Effect of pH Cycling on Lipid Production by *Yarrowia lipolytica*

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Abstract

The present study aims to investigate the effect of pH cycling on lipid production by nitrogen limited batch cultures of oleaginous yeast Yarrowia lipolytica NCIM 3472. D-glucose was used as a carbon source in all experiments. C/N ratio of 120 (mol/mol) was found to be optimum for lipid production among tested ratios in shake flask experiments, however lipid was produced in very low quantities in all trials. Lipid production in optimum C/N ratio was then examined in culture propagating in 3.7 L bioreactor under three different pH profiles. The maximum lipid content obtained was 34.57% (w/w) when pH was not controlled whereas it was 34.78% when pH was maintained at 6. Cycling pH in the third experiment resulted in the maximum lipid content of 36.92%. Lipid production rate and glucose to lipid conversion yield remained almost unchained in all the three experiments. The results indicate that the deliberate modification in the pH profile of the culture during lipogenic phase leads to a small increment in the lipid content of the cells.

Keywords: Lipid; Oleaginous; Single Cell Oil; *Yarrowia lipolytica*; pH Cycling

1.Introduction

Few microorganisms are known to accumulate substantial amount of lipids, usually in the form of triacylglycerols, when grown under appropriate conditions. Such species are known as oleaginous and the lipids so produced are known as single cell oils (SCO). Lipid accumulation in oleaginous microbes begins when a key nutrient (nitrogen, phosphorus or sulfur) becomes exhausted and an excess of assimilatable carbon source is present in the growth medium. Accumulated lipids are then not only useful to the producing microorganism as an intracellular reserve supply of both carbon and energy to rescue during any subsequent period of starvation, but also may be considered as sources of these commodities.

Extensive research on biochemistry of lipid synthesis in oleaginous microalgae and yeast under stress conditions has opened new avenues in lipid biotechnology. Economic production of microbial lipids is the major concern especially when these lipids are to be used for low value products like biodiesel. Unlike microalgae, yeast is fast growing, with high lipid content resulting in comparatively better lipid productivities. Various strategies were investigated for the economic production of SCO from oleaginous yeast. Several studies on the use of inexpensive carbon sources, growth medium optimization, effect of environmental conditions and fermentation configurations on lipid production by oleaginous species explored the possibilities of making this production process economically and therefore commercially viable [1-9].

Citrate accumulates as soon as Isocitrate dehydrogenase activity drops when cells are nitrogen starved, which is the most favored condition for inducing lipogenesis, to stop energy generation. In oleaginous yeast, citrate is then converted to acetyl-CoA and oxaloacetate by ATP citrate lyase in the cytosol. Malate dehydrogenase converts oxaloacetate to malate which is then catalyzed to pyruvate by malic enzyme to produce NADPH required for lipid biosynthesis [10]. Accumulation of TCA cycle acids during lipogenic phase decreases the pH of culture making necessary to control it as pH dramatically affects microbial growth. Extracellular pH has a strong influence on the pathways of metabolisms and products generated by microorganisms.

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We worked on oleaginous yeast *Yarrowia lipolytica* which has been extensively studied as a model for lipid accumulation and has also been subjected to genetic manipulations for enhanced lipid production [11]. Nitrogen-limited cultures of *Y.lipolytica* secretes variety of organic acids, including significant quantities of citric acid, from a wide range of carbon sources [2,12,13]. Studies have shown that pH effects the lipid content to significant levels but has less influence over the fatty acid composition of the accumulated lipids [9,14,15]. Significant changes in the lipid content of *Rhodotorula glutinis* with the change in pH was reported by Johnson V et al. [9]. The aim of the present study was to investigate the changes in the lipid content of *Yarrowia lipolytica* NCIM 3472 when grown under fluctuating pH with tolerable upper and lower limits.

