design of experiment (DOE) was done by Taguchi method for evaluating the effects of different physical and chemical parameters on lipid production. These parameters included glucose and nitrogen concentrations, pH, agitation rate, time, and temperature of incubation. This method can predict optimized conditions and optimum lipid productions in the predicted conditions. Glucose, time and pH had 4 levels, nitrogen had 3 levels, agitation rate and temperature had two levels (Table 1). Qualitek-4 software designed an experimental plan (L16) which contained 16 experiments for each strain (Table 2). The effective percentage of each factor can be shown by the analysis of variance (ANOVA). The best optimum condition for the highest lipid

Table 1. Setting of different factors and their level for lipid production by Taguchi design.

Number	Variables	Level 1	Level 2	Level 3	Level 4
1	Glucose	55	75	95	115
2	$(NH_4)_2SO_4$	0.5	1	1.5	----
3	Time	24	48	72	96
4	Temperature	25	35	----	----
5	pH	5	5.5	6	6.5
6	rpm	150	200	----	----

Table 2. L16 array of Taguchi design.

Number of trials	Nitrogen (g L^{-1})	Glucose (g L^{-1})	Temperature (ºC)	Time (h)	pH	rpm
1	0.5	55	25	24	5	150
2	0.5	75	25	48	5.5	200
3	0.5	95	35	72	6	150
4	0.5	115	35	96	6.5	200
5	1	55	25	96	6	200
6	1	75	25	72	6.5	150
7	1	95	35	48	5	200
8	1	115	35	24	5.5	150
9	1.5	55	35	48	6.5	150
10	1.5	75	35	24	6	200
11	1.5	95	25	96	5.5	150
12	1.5	115	25	72	5	200
13	0.5	55	35	72	5.5	200
14	0.5	75	35	96	5	150
15	0.5	95	25	24	6.5	200
16	0.5	115	25	48	6	150

production for each strain was achieved by Taguchi method.

Lipid Production Using Wheat Straw and Corn Stalk as Sole Carbon Sources

Before using wheat straw and corn stalk, they had to be prepared by acid hydrolysis. To this end, the materials were ground and then hydrolyzed 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 minutes. Then, the suspension was centrifuged to remove un-hydrolyzed residues (Dai *et al*., 2007). Ten 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. Then, 5 mL of the inoculation was added and incubated at the optimum condition (Enshaeieh *et al*., 2012a; Enshaeieh *et al*., 2013a).

Identification of the Yeast by Sequencing Ribosomal RNA

The best isolation was identified by sequencing a fragment of genome. Internal Transcribed Spacer (ITS) was used as primer in this study. In order to extract the DNA from the yeast, it was initially cultured in yeast extract-peptone-glucose broth (YPD), and then the harvested cells were washed with distilled water. At the next stage, the cells were mixed with lysing buffer, glass beads (425-600 microns), phenol-chloroform-Amyl Alcohol (1:24:25) solution and the mixture were vortexed vigorously for 3-5 min in order to break the cells. The lysing buffer contains X-100 Triton, Sodium Dodecil Sulphate (SDS), sodium chloride, Tris-HCL and ethylenediaminetetra-acetic acid (EDTA). Then, Tris-EDTA buffer (TE) was added to the solution. To dissolve the DNA in TE buffer mixing was done gently. After centrifugation at the rate of 10,000 rpm for 10 minutes, the supernatant was separated by adding cold isopropanol twice its volume and the mixture was inverted gently several times. Quick motion at this step can cause DNA fragmentation. After centrifugation, the upper solution was discarded and the isopropanol was evaporated under the hood. Next, 100 µL of TE buffer was added to precipitate and followed by gentle vortex and micro-centrifugation. It was then kept at -20°C for PCR (Hoffman, 1997). The PCR was done using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') as a forward primer and ITS4 (5'TCCTCCGCTTATTGATATGC3') as a reverse primer (Deak *et al*., 2000). Purity and relative size of the PCR product were obtained on agarose gel (1.5 percentages), then, the sequence was determined and searched in NCBI Site to identify the genus and species of yeast. Polymerase chain reaction contained three stages as follows: the initial denaturation (95°C, 5 minutes), 30 cycle (95°C for 30 seconds), then at 55°C for 30 seconds, and 72°C for 1 minute, and the final extension (72°C, 10 minutes).

