RESEARCH PAPER

Asian Journal of Bio Science (April, 2010) Vol. 5 Issue 1 : 87-91

Influence of nitrogen on lipid and biomass production by oleaginous yeast cultures

S. VIJAYA KUMAR, **K. KUMUTHA**, P. SANTHANA KRISHNAN AND H. GOPAL
Department of Agricultural Microbiology, Tamil Nadu Agriculture University, COIMBATORE (T.N.) INDIA

(Accepted : February, 2010)

This study was undertaken to investigate the influence of nitrogen sources on lipid production by different yeast cultures. Among the four nitrogen sources, yeast extract and peptone showed maximum lipid production in biomass (22.85 and 20.03 per cent, respectively) and they were at par with each other, while ammonium sulphate (16.70 per cent) and sodium nitrate (13.34 per cent) exhibited poor lipid accumulation. Yeast extract containing broth supported to produce maximum lipid in *Rhodotorula glutinis* (23.82 per cent), followed by *Rhodosporidium toruloides* (22.53 per cent) and *Lipomyces starkeyi* (22.21 per cent). High amount of biomass as 10.20, 10.16 and 10.14 g l^{-1} was also observed using yeast extract as nitrogen source, respectively of the above cultures. Further experiment on optimization of the concentrations ranging from 0.5 to 1.75 per cent revealed the production of higher lipids was under 0.75 per cent. Among the various concentrations evaluated, maximum lipid and biomass production was observed in *Rhodotorula glutinis* (31.3 per cent and 11.5 g l^{-1}), *Rhodosporidium toruloides* (29.72 per cent and 11.10 g l^{-1}) and *Lipomyces starkeyi* (28.50 per cent and 11.05 g l^{-1}), respectively at 0.75 per cent level. Reduction in biomass as well as lipid yield was observed when increase in concentrations of yeast extract beyond 1.0 per cent.

Key words : Nitrogen, Oleaginous yeast cultures, Cell biomass

INTRODUCTION

Lipids are indispensable for growth and survival of all organisms. They are important structural components of membranes and in many organisms play a crucial role in carbon and free energy storage. Lipids have high free-energy content and a tendency to form aggregates in water, which allow the compact unhydrated intracellular packing. In biological systems, fatty acids are mostly encountered as components of lipids. Lipids are organic compounds that are insoluble in water and soluble in organic solvents. Chemically, lipids vary to such a great extent that no structural definition is available (Gurr and Harwood, 1991).

Lipid accumulation is a dynamic process, which depends on the microorganism, the growth conditions (like pH, temperature, nutrients and aeration) and the growth phase. Most oleaginous microorganisms start to accumulate oil whenever excess carbon source is present. While, at the same time, growth is limited by another nutrient (Kessell, 1968; Ratledge and Evans, 1989). Lipid accumulation in an oleaginous microorganism begins when it exhausts a nutrient from the medium, usually this is nitrogen but with a surfeit of carbon, usually in the form of glucose, still remaining. However, the limitation in the supply of nitrogen arises, the cell proliferation is prevented, and the lipid that is now formed has to be stored within the existing cells which can no longer divide. In yeast, lipid bodies do not serve simply as inert lipid stores but play an important role in the biosynthesis, mobilization and trafficking of intracellular neutral lipids (Leber *et al*., 1994). Hence, by knowing the importance of N limitation on lipid production, this present study was undertaken to optimize the best source of nitrogen and concentration of N source for increasing the lipid production in oleaginous yeast cultures.

MATERIALS AND METHODS

In this experiment, influence of nitrogen on the ability of oleaginous yeast cultures on lipid production was tested with different nitrogen sources like yeast extracts, ammonium sulphate, sodium nitrate and peptone. Screening broth (Dai *et al*., 2007) containing Yeast extract - 15.0g/l, Peptone - 5.0g/l with 0.22 M carbon source was used for this experiment. Yeast extract in the broth was replaced with other nitrogen sources. The pH of the broth was adjusted to 6.0 and three replications were maintained for each nitrogen source. The broth was inoculated with 24 hrs old cultures grown in YEPG broth at 10 per cent level containing $28x10^4$ cfu/ml in the broth. (Saxena *et al*., 1998).

