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Harnessing Indigenous Plant Seed Oil for the Production of Bio-fuel by an Oleaginous Fungus, *Cunninghamella blakesleeana*- JSK2, Isolated from Tropical Soil

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Abstract *Cunninghamella blakesleeana*- JSK2, a gamma-linolenic acid (GLA) producing tropical fungal isolate, was utilized as a tool to evaluate the influence of various plant seed oils on biomass, oleagenicity and bio-fuel production. The fungus accumulated 26 % total lipid of their dry biomass (2 g/l) and 13 % of GLA in its total fatty acid. Among the various plant seed oils tested as carbon sources for biotransformation studies, watermelon oil had an effect on biomass and total lipid increasing up to 9.24 g/l and 34 % respectively. Sunflower, pumpkin, and onion oil increased GLA content between 15–18 %. Interestingly, an indigenous biodiesel commodity, *Pongamia pinnata* oil showed tremendous effect on fatty acid profile in *C. blakesleeana*- JSK2, when used as a sole source of carbon. There was complete inhibition of GLA from 13 to 0 % and increase in oleic acid content, one of the key components of biodiesel to 70 % (from 20 % in control). Our results suggest the potential application of indigenous plant seed oils, particularly *P. pinnata* oil, for the production of economically valuable bio-fuel in oleaginous fungi in general, and *C. blakesleeana*- JSK2, in particular.

Keywords *Cunninghamella blakesleeana*- JSK2 · Biotransformation · Gamma-Linolenic Acid · Biodiesel

Introduction

Global warming, increase in demand for non-renewable fossil fuels and uncertainty in their availability are the three major problems confronted by today's world. This has led to stimulated research in finding alternative sources for bio-oil production. Biodiesel, mono alkyl esters of fatty acids, from oleaginous fungi presents a pivotal approach and has marked a breakthrough for next-generation fuels from sustainable resources [1]. It is probably the most researched alternate fuel [2]. 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 produced from an array of inexpensive

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and renewable raw materials such as lignocellulosic biomass [4], plant seed oils [5, 6], waste glycerol [7], corn cob waste liquor [8], wheat straw [9], and agro-residues [10]. Utilization of these by-products will make an important contribution in the reduction of overall production costs of biodiesel in the global market which is the major reason for researchers focusing their attention on oleaginous fungi [11, 12].

An important bio-oil is gamma-linolenic acid (GLA, 18:3 (n −6) Δ 6, 9, 12-octadecatrienoic acid), a ω-6 polyunsaturated fatty acid (PUFA)-derived from linoleic acid. Gamma-linolenic acid is of particular interest owing to its anti-inflammatory [13] and anticancer property [14]. Besides this, it is used in the treatment of multiple sclerosis [15] and rheumatoid arthritis [16]. Distribution of GLA among zygomycetous fungi (*Mortierella spp*., *Cunninghamella spp*., *Mucor spp*., *Syzygites spp*., and *Rhizopus spp*.), is well documented [17–20]. The first trial of GLA production was pioneered in the United Kingdom and Japan using *Mucor spp*. [21, 22]. Several benefits can be envisioned from these fungi due to their advantages over higher plants such as similarity in fatty acid profiles with plant seed oils, easy to grow, simple cultural conditions, and consistency of the product yield [23]. In our laboratory, we have identified three fungi (*Rhizopus oryzae*- JSK1, *Cunninghamella blakesleeana* - JSK2, and *Cunninghamella nodosa*- JSK3) showing oleagenicity and accumulating GLA in a considerable amount. Optimization of GLA production was carried out in *C. blakesleeana*- JSK2 by altering the physiological growth conditions. Further, an enhancement program was carried out by transformation studies with indigenous plant seed oil which led to some interesting findings and they are presented in this paper.

Materials and Methods

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.

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 10 ml of sterile distilled water, serially diluted (10^{-6}) and plated on to potato dextrose agar (PDA) [24] and incubated at 28 °C for 72 h. Individual fungal colony was transferred onto PDA slants, incubated at 28 °C, assessed for purity after 4 days of incubation and stored at 4 °C, till further use.

Identification of Fungi

The fungal isolates were identified initially by observing their morphological and microscopic characteristics [24] and molecular identification by sequencing the gene coding for ribosomal RNA for the selected fungus was done as per White [25].

