

Optimization of Cultivation Conditions for Microbial Lipid Production by *Rhodotorula glutinis*, an Oleaginous Yeast

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ABSTRACT

Microbial fuels are the 3rd generation biofuel. These fuels are made by the conversion of microbial lipid into fuel. *Rhodotorula glutinis*, a pink oleaginous microbe has the capacity to produce microbial lipids from culture medium containing carbon and nitrogen sources. These microbial lipids can further be converted in to fuel. In this work, the growth conditions for *R. glutinis* were optimized with different C:N ratios by having various concentrations of Carbon and Nitrogen sources. Glucose, Fructose, Sucrose, Galactose and Xylose were evaluated as carbon sources (70 g/L). (NH₄H₂PO₄, NH₄NO₃, (NH₄)₂SO₄, Urea, Asparagine were evaluated as Nitrogen source (20g/L). Subsequently, the influence of surfactants (Tween 20 and Tween 80 (0.5 ml/L), pH (3, 4,5) and incubation temperature °C (25,30,35 and 40) were also analysed in initial media composition. *Rhodotorula glutinis* showed significant growth with maximum biomass and lipid production in the media containing sucrose as carbon source, NH₄NO₃ as nitrogenous source at pH 4 and temperature 30 °C. The surfactant has shown no effect in lipid production. Thus, the results of this study indicated that these optimized conditions could be used to produce maximum production of lipids as biodiesel feedstock.

KEY WORDS: *RHODOTORULA GLUTINIS*, MICROBIAL LIPIDS, MICROBIAL FUELS, OLEAGINOUS MICROBE

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INTRODUCTION

The price of fuel rises every year with a worrying concern of depletion and complete loss of the available natural resources. With the idea of sustainable development and cutting the economic values of the fuels other options are like usage of plants, animals, aqua life and microbes are acquitted for research and development. Biofuels, namely bioethanol, butanol produced from cellulose are therefore of considerable interest to various researchers as well as governments (Demian *et al.*, 2005; Hill *et al.*, 2006; Zhou *et al.*, 2014; Choudhary *et al.*, 2017).

Yeast is generally being employed on a large scale yielding vitamins, industrial enzymes and pharmaceutical polypeptides. The oleaginous attribute of yeast renders it advantage over its proponent viz. bacteria, molds, and alga, primarily due to higher rates of proliferation & also due to the propensity to greater lipid yields (Saenge *et al.*, 2011). High degree of lipid accumulation is due to carbon surplus and a nitrogen or some other nutrient deficit in the growth medium. (Ratledge & Boulton, 1985; Ratledge, 1986). Hence fatty acid profile of the microbial oil like the oleaginous yeast makes it potential host for biodiesel industry due to the accumulation of polyunsaturated fatty acid triacylglycerol inside the cells, which is similar to vegetable oils (Dai *et al.*, 2007; Kot *et al.*, 2016).

Jiru *et al.*, (2017) optimized the biotechnological production of lipid by *Rhodotorula kratochvilovae* (syn, *Rhodospiridium kratochvilovae*) SY89 for biodiesel preparation. It could serve as a replacement for conventional oil sources like crude oil replenishing the necessities of the energy sector & also industries like food, pharmaceutical or cosmetic. *Rhodotrula glutinis* is aerobic yeast characterized by pink, smooth colonies with a moist appearance, sexually reproduce via basidiospores arising from a teliospore developed primarily due to the existence of mycelial clamp connection. Multipolar budding serve as reproductive avenue. The significance of *Rhodotorula glutinis* yeast is being acknowledged & published in different journals for the production of numerous useful metabolites such as lipid (Li, *et al.*, 2013), carotenoids having anticancerous and antioxidant properties (Gupta *et al.*, 2012; Yen *et al.*, 2015) and many industrially useful enzymes. *Rhodotorula glutinis* can produce brewery effluent (Yen *et al.*, 2012), crude glycerol (Schneider *et al.*, 2013), microbial lipids, carotene, cellulase and carotenoids by using cheap energy sources like carbon, nitrogen, Sulphur, (Karamerou *et al.*, 2016; Pi *et al.*, 2018; Elfeky *et al.*, 2019). In this research Optimization of Microbial lipid production by *Rhodotrula glutinis* was done by using various carbon and nitrogen sub-

strates at different pH, Temperature and time of cellular growth.