Inoculated flasks were incubated at 30^0C for 7 days in an incubator shaker at 200 rpm (Innova 4320, New Brunswick, USA) for the growth of culture. After seven days of growth, cultures were harvested by centrifugation

and the cell pellets were obtained. Lipid content and biomass production of dried yeast cells were estimated by adopting the standard procedures

Cell biomass determination :

For dry weight determination, culture sample was centrifuged at 6000 rpm for 10 min at room temperature and cell pellet was washed first with 0.1 M phosphate buffer at pH 7.0. The supernatant was discarded and the cell pellet was dried at 65^0C for 48 h. After cooling, the cell dry weight was estimated (Esther *et al*., 1998).

Extraction of yeast lipid :

Yeast lipid was extracted from the dried cells as described by Bligh and Dyer (1959). Ten gram of dried cells were homogenized with 15 ml of methanol chloroform mixture 2:1 (v/v). Homogenized contents were filtered on a Buchner funnel through Whatman No.1 filter paper. While filtering, water in the contents was removed by adding one per cent anhydrous Na_2SO_4 to the filter paper. The filtrate was transferred to a graduated glass cylinder. Small quantities of yeast cells remaining in the blender and in the filter paper were washed with 10 ml of chloroform and the contents were transferred to the graduated cylinder and allowed for few min for the phases to separate. The lower volume of chloroform layer (20 ml added above) was recorded as 'x' ml. Upper methanol - water layer was removed by pipetting out along with layer of chloroform to ensure complete removal of methanol-water layer. Again the remaining lower volume of chloroform layer was recorded as 'y' ml. This volume was transferred quantitatively into a pre-weighted conical flask denoted as 'a' g. The contents in the conical flasks were evaporated at 40-50^0C in water bath under stream of nitrogen gas. Remaining residues were cooled and dried over phosphoric anhydride in a vacuum desiccator. Weight of the conical flask was taken again and denoted as 'b' g.

Five ml of chloroform was added three times to dissolve the evaporated lipid. Again flasks were evaporated and dried as above. Weight of the flask was taken at third time and denoted as 'c' g. Amount of lipid present in the yeast cells was calculated by using the following formula:

Weight of lipid (g) = (b-a) - (c-a) = 'd' g.

where a - is the weight of empty flask.

$$\text{Total lipid (g)} = \text{Wt. of lipid (d)} \times \frac{\text{Total vol. of chloroform layer (x ml)}}{\text{Vol. of chloroform layer evaporated (y ml)}}$$

$$\text{Total lipid (per cent)} = \frac{\text{Total lipid (g)}}{\text{Weight of sample (g)}} \times 100$$

Based on the results, another experiment was conducted to assess the influence of different concentrations of yeast extract on the production of lipids. Yeast extract was added at varying concentrations *viz.*, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 17.5 g l^{-1} to the screening broth, pH of the broth was adjusted to 6.0 and three replications were maintained for each nitrogen source. Inoculation of broth and incubation was carried out as per the earlier experiment (given above). After seven days of growth, the cultures were harvested and analyzed for the production of lipids, as well as biomass. The data pertaining to the above experiments were analyzed statically in completely randomized design.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Effect of nitrogen sources on lipid production by three oleaginous yeast cultures :

Lipid production was determined for yeast cultures grown at different nitrogen sources at 1.5 per cent concentration in culture broth. Among the four nitrogen sources tested, yeast extract supported maximum lipid in *Rhodotorula glutinis* (23.82 per cent), followed by *Rhodosporidium toruloides* (22.53 per cent) and *Lipomyces starkeyi* (22.21 per cent). High amount of biomass as 10.20, 10.16 and 10.14 g l^{-1} was also observed using yeast extract as nitrogen source, respectively of the above cultures. Among the four nitrogen sources yeast extract and peptone showed maximum lipid production in biomass (22.85 and 20.03 per cent, respectively) and they were at par with each other, while ammonium sulphate (16.70 per cent) and sodium nitrate (13.34 per cent) exhibited poor lipid accumulation. When the nitrogen sources were considered, yeast extract exhibited higher biomass, lipid yield as well as lipid in biomass in all the cultures tested. Though peptone was in the second place, no significant differences were noticed between yeast extract and peptone utilization. Inorganic sources of nitrogen such as $(NH_4)_2SO_4$ and $NaNO_3$ registered comparatively lesser biomass and lipid yield than the organic sources tested. Yeast extract and *Rhodotorula glutinis* combination resulted better lipid yield than other combinations tested (Table 1 and Fig. 1).

