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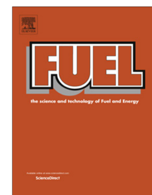

Full Length Article

Process optimization for cultivation and oil accumulation in an oleaginous yeast *Rhodosporidium toruloides* A29

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ABSTRACT

Biodiesel is a renewable and environment friendly energy source, which is a potential alternative to petro-diesel. In the present study, mass production of microbial lipid by the yeast strain *Rhodosporidium toruloides* A29 was studied for biodiesel production. Realizing the importance of microbial lipids as a potential source for biodiesel, the strain was evaluated for higher biomass production using statistical modelling approach, which resulted in a lipid yield of 0.436 g/g cell dry weight. This high lipid content was achieved using RSM optimized medium containing 2.5 g/L of yeast extract, 2.75 g/L of $NaNO_3$, 0.5 g/L of $MgSO_4$ and 75 g/L of glucose. The production of *R. toruloides* was successfully scaled up in a 30 L bioreactor. In the reactor, lipid yield increased to 0.535 g/g CDW leading to a 22-fold increase in oil content after scale up. Fatty acid characterization of the oil by GC revealed that *R. toruloides* A29 lipids consist of 34.59% saturated fatty acids, 46.49% monosaturated fatty acids and the rest polyunsaturated fatty acid. Transesterification of the extracted yeast oil revealed that the FAME (biodiesel) formed was similar in composition to the biodiesel produced from vegetable oil. The physico-chemical properties of the transesterified SCO were in range of the biodiesel standard specifications. Thus, this makes the microbial lipids obtained from *R. toruloides* A29 as potential alternative oil for sustainable production of biodiesel to meet the escalating energy demands.

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1. Introduction

Increasing industrialization in today's world has led to a situation, where the fossil fuel reservoirs are continuously depleting which has lead to high energy prices. Being a non-renewable resource these fossil fuels are generating various environmental concerns like global warming, elevated levels of greenhouse gas (GHG) emissions, drought melting of glaciers, etc. This situation has given rise to a growing worldwide interest in renewable energy such as biomass-based bio-fuels [1,2].

One of these is biodiesel, which offers several advantages over petroleum-derived diesel and can be directly used in the existing diesel engines [2]. Recently, the National Bio-fuel Policy of India proposed an indicative target of 20% blending for biodiesel by the year 2017 and the United States plans to increase the amount of biodiesel to 136 million cubic meters by 2022 [3].

Biodiesel consists of fatty acid methyl esters (FAMEs), which are obtained by transesterification of oils. The oils are primarily of plant origin. One of the major hurdles and important drawbacks of producing biodiesel is the high production cost, which is mainly due to the high cost of the vegetable oil, from which it is produced. About 70–90% of the biodiesel production cost corresponds to raw vegetable oil.

Oil production using oleaginous microorganisms is a sustainable alternative to conventional oil for biodiesel production. Oil produced from microorganism have several advantages like shorter production time, easy scale up, does not compete with the controversy of food and fuel, requires less labour and land resources, and is environment friendly [4–6]. Therefore, instead of using vegetable oils, oleaginous microorganisms (also called single-cell oils) can be used as an alternative for producing biodiesel [7]. Oleaginous microorganisms accumulate lipids >50% of their dry biomass [5,8,9] as a means of intracellular reserve supply of carbon and energy to be used at the time of nutrient starvation. Among the various microorganisms, yeasts are considered as favourable oleaginous microorganisms since 1980's [10]. The advantages of using yeasts as lipid producers are that yeasts are unicellular and unlike, algae and fungi their lipid can be produced in a short time at a larger scale. Also, their fatty acid composition is very similar to that of vegetable oils and animal fats already used

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as a feedstock for biodiesel. Some yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp. and *Lipomyces* sp. can accumulate intracellular lipids as high as 70% of their cell dry weight under optimum conditions [11–13]. In order to achieve optimum conditions, Response Surface Methodology (RSM) has been used for several biotechnological and industrial processes [14–17]. RSM has an advantage over the traditional approach of dealing with one variable at a time and because the latter is a time consuming process and does not account for the interactions among various physicochemical parameters [14].

Realizing the importance of lipid production by oleaginous yeasts and the necessity of developing bio-based routes using fermentation, the present investigation was carried out with an objective of increasing the amount of lipid accumulated within the selected oleaginous yeast *R. toruloides* A29 using process optimization using (a) One variable at a time approach (b) Response Surface Methodology and scale up to 30 L bioreactor. Further the lipids were extracted and analysed.