RESULTS AND DISCUSSION

Isolation of Oleaginous Yeasts

A total of 138 yeasts were isolated from which 35 were oleaginous based on the first qualitative analyses by Sudan Black B staining. The yeast lipid bodies were observed 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 results of lipid extraction, namely, the dry biomass, lipid productivity, and the ability of xylose assimilation are shown in Table 3.

As the results show, the strain *Rhodotorula mucilaginosa* (strain number nine) produced the highest amount of lipid. Also, this strain could produce lipid in a medium with xylose as a sole carbon source.

Table 3. Results of lipid extraction, dry biomass, lipid productivity and Xylose assimilation for oleaginous colonies.

Strain	Lipid yield (g L^{-1})	Dry biomass (g L^{-1})	Lipid productivity (%)	Xylose assimilation
1	4.45	14.21	31.35	w [a]
2	2.13	8.61	24.78	++ [b]
3	2.87	11.19	25.67	+ [c]
4	2.19	9.18	23.88	++
5	1.97	8.90	22.13	-
6	2.06	9.17	22.53	++
7	2.36	9.78	24.18	++
8	2.35	10.06	23.35	-
9	6.17	17.82	34.62	+
10	2.16	8.08	26.78	+
11	1.94	8.16	23.81	-
12	2.29	10.18	22.45	++
13	2.36	10.02	23.57	+
14	6.04	18.25	33.09	-
15	3.86	13.87	27.88	+
16	1.71	7.53	22.68	+
17	1.92	8.37	22.92	+
18	2.03	8.00	25.38	+
19	3.04	10.72	28.37	+
20	3.67	14.22	25.85	++
21	3.95	16.91	23.39	-
22	2.91	11.80	24.33	+
23	3.18	12.02	26.50	++
24	4.97	15.47	32.15	+
25	3.99	16.09	24.82	+
26	1.95	8.83	22.17	++
27	5.35	17.98	29.77	++
28	5.09	15.15	33.62	-
29	3.33	12.74	26.18	-
30	4.88	19.93	24.48	+
31	3.90	16.18	24.15	+
32	3.14	13.12	23.92	+
33	4.13	15.08	27.38	w
34	4.07	16.35	24.89	++
35	3.30	14.04	23.55	++

[a] Weak assimilation; [b] High assimilation of xylose, [c] Positive (strains with ability of xylose assimilation); [d] Negative (strains that could not grow on xylose as sole carbon source).

This strain produced 6.17 g L^{-1} lipid per 17.82 g L^{-1} dry biomass and its lipid productivity was 34.62%. Therefore, it was selected for further investigation.

Then, the experiments were set for L16 design of Taguchi method. The merit of Taguchi design lies in the fact that many arrays of experiments can be evaluated without actually doing all of them (Enshaeieh *et al.*, 2013c). Software evaluates all the parameters based on the results of 16 experiments. Table 4 shows the result of Taguchi experiments for *Rhodotorula mucilaginosa*. 'Lipid productivity' is a common term in related research about microbial oil and indicates the percentage of lipid production in the biomass. The terms 'growth yield efficiency' and 'SCO yield efficiency' are two other common related terms that show

Table 4. Results of Taguchi design, dry biomass, lipid productivity, growth yield efficiency, SCO yield efficiency, and nitrogen concentration for *Rhodotorula mucilaginosa.*

Number	Lipid production (g L^{-1})	Dry biomass (g L^{-1})	Lipid productivity (%)	Growth yield efficiency	SCO yield efficiency
1	4.15	13.17	31.5	43.9	13.83
2	5.98	16.98	35.2	39.12	13.77
3	5.12	14.88	34.4	37.95	13.06
4	4.83	15	32.2	40	12.88
5	5.82	16.62	35	37.57	13.15
6	10.97	18.84	58.2	25.8	15.02
7	4.13	13.12	31.47	42.02	13.22
8	5.16	14.95	34.5	37.75	13.03
9	5.1	14.86	34.3	38.9	13.35
10	4.01	12.93	31	43.1	13.36
11	6.36	16.82	37.8	37.79	14.29
12	6.14	16.59	37	36.06	13.34
13	4.03	12.93	31.15	42.22	13.16
14	5.89	16.82	35	38.05	13.32
15	5.34	15.34	34.8	37.87	13.18
16	4.71	14.67	32.1	41.09	13.19

the rate of growth and lipid production to the sugar consumed by the yeasts, respectively.