Findings from the present study suggested that yeast extract to be the best nitrogen source for higher lipid and biomass production. Present study was in agreement with

Table 1 : Effect of nitrogen sources on lipid production by three oleaginous yeast cultures

Nitrogen source (%)	Biomass (g l^{-1})				Lipid yield (g l^{-1})				Lipid biomass (%)			
	C_1	C_2	C_3	Mean	C_1	C_2	C_3	Mean	C_1	C_2	C_3	Mean
$(NH_4)_2SO_4$	8.90	8.65	8.60	8.71	1.57	1.41	1.39	1.45	17.64	16.30	16.16	16.70
$NaNO_3$	8.00	7.93	7.81	7.91	1.15	1.02	1.00	1.05	14.36	12.86	12.80	13.34
Yeast extract	10.20	10.16	10.14	10.16	2.43	2.29	2.25	2.32	23.82	22.53	22.21	22.85
Peptone	9.65	9.55	9.50	9.56	2.05	1.87	1.83	1.91	21.24	19.58	19.26	20.03
Mean	9.18	9.07	9.01		1.80	1.64	1.61		19.26	17.81	17.60	
	S.E. ±		C.D. (P=0.05)		S.E. ±		C.D. (P=0.05)		S.E. ±		C.D. (P=0.05)	
C	0.30		NS		0.15		NS		1.16		NS	
T	0.35		0.72		0.16		0.36		1.34		2.77	
C X T	0.61		NS		0.30		NS		2.33		NS	

Nitrogen source at 1.5 per cent was used. C_1-*Rhodotorula glutinis* C_2- *Rhodosporidium toruloides*
C_3- *Lipomyces starkeyi* C- Cultures T- Nitrogen source NS- Non significant

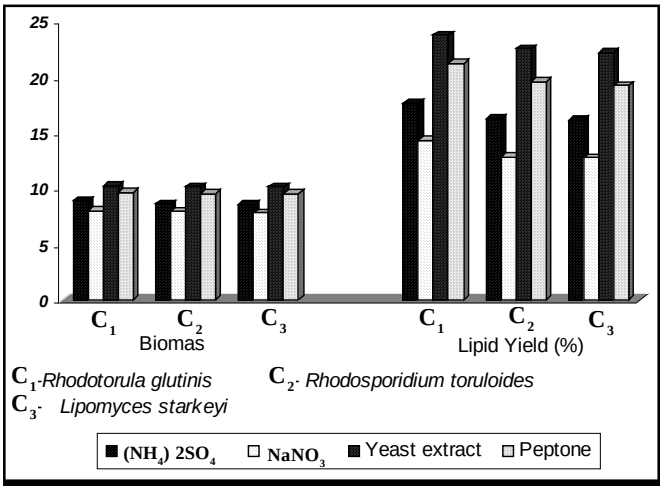

Fig. 1 : Effect of nitrogen sources on lipid production by three oleaginous yeast cultures

Li *et al*. (2007) who used yeast extract and peptone as nitrogen sources in the initial medium for higher lipid production in yeast cultures and observed poor synthesis of fat on media containing amino acids or urea as nitrogen source. Lipid accumulation was found higher in *Rhodosporidium toruloides* when organic nitrogen source was employed (Evens and Ratledge, 1984 a). Jang *et al*. (2005) also reported that yeast extract as the best N-source for lipid production. Oladipo *et al*. (2007) observed good lipid yield in *Lipomyces starkeyi* when cultured in yeast extract broth, while in nitrogen free broth the isolate produced less of lipids. *Rhodotorula glutinis* accumulated lipids up to 49 per cent on a cellular biomass basis with glucose as carbon source and yeast extract and peptone as nitrogen sources (Dai *et al*., 2007).

