هشتمین همایش بین المللی علوم و تکنولوژی با رویکرد توسعه پایدار
8th International Conference on Science & Technology with sustainable development approach
tech.sdcongress.ir

A promising oleaginous yeast strain for single cell oil production as biodiesel feedstock

Oleaginous yeast strain as biodiesel feedstock

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Abstract

Single cell oil is a promising alternative feed stock in biodiesel production. Application of SCO is the subject of many studies which seek to optimize fermentation process and to reduce production cost. Utilization of plant wastes as carbon source and optimization of their physical and chemical condition can reduce the processing cost. In this study an oleaginous yeast strain, *Rhodosporidium babjevae* , was utilized for SCO production. The experiment design is applied in optimizing process and Qualitek-4 (W32b) software is used for analyzing the experiments. The obtained results indicate that the following levels of 10.97 g/L, 18.84 g/L and 58.2% are available as lipid, dry biomass and lipid productivity, respectively. It is revealed here that *Rhodosporidium babjevae* is a very appropriate candidate for SCO production and the physicochemical properties of the obtained biodiesel was confirmed according to the US and EU standards. In this case application of oleaginous yeast and mold would be an appropriate solution to prevail feed stock for biodiesel production.

Keywords: Rhodosporidium babjevae, biodiesel production, Taguchi method, Optimization

1. Introduction

Oleaginous microorganisms can accumulate lipid more than 20% of their biomass and some of them have the ability of using different kind of carbon sources like wastes from olive oil and soybean production, whey from cheese and glycerol from biodiesel production. This ability is valuable from economical point of view [1-3]. The important part of microbial oil is TAG that contains long chain fatty acids and is similar to conventional plant oil [4-6]. By optimization of medium condition, oleaginous yeast types can accumulate high amounts of lipids. This lipid gained interest because of its similarity to the oil extracted from plants which can be consumed as a substrate in biodiesel production [7-10].

One of the advantages of biodiesel is that it does not contain polycyclic hydrocarbons which can be converted into different substances hence it is nontoxic and can be mixed with petroleum diesel in any ratio. Moreover, biodiesel has higher energy density, lower sulfur content, sufficient burning potential lubricating characteristic and low explosive potential. Hemicelluloses contain pentose (xylose in specific) and hexoses (glucose in specific); depending on the pre-treatment method, hemicelluloses can be converted into monomeric and oligomeric forms which constitute the main substrates for biodiesel production [11-13].

The objection of this study is application of oleaginous yeast with high capacity of lipid production and optimize the medium condition by applying two steps of design of experiments through Taguchi method.

2. Materials and Methods

2.1. Yeast cultivation for lipid production & lipid extraction

Oleaginous yeasts are cultured in nitrogen-limited medium for 5 days. This medium contains (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. From this nitrogen limited medium 50 mL is poured in 250 Erlenmeyer flask put on a shaker at 180 rpm and 28 ºC [4, 14]. Lipid extraction is carried out based on Bligh and Dyer method subject to few modifications [4, 15].

2.2. Determining the dry biomass, SCO productivity, Growth yield efficiency and SCO yield efficiency

Portions of 5 mL cultures are harvested by centrifugation at 6000 rpm for 20 min. The harvested biomass is washed twice with 5 mL of distilled water and then dried at 80 ºC to generate a constant mass. This biomass is determined gravimetrically [16-19].

Lipid content in each one of the trial conditions is determined through the following equation introduced by Kraisintu et al. [14]:

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

This supernatant is analyzed for glucose consumption by dinitrosalicylic acid (DNS) solution through the following equitation:

Growth yield efficiency = Cell dry weight (g L^{-1}) / Sugar consumed × 100 (2)

SCO yield efficiency = SCO Weight (g L^{-1}) / Sugar consumed × 100 (3)

2.3. SCO production analysis through gas chromatography- mass spectrometry (GC-MS)

After the extracted oil is analyzed by FTIR spectrometry, the GC-MS is applied for further confirmation. The extracted oil is used for trans-esterification mixed with methanol at 30:1 molar ratio, at 55°C, at 180 rpm, for 5.5h as reaction time. In proportion to the extracted oil weight, 80% sulfuric acid is added as catalyst [20, 21]. Following this step, the upper layer containing biodiesel is separated by petroleum ether. Fatty acid methyl esters are analyzed by GC-MS (HP5890, serieII gas chromatography, HP 5972 mass selective detector) [22].

