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Cultivation of Oleaginous Yeast on Extracts of Unedible Parts of Vegetables/Fruits for Lipid Production

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Abstract—*Rising concerns of energy price and environmental concerns has led to consideration of biodiesel as one of the most promising potential substitute for traditional energy oil. Oleaginous yeasts, that accumulate lipids upto 50% of its body weight, has been reported to grow on various cheap carbon sources including industrial effluents, are considered as invaluable sources of triglycerides. Using cheap sources of growth has been a challenge. We obtained waste material such as peels of fruits and unedible/discarded parts of vegetables e.g. cauliflower stubs, pea pods, etc which pollute vegetable markets (mandis) in India for using as a fermentation medium. Pretreatment of waste by steaming and subsequent cultivation of yeasts in this extract has been studied and accumulation of lipids has been analysed at various growth stages.*

1. INTRODUCTION

Under the premise of high energy price with estimated petroleum crisis, biodiesel extracted from plant oil, animal fat and microorganisms is considered as a promising replacement for fossil raw material considering as 1st generation, 2nd generation and 3rd generation biodiesel respectively. Biodiesel production of the 3rd generation using fatty acids produced by oleaginous microorganisms for the trans-esterification into the FAMEs/FAEEs. In general, microorganisms that accumulate more than 20–25% of their cell dry weight in the form of lipids are referred to as oleaginous species [1]. Especially the use of oleaginous yeast is a promising approach since they stand out from other heterotrophic microorganisms in terms of fast growth rate and high lipid content in fermentation bioreactors of batch and fed batch culture medium [2-4]. In addition, their lipids are mainly composed of triacylglycerols, which make their chemical characteristics and thus the potential applications comparable to plant oil [2]. A variety of fermentation substrates have been tested for their ability to serve oleaginous yeast as adequate growth and production media, including, for example, effluent from steam fish processing [5], whey [6], municipal wastewater and sewage sludge [7-9], lignocellulosic materials [10], molasses [11-13], glycerol [14] and starch wastewater [15]. Carbohydrates derived from lignocellulosic materials have been used to culture oleaginous yeasts for microbial lipids [1, 3-5, 16].

Oleaginous yeasts such as *Rhodotorula glutinis* is able to accumulate up to 70% of its cell dry weight in the form of lipids [17] with a biomass production up to 180 gL^{-1} in rich fermentation media [18]. Additionally, the fatty acid profile is rich in palmitic, oleic, and linolenic acid [19] and therefore comparable to vegetable oils allowing biodiesel production. In addition, it is comparatively unproblematic to handle, being nontoxic and easy to grow and harvest [13].

2. MATERIALS AND METHODS

2.1 Isolation and culture medium

To isolate new strains, the medium used contained :($(NH_4)_2SO_4$– 1g/l; Glycerol-100g/l; KH_2PO_4-1g/L; $MgSO_4.7H_2O$ – 0.5gm/L; Yeast extract – 0.2g/l) containing antibiotic streptomycin. Strains also grew in xylose (2%) containing medium.

Soil sample was collected from areas where we expected enrichment of carbon sources e.g. sewage and road side restaurants. 5gm of soil was inoculated in 50mL growing medium and was incubated for 24hours. Isolated new strains were then spread on to the xylose as well as glucose plates with concentration 100µl and 500µl of the sample.

Strains were then maintained on minimal media (glucose 5 g/l ; Na_2HPO_4 6 g/l ; NaCl 5 g/l ; KH_2PO_4 3 g/l ; NH_4Cl 2 g/l ; $MgSO_4$ 0.1 g/l ; yeast extract 2 g/l) solidified with 1.5% agar**.**

2.2 Culture conditions

Cells were maintained on agar- solidified medium in petri dishes. Inoculum for production of cultures on waste was grown in 100mL flasks containing 10mL medium and agitated at 120 rpm, 30^0C.

2.3 Pre-treatment for obtaining sugar (carbon) from waste

Three different types of pretreatment methods were tried. Pretreatment1 included steaming under pressure using an autoclave for preparation of extract in which various amounts (25gm, 50gm and 100 gm) of fresh orange peels and pea pods were taken in a beaker separately. 100 ml of distilled water was added to each beaker and then subjected to autoclaving for 15 minutes at 10 psi.

Preateatment 2 was conducted by using chemical treatment followed by streaming as in pretreatment 1. For this, 50gm of orange peels, banana peels and mango peels each were soaked in (a) 3% H_2O_2 and (b) 3% H_2O_2 + 2% NaOH solution in a ratio of 1:10 (solid:liquid) for 2 hours at room temperature, followed by autoclaving for 15 minutes at 10 psi. The extracts were obtained after filtering through the muslin cloth and stored in the refrigerator for future use.