The result of glucose concentrations as well as growth yield efficiency and SCO yield efficiency are shown in Table 4. The best experiment for *Rhodotorula mucilaginosa* was array 6 and the best lipid production was in this array. Table 5 shows the ANOVA results for *Rhodotorula mucilaginosa*. The last column of the table shows the influence percentage for each factor. The analysis revealed that glucose concentration, time of incubation, and temperature all had significant effects on lipid production, respectively, whereas pH and nitrogen concentration had smaller effect on lipid production for this strain. The agitation rate had the least effect among the parameters. The last array i.e. 'other/error' indicates the error level in the experiment, which is significantly the smallest amount. Figure 1 shows the effect of different parameters on lipid production by this strain. *Y*-axis shows the percentage of each parameter that was obtained from ANOVA, and the *X*-axis shows the related parameters. Figure 2 shows the plot of each parameter. It shows how each factor can affect lipid production in this strain. Table 6 shows the optimum condition predicted by Taguchi

Table 5. Analysis of variance (ANOVA) of Taguchi results for *Rhodotorula mucilaginosa*.

Factors	DOF	Sums of squares (S)	Variance (V)	F-ratio (F)	Pure sum (S´)	Percent P (%)
Nitrogen	2	6.145	3.072	52.478	6.028	14.65
Glucose	3	8.59	2.863	48.905	8.415	20.45
Temperature	1	7.84	7.84	133.891	7.781	18.91
Time	3	8.605	2.868	48.989	8.43	20.486
pH	3	6.628	2.209	37.732	6.452	15.68
RPM	1	3.221	3.221	55.025	3.163	7.687
Other/Error	2	0.116	0.058	------	------	2.137
Total	15	41.149	------	------	------	100

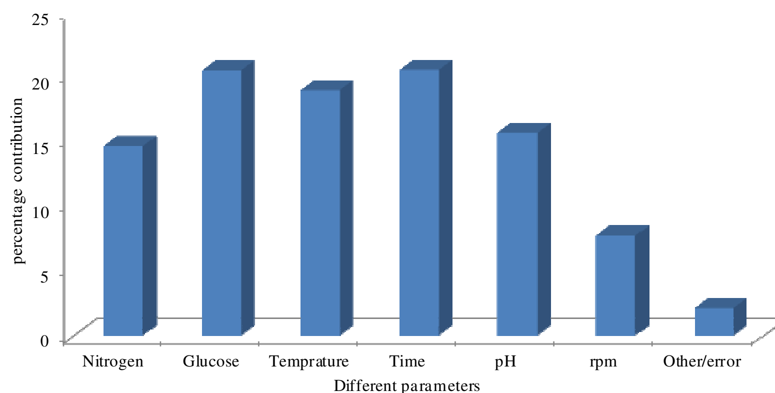

Figure 1. The percentage contribution for each factor in lipid production by *Rhodotorula mucilaginosa* obtained from Qualitek-4 software. The last column shows the error level which is by far the smallest amount. Time of incubation and glucose concentration had the highest effect on lipid production in this strain (20.486 and 20.45%, respectively).

design. The obtained amount of oil was close to the expected result.

Lipid Production Using Agricultural Residues

The results of lipid production on xylose and agricultural residues in optimized conditions are shown in Table 7. The results obtained in this study were significant and showed high potential of lipid production by the evaluated yeast strain.

FTIR Spectroscopy Analysis of Lipid Products

Microbial lipid graphs obtained from the FTIR analysis are shown in Figure 3. Comparison of the two graphs showed the highest similarity between oil extracted from oleaginous yeast and the standard oil of triolein. Significant peaks were created between 1,670 to 1,820 cm^{-1}, confirming presence of carbonyl groups. There were peaks between 2,850 to 2,929 cm^{-1} that showed presence of methyl groups. All of the aforementioned peaks confirmed that the produced oil can potentially be converted to biodiesel (Elumalai *et al*., 2011; Lin-Vien, 1991). Biodiesel compounds were analyzed based on the European standard of EN 14078 (European Standard EN 14078). FTIR also was used for analysis and confirmation of biodiesel based on the methy and ethyl ester of long chain fatty acids in productsfrom *Chloralla vulgaris* and *Senedesmis* sp. (Elumalai *et al*., 2011).