MATERIALS AND METHODS

Yeast *Rhodotorula glutinis* strain was obtained from IMTECH (Chandigarh, India). The lyophilized culture was hydrated in medium containing Glucose (70g/l), $(\text{NH}_4)_2\text{SO}_4$ (20g/l), a slightly modified basal medium recommended by Bhosale and Gadre (2001). Then a culture was transferred to slant tubes containing medium with the same composition, including 15 g/l agar and incubated at 30° C for 60 hr. After growth, the slants were kept at 4° C and were subcultured each 2 months. The inoculum was prepared in Erlenmeyer flasks of 250-ml with 100 ml of medium.

Optimization of Growth Conditions: The growth conditions for *R. glutinis* were optimized with different C:N ratios by having various concentrations of Carbon and Nitrogen sources. Glucose, Fructose, Sucrose, Galactose and Xylose were evaluated as carbon sources (70 g/l) with Ammonium Sulphate (20 g/l) as nitrogen source. $\text{NH}_4\text{H}_2\text{PO}_4$, NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, Urea, Asparagine were evaluated as Nitrogen source (20g/l) with glucose (70g/l) as carbon source. Subsequently, the influence of surfactants (Tween 20 and Tween 80 (0.5 ml/l), pH (3, 4, 5) and incubation temperature °C (25,30,35 and 40) were also analysed in initial media composition.

R. glutinis were inoculated and incubated at 30°C for 60 Hr. The dry weight of *R. glutinis* was measured after each 12Hr. Dry mass of the cell was taken by centrifugation of one ml culture medium and dried the pallet at 80 °C for 12 hr. then the weight of dried mass was taken and converted into g/l.

Isolation of lipid from *R. glutinis*: Extraction of lipids was done by Soxhlet method in which hexane was used as solvent for the extraction from *R. glutinis*. The dried yeast was used for the isolation of lipid. The moisture content of the yeast sample should not exceed 10%. Weighed exactly 1 g of yeast sample which was kept on Whatman filter. Covered the top of each thimble with glass wool to prevent floating. Weighed the pre-dried flat-bottom extraction flask with a few boiling chips or glass beads. Extract lipids with 150 to 200 ml of hexane at the boiling point for 7 to 12 h in a Soxhlet extractor using a heating mantle. The condensation rate for the solvent was set at about 2 to 6 drops per second, depending on the extraction period envisaged. For longer extraction periods, a lower condensation rate was selected and vice versa. Usually, an extraction period of 8 hr at a rate of 150 drops per min was considered adequate. The sample was made to cool. Finally solvent was removed from

the sample extract using rotary evaporator at 40°C under reduced pressure. Further calculations were performed for the amount of lipid recovered and its percentage in the original sample using following equation: –

$$\text{Mass of lipid} = (\text{weight of the flask} + \text{boiling chips} + \text{extracted oil}) - (\text{weight of the Flask} + \text{boiling chips})$$

RESULTS AND DISCUSSION

The *Rhodotorula glutinis* was grown on different carbon source (Glucose, Fructose, Sucrose and Xylose) for different incubation time (12, 24, 36, 48, 60 hr.) As we can see from the Table 1 & Figure 1, both carbon source and incubation time play an important role in the production of lipid. The maximum biomass (17.14 g/l), lipid production (8.82 g/l) and % lipid yield (51.45%) were found with Sucrose in the incubation period of 60 hrs followed by glucose, xylose and fructose.

Table 1. Growth of *Rhodotorula glutinis* and production of lipid at different carbon sources.