Culture Media and Growth Conditions

C. blakesleeana -JSK2 was grown on PDA for 24 h at 28 °C before being transferred to nitrogen-limited medium of the following composition [18] (gram per liter): 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, jatropha, 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 (120 rpm) for 6 days.

Extraction of Plant Seed Oils

Fifteen grams of dry seeds was weighed, coarsely ground using a blender and was subjected to Soxhlet extraction for 10 h using 300 ml of hexane at 60 °C [26].

Biomass and Lipid Analysis

Cell Dry Biomass Determination and Lipid Extraction

After 6 days (144 h) of 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 %) was done by following the method of Folch et al. [27]. 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 [28]. Results were expressed as percentage of fatty acid composition in total lipid (%).

Preparation of Fatty Acid Methyl Esters

The fatty acids of total lipid were analyzed as their methyl esters by gas chromatography according to Certík et al. [29]. The gas chromatograph (GC-6890N, Agilent Technologies) was equipped with a capillary column DB-23 (60 m×0.25 mm, film thickness 0.25 μm, Agilent Technologies) and a FID detector (constant flow, hydrogen 35 ml/min, air 350 ml/min, 250 °C). Analysis was carried out under a temperature gradient (130 °C for 1 min;130–170 °C at program rate 6.5 °C/min; 170–215 °C at program rate 2.7 °C/min; 215 °C for 7 min; 220–240 °C at program rate 2 °C/min; 240 °C for 2 min) with hydrogen as a carrier gas (flow 2.1 ml/min, velocity 49 cm/s, pressure 174 kPa) and a split ratio of 1/50 (inlets: heater 230 °C, total hydrogen flow 114 ml/min, pressure 174 kPa). The fatty acid methyl ester peaks were identified by authentic standards for a C4–C24 fatty acid methyl ester mixture (Supelco, USA) and quantified by an internal standard of heptadecanoic acid (C17:0, Supelco, USA). The fatty acid concentration was evaluated with ChemStation software B0103 (Agilent Technologies, USA). All values were means of triplicate determination.

Results and Discussion

Screening of Soil Fungi for GLA Production

A total number of 200 fungi were isolated and among these, only zygomycetous fungi (identified by morphological and microscopical observation) were selected to assess the oleagenicity by

estimating the total lipid in general and bio-fuel and GLA in particular (Table 1). The isolate JSK2, identified as *C. blakesleeana* accumulated 26 % total lipid, 20 % oleic acid, and 13 % of GLA in total fatty acid, the highest producer among the zygomycetous fungi tested and therefore selected for further study.

Molecular Identification of *Cunninghamella blakesleeana*- JSK2

Molecular identification was done by sequencing the gene encoding for 18srRNA (Fig. 1). The sequence was queried in blast search (NCBI) to find out the homology with the existing species of *Cunninghamella* and showed 96 % similarity with *C. blakesleeana*- JSK2.

Effect of Different Plant Seed Oil on Biomass and TL (%) 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 [30]. A growing body of evidence suggests that plant seed oil, in general, as a sole source of carbon, substantially increases the biomass and total lipid content in fungi [31]. Our study also revealed similar such results (Table 2). Pumpkin, watermelon, muskmelon, cucumber, onion, tomato, bottle guard, and knol khol oil (1 %) increased biomass (7–9 g/l) whereas pongamia, jatropha, bauhinia, dhania, sesame, niger, and coconut oil decreased biomass production (1.70–2.87 g/l). Watermelon oil enhanced the lipid synthesis and the lipid content increased from 26 to 34 %, whereas, sesame oil decreased the lipid synthesis, by bringing down the lipid content from 26 to 14 %. These differences in biomass and lipid accumulation in fungi, when oil is used as sole carbon source could be attributed to the assimilation of exogenous oils 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 [32, 33].