Results of Lipid Analysis by GC-MS

The result of GC-MS indicated the potential for industrial application of microbial oil. The composition of fatty acids methyl esters was as follows: Palmitic acid 16.62%, Oleic acid 69.45%, Myristic acid 1.98%, Stearic acid 1.63%, Linoleic acid

Table 6. Optimum condition predicted by Taguchi for the highest production by *Rhodotorula mucilaginosa.* Expected result at optimum condition was11.052 g L^{-1} lipid. This amount is close to 10.97 g L^{-1}.

Factor	Optimized level	Leve l
Nitrogen	1	2
Carbon	75	2
Temperature	25	1
Time	72	3
pH	6.5	4
RPM	150	1

Table 7. Lipid production on agricultural residues as sole carbon sources.

Condition	Lipid production ($g\ L^{-1}$)	Biomass ($g\ L^{-1}$)	Lipid productivity (%)
Xylose	6.98	16.11	43.32
Wheat straw	6.64	15.88	41.81
Corn stalk	5.74	15.55	36.91

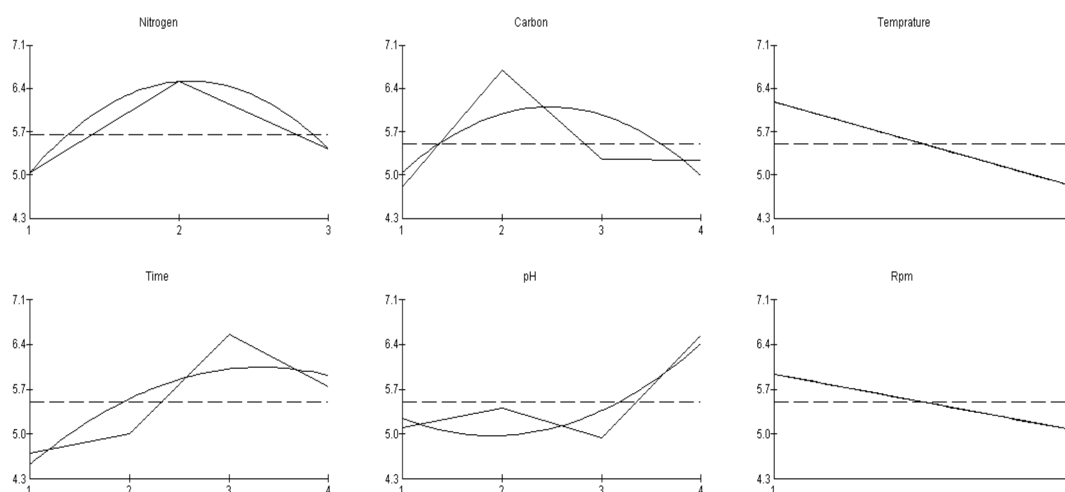

Figure 2. The plot obtained from Qualitek-4 software that shows the effect of each factor on lipid production by *Rhodotorula mucilaginosa*. *Y*-axis of the plot shows the average lipid production in each level of different factors. *X*-axis indicates the number of levels for different factors.

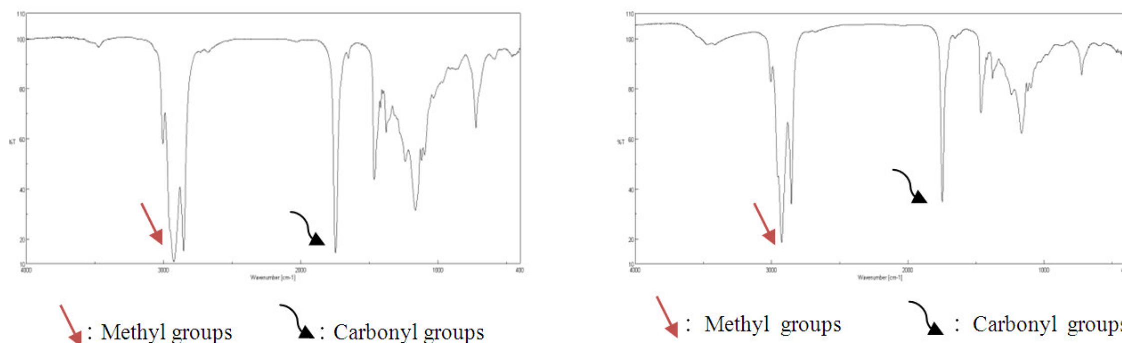

Figure 3. FTIR analytical graphs performed on the standard oil triolein (a) and the oil product from *Rhodotorula mucilaginosa* (b).