2.4 Staining with Sudan Black B

To prepare SBB dye 0.015gm of Sudan B black powder was dissolved in 10ml of 70% ethanol. For staining, added 1ml HCl and 0.4ml of SBB dye to 5ml cell suspension (obtained at various growth intervals) and incubated at 100°C in a waterbath for 30 seconds. After cooling to room temperature the cells were centrifuged at 3000rpm for 10 minutes, washed 3-4 times with 50% ethanol and finally resuspended in distilled water. Lipid molecules were observed under light microscope (100X) and photographed.

2.5 Analytical methods

Phenol- Sulfuric acid test (Dubois et al. 1956), and 3,5 dinitrosalysilic acid test (Miller,1959) were performed to estimate total sugars and reducing sugars, respectively, in the samples. Nitrogen estimation was carried out by Kjeldahl method, 1883. FAME analysis was conducted at IMTECH Chandigarh using MIDI, Inc.

3. RESULTS

The extracts from different agricultural wastes were prepared using various pretreatments. All the extracts prepared were analysed for various constituents such as total sugar(TS), reducing sugar (RS) and nitrogen (N) content (Table1).

Table 1: Comparison of pretreatment methods

Treatment	TS(gm)	RS(gm)	N(gm)
Pretreatment I	1.056	0.304	0.09
Pretreatment IIa	3.9	2.8	ND*
Pretreatment IIb	8.1	ND*	ND*

From Pretreatment I (steaming alone) the maximum concentration of total sugar was found to be present in 50gm of orange peel extract (i.e.1.932 gm (w/w)) and 25gm of pea pod extract (i.e. 0.834gm (w/w)). The nitrogen content was found to be low in orange peel extract as compared to pea pod extract. Studies suggest that for optimum accumulation of lipids by oleaginous yeasts, a high concentration of sugar and low amounts of nitrogen are desired [20]. Therefore, we used 50 g of orange peels for chemical treatment via pretreatment II. The maximum concentration of total sugar was found to be present in 50gm of banana peel extract (i.e.9.75 gm (w/w)) and maximum concentration of reducing sugar in 50gm of mango peel extract (i.e. 3.6gm (w/w)) using pre treatment II. Nitrogen estimation of the extracts was however not possible because of the presence of hydrogen peroxide in the extracts which hindered nitrogen estimation by Kjeldahl method and gave negative results. Yet another pretreatment IIb (Treatment with H_2O_2 and NaOH followed by autoclaving) was used to estimate the amount of total sugar present in each one of the extracts. The maximum concentration of total sugar was found to be present in 50 gm of banana peel extract (i.e.9.75 gm (w/w)). DNS test to check the presence of reducing sugars gave no results probably because of the presence of NaOH, which is an oxidising agent and thus has a counter effect on the chemistry of the reaction which involves reduction of DNS by reducing sugars to give a colour change. Nitrogen estimation was not possible due to the above mentioned reason.

Among various pretreatments given, pretreatment 3 i.e. soaking in 3% H_2O_2 and 2% NaOH followed by autoclaving, released the maximum amount of total sugars in the extract as the major action of NaOH is to degrade the lignin by breaking ester bond cross linking, thus creating porosity in biomass [21] and addition of H_2O_2 is to detach and loosen the lignocellulosic matrix [22], therefore aiding the release of sugars from lignocellulosic biomass. Also, an increase in the amount of reducing sugars is observed on giving pretreatment 2a i.e. soaking in 3% H_2O_2 prior to autoclaving.

For making the waste extract for fermentation medium we used pretreatment method I because downstream processing i.e. analysis of sugars and nitrogen and growing of the culture was best possible when chemical treatment is not included.

3.1 Staining

Samples 1 and sample 4 were collected from sewage area and is marked as S1 and S2 respectively. Another sample was taken from chaat vendor (street side restaurant) as CV1. Slides of S1, CV1 and S4 shows SBB results where the fat globules were stained black. A slide of negative control shows no staining which refers to no fat globules was stained (Fig 1).

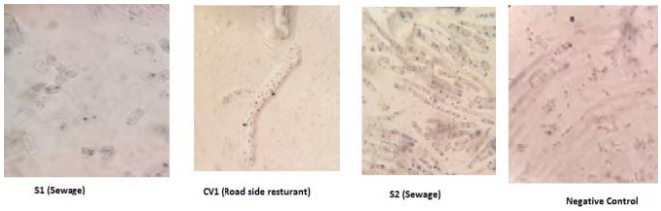

Fig. 1: Slide showing lipid globules in cells of oleaginous strains, stained with Sudan Black B.

