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BIO-PROSPECTING INDIGENOUS PLANT SEED OILS FOR THE PRODUCTION OF BIO-OILS FROM ZYGOMYCETOUS FUNGI, ISOLATED FROM TROPICAL SOIL

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ABSTRACT

Rising crude oil prices, uncertainty over oil reserves have stimulated interest globally in renewable energy sources for sustainable energy production. *Cunninghamella nodosa*-JSK1 and *Rhizopus oryzae*-JSK3, a Gamma-linolenic acid (GLA) producing tropical fungal isolates, were used to evaluate the influence of various indigenous plant seed oils on biomass, oleagenicity and bio-oil production. The fungal isolates accumulated total lipid (TL %) in the range of 16-17% of their dry biomass (2-3 g/l) and 14-18% of GLA in its total fatty acid. Among the various plant seed oils used for biotransformation studies, watermelon and pumpkin seed oil increased biomass up to 5g/l in both the fungal isolates. However, maximum TL (27% and 23%) was obtained with pumpkin and sunflower seed oil. Sunflower, pumpkin, watermelon, niger and onion seed oil increased GLA content to 15-18%. Interestingly, addition of *Pongamia pinnata* oil (1%), an indigenous biodiesel commodity, led to complete inhibition of GLA from 18 to 0% and 14 to 0% and on the contrary, increased in oleic acid content from 32 to 55% in *Cunninghamella nodosa*-JSK1 and 20 to 63% in and *Rhizopus oryzae*-JSK3.

Keywords: *Cunninghamella echinulata var nodosa*-JSK1, *Rhizopus oryzae*-JSK3, Biotransformation, Biodiesel, Gamma-Linolenic Acid

1 INTRODUCTION

Modern way of life intimately depends on fuels that are derived from fossil resources. With the continued depletion of fossil fuels and to meet the growing demand of the global diesel fuel industry, alternate bio-oil sources have been explored and developed in recent days [1, 2]. Biodiesel, a mono alkyl esters of fatty acids derived from renewable biomass and organic matter could minimize the depletion of natural resources and reduce the dependency on fossil fuel. It is eco-friendly, non-toxic, bio-degradable, stable, reduces the level of potential or probable carcinogens and has a favorable emission profile [3]. It is probably the most researched alternate biofuel [4]. Oleaginous microorganisms are attractive candidates for biodiesel production and confer several advantages over plants such as similarity in fatty acid profiles with plant seed oils, easy to grow, simple cultural conditions and consistency of the product yield [5, 6].

However, the limiting factor for microbial lipid production is often the low productivity coupled with high expense of the glucose substrate [7]. In order to overcome the cost barrier, an array of inexpensive agro-industrial by-products such as such as lignocellulosic biomass [8, 9], plant seed oils [10,11], waste glycerol [12], corn cob waste liquor [13] and agro-residues [14] has been widely used for biodiesel production. Oleaginous fungi such as *Rhizopus* spp [15], *Mucor* spp [16], *Cunninghamella* spp [10] with 20% or more lipids in their cell mass have emerged as a potential candidate for biodiesel production.

An important bio-oil is microbial oil containing Gamma-linolenic acid (GLA, 18:3 (n-6) Δ6, 9, 12-octadecatrienoic acid), a ω-6 polyunsaturated fatty acid (PUFA) derived from linoleic acid. Gamma-linolenic acid has been paid close attention due to its anti-inflammatory property [17, 18]. Recent studies have tried to decipher the protective role of GLA such as lipid metabolism disorder [19], Ionizing radiation [20], cancer [21, 22]. Distribution of GLA among zygomycetous fungi such as *Cunninghamella* spp, *Mortierella* spp, *Mucor* spp and *Rhizopus* spp is well documented [23, 24, 25, 26, 27]. The first trial of GLA production was pioneered in the United Kingdom and Japan using *Mucor* spp [28]. The aim of this work was to isolate soil fungal isolates from tropical areas and identify economically valued bio-oil producing fungi.

2 MATERIAL AND METHODS

2.1 Chemicals

Media components used in the experiment were procured from Merck (India). Solvents were of reagent grade (AR). The fatty acid standards were obtained from Supelco, USA.

2.2 Isolation and Identification of fungi from soil samples

One hundred soil samples were collected from different parts of Karnataka, India. One gram of each soil sample was suspended in 10ml of sterile distilled water, serially diluted (10^{-6}) and plated on to Potato Dextrose Agar (PDA) and incubated at 28ºC for 72 hours. Individual fungal colony was transferred onto PDA slants, incubated at 28ºC, assessed for purity after four days of incubation and stored at 4ºC, till further use [29].