The physiochemical features of single cell oil and biodiesel were identified according to ASTM D6751-02 (US Standard ASTM).

2.4. Medium optimization through Taguchi design

Design of experiment (DOE) is based on by Taguchi method for evaluating the effects of different physical and chemical parameters on lipid production. L27 design is applied in optimizing the concentrations of materials and the physical parameters of the medium. These parameters were carbon, nitrogen, $MgSO_4$, KH_2PO_4, $Fecl_3$ and $Cacl_2$ concentrations, Inoculum, pH, agitation rate, time and temperature of incubation. All the parameters are of 3 levels, Table 1. Qualitek-4 software designed an experimental plan of L27 which contains 27 experiments, Table 2. Statistical analysis is run through ANOVA and Taguchi design. The best optimum condition for the highest lipid production is achieved through Taguchi method.

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

Variables	Level 1	Level 2	Level 3
Glucose (g)	60	80	100
Ammonium sulfate (g)	0.5	1	1.5
Time (h)	72	96	120
pH	5	6	7
Rpm	120	150	180
Temperature (°C)	15	20	25
$MgSO_4$ (g)	1	1.5	2
KH_2PO_4 (g)	1	1.5	2
Inoculum (ml)	4	6	8
$FeCl_3$ (g)	0.15	0.3	0.45
$CaCl_2$ (g)	0.15	0.3	0.45

Table 2. L27 array of Taguchi design.

Number of trials	Glucose	$(NH_4)_2SO_4$	Time	pH	rpm	Temperature	$MgSO_4$	KH_2PO_4	Inoculum	$FeCl_3$	$CaCl_2$
1	60	0.5	72	5	120	15	1	1	4	0.15	0.15
2	60	0.5	72	5	150	20	1.5	1.5	6	0.3	0.3
3	60	0.5	72	5	18	25	2	2	8	0.45	0.45
4	60	1	96	6	120	15	1	1.5	6	0.3	0.45
5	60	1	96	6	150	20	1.5	2	8	0.45	0.15
6	60	1	96	6	18	25	2	1	4	0.15	0.3
7	60	1.5	120	7	120	15	1	2	8	0.45	0.3
8	60	1.5	120	7	150	20	1.5	1	4	0.15	0.45
9	60	1.5	120	7	180	25	2	1.5	6	0.3	0.15
10	80	0.5	96	7	120	20	2	1	6	0.45	0.15
11	80	0.5	96	7	150	25	1	1.5	8	0.15	0.3

12	80	0.5	96	7	180	15	1.5	2	4	0.3	0.45
13	80	1	120	5	120	20	2	1.5	8	0.15	0.45
14	80	1	120	5	150	25	1	2	4	0.3	0.15
15	80	1	120	5	180	15	1.5	1	6	0.45	0.3
16	80	1.5	72	6	120	20	2	2	4	0.3	0.3
17	80	1.5	72	6	150	25	1	1	6	0.45	0.45
18	80	1.5	72	6	180	15	1.5	1.5	8	0.15	0.15
19	100	0.5	120	6	120	25	1.5	1	8	0.3	0.15
20	100	0.5	120	6	150	15	2	1.5	4	0.45	0.3
21	100	0.5	120	6	180	20	1	2	6	0.15	0.45
22	100	1	72	7	120	25	1.5	1.5	4	0.45	0.45
23	100	1	72	7	150	15	2	2	6	0.15	0.15
24	100	1	72	7	180	20	1	1	8	0.3	0.3
25	100	1.5	96	5	120	25	1.5	2	6	0.15	0.3
26	100	1.5	96	5	150	15	2	1	8	0.3	0.45
27	100	1.5	96	5	180	20	1	1.5	4	0.45	0.15

3. Results and Discussion

3.1. Lipid production by the obtained yeast train

Lipid production, Biomass and sugar consumption by *Rhodosporidium babjevae KB 649* before optimization during 5 days are shown in Fig (1).