2.Materials and Methods

Microorganism and culture conditions

Yarrowia lipolytica strain NCIM 3472 used in this study was provided by National Collection of Industrial Microorganisms, Pune, India. Colonies of the strain were maintained at 4 ± 1°C on YEPD slants containing (in g/L): D-glucose 20, Yeast extract 10, Peptone 20, and Agar 15. The nitrogen-limited fermentation medium used contained (g/L): $(NH_4)_2SO_4$ 0.5, KH_2PO_4 7, $Na_2HPO_4.2H_2O$ 3.13, $MgSO_4.7H_2O$ 1.5, $CaCl_2.2H_2O$ 0.2, $ZnSO_4.7H_2O$ 0.02, $MnSO_4.H_2O$ 0.06, Yeast extract 0.5, D-glucose – depending on the C/N ratio (pH after sterilization 6 ± 0.1).

To determine the effect of C/N ratio on the lipid yield and production rate, shake flask experiments were performed for different C/N ratios in 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium inoculated with 1 mL of a 21 h exponential phase shake flask culture giving an initial concentration of 0.1 g/L. To vary the C/N ratio, following initial glucose concentrations were used: 5.90, 11.94, 17.99, 24.03, 30.08, 36.12, and 42.17 g/L, corresponding to C/N ratios of 20, 40, 60, 80, 100, 120, and 140, respectively. Culture flasks were incubated in an orbital shaker (Adolf Kühner AG, Switzerland) at incubation temperature of 28 ± 1°C, agitation rate of 180 rev/min and the experiments were done with triplicates.

Fermenter experiments were performed in a 3.7 L capacity bioreactor (Bioengineering AG, Switzerland). In all fermenter experiments, 2 L of fermentation medium was inoculated with 200 mL of 26 h exponential phase shake flask culture. The pH was initially adjusted to 6 by addition of 2N NaOH. Air was sparged at a flow rate of 1 vvm and the stirrer speed was 300 rpm. Incubation temperature was kept at 28 ± 1°C throughout the fermentation period. Sterilized silicone oil was used to control foaming.

To elucidate consequences of pH cycling three fermenter experiments were performed, each with a different objective. Correlation between pH change and lipid production was determined in the first fermenter run when pH was not controlled. pH was maintained at 6 throughout the second run whereas in the third run pH was controlled only in the lipogenic phase by increasing the pH to 6 every time it falls to 5.5. Samples were tested for lipid production during lipogenic phases.

Analytical methods

To determine dry biomass a known volume of culture was harvested by centrifugation at 5000g for 5 min. Supernatants were analyzed for glucose concentration. Harvested biomass was washed twice with distilled water and then dried at 60°C to constant mass (about 24 h.) after which the biomass was determined gravimetrically [3]. Glucose concentration in the culture medium was determined with the dinitrosalicylic acid (DNS) method.

To determine the lipid content, a 100 mL. sample was centrifuged at 5000g for 5 min. Harvested biomass was washed twice with 100 mL of distilled water, then added into 20 mL of 4 M HCl, and incubated for 1 to 2 h at 60°C. Mixture was stirred with 40 mL of chloroform/methanol mixture (1:1) at room temperature for 2 to 3 h, followed by centrifugation at 2000g for 5 min at room temperature. Lower phase containing lipids was recovered with a Pasteur pipette, and solvent was evaporated. Lipids were then weighed [3].

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3.Results and Discussion

Yarrowia lipolytica can metabolise diverse carbon sources [13-16]. We grew it on glucose and there was no glucose inhibition even at high initial glucose concentrations (42.17 g/L). Exponential growth occurred during balanced growth phase with the same specific growth rate (μ = 0.2 h^{-1}) in all batches. Results of shake flask experiments (Table 1) clearly indicate that biomass concentration and lipid percentage increased with the increase in C/N ratio, however very low quantities of cellular lipids were accumulated with a maximum lipid content of 9.1% (w/w) corresponding to maximum biomass concentration of 11.7 g/L at C/N ratio of 120. Similar findings on low quantities of cellular lipids (5-12% w/w) at C/N ratios of 110-500 (mol/mol) when *Yarrowia lipolytica* was grown under similar conditions were earlier reported [2]. Progressive increase in final biomass concentration may be due to increase in lipid percentage with increase in C/N ratio. Carbon gets consumed rapidly at high C/N ratios due to increase in rate of production of organic acids and this is evident from the fact that process time is significantly reduced at C/N ratios of 120 and 140. Significantly low values of lipid production rate and glucose to lipid conversion yield were obtained which remained almost constant throughout the range of C/N ratios tested and were too low to be used for determining optimum C/N ratio. These results suggest that C/N ratio of 120 is optimum for lipid production and therefore this ratio was used in all fermentations in the second phase of investigation.