Carbon Source	Time (Hr.)	Biomass (g/l)	Lipid (g/l)	% Lipid yield
Glucose	12	0.76	0.29	38.15
	24	3.71	1.41	38.00
	36	8.73	4.11	47.07
	48	14.13	6.91	48.90
	60	14.63	7.36	50.30
	72	13.95	6.92	49.60
Fructose	12	0.97	0.26	26.80
	24	4.65	1.94	41.72
	36	7.23	3.63	50.20
	48	10.84	5.21	48.06
	60	17.32	5.62	32.44
	72	16.82	5.34	31.74
Sucrose	12	1.64	0.47	28.65
	24	8.81	3.25	36.88
	36	13.21	6.63	50.18
	48	16.62	8.38	50.42
	60	17.14	8.82	51.45
	72	16.74	8.55	51.07
Xylose	12	0.83	0.31	37.34
	24	7.65	3.13	40.91
	36	10.93	5.32	48.67
	48	13.66	6.89	50.43
	60	13.79	6.91	50.10
	72	13.67	6.63	48.50

Among the various nitrogen sources Table 2 & Figure 2, NH_4NO_3 was the best nitrogen source for production of biomass (17.11 g/l), lipid production (8.62 g/l) and % lipid yield (50.37%). Asparagine showed the poorest result.

Various nitrogenous sources are taken for the optimization of cultivation conditions viz. ammonium dihydrogen phosphate, ammonium nitrate, ammonium sulphate, urea & asparagine in which the best possible nitrogenous substrate was found out to be ammonium nitrate (NH_4NO_3) at an optimal temperature of 30 °C at the 60th hour incubation as far the microbial proliferation with respect to lipid yield is concerned.

The cell dry weight increased gradually with an increase in the pH of the broth (at pH 3 & 4) later got decreased by increasing the pH (pH 5). The optimum pH were found to be 4 & 3 as per Table 3 & Figure 3 for maximum yield of biomass, lipid production and % of lipid yield.

R. glutinis was able to grow at all the temperatures examined °C (25, 30, 35, 40). A considerably increased biomass (18.65 g/L) 30°C; whereas enhancement of temperature 35°C to 40°C causes reduction in the biomass. The reduction in the biomass primarily is an indicator of microbial competency to withstand the temperature alterations (Table 4 and Figure 4). Biomass produced efficiently at 30°C suggests that the rate of replication has increased significantly during the or in other words the doubling time of oleaginous organism has decreased vis-à-vis to all the other incubation temperature. The sampling time of incubated organism in order to assess the biomass yield as well as the lipid content is strategically being conceived at a time interval of 12 hours depending upon the microbial growth curve of the organism.

The illustration in the graphs primarily suggests the data which is being tabulated. The rate of production of the biomass is directly correlated lipid yielding efficiency of the biomass. As one can easily decipher from the depiction maximal biomass yield is being acquired at optimal mesophilic temperature i.e., 30°C, invariably also suggests the ratio of production of biomass vis-à-vis lipid content which is 1:2

The effect of surfactant (0.5ml/l) on the growth of *R. glutinis* was measured at different time intervals and observed that surfactants did not effected much. The production of Biomass and lipid were almost same for both the surfactants (Table 5 & figure 5)

The table illustrates the implication of two surfactants being used viz. tween 20 & tween 80 which had no negative or positive effect on the biomass yield with respect to the lipid content. Hence one can imply the microbial culture or lipid yield remain unaffected with the aforementioned concentration (0.5ml/l) of surfactants being used

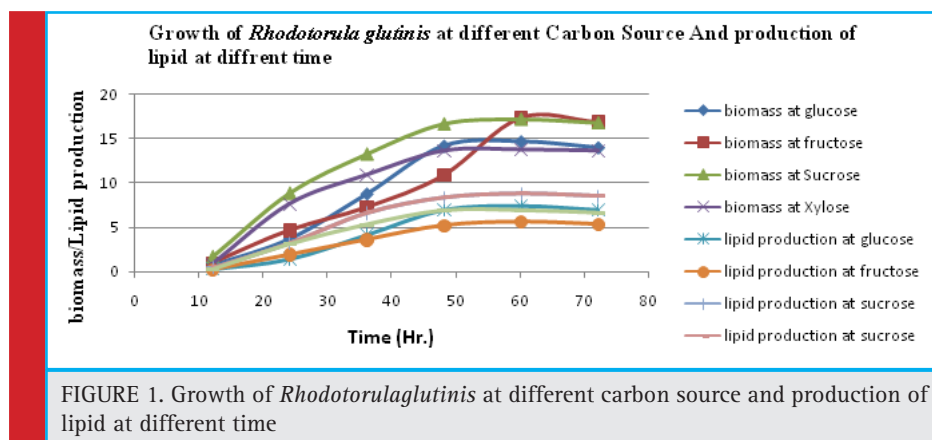


Table 2. Growth of *Rhodotorula glutinis* and production of lipid at different nitrogen sources.