The result of the optimization process where the physical and chemical parameters that affect lipid production are optimized are tabulated in table 3 and figure 2.

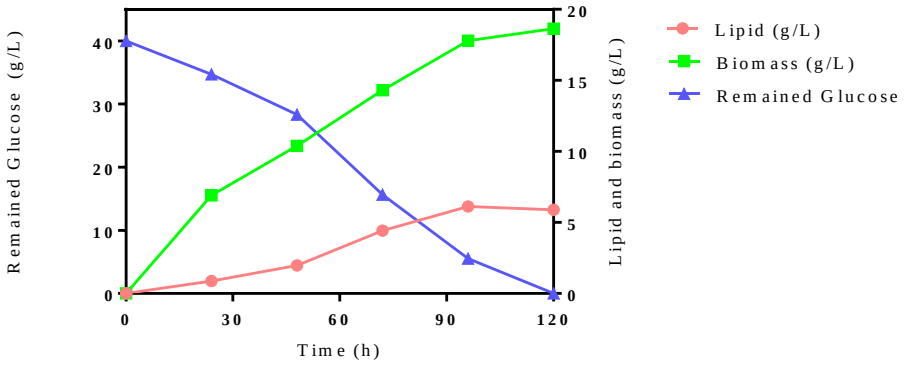

Figure 1. Lipid production, Biomass and sugar consumption by *Rhodosporidium babjevae KB 649*

Table 3. Results of Taguchi design, dry biomass, lipid productivity, growth yield efficiency, SCO yield efficiency and nitrogen concentration for *Rhodosporidium babjevae KB 649*in L27 array

Number	Lipid production (g L^{-1})	Dry biomass (g L^{-1})	Lipid productivity (%)	Growth Yield Efficiency	SCO Yield Efficiency
1	5.68	13.40	42.38	36.12	14.36
2	7.34	14.11	52.02	38.52	15.87
3	5.49	12.86	42.69	29.21	14.16
4	6.66	13.87	48.01	37.21	15.45
5	7.19	13.98	51.43	36.04	15.98
6	7.09	14.13	50.17	41.39	16.54
7	5.48	13.36	41.01	36.14	14.22
8	6.39	13.45	47.50	31.15	15.43
9	5.23	12.47	41.94	26.78	14.13
10	7.41	14.46	51.24	28.56	16.31
11	9.48	15.77	60.11	32.12	17.86
12	7.01	14.12	49.64	28.90	16.28
13	6.34	13.42	47.24	34.26	15.02
14	6.69	13.93	48.02	32.60	15.52
15	6.18	13.17	46.92	30.07	15.23
16	6.31	13.41	47.05	27.15	15.49
17	7.08	14.03	50.46	30.24	16.00
18	5.78	13.48	42.87	31.56	14.98
19	6.41	13.64	46.99	34.17	15.48
20	6.80	14.26	47.68	38.50	15.92
21	8.19	14.59	56.13	38.89	17.01
22	5.17	12.50	41.36	27.48	14.12
23	6.30	13.28	47.44	26.60	15.47
24	6.34	13.53	46.85	26.93	15.49
25	7.60	14.54	52.27	39.25	16.72
26	5.49	12.93	42.45	26.39	14.65
27	6.38	13.72	46.50	29.86	15.47