Profile of pH (Fig. 1) reported during first fermenter run shows that pH is decreasing with time. Maximum drop in pH was between 22 h and 23 h and there was no change in pH after 28 h when pH was 3.9. Sampling was done after every 2 h upto 22 h after which the sampling period was reduced to 1.5 h to more closely monitor the changes in the lipogenic phase. Lipid analysis was started from 22 h. Log phase specific growth rate was 0.25 h^{-1}. Lipid content (Table 2) increased from 29.38% in 22 h to maximum lipid content of 35.10% in 25 h. Moreover, Glucose to lipid conversion yield and lipid production rate was significantly low and remained unchanged. The results clearly indicate that the culture was in the lipogenic phase during this period. It is evident from biomass concentration curve (Fig. 1) that the culture moved from balanced growth phase to lipogenic phase after 14 h and therefore it was proposed to start pH cycling in the third run after 19 h when the culture must have completely entered into the lipogenic phase.

In the second run where the pH was kept constant, we began sampling for lipid analysis from 19 h to compare with the corresponding results of third run. There was no change in log phase specific growth rate (0.25 h^{-1}) and it appears that like the first run, the culture is in lipogenic phase after 14 h (Fig. 2). Lipid content increases to a maximum of 34.78% in 25 h after which it remained constant (Table 3). As in the first run, Glucose to lipid conversion yield and lipid production rate was considerably low.

Growth in the third experiment was similar to the first experiment up to 19 h when pH was not controlled. The first addition of base to increase the pH to 6 was done at 19 h when pH was fallen to 5.41 (Fig. 3). After 2.3 hours base was added again when pH was 5.5. Third and fourth addition of base was at 23.43 h and 26.65 h respectively. Biomass concentration dropped and there was no change in pH after 28h making us to terminate the process at 28 h as we did in the first and second experiment. Maximum lipid content was 36.92% in 25 h. Conversion rate and yield presented very low values (Table 4).

Like shake flask, lipid yield and production rate in fermenter experiments were insignificant in determining performance of the process. In the first 19 h of cultivation it seems that lipid accumulation is unaffected by the pH profile as it dropped by only 0.6 units in the third experiment. Three hours after the first addition of base; lipid contents of cells in the third fermentation was same as that of cells in the second fermentation and was 2% more than the cells in the first fermentation at the same time. This can be reasoned as fluctuating pH in the third experiment is averaging its own effect and has brought similar results as of constant pH 6. Also it is evident that the uncontrolled process is producing less lipids comparatively. At 25 h; in the third fermentation, the strain accumulated the highest percentage of lipids (36.92%) when 34.57% and 34.78% lipids were accumulated in the first and second experiment, respectively. It is at this instant stimulating effects of such

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fluctuations on the lipid synthesis can be postulated. Thereafter it appears that the cells experiencing pH fluctuations are now metabolizing their own lipids as lipid percentage fell and the cells in the first two fermentations have lost their potential to accumulate more lipids.