5.78% and very low concentration of other fatty acid methyl esters.

Identification of Yeast by Sequencing Ribosomal RNA

Fragments obtained from the yeast after PCR were used on agarose gel electrophoresis to determine their relative size and purity. The length of fragments obtained was different using primers ITS1 and ITS2 in various yeasts and included "a part of ITS2-5.8srRNA-ITS1-18srRNA and a part of 28srRNA". Figure 4 shows agarose gel electrophoresis of PCR products of the yeast isolated in this study. After sequencing and matching the specified isolated on NCBI site, it was identified as *Rhodotorula mucilaginosa*, which had soft, smooth,

mucoid and red-orange colonies on PDA medium.

Taguchi method limited the number of experiments, therefore, it was a powerful tool for investigating the effect of all parameters. The effects of a number of different factors were investigated simultaneously by Taguchi method. Another advantage of this software was the suggestion of the optimum condition, thus, by using this method less time and cost were spent for optimization process in this study. Many investigators have already done optimization of lipid production in different yeast strains. Most of them have used one factorial method for performing the optimization process (Enshaeieh *et al*., 2014). Kraisintu *et al*. (2010) evaluated different chemical and physical parameters on lipid production in *Rhodosporidium toruloides* DMKU3-TK16 and obtained 9.26 g L^{-1} lipid production in nitrogen-limited medium containing 70 g L^{-1} glucose, 0.55 g L^{-1} $(NH_4)_2SO_4$ with a pH of 5.5 at 150 rpm and 28°C. Optimizations were done step by step for each factor, which required many experiments and were more time consuming. Many other investigators evaluated the type of carbon and nitrogen sources, their concentrations, pH, time, and temperature of incubation separately. However, using Taguchi or other methods of designing experiments, all the parameters can be evaluated simultaneously. During each production process, optimization was necessary to raise the level of efficiency and to reduce the cost. In this study, *Rhodotorula mucilaginosa* had high potential in the field of lipid synthesis; lipid content and lipid production of this strain were 58.2% and 10.97 g L^{-1}, respectively, in optimized condition. Pan *et al*. (2009) isolated oleaginous yeasts with assimilating capacity of xylose and the best yeast strain could produce 5.8 g L^{-1} lipid using 40 g L^{-1} xylose. Li *et al*. (2005) reported that lipid production in *Rhodosporidium toroloides* AS2.1389 was 10.6 g L^{-1} when using 100 g xylose. In the present study, lipid production on xylose with *Rhodotorula mucilaginosa*, was 6.98 g L^{-1}. Through optimization of cultivation condition, even higher lipid production can be achieved.

Lignocellulosic biomasses are the most abundant organic sources and various oleaginous micro-organisms, especially yeasts, have attracted attentions for converting them to microbial oil (Xu *et al*., 2012). SCO production from lignocellulosic materials containing xylose have been carried out by several investigators (Meester *et al*., 1996; Zhao, 2005; Papanikolaou, 2008; Gong *et al*., 2013; Enshaeieh *et al*., 2013b). Yeast strains that can use xylose in lignocellulosic hydrolysate have potential for industrial application (Dai *et al*., 2007; Huang *et al*., 2012). Yu *et al*. (2011) used *Cryptococcus curvatus* on wheat straw as carbon source and the lipid content was reported 33.5%. Huang *et al*. (2009) reported 40.1% lipid content by using *Tricosporon fermentens* and rice straw as the sole carbon source. Therefore, it is suggested that lignocellulosic materials are a good substrate for microbial oil production due to their low cost and abundance (Zheng *et al*., 2012, Khot *et al*., 2012). Enshaeieh *et al*. (2012b) used *Rhodotorula mucilaginosa* and a novel carbon source, i.e. grass hydrolysate, and reported 55% lipid content, which showed high potential of this strain in lipid production. In this study, *Rhodotorula mucilaginosa* had lipid content of 41.81 and 36.91% on wheat straw and corn stalk, respectively. According to the results, further attention to this bio-converting process via oleaginous yeasts is suggested.