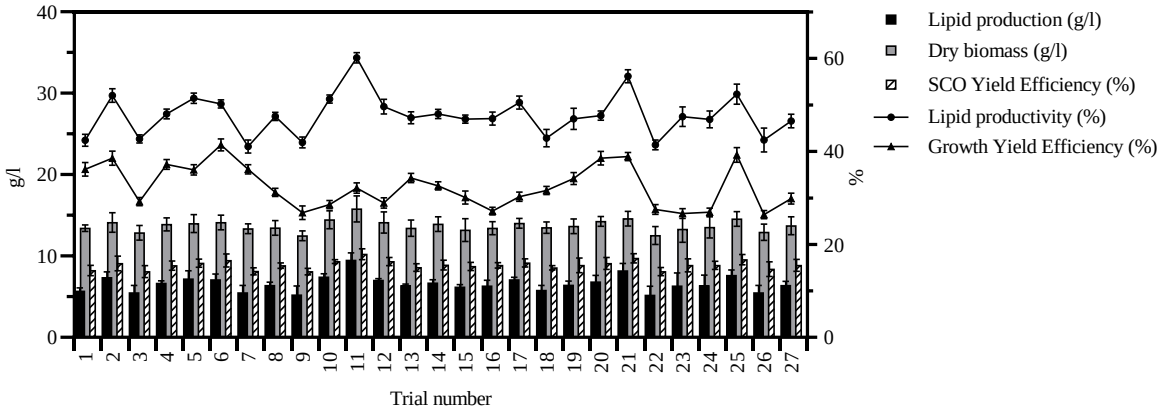

Figure 2. Dry biomass, lipid productivity, growth yield efficiency, SCO yield efficiency and nitrogen concentration for *Rhodosporidium babjevae KB 649*in L27 array

The best experiment for *Rhodosporidium babjevae KB 649* is the trial 11 in L27 array where lipid production and lipid content reach 9.48 g/l and 60.11%, respectively. The ANOVA results for *Rhodosporidium babjevae KB 649* L27 array are tabulated in Table 4. The last columns of both the tables show the influence percentage of each factor. The effect of different parameters on lipid production through this strain is illustrated in Fig. (3). The Y- axis represents the percentage of each parameter that obtained from ANOVA, and the X- axis represents the related parameters. The optimum values predicted by Taguchi method are tabulated in Table 5. Taguchi can estimate the optimum condition even though the given condition is not between the trials and its mount can be predicted. Lipid production is analyzed in the estimated condition and the obtained lipid is 10.34 g/L which is close to the estimated amount.

Table 4. Analysis of variance (ANOVA) of Taguchi results for *Rhodosporidium babjevae* KB 649 in L27 array

Factors	DOF	Sums of squares (S)	Variance (V)	F-Ratio (F)	Pure sum (S´)	Percent P(%)
Glucose (g/L)	2	1.864	0.932	55.181	1.83	7.684
$(NH_4)_2SO_4$ (g/L)	2	3.861	1.93	114.296	3.827	16.069
Time (h)	2	4.677	2.338	138.46	4.643	19.496
pH	2	1.057	0.528	31.315	1.024	4.299
rpm	2	2.17	1.085	64.244	2.136	8.97
Temperature (°C)	2	2.544	1.272	75.325	2.51	10.542
$MgSO_4$ (g)	2	1.693	0.846	50.141	1.66	6.97
KH_2PO_4 (g)	2	0.266	0.133	7.879	0.232	0.975
Inoculum (ml)	2	1.337	0.668	39.596	1.303	5.474
$FeCl_3$ (g)	2	2.261	1.13	66.939	2.227	9.352
$CaCl_2$ (g)	2	2.015	1.007	59.657	1.981	8.319
Other/Error	4	0.067	0.016	-	-	1.85
Total	26	23.817	-	-	-	100