4. Tables

Table 1: Shake flask results

	C/N (mol/mol) Ratio*						
	20	**40**	**60**	**80**	**100**	**120**	**140**
X_{final} **(g/L)**	10.21	10.38	10.63	11.20	11.47	11.70	11.30
Total fermentation time (h)	56	76	98	102	116	76	66
% Lipid (wt./wt. dry biomass)	1.70%	3.11%	5.19%	6.79%	8.53%	9.10%	8.87%
Lipid production rate (g/L/h)	0.0031	0.0042	0.0056	0.0075	0.0084	0.0140	0.0152
$Y_{L/S}$ **(wt./wt.)**	0.031	0.030	0.034	0.035	0.033	0.032	0.026

* it was assumed that yeast extract contained 12% wt./wt. of carbon and 7% wt./wt. of nitrogen.

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Table 2: First fermenter run results

	Time (h)				
	22h	**23.5h**	**25h**	**26.5h**	**28h**
X **(g/L)**	13.61	13.69	13.80	13.83	13.57
L **(g/L)**	4	4.51	4.77	4.75	4.73
S **(g/L)**	2.32	1.84	1.6	1.28	1.20
% Lipid (wt./wt. dry biomass)	29.39%	32.94%	34.57%	34.35%	34.86%
Lipid production rate (g/L/h)	0.18	0.19	0.19	0.18	0.17
$Y_{L/S}$ **(g/g)**	0.12	0.13	0.14	0.14	0.14

Table 3: Second fermenter run results

	Time (h)						
	19h	**20.5h**	**22h**	**23.5h**	**25h**	**26.5h**	**28h**
***X* (g/L)**	13	13.7	14.6	15.12	15.18	14.4	14.24
***L* (g/L)**	2.97	3.90	4.67	5.25	5.28	5.00	4.91
***S* (g/L)**	6.42	3.62	2.76	1.12	0.92	0.41	0.4
% Lipid (wt./wt. dry biomass)	22.81%	28.50%	32%	34.52%	34.78%	34.72%	34.48%
Lipid production rate (g/L/h)	0.16	0.19	0.21	0.22	0.21	0.19	0.18
$Y_{L/S}$ (g/g)	0.10	0.12	0.14	0.15	0.15	0.14	0.10

Table 4: Third fermenter run results

	Time (h)						
	19h	**20.5h**	**22h**	**23.5h**	**25h**	**26.5h**	**28h**
***X* (g/L)**	12.9	13.4	14.18	13.91	14.3	14.11	13.73
***L* (g/L)**	2.87	3.57	4.43	4.80	5.28	5.12	4.81
***S* (g/L)**	4.23	3.67	2.04	1.83	0.92	0.45	0.42
% Lipid (wt./wt. dry biomass)	22.25%	26.64%	31.24%	34.51%	36.92%	36.29%	35.03%
Lipid production rate (g/L/h)	0.15	0.18	0.20	0.20	0.21	0.19	0.17
$Y_{L/S}$ (g/g)	0.09	0.11	0.13	0.14	0.15	0.14	0.13