In order to find out the optimum concentration of yeast extract for maximum lipid production, the cultures were grown in varying concentration ranging from 0.5 to 1.75 per cent. Maximum lipid and biomass production was observed in *Rhodotorula glutinis* (31.3 per cent and 11.5 g l^{-1}), *Rhodosporidium toruloides* (29.72 per cent and 11.10 g l^{-1}) and *Lipomyces starkeyi* (28.50 per cent and 11.05 g l^{-1}), respectively at 0.75 per cent level. Interestingly it was observed that, 0.75 per cent concentration resulted more of cell biomass and lipid accumulation. Beyond that a gradual decline in lipid yield was noticed. Reduction in biomass as well as lipid yield was observed when increase in concentrations of yeast extract beyond 1.0 per cent. Irrespective of the cultures used, yeast extract concentration of 0.75 per cent was found superior and *Rhodotorula glutinis* was excelling in its performance with the utilization of yeast extract in terms of biomass and lipid yield (Table 2 and Fig. 2).

Lipid accumulation process which requires exhaustion of nutrient usually nitrogen, to allow excess carbon to be channeled in to lipids (Colin *et al*.,1977). Nutrient imbalance in the culture medium has long been known for trigger lipid accumulation by oleaginous microorganisms. When cells out of key nutrients, usually nitrogen, excess substrate continues to be assimilated by the cells and converted in to fat for storage (Li *et al*., 2007). The present results were in line with the above reports, and reveals that excess nitrogen may cause reduction in storage of lipids. Under nitrogen limited conditions, C: N ratio was found higher, hence the excess carbon may be routed for the conversion in to lipid bodies.

From a physiological view point, however, lipid accumulation is not only caused by the synthesis of lipids in nitrogen limited conditions, but also due to the reduction in synthesis of nucleic acids and proteins leads to the cells, which indirectly leads to lipid accumulation.(Colin *et al*., 1977).

Table 2 : Effect of different concentration of yeast extract on lipid production by three oleaginous yeast cultures

Concentration of yeast extract (%)	Biomass (g l^{-1})				Lipid yield (g l^{-1})				Lipid biomass (%)			
	C_1	C_2	C_3	Mean	C_1	C_2	C_3	Mean	C_1	C_2	C_3	Mean
0.50	11.10	10.75	10.75	10.685	3.23	2.90	2.52	2.88	29.10	27.75	23.46	23.98
0.75	11.50	11.10	11.05	11.21	3.60	3.30	3.15	3.92	31.30	29.72	28.50	34.97
1.00	10.90	10.85	10.85	10.86	3.22	2.89	3.25	3.38	30.10	30.03	29.95	30.10
1.25	10.50	10.50	10.40	10.46	2.84	2.45	2.81	2.89	28.70	27.15	27.04	27.63
1.50	10.21	10.18	10.16	10.18	2.42	2.04	2.26	2.32	23.75	22.59	22.26	22.86
1.75	9.65	9.60	9.50	9.58	2.04	1.80	1.95	1.99	20.10	20.60	20.52	20.40
Mean	10.65	10.44	10.45		2.90	2.55	2.75		27.16	26.34	26.20	
	S.E. ±		C.D. (P=0.05)		S.E. ±		C.D. (P=0.05)		S.E. ±		C.D. (P=0.05)	
C	0.19		NS		0.15		0.30		1.19		NS	
T	0.26		0.53		0.21		0.42		1.30		3.41	
C X T	0.45		NS		0.36		NS		2.92		NS	

C_1-*Rhodotorula glutinis* C_2- *Rhodosporidium toruloides* C_3- *Lipomyces starkeyi*
C- Cultures T- Concentration of Yeast extract (%) NS-Non significant