3.2 Growth profile of the organism(s) in minimal media

The strains (S1, S2) were cultured in 50 ml minimal media in 250 ml flasks and the growth profile was plotted (Fig 2). The lipid was extracted at point 24hours and 30hours.

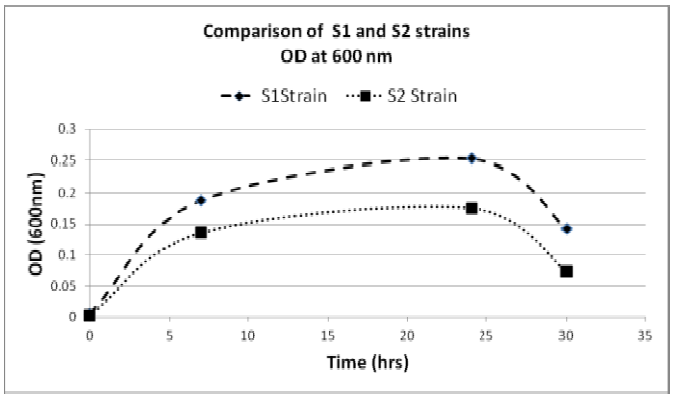

Fig. 2: Growth profile of the yeast samples – strain S1 and S2 in minimal medium.

3.3 Lipid estimation

The maximum amount of lipids (in percentage dry cell weight) was found to be 50%-60% in the cells grown in orange and pea extracts, whereas when grown in Minimal medium they accumulated 31-34% lipids after 24 hrs of growth. The lipids were consumed after this and at 30 hrs, the samples showed 0.5-3% lipids only. Since the waste extracts contained xylose and other sugars apart from minimal medium, more lipids accumulated in waste extract cells as compared to minimal medium. The FAME analysis showed the presence of various types of fatty acids such as palmitic acid (C16) and oleic acids (C18:1) which is desirable for transesterification to biodiesel (fig 3).

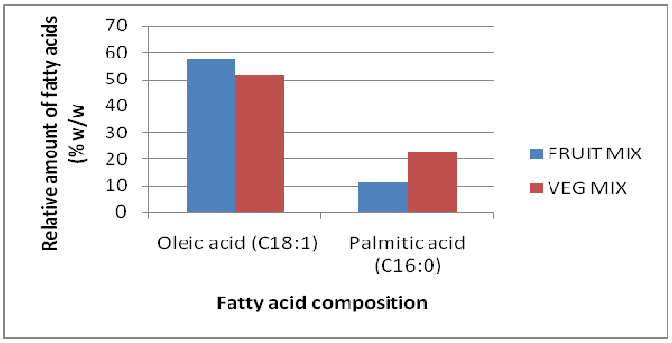

Fig. 3: Fatty Acid Methyl Esters analysis showing the presence of palmitic acid (C:16) , oleic acid (C18:1) and other FAMEs (C18.2 w6,9c / C18.0 ANTI C18.0 ANTI / C18.2 w6,9c) in the lipid extracted from the oleaginous isolates.

4. CONCLUSION

Pre-treatment I method for preparing waste extract was found to be effective for growing the isolated oleaginous strains. This extracts produced by this method was analysed for total sugars, reducing sugars and nitrogen content. Oleaginous species were isolated form soil obtained from various sites and assessed for lipid accumulation. Results showed that waste extracts were very suitable as growth medium for the oleaginous yeasts as it contained more than one type of carbon source, thus allowing quicker growth, more biomass production and better lipid production and accumulation. This is a preliminary work and further studies are required to standardise growth and lipid accumulation by these strains.

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REFERENCES

[1] Ratledge C., Acta Biotechnologica, “Microorganisms for lipids” vol. 11, no. 5, 1991 pp. 429–438.

[2] Subramaniam R., Dufreche S., Zappi M., and Bajpai R., Journal of Industrial Microbiology and Biotechnology, “Microbial lipids from renewable resources: production and characterization” vol. 37, no. 12, 2010, pp. 1271–1287.

[3] Ageitos J.M., Vallejo J.A., Veiga-Crespo P., and Villa T.G., Applied Microbiology and Biotechnology, “Oily yeasts as oleaginous cell factories” vol. 90, no. 4, 2011, pp. 1219–1227.

[4] Li Q., Du W., and Liu D., Applied Microbiology and Biotechnology, “Perspectives of microbial oils for biodiesel production” vol. 80, no. 5, 2008, pp. 749–756.