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5. Figures

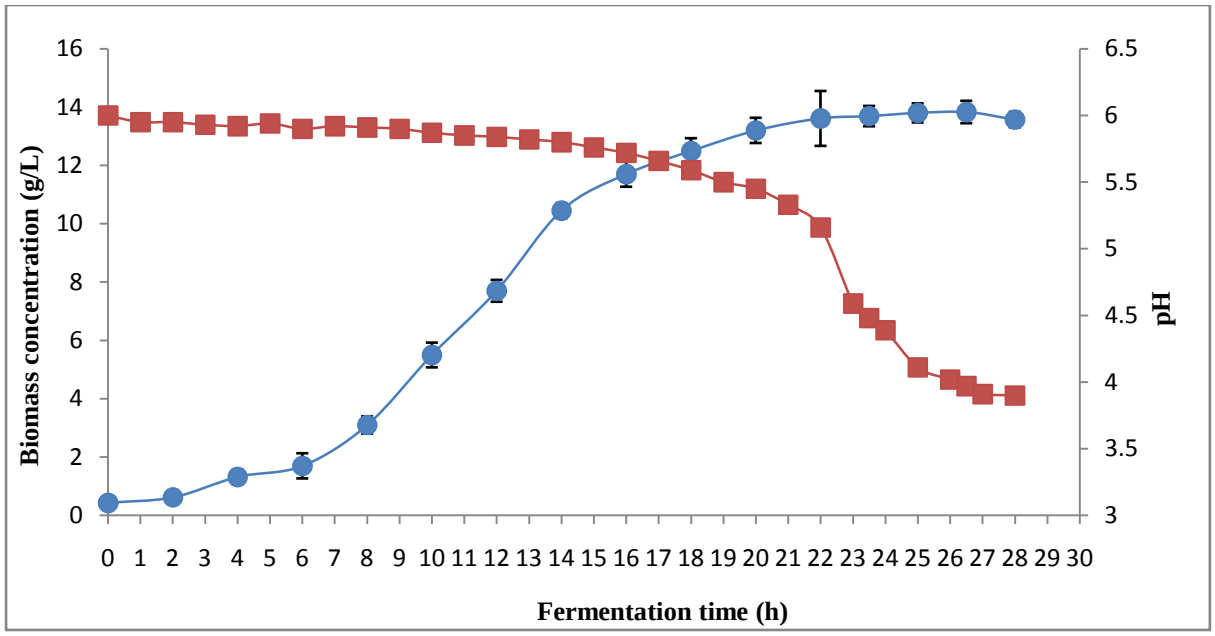

Fig.1. Biomass concentration and pH profile in first fermenter run (Biomass concentration g/L (●), pH (■))

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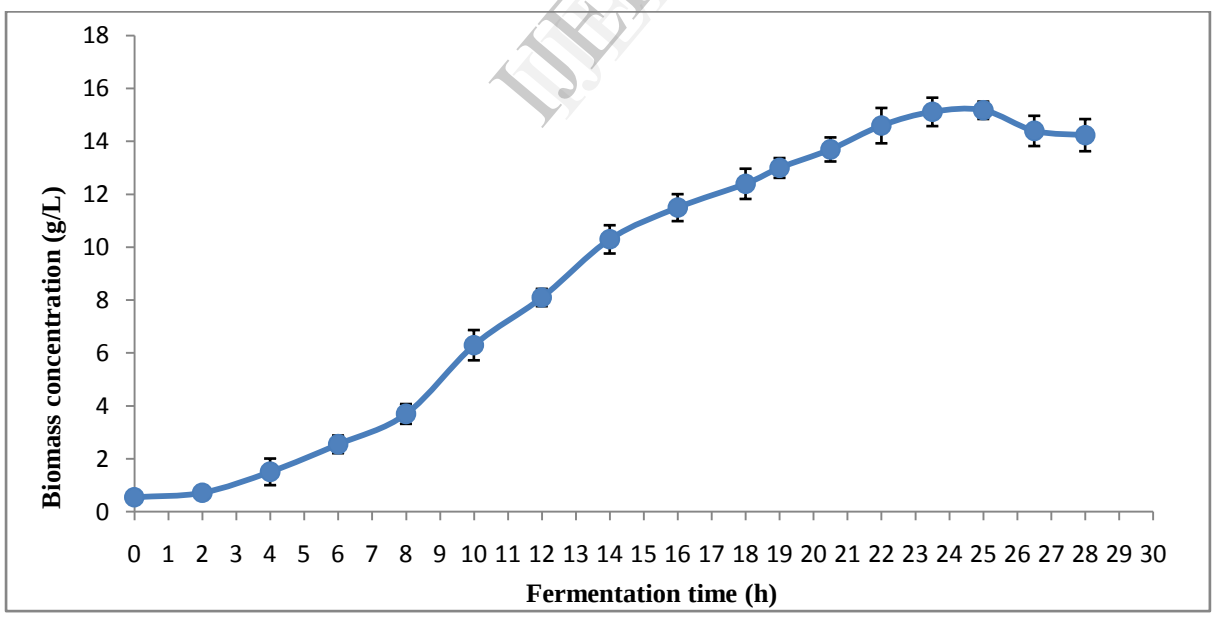