Nitrogen Source	Time (Hr.)	Biomass (g/l)	Lipid Content (g/l)	Lipid Yield (%)
NH ₄ H ₂ PO ₄	12	1.21	0.31	25.61
	24	5.62	2.19	38.96
	36	11.63	5.08	43.68
	48	14.25	7.11	49.89
	60	14.83	7.49	50.5
	72	14.52	7.29	50.2
NH ₄ NO ₃	12	1.93	0.61	31.6
	24	8.65	4.09	47.28
	36	12.82	6.32	49.29
	48	16.89	8.53	50.5
	60	17.11	8.62	50.37
	72	16.79	8.41	50.08
(NH ₄) ₂ SO ₄	12	1.21	0.51	42.14
	24	3.18	1.21	38.05
	36	5.62	2.31	41.1
	48	8.93	4.31	48.26
	60	9.32	4.19	44.95
	72	9.12	3.96	43.42
Urea	12	0.919	0.27	29.37
	24	8.82	3.89	44.1
	36	9.12	4.19	45.94
	48	9.16	4.41	48.14
	60	10.14	4.62	45.56
	72	9.83	4.3	43.74
Asparagine	12	0.33	0.06	18.18
	24	0.81	0.31	38.27
	36	1.96	0.68	34.69
	48	3.92	1.56	39.79
	60	5.72	2.22	38.81
	72	5.65	2.15	38.05

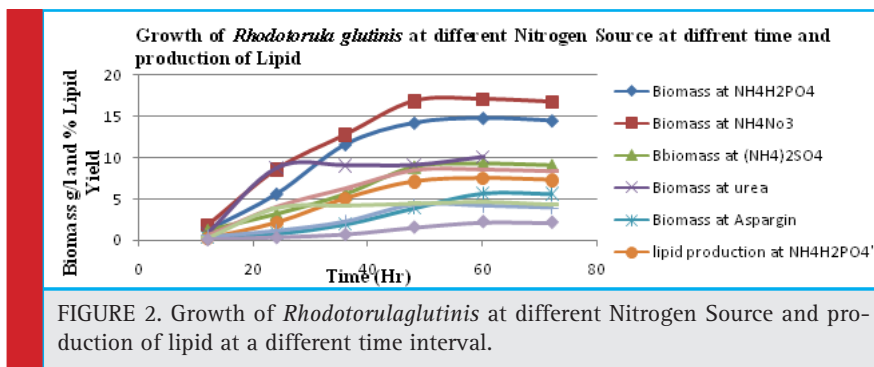


Table 3. Growth of *Rhodotorula glutinis* and production of Lipid at different pH and different time interval.

S. No.	pH of the media	Time Duration Hr.	Biomass g/l	Lipid production g/l	Lipid Yield %
1	3	12	0.61	0.23	37.70
		24	2.93	1.15	39.24
		36	7.63	3.43	44.95
		48	14.22	7	49.22
		60	15.61	7.97	51.05
		72	15.34	7.4	48.23
2	4	12	1.92	0.72	37.50
		24	4.32	2.17	50.23
		36	12.33	6.12	49.63
		48	17.61	8.92	50.65
		60	18.98	9.68	51.00
		72	18.56	9.32	50.21
3	5	12	1.62	0.41	25.30
		24	9.82	4.13	42.05
		36	11.31	5.67	50.13
		48	16.13	8.12	50.34
		60	16.92	8.42	49.76
		72	16.82	8.32	49.46

The graphical illustrations of tabulated data is indicative of the fact aforesaid i.e., surfactant remains inert to the microbial proliferation & hence lipid yield unaffected, hence the indictment for the biomass production

& lipid yield remains stable as for as the exposure to surfactant is concerned.