CONCLUSIONS

The results showed that the strain *Rhodotorula mucilaginosa* was a high lipid producing yeast with potential of industrial applications. Its lipid yield reached 10.97 g L^{-1} with lipid content of 58.2% after 72 hours at 25°C when cultivated in nitrogen limited medium. This medium included: 75 g L^{-1} glucose, 1 g L^{-1} ammonium sulfate, and 1 g L^{-1} yeast extract with pH adjusted at 6.5

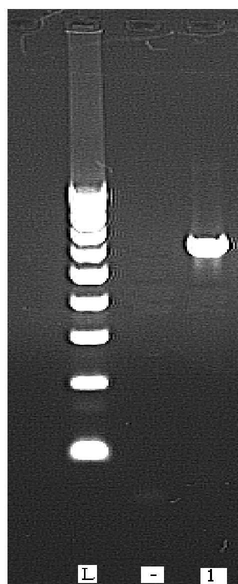

Figure 4. Image of gel electrophoresis of PCR products of the isolated strain. L: Ladder (Fermentas, 100 base); (-): Negative control (distilled water), (1): *Rhodotorula mucilaginosa.*

in shaking flask at 150 rpm. It could assimilate xylose and produce lipid on lignocellulosic hydrolysate as the sole carbon source. This ability shows the potential of this native strain in industrial lipid production.

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تولید روغن تک یاخته توسط *Rhodotorula mucilaginosa* و فواید زیست محیطی آن

م. انشائیه، آ. عبدلی، م. مدنی

چکیده

روغن میکروبی به لیپید به دست آمده از گیاهان و حیوانات شباهت زیادی دارد. این منابع قابل تجدید برای مقاصد گوناگونی نظیر تولید سوخت زیستی قابل استفاده هستند. سوخت های زیستی تجدید پذیر، قابل تجزیه و غیر سمی بوده بنابراین دارای فواید زیست محیطی فراوانی هستند. در نتیجه جداسازی مخمر های مولد چربی با قابلیت بالای تولید لیپید ارزشمند است. در این مطالعه ۱۳۸ مخمر جداسازی شده که ۳۵ عدد از آن ها توانایی تولید لیپید را دارند. پس از استخراج لیپید بهترین سویه انتخاب شده و با استفاده از PCR تحت عنوان *رودوتورولا موسیلاژینوزا* شناسایی شد. بهینه سازی با استفاده از روش طراحی آزمایش ها انجام داده شد؛ نرم افزار Qualitek-4 (W32b) برای آنالیز آزمایش ها براساس روش تاگوچی مورد استفاده قرار گرفت. طبق نتایج به دست آمده *رودوتورولا موسیلاژینوزا* به ترتیب دارای تولید لیپید، بیومس خشک و محتوای لیپیدی $g.L^{-1}$ ۱۰/۷، $g.L^{-1}$ ۱۸/۸۴ و ۵۸/۲٪ در شرایط بهینه بود. محتوای لیپیدی بر روی ساقه ذرت و پوشال گندم هیدرولیز شده به ترتیب ۳۶/۹٪ و ۴۱/۸٪ بود. لیپید استخراج شده با استفاده از تکنیک اسپکتروسکوپی FTIR و کروماتوگرافی گازی – اسپکترومتری توده ای مورد آنالیز قرار گرفت. این مطالعه قابلیت بالای تولید لیپید در *رودوتورولا موسیلاژینوزا* و علاوه بر آن تاثیر زیاد طراحی تاگوچی در بهینه سازی محیط کشت را نشان می دهد؛ بنابراین با استفاده از این روش پروسه ی بهینه سازی به بهترین وجه ممکن قابل انجام خواهد بود. ارزش اقتصادی تولید لیپید میکروبی زمانی بیشتر قابل توجه است که ضایعاتی با ارزش اقتصادی صفر و یا منفی به عنوان منبع کربن استفاده شوند. با استفاده از این پروسه های زیستی، تولید لیپید میکروبی از ضایعات، مشکل کمبود منابع انرژی و آلودگی هوای ناشی از سوخت های فسیلی، قابل حل خواهد بود.