2.3 Identification of fungi

The fungal isolates were identified initially by observing their morphological and microscopic characteristics [29].

2.4 Culture media and growth conditions

Cunninghamella echinulata var nodosa-JSK1 and *Rhizopus oryzae*-JSK3 were grown on PDA for 24 hours at 28ºC before being transferred to nitrogen limited medium of the following composition (g l^{-1}): KH_2PO_4 2.5; $ZnSO_4.7H_2O$ 0.01; $CuSO_4.5H_2O$ 0.002; $MnSO_4$ 0.01; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.02; $CaCl_2$ 0.1; yeast extract 5.0; KNO_3 1.0 and glucose 30.0. The effect of various plant seed oils (Sunflower, Safflower, Castor, Jatropa, Pongamia, Sesame, Niger, Coconut, Watermelon, Pumpkin, Tomato, Muskmelon, Cucumber, Onion, Black Cumin, Bauhinia, Knol Khol, Bottle Guard, Radish, Dhania) was tested by replacing glucose of the above medium with 1% oil. The culture was incubated at 28°C under shaken condition (120rpm) for 6 days [30].

2.5 Extraction of plant seed oils

Fifteen grams of dry plant seeds was weighed, coarsely ground using a blender and was subjected to Soxhlet extraction for 10 hours using 300mL of hexane at 60°C [31].

2.6 Biomass and lipid analysis

2.6.1 Cell dry biomass determination and lipid extraction

After growth, biomass was harvested by filtering through muslin cloth and washed thoroughly with distilled water to remove any media ingredients and vigorously with alcohol and hexane for a minute to remove the oil adhering to the mycelia and then dried at 50-55°C until the constant weight was obtained. The dried mycelial mat weight was recorded. The dried mycelia were made into fine powder using mortar and pestle. The extraction of total lipid (TL/ DBM, %) was done by following the method of Folch et al 1957 [32]. Known amount of powdered mycelial mat was mixed with appropriate volume of chloroform: methanol (2:1) and the lipid was extracted using separating funnel. The lower fraction was collected in a beaker and allowed to evaporate till dryness. The residue containing lipid was dissolved in diethyl ether and later was transferred to pre-weighed sterilized vials, allowed to evaporate till dryness and weight of the vials were recorded again [33].

2.6.2 Preparation of fatty acid methyl esters (FAMEs)

Fatty acids methyl esters were prepared for analysis from a total lipid sample with heptadecanoic acid as an internal standard. Fatty acid methyl esters were analysed by gas chromatography (GC-6890 N, Agilent Technologies) using a capillary column DB-23 under a temperature gradient with hydrogen as the carrier gas and detected by a flame ionization detector (FID), as described previously [34,35]. A split ratio of 1/20 was applied during analysis. Identification of the fatty acid methyl ester peaks was performed by comparison with authentic standards of C4-C24 fatty acid methyl ester mixtures (Supelco, USA). Quantitative evaluation of individual fatty acids was calculated by ChemStation B0103 (Agilent Technologies). All values were means of triplicate determination.

3 RESULTS AND DISCUSSION

3.1 Screening of soil fungi for GLA production

A total number of two hundred fungi were isolated and among these, only Zygomycetous fungi such as *Cunninghamella* spp, *Rhizopus* spp and *Mucor* spp (identified by morphological and microscopical observation) were selected to assess the oleagenicity by estimating the total lipid in general, bio-fuel and GLA in particular [Table 1]. The isolates JSK1 and JSK3, identified as *Cunninghamella echinulata var. nodosa* and *Rhizopus oryzae* accumulated 16% and 17% total lipid in microbial cells, 32% and 20% oleic acid, 18% and 14% of GLA in their total fatty acid respectively.

3.2 Effect of different plant seed oil on biomass and TL/DBM (%) production

In general, plant seed oil, influences the growth of fungal strains and lipid production, which varies with the fatty acid composition of the oil [36]. Our study also revealed similar such results [Table 2a and 2b]. Pumpkin, watermelon, onion, tomato, radish, sunflower, niger and castor seed oil (1%) increased biomass production (3-7g/l) in *C echinulata var. nodosa*-JSK1, whereas pumpkin, watermelon, muskmelon, onion, tomato, pongamia, radish, sunflower, safflower and castor seed oil (1%) increased biomass to 2-5g/l in *R oryzae*-JSK3. However, muskmelon, cucumber, pongamia, jatropa, black cumin, bauhinia, bottle guard, knol khol, dhania, sesame, coconut and safflower seed oil (1%) decreased biomass production (0.9-2-1g/l) in *C echinulata var. nodosa*-JSK1, whereas in *R oryzae*-JSK3, addition of cucumber, jatropa, black cumin, bauhinia, bottle guard, knol khol, dhania, sesame, niger, coconut seed oil (1%) decreased biomass production (0.7-1.2 g/l).