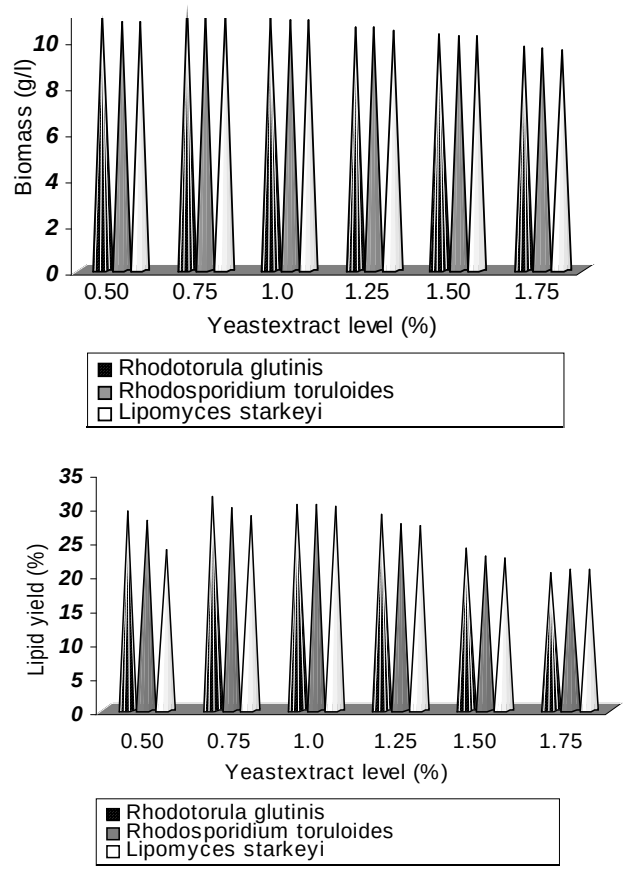

Fig. 2 : Effect of different concentration of yeast extract on lipid production by three oleaginous yeast cultures

REFERENCES

Bligh E.G. and Dyer, N.J. (1959). A rapid method of total lipid extraction and purification. *Canadian J. Biochem. Physiol.*, **37**: 911-917.

Colin, G. O., Michele, J.H. and Ratledge, C. (1977). Lipid accumulation in oleaginous yeast (*Candida* 107) growing on glucose under various conditions in a one and two stage continuous culture. *Appl. Environ. Microbiol.*, **33**: 577-584.

Dai, C.J., Lengxie Tao, Y. and Mozhao, R. (2007). Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity. *African J. Biotechnol.*, **6** : 2130-2134.

Esther, Z., Bonita, V., Glatz, A. and Hammond, E.G. (1998). Optimization of banana juice fermentation for the production of microbial oil. *Appl. Environ. Microbiol.*, **54** :748-752.

Evans, C.T. and Ratledge, C. (1984a). Influence of nitrogen metabolism on lipid accumulation in oleaginous yeasts. *J. Gen. Microbiol.*, **130** : 1693–1704.

Gurr, M.I. and Harwood, J.L. (1991). *Lipid biochemistry*, Chapman and Hall, 4th ed. London. pp. 12-131.

Holdsworth, J.E. and Ratledge, C. (1988). Lipid turnover in oleaginous yeasts. *J. Gen. Microbiol.*, **134** : 339–346.

Jang, H.D., Lin, Y. and Yang, S.S. (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpine*. *Bioresour. Technol.*, **96** : 1633-1644.

Kessell, R.H.J. (1968). Fatty acids of *Rhodotorula gracilis*: fat production in submerged culture and the particular effect of pH value. *J. Appl. Bacteriol.*, **31**:220-231.

Leber, R., Zinser, E., Zellnig, G., Paltauf, F. and Daum, G. (1994). Characterization of lipid particles of the yeast, *Saccharomyces cerevisiae*. *Yeast*, **10**:1421–1428.

Li, Y.H., Zhao, Z.B. and Bai, F.W. (2007). High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme Microbiol. Technol.*, **41**: 312–317.

Oladipo, I.C., Adebiyi, A.O., Ayandele, A., Adebiyi, A.P. and Adelowo, O.O. (2007). Determination of the growth rate and volume of lipid produced by *Lipomyces* species isolated from shear butter leaf (*Vitellaria paradoxa*). *African J. Biotechnol.*, **618** : 2174-2176.

Ratledge, C. and Evans, C.T. (1989). *Lipids and their metabolism*. In: The yeasts. Rose, A.H and J.S. Harrison (2nd Ed.) Academic Press, London, pp. 367-455.

Saxena, V., Bhagat, C.D., Saini, V.S. and Adhikari, D.K. (1998). Lipid and fatty acid synthesis in *Rhodotorula minuta*. *JACOS*, **75**: 501-505.