Effect of Plant Seed Oil on Fatty Acid Production

The fatty acid profile of the lipid in glucose-grown *C. blakesleeana*- JSK2 was as follows (Table 3): lauric acid (C14:0, 2 %), palmitic (C16:0, 28 %), stearic (C18:0, 13 %), oleic (C18:1,

Table 1 Production of dry biomass (DBM, g/l), total lipid (TL, %), Oleic acid and gamma-linolenic acid (GLA, %) content, grown under nitrogen-limiting medium in selected zygomycetous fungi isolated from soil

SL No.	Fungal isolates	DBM (g/l)	TL (%)	Oleic acid (%) (C18:1)	GLA (%) (C18:3,γ)
1	*C. nodosa*- JSK1	2.58±0.50	16±0.12	16.23±0.98	18.25±0.28
2	*C. blakesleeana* -JSK2	3.00±0.28	26±0.46	20.20±0.38	13.80±0.19
3	*R. oryzae* - JSK3	1.59±0.36	17±0.18	27.19±0.57	14.54±0.61
4	*Mucor spp*. -JSK4	0.89±0.68	10±0.85	12.60±1.34	5.48±0.95
5	*Mucor spp*.- JSK5	5.95±0.47	13±0.46	14.71±2.32	6.75±0.18
6	*Rhizopus spp*.-JSK6	6.73±0.79	15±0.69	10.25±0.92	8.19±0.14
7	*Mucor spp*.- JSK7	4.81±0.25	11±0.46	13.67±0.85	6.48±0.43
8	*Rhizopus spp*.-JSK8	1.25±0.61	10±0.35	17.84±1.78	4.35±0.34
9	*Rhizopus spp*.-JSK9	2.56±0.94	12±0.36	9.34±0.67	8.24±0.63
10	*Mucor spp*.- JSK10	4.94±0.27	13±0.26	14.52±1.23	4.84±0.17

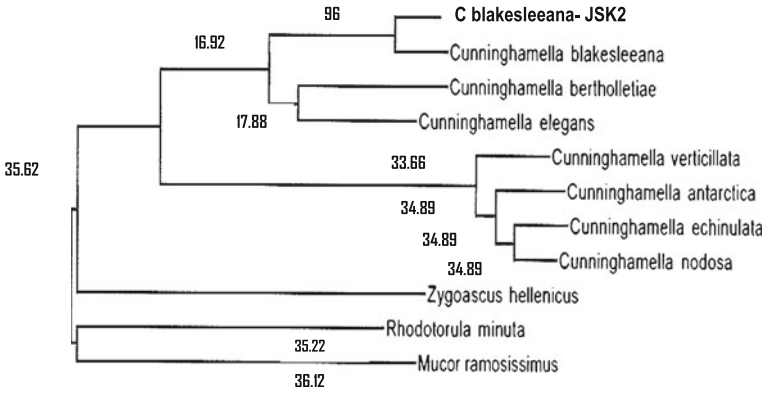

Fig 1 Molecular phylogenetic analysis of *Cunninghamella blakesleeana*- JSK2 by Internal transcribe sequence (ITS) 18srRNA sequence analysis

20 %), linoleic (C18:2, 15 %), and gamma-linolenic acid (C18:3γ, 13 %), whereas the major fatty acid in *C. blakesleeana*- JSK2, grown in plant seed oil, as a sole source of carbon was lauric acid (C14:0, 0.1–1 %), palmitic (C16:0, 1–22 %), stearic (C18:0, 0.3–3 %), oleic (C18:1, 8–70 %), linoleic (C18:2, 6–65 %), and gamma-linolenic acid (C18:3γ, 0–18 %). Biotransformed *C. blakesleeana*- JSK2 produced GLA in the range of 4–18 %. Although, pumpkin, black cumin,

Table 2 Effect of different oil supplements on dry biomass (DBM) and total lipid (TL, %) production in *Cunninghamella blakesleeana*- JSK2

SL No.	Plant oil (1 %)	DBM (g/l)	TL (%)
1	Control	3.07±0.24	26.04±0.11
2	Pumpkin	6.75±0.57	28.2±0.25
3	Watermelon	9.24±0.31	34.2±0.19
4	Muskmelon	7.56±0.44	30.2±0.24
5	Cucumber	7.45±0.15	31.4±0.32
6	Onion	8.18±0.23	33.2±0.27
7	Tomato	7.56±0.18	30.2±0.31
8	Pongamia	1.70±0.29	33.1±0.13
9	Jatropha	2.87±0.62	31.2±0.23
10	Black cumin	3.94±0.14	19.7±0.20
11	Bauhinia	2.12±0.41	22.3±0.28
12	Bottle guard	7.14±0.17	24.4±0.36
13	Radish	3.56±0.28	24.8±0.23
14	Knol Khol	7.94±0.41	23.5±0.10
15	Dhania	2.86±0.18	21.6±0.38
16	Sunflower	3.27±0.52	32.5±0.61
17	Sesame	1.91±0.30	14.2±0.54
18	Niger	1.99±0.28	24.4±0.26
19	Coconut	1.76±0.52	24.7±0.39
20	Safflower	3.58±0.41	27.5±0.34
21	Castor	4.56±0.19	28.29±0.67