Fig. 2. Biomass concentration profile in second fermenter run (Biomass concentration g/L (●))

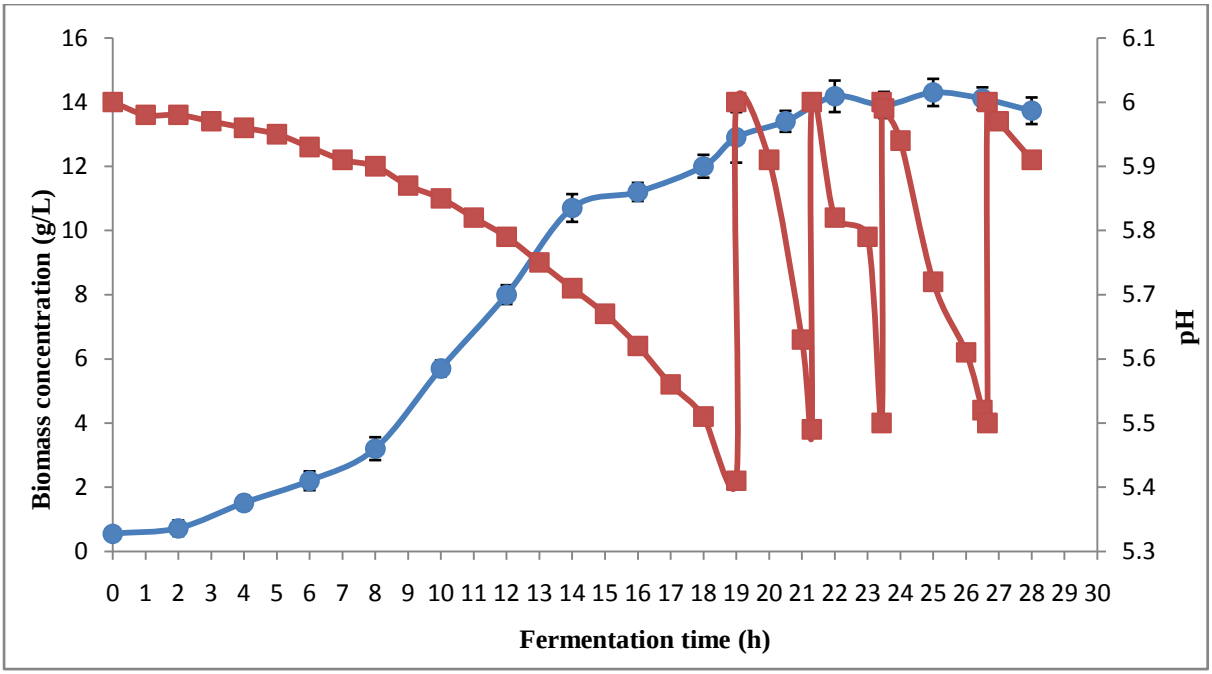

Fig. 3. Biomass concentration and pH profile in third fermenter run (Biomass concentration g/L (●), pH (■))

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6. Legends

μ	Specific growth rate	h^{-1}
L	Lipid concentration	g/L
S	Substrate (Glucose) concentration	g/L
X	Biomass concentration	g/L
X_{final}	Final biomass concentration	g/L
$Y_{L/S}$	Yield of Lipid on sugar	g/g
DNS	3,5-Dinitrosalicylic acid	
vvm	volume of gas/volume of aerated liquid/ minute	L/L/min
YEPD	Yeast extract Peptone Dextrose	

7. Conclusion

Results of our experiments have shown that:

- C/N ratio has an influence on the amount of lipid accumulated and the culture accumulated maximum lipid in shake flask when the ratio was 120 (mol/mol) under reported conditions.
- Fluctuating pH can be used as a strategy to improve lipid production. This will not only increase the production of lipids but will also profoundly reduce the amount of base required which will be quite economical in large scale operations.

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