When *R. glutinis* was incubated with the medium containing sucrose as a carbon source (70 g/l), NH₄NO₃ as

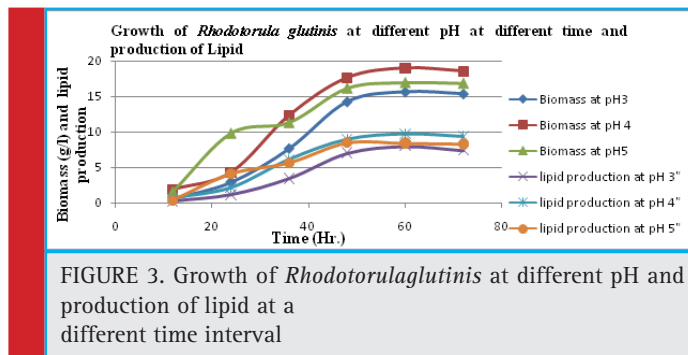


Table 4. Growth of <i>Rhodotorula glutinis</i> and production of Lipid at different Temperature and different time interval.					
S. No.	Temp. (C)	Time Duration (Hr.)	Biomass (g/l)	Lipid Content (g/l)	Lipid Yield %
1.	25	12	1.01	0.28	27.72
		24	13.13	3.72	28.33
		36	14.99	4.43	29.55
		48	16.43	5.43	33.04
		60	17.34	6.32	36.44
		72	16.98	6.12	36.04
2.	30	12	1.21	0.35	28.92
		24	15.08	7.18	47.61
		36	15.98	8.17	51.12
		48	18.32	9.24	50.43
		60	18.65	9.41	50.45
		72	18.43	9.21	49.97
3.	35	12	1.09	0.31	28.44
		24	2.93	1.29	44.02
		36	13.17	6.48	49.20
		48	14.68	7.32	49.86
		60	15.73	7.95	50.54
		72	15.21	7.12	46.81
4.	40	12	0.66	0.21	31.81
		24	4.32	2.22	51.38
		36	8.11	3.94	48.58
		48	12.13	5.93	48.88
		60	12.56	6.45	51.35
		72	12.09	6.38	52.77

a nitrogen source (20 g/l), yeast (15g/l) at 30°C with pH 4. The yield of lipid was increased and reached 57.23% of total dry biomass with inoculum size 6%. (Table 6 and Figure 6)

Again the the table illustrates the significance of the optimized inoculum size with respect to optimal temperature & media components making growth maximal to

its competency. The maximal biomass yield is achieved at pH 4 indicative of the operability of oleaginous organism to produce lipid under the influence of the acidic condition. Moving towards the alkaline scale the rate of microbial proliferation decrease & withit the lipid content is invariably effected. The best possible microbial proliferation & lipid content is being perceived for the sucrose

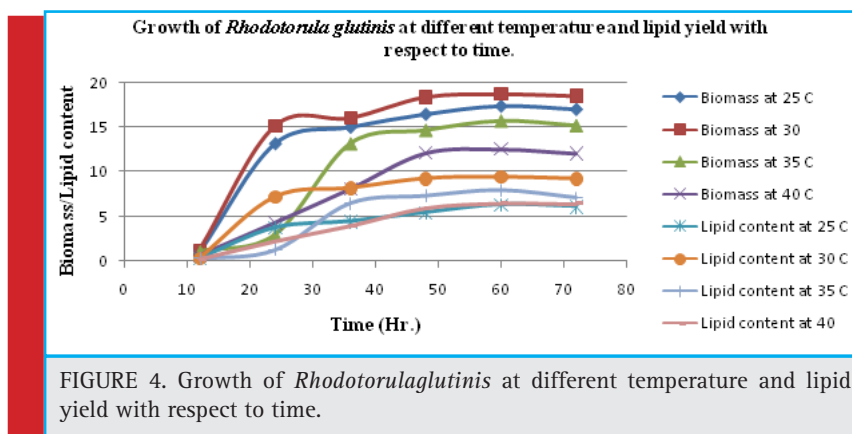
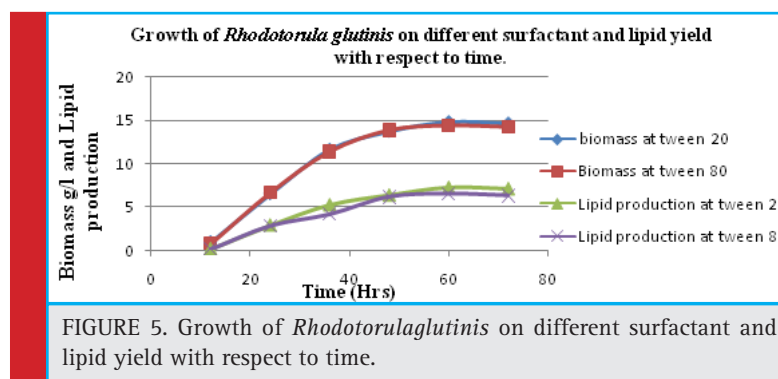