[5] Cheirsilp, B., Suwannarat, W., and Niyomdecha, R., New Biotechnology, “Mixed culture of oleaginous yeastRhodotorula glutinis and microalga Chlorella vulgaris for lipid production from industrial wastes and its use as biodiesel feedstock” vol. 28, no. 4, 2011, pp. 362–368.

[6] Akhtar, P., Gray, J.I., and Asghar, A., Journal of Food Lipids, “Synthesis of lipids by certain yeast strains grown on whey permeate” vol. 5, no. 4, 1998, pp. 283–297.

[7] Chi, Z., Zheng, Y., Jiang, A., and Chen, A., Applied Biochemistry and Biotechnology, “Lipid production by culturing oleaginous yeast and algae with food waste and municipal wastewater in an integrated process,” vol. 165, no. 2, 2011, pp. 442–453.

[8] Hall, J., Hetrick, M., French, T., et al., Journal of Chemical Technology and Biotechnology, “Oil production by a consortium of oleaginous microorganisms grown on primary effluent wastewater,” vol. 86, no. 1, 2011, pp. 54–60.

[9] Angerbauer, C., Siebenhofer, M., Mittelbach, M., and Guebitz, G.M., Bioresource Technology, “Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production,” vol. 99, no. 8, 2008, pp. 3051–3056.

[10] Hu, C., Zhao, X., Zhao, J., Wu, S., & Zhao, Z. K. Bioresource Technology.” Effects of biomass hydrolysis byproducts on oleaginous yeast Rhodosporidium toruloides” Vol.100, No. 20, 2009, pp. 4843-4847, ISSN 0960-8524.

[11] Karatay, S.E., and Dönmez, G., Bioresource Technology, “Improving the lipid accumulation properties of the yeast cells

for biodiesel production using molasses" vol. 101, no. 20, 2010, pp. 7988–7990.

[12] El-Fadaly, H.A., El-Naggar, N.E.A. and Marwan, E.S.M., Research Journal of Microbiology, "Single cell oil production by an oleaginous yeast strain in a low cost cultivation medium" vol. 4, no. 8, 2009, pp. 301–313.

[13] Alvarez, R.M., Rodríguez, B., Romano, J.M. et al., "World Journal of Microbiology & Biotechnology, "Lipid accumulation in Rhodotorula glutinis on sugar cane molasses in single-stage continuous culture," vol. 8, no. 2, 1992, pp. 214–215.

[14] Easterling, E.R., French, W.T., Hernandez, R. and Licha, M., Bioresource Technology, "The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of Rhodotorula glutinis," vol. 100, no. 1, 2009, pp. 356–361.

[15] Xue, F., Gao, B., Zhu, Y., Zhang, X., Feng, W., and Tan, T., Bioresource Technology, "Pilot-scale production of microbial lipid using starch wastewater as raw material.," vol. 101, no. 15, 2010, pp. 6092–6095.

[16] Papanikolaou, S. and Aggelis, G., Journal of Lipid Science and Technology, "Lipids of oleaginous yeast. Part I. Biochemistry related with single cell oil production," vol. 113, no. 8, 2011, pp. 1031–1051.

[17] Meng, X.,Yang, J., Xu, X., Zhang, L., Nie, Q., & Xian, M. (2009) Renewable Energy. "Biodiesel production from oleaginous microorganisms," Vol. 34, No. 1, 2009, pp. 1-5, ISSN 0960-1481.

[18] Pan, J. G., Kwak, N. Y., & Rhee, J. S. (1986) Biotechnology Letters. Vol. 8, No. 10, pp. 715–718, ISSN 0141-5492, "High density cell culture of Rhodotorula glutinis using oxygen-enriched air."

[19] Misra, S., Ghosh, A., and Dutta, J., Journal of the Science of Food and Agriculture, "Production and composition of microbial fat from Rhodotorula glutinis," vol. 35, no. 1, 1984, pp. 59–65.

[20] Braunwald, T., Schwemmlein, L., Graeff- Honninger, S., French, WT., Hernandez, R., Holmes, WE., Claupein. W., pp. 6581-8, 2013 "Effect of different C/N ratios on carotenoid and lipid production by Rhodotorula glutinis."

[21] Rebeeca, A.S., Ye, C., Ratna, R.S-S., Michael, D.B. and Jason, O., 2007, Bioresource Technology.98, 3000-3011, "A Comparison of Chemical Pretreatment Methods for Improving Saccharification of Cotton Stalks."

[22] Playne, M.J., 1984, Biotechnology and Bioengineering.26(5), 426-433, "Increased Digestibility of Bagasse by Pretreatment with Alkalis and Steam Explosion."