Table 3 Relative percentage of fatty acid composition of *Cunninghamella blakesleeana*- JSK2 grown under different vegetable and plant seed oils [C14:0 (lauric acid), C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3(γ) (gamma-linolenic acid)]

SL no.	Seed oil	C14:0 (%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	C18:2 (%)	C18:3 (γ)
	Control	2.4±0.24	28.3±0.08	0.8±0.56	20.2±0.38	15.6±0.29	13.8±0.19
1	Pumpkin	0.6±0.54	20.5±0.41	0.3±0.27	18.6±0.38	42.1±0.17	15.2±0.28
2	Watermelon	0.6±0.16	9.2±0.21	1.8±0.10	13.2±0.17	59.1±0.44	13±0.31
3	Muskmelon	0.5±0.26	14.1±0.38	2.2±0.14	33.2±0.46	40.8±0.17	6.75±0.22
4	Tomato	0.3±0.31	13.4±0.23	2.9±0.10	32.4±0.36	44.5±0.37	6.55±0.28
5	Onion	0.1±0.61	11.8±0.49	1.5±0.21	37.8±0.32	31.4±0.52	16.75±0.31
6	Cucumber	0.2±0.34	17.6±0.48	0.4±0.61	8.4±0.17	65.3±0.29	6.8±0.18
7	Radish	0.7±0.15	9.4±0.21	1.1±0.18	40.7±0.31	20.2±0.24	3.75±0.51
8	Knol Khol	0.6±0.09	8.6±0.45	1.8±0.36	32.6±0.22	40.3±0.29	8.45±0.48
9	Dhania	0.9±0.23	7.1±0.16	0.5±0.54	52.6±0.26	27.4±0.19	6.25±0.31
10	Bottle guard	0.5±0.49	5±0.38	0.4±0.14	37.1±0.08	45.9±0.24	9.65±0.16
11	Sesame	0.5±0.28	6.4±0.25	1.9±0.18	45.6±0.49	40.9±0.18	–
12	Castor	0.9±0.51	10.6±0.29	0.4±0.17	53.1±0.34	22.4±0.26	10.5±0.23
13	Safflower	0.4±0.19	12.7±0.34	1.6±0.27	31.3±0.47	42.6±0.48	11.24±0.24
14	Niger	0.5±0.18	38.2±0.29	1.9±0.14	33.5±0.19	18.6±0.47	5.25±0.34
15	Jatropha	0.3±0.22	21.4±0.20	2.9±0.28	15.5±0.42	54.7±0.02	5.5
16	Pongamia pinnata	0.4±0.24	10.3±0.29	2.1±0.52	69.5±0.38	13.6±0.29	–
17	Black cumin	0.4±0.30	22.2±0.38	2.8±0.33	22.8±0.51	33.4±0.29	17.25±0.32
18	Coconut	0.8±0.19	2.4±0.20	3.2±0.34	16.2±0.50	6.5±0.60	4±0.17
19	Sunflower	0.5±0.41	1.3±0.20	2.1±0.38	27.1±0.42	48.1±0.28	18±0.16
20	Bauhinia	0.3±0.24	15.1±0.14	2.3±0.30	23.7±0.31	52.4±0.28	8.23±0.17

Table 4 Relative percentage of fatty acid composition of *Cunninghamella nodosa* – JSK1 and *Rhizopus oryzae*- JSK3 grown under *Pongamia pinnata* oil [C14:0 (lauric acid), C16:0 (palmitic acid), C18:0 (stearic acid), C18:1(oleic acid), C18:2 (linoleic acid), C18:3(γ) (gamma-linolenic acid)]