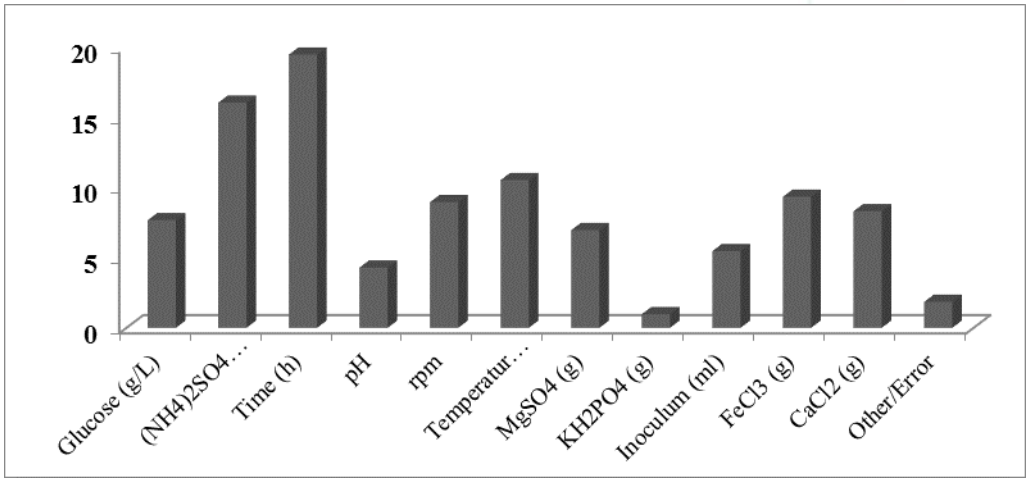

Figure 3. The Percentage contribution for each factor in lipid production by *Rhodosporidium babjevae* KB 649 obtained from Qualitek-4 software in L27 array. As observed, the last Column indicated the error level which is by far the smallest amount. Time of incubation and glucose concentration have the highest effect on lipid production in this strain at 20.486% and 20.45%, respectively).

Table 5. Optimum condition predicted by Taguchi for the highest production by *Rhodosporidium babjevae* KB 649 Expected result at optimum condition was 10.506 g L^{-1}lipid.

Factor	Optimized level	Level	Contribution
Glucose (g/L)	80	2	0.345
$(NH_4)_2SO_4$ (g/L)	0.5	1	0.515
Time (h)	96	2	0.571
pH	6	2	0.259
rpm	150	2	0.398
Temperature (°C)	20	2	0.302
$MgSO_4$ (g)	1	1	0.312
KH_2PO_4 (g)	2	3	0.121
Inoculum (ml)	6	2	0.313
$FeCl_3$ (g)	0.15	1	0.408
$CaCl_2$ (g)	0.3	2	0.383

3.2. Results of lipid analysis by GC-MS

The result of GC-MS indicates that the potential for industrial application of microbial oil. The composition of fatty acids methyl esters is: Myristic acid 1.98%, Palmitic acid 24.62%, Palmitoleic acid 0.9, Stearic acid 6.63%, Oleic acid 54.2%, Linoleic acid 7.78% and very low concentration of other fatty acid methyl esters similar to the composition reported by Bonturi et al. [23] (1.6% of Myristic acid, 28.6% of Palmitic acid, 0.8% of Palmitoleic acid, 8.6% of Stearic acid, 47.1% of Oleic acid, and 5.8% of Linoleic acid). The results of characteristic analysis for the extracted oil and biodiesel production are shown in Table 6. For better comparison, values of both the United States and European standards are reported as well.