Table 5. Growth of *Rhodotorula glutinis* at different surfactant and production of lipid at different time interval

S. No	Surfactant	Time (hr.)	Biomass (g/l)	Lipid production (g/l)	Lipid content (%)
1	Tween 20	12	0.95	0.21	22.10
		24	6.52	2.91	44.63
		36	11.62	5.24	45.09
		48	13.73	6.36	46.32
		60	14.81	7.23	48.81
		72	14.65	7.09	48.39
2	Tween 80	12	0.81	0.17	20.98
		24	6.66	2.84	42.64
		36	11.38	4.15	36.46
		48	13.84	6.15	44.43
		60	14.38	6.9	46.52
		72	14.22	6.3	44.30



as the carbon substrate followed by glucose & fructose. Xylose is least catabolized substrate as far as microbial replication & lipid production is concerned

CONCLUSION

In this present work, we optimize the growth conditions of *Rhodotorula glutinis* to get maximum production of Biomass and lipid content. Sucrose was found to be the

best carbon source (70 g/l), NH_4NO_3 was found to be best nitrogen source (20g/l) with optimum pH 4, optimum temperature 30 °C and optimum time period 60 hrs. Surfactant does not have any effect on the growth and lipid production of *Rhodotorula glutinis*. This study reveals that cultivation conditions had an influence on lipid production. This study provides a valid and potential strategy for optimizing yeast cultivation conditions to improve the production of lipid with possibly broad biotechnological applications.

Table 6. The growth of *Rhodotorula glutinis* at the optimized condition, and effect of inoculums size and production of lipid at a different time interval

Media	pH	Temperature	Inoculum Size (%)	Biomass after 60 hrs of incubation (g/l)	Lipid production (g/l)	% Yield of lipid
70 g/l Sucrose	4	30 °C	5	19.5	10.3	52.82
20 g/l NH_4NO_3			6	22.2	12.6	57.27
15 g/l Yeast Extract						
7 g/l KH_2PO_4						
1 g/l MgSO_4						
15 g/l Agar			7	20.1	10.7	53.23

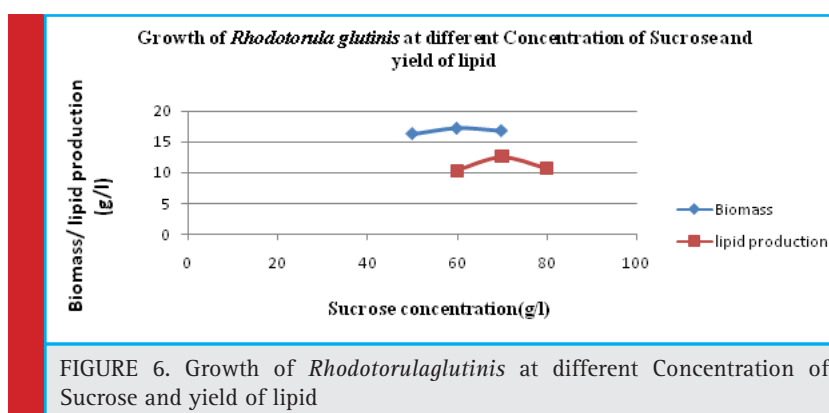


FIGURE 6. Growth of *Rhodotorulaglutinis* at different Concentration of Sucrose and yield of lipid

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