2. Materials and methods

2.1. Materials

Molecular grade fatty acids (capric, myristic, linoleic, linolenic, palmitic, stearic, oleic acids) and their methyl esters, Dinitrosalicylic acid (DNS) were purchased from Sigma. Nile red (Nile blue A oxazone) was purchased from Hi Media. All other materials were of analytical grade.

2.2. Screening and isolation of oleaginous yeasts

Decaying peel of banana, mango, litchi, grapes, melon, and plums were collected from fruit markets of New Delhi, India. The fruit samples were inoculated into 80 ml enrichment medium containing (g/L): glucose, 20; peptone, 15; yeast extract, 10; KH_2PO_4, 1; K_2HPO_4, 3; malt extract, 5 and pH 6 in a 250 ml Erlenmeyer flask and incubated at 30 °C for 120 h at 200 rpm. Ampicillin (40 μg/ml) was added to the media to minimize bacterial growth. The enriched cultures were serially diluted with sterile distilled water and plated onto Malt extract glucose yeast extract peptone (MGYP) agar plates and incubated at 30 °C for 48 h. Colonies with the morphology typical of yeasts were picked up for further study.

The identification of the yeast isolate up to species level was carried out on the basis of standard morphological and physiological/biochemical tests by the procedure described Saran et al. [17].

The yeast strain A29 showing significant lipid production was identified on the basis of 500 bp sequence of 18S rDNA analysis done at Central Instrumentation Facility (CIF), University of Delhi South Campus, India using the universal primers ITS 1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS 4 (5′ TCCTCCGCTTATTGATATGC 3′) [18].

2.3. Selection of oleaginous microorganisms by Nile red staining

All yeast strains were checked for lipid accumulation by Nile red fluorescence [19]. Nile red (Nile blue A oxazone) was prepared in acetone and stored away from light. Isolated cells (1–2 × 10~/ml) were suspended in PBS and the dye was added directly to the preparation to achieve a l:100 dilution. The preparation was incubated for 5 min and covered with a cover slip. Fluorescence microscopy studies was performed with Olympus BX 51 microscope equipped for epi-illumination using an HBO 200 high pressure mercury light source. Nile red fluorescence was viewed at green beam of light at two spectral settings: yellow-gold fluorescence, using a 450–500 nm excitation filter and a 510-rim center wavelength chromatic beam splitter. The stained cells were photographed in color using Olysia software.

2.4. Plackett-Burman statistical approach for process optimization of biomass

The Plackett-Burman design was used to determine the role of media components on biomass production. All the experiments were carried out according to the matrix designed by Design–Expert ® 8.0 Stat-Ease, Inc., (Minneapolis, USA). Five independent factors screened were (A) Yeast extract (B) Glucose (C) $NaNO_3$ (D) $MgSO_4$ (F) KH_2PO_4. The low and high values of each variable are presented in Table 1.

A Pareto-Plot was plotted indicating the positive and negative effect of the factors. The effect of each factor was determined with the following equation:

$$\sum x_i = \left(\sum X + i - \sum X - i\right)/N$$

where $\sum x_i$ is the main effect of the factor x + i and x − i, which is high and low variable respectively. Biomass in terms cell dry weight (g/L) produced from each combination of factors was treated as the response.

2.5. Experimental design for maximum biomass production using RSM

RSM was employed to optimize the concentrations of parameters selected by Packett-Burman design on biomass yield using a face centered central composite design (FCCCD). The four independent variables studied were Yeast Extract (A), $NaNO_3$ (B), $MgSO_4$ (C), and Glucose (D) (Table 2). A set of 30 experiments were generated (Table 3) using the statistical software package 'Design–Expert ® 8.0 Stat-Ease, Inc., (Minneapolis, USA), where the different variables were set at five levels (−α, −1, 0, +1, +α). All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were investigated.

The data obtained on biomass production was subjected to Analysis of Variance (ANOVA) appropriate to the design of the experiments. The mathematical relationship of the independent variable and the response cell dry weight was calculated by the second order polynomial equation (Eq. (1)).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

where Y is the predicted response; β_0, intercept; β_1, β_2, β_3, β_4, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, squared coefficients; β_{11}, β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} interaction coefficients. This equation is used to evaluate the linear, quadratic and interactive effect of independent variables on the selected response. The model was later put to validation by performing the sets of 6 experiments randomly generated by design expert.

Table 1
Experimental factors at different levels used for yeast biomass production using Plackett-Burman Design.

Factor code	Factor name	Level	
		Low (−1)	High (+1)
A	Yeast extract	0.5	1.5
B	Glucose	40	60
C	$NaNO_3$	0.5	1.5
D	$MgSO_4$	0.25	0.75
E	KH_2PO_4	7	9

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