Table 6. Properties of the extracted oil from *Rhodosporidium babjevae* KB 649, biodiesel from single cell oil and ASTM/EN standards

Properties	Units	extracted oil	biodiesel from extracted oil	US Standard ASTM D6751 standard	EU Standard EN 14214
Density	g/cm^3	0.883	0.889	NS	0.86-0.90
Moisture content	% wt	0.028	0.028	0.050max	0.25max
Acid value	mgKOH/g	0.19	0.20	0.5max	0.5max
FFA	%	0.09	0.10	NS	NS
Saponification value	mg/g	182	196	NS	NS
Iodine value	$mgI_2/100g$	75.31	113.44	NS	120max
Peroxide value	meq/kg	93.12	33.21	NS	NS
Kinematic viscosity at 40 ºC	Mm^2/s	4.65	3.86	1.9-6.0	3.5-5
Colour	-	Light brown	Dark yellow	-	-
Cetan number	-	-	57	48-65	51min
Flash point	ºC	149	156	100-170	>101
Cloud point	ºC	2	9	-3-12	NS
High heating value	mJ/KJ	43.4	42.7	NS	NS

NS: Not Specified

Taguchi method, in addition to the analysis, provides a procedure for designing and implementing experiments. This software can estimate the optimum condition even though the given optimum condition is absent during the experiments design. Less time and low cost are the advantages of this method [24]. Kraisintu et al. optimized chemical and physical condition for lipid production in *Rhodosporidium toruloides* DMKU3-TK16 and obtained 9.26 gL^{-1} lipid production in the 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 at 28°C. Optimization was achieved step by step for each parameter which requires many trials hence, more time and cost [14]. By using a method of designing experiments all the factors can be evaluated simultaneously. In this study *Rhodosporidium babjevae KB 649* has high potential in the field of lipid synthesis; lipid content and lipid production of this strain were 60.11% and 9.48 g L^{-1}, respectively in optimized condition.

In this study the optimum glucose concentration for lipid production is 80 g/L indicating no need for higher concentrations for optimum condition.

Huang et al. reported that organic nitrogen compounds are appropriate for Lipid accumulation and misappropriate for cell growth, while mineral nitrogen compounds are appropriate for cell growth, and misappropriate for lipid accumulation, thus both of the organic and inorganic nitrogen sources are essential in lipid production [25]. It was reported that lipid production in a medium containing yeast extract is higher than the medium containing peptone. According to this reports the yeast extract is the best nitrogen source for biomass and lipid production [26].

Li et al. [27], Yong Huang et al. [28] and Papanickolao and aggelis [29] reported that $(NH_4)_2SO_4$ is preferred as nitrogen source for lipid accumulation. Zhao et al. reported that the yeast extract is the best organic nitrogen source among the assessed nitrogen sources such as yeast extract, corn extract and urea in *lipomyces starkey* [30]. Karaty et al reported that an increase in ammonium sulfate concentrations by more than 1.5 g/L, the lipid production decreases [31]. It was revealed that phosphorus limitation benefits on lipid accumulation [21]. Kraisintu et al. assessed the effect of KH_2PO_4 at concentrations of 0.4, 1.6, 2.8 and 4 g/L on lipid production [14]. The highest lipid production in this study is 8.85 g / L in 0.4 g/L of KH_2PO_4. Hence, an increase in KH_2PO_4 more than 1.5 g/L leads to a reduction in lipid production.

4. Conclusion

The results indicated the effect of Taguchi method in optimization process and that the two stages of setting experiments contribute in obtaining optimized condition. The *Rhodosporidium babjevae KB 649* is a high lipid producing yeast with the potential of industrial applications. The lipid yield of this strain reaches 9.48 g L^{-1} with lipid content of 60.11% after 96 h at 25 $^{\circ}C$ when cultivated in nitrogen limited medium. This medium includes: 80 g L^{-1}glucose,0.5 g L^{-1} ammonium sulfate, 1 g L^{-1} yeast extract, 1 g L^{-1} $MgSO_4$, 1.5 g L^{-1} KH_2PO_4, 0.15 g L^{-1} $FeCl_3$, 0.3 g L^{-1} $CaCl_2$ with 8 ml inoculums and pH adjusted at 7 in shaking flask at 150 rpm. Evaluating all the parameters simultaneously and obtaining a reliable result determine the effectiveness of this adopted method.

Conflict of interest

The authors declare that there is no conflict of interest between them.

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