Pumpkin and sunflower oil influenced lipid accumulation from 16% to 27% and 17% to 24% in *C echinulata var. nodosa*-JSK1 and *R oryzae*-JSK3 respectively. However, sesame and coconut oil decreased the lipid synthesis, by bringing down the lipid content from 16% to 7% and 17% to 8% in both the fungi used in the present study. These differences in biomass and lipid accumulation in fungi, when plant seed oil is used as sole carbon source could be attributed to the assimilation of exogenous oil through the production of extracellular lipases, which cleave fatty acid residues from exogenous oil and the free fatty acids thus released can either be incorporated to lipid structures for desired PUFA production or degraded to basic skeletons for biomass synthesis [37].

3.2.1 Effect of plant seed oil on fatty acid production

The fatty acid profile of the lipid in glucose-grown *C echinulata var. nodosa*-JSK1 and *R oryzae*-JSK3 was as follows [Table 3a and 3b]: lauric acid (C14:0, 8% and 2%), palmitic (C16:0, 15% and 29%), stearic (C18:0, 7% and 13%), oleic (C18:1, 33% and 20%), linoleic (C18:2, 20% and 16 %) and gamma-linolenic acid (C18:3γ, 18% and 14%).The major fatty acids in *C echinulata var. nodosa*-JSK1 and *R oryzae*-JSK3, grown in plant seed oil, as a sole source of carbon was lauric acid (C14:0, 0.1-0.6% and 0.2-15%), palmitic (C16:0, 7-17% and 4-19%), stearic (C18:0, 2-9% and 0.6-11%), oleic (C18:1, 11-55% and 14-63%), linoleic (C18:2, 11-50% and 11-49%) and gamma-linolenic acid (C18:3γ, 0-23% and 0-19%). Biotransformed *C echinulata var. nodosa*-JSK1 and *R oryzae*-JSK3 produced GLA in the range of 3-23% and 5-19%. Although, pumpkin and onion oil (1%) also increased GLA production (16-20%) to some extent in the fungi tested by us, maximum GLA production was obtained when sunflower oil (23% and 19%) was used as a sole source of carbon. This is in accordance with the results obtained in our previous study with the fungus *C blakesleeana*-JSK2, isolated from the tropical soil [10]. Similar results were obtained in *Mucor mucedo* and *C echinulata* respectively [23]. When sesame oil was used as a sole source of carbon, complete inhibition of GLA production was observed in the fungus. Such findings were also reported in *C blakesleeana*-JSK2 [10], *Mucor rouxii* and *Mucor sp.1b* [32].

Among the monounsaturated fatty acids, oleic acid, one of the key components of biodiesel was dominant in biotransformed *C echinulata var. nodosa*-JSK1 and *R oryzae*-JSK3, ranging between 10-55% and 14-63% of the total fatty acids. This is the first report to show that, addition of *Pongamia pinnata* oil (1%), a non-edible, indigenous biodiesel oil, led to trivial increase in oleic acid content from 32-55% and 20 to 63% and on the contrary, apparent repression of gamma-linolenic acid in the fungi used in this study. These results were in accordance with another oleaginous fungi (*C blakesleeana*- JSK2, ranging between 20-70%) isolated by us from tropical soil, when *Pongamia pinnata* oil was used as a sole source of carbon [10]. The most interesting and significant finding of our present study is the increase in the

percentage of oleic acid content when grown on *Pongamia pinnata* oil as sole source of carbon. In contrast, production of oleic acid in *C echinulata var. nodosa*-JSK1 was less when; tomato, niger, coconut and bauhinia oil (8-19%) was used as a sole source of carbon. However, in *R oryzae*-JSK3, addition of muskmelon, tomato, onion, knol khol and dhania plant seed oil as sole source of carbon reduced oleic acid content to 14-18%.

4 CONCLUSION

Driven by the mounting demand for high value lipid products, its production process has been paid close attention all over the world. The present study uncovered the potential application of indigenous plant seed oils for the production of value added lipids in oleaginous fungi; *C echinulata var. nodosa*-JSK1and *R oryzae*-JSK3, isolated from tropical soil. The results obtained in our study further warrants research on how best they could be produced and tailor made to meet the growing demands of bio-oils: for sustainable biodiesel production and oils for human consumption.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The authors declare no conflicts of interest regarding this manuscript.

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