SL no.	Code	C14:0 (%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	C18:2 (%)	C18:3(γ)
	Pongamia pinnata oil	0.4±0.42	11.6±0.26	7.5±0.31	50.5±0.36	19.2±0.18	–
1	*Cunninghamella nodosa* – JSK1 (Control)	0.8±0.31	19.4±0.16	8.8±0.44	16.2±0.25	20.9±0.36	18.7±0.28
2	*Cunninghamella nodosa* – JSK1 (*Pongamia pinnata*)	0.4±0.26	17.2±0.38	9.3±0.24	55.2±0.38	11.3±0.41	–
3	*Rhizopus oryzae*- JSK3 (Control)	0.7±0.37	19.7±0.15	9.5±0.24	27.3±0.39	21.5±0.23	14±0.16
4	*Rhizopus oryzae*- JSK3 (*Pongamia pinnata*)	0.3±0.24	12.3±0.26	7.4±0.32	63.1±0.19	11.4±0.36	–

and onion oil (1 %) too increased GLA production (15–17 %) to some extent, maximum GLA production was observed in the medium supplemented with sunflower oil (18 %). This is in accordance with the results obtained in *Mucor mucedo* and *Cunninghamella echinulata*, respectively [32]. When sesame oil was used as a sole carbon source, complete inhibition of GLA production was observed in the fungus. Such finding was also reported in *Mucor rouxii and Mucor sp. 1b* [18]. This could be due to the strong repression of Δ^6 desaturase and elongase enzymes essential for GLA production. This finding, however, is not new, as Shimizu et al. in 1989 has reported similar finding in arachidonic acid (ARA), a ω-6 PUFA-producing *Mortierella alpina* [34]. He reported that, a non-oil fraction of sesame oil as carbon source repressed Δ^5 desaturase enzyme, essential for ARA production and thereby increasing the dihomo-gamma-linolenic acid content in *M. alpina*.

Among the monounsaturated fatty acids, oleic acid, one of the key components of biodiesel was dominant in biotransformed *C. blakesleeana*- JSK2, ranging between 20–70 % 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 substantial increase in oleic acid content from 20 to 70 % and on the contrary, apparent repression of Gamma-linolenic acid. These results were in concordant with the other two oleaginous fungi (Table 4, *R. oryzae*- JSK1, from 27 to 63 % and *Cunninghamella nodosa*- JSK3, from 16 to 55 %, respectively) isolated by us from tropical soil, when *P. pinnata* oil was used as a sole source of carbon. Amongst the many plant species, which can yield oil as a source of energy in the form of bio-fuel, *P. pinnata* has been found to be one of the most suitable species in India [35]. Therefore, the most interesting and significant finding of our present study is the increase in the percentage of oleic acid content to 70 %, when *C. blakesleeana*- JSK2 and other two oleaginous fungi (*R. oryzae*- JSK1 and *C. nodosa*- JSK3) were grown on *P. pinnata* oil as

Table 5 Properties of crude biodiesel in *Cunninghamella blakesleeana*- JSK2

Property	Unit	Crude fungal oil
Density at 15 °C	g/cm3	0.875
Acid value	mg/KOH/g oil	22.36
Free Fatty acid	%	1.15 %
Iodine value	gI_2/100 g of oil	96.8
Saponification number		205.16

carbon source. Although, the exact reason for this phenomenon could not be explained at this time, it warrants further research. In contrast, production of oleic acid was less, when, watermelon, pumpkin, cucumber, jatropha, and coconut oil (8–19 %) was used as a sole source of carbon. This can be attributed to the reduced efficacy of the fungus to assimilate oils or due to poor incorporation of exogenous fatty acids into mycelium [36].

Biodiesel Characterization

A basic study was also undertaken to characterize the physico-chemical nature of *C. blakesleeana*-JSK2 oil to ascertain whether this fungal oil falls under the category of biodiesel. Physico-chemical properties such as density, acid value, free fatty acid, iodine value, and saponification number were determined for oil obtained from the fungus, *C. blakesleeana*- JSK2, grown in *P. pinnata* oil (Table 5). These values are in the acceptable range of international biodiesel standard norms [37] (US biodiesel standards ASTM D6751, European biodiesel standards EN14214, and Indian biodiesel standards IS15607) suggesting the possible use of biotransformed oil obtained from *C. blakesleeana*- JSK2 as a potential feedstock for biodiesel production.

Conclusion

Uncertain supplies and frequent price hikes of fossil fuels in the global market is posing a serious economic threats for developing countries. Although the avenue of using microbes for biodiesel production is not totally a new concept, the present study has given an ancillary platform for the potential application of plant seed oils, as an alternate feed stock for the production of high value lipid products using microorganisms such as our fungal isolate *C. blakesleeana*- JSK2.

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Conflict of interest Authors declare no conflict of interest.

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