Optimization of Physiological Growth Conditions for Maximal Gamma-linolenic Acid Production by *Cunninghamella blakesleeana*-JSK2

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Abstract Effects of various cultural conditions on biomass, lipid and Gamma-linolenic acid (GLA) production were investigated in the oleaginous fungus *Cunninghamella blakesleeana*-JSK2 isolated from soil. The GLA production was influenced by various factors such as growth condition (static and shaken), incubation time, pH, temperature, carbon and nitrogen sources. The results indicated that optimum GLA production (21 %) was obtained when the fungus was grown under shaken condition at 120 rpm for 6 days with optimum pH and temperature of 6 and 28 °C, respectively. Glucose and potassium nitrate enhanced the GLA production. Urea and sucrose were poor substances for biomass, lipid and GLA production.

Keywords Gamma-linolenic acid · Submerged fermentation · *Cunninghamella blakesleeana*-JSK2

Introduction

There are two families of polyunsaturated fatty acids (PUFA) namely omega-3 and omega-6 fatty acids. Gamma-linolenic acid (GLA, 18:3(n-6) Δ 6,9,12-octadecatrienoic acid) belongs to omega-6 fatty acid family. It

synthesizes pro-inflammatory mediators such as series 1 of prostaglandins and thromboxanes which helps in reducing inflammation. Gamma-linolenic acid is used in the treatment of rheumatoid arthritis [1], multiple sclerosis [2], schizophrenia [3], atopic eczema [4] and premenstrual syndrome [5]. It should be noted that GLA was the first PUFA to be commercially available in the market [6].

Conventionally, GLA is produced from plant sources such as borage, evening primrose, safflower and black currant. Increasing demand in the nutraceutical and pharmaceutical companies has further led to stimulated research in GLA production from microbial sources, specifically from fungi. Microorganisms are attractive candidates and possess several advantages over plants such as owing to their ease with which they can be utilized in bioprocess reactors, relatively simpler cultural conditions and nutrients for growth, no requirement of agricultural land and sunlight and consistency of the product yield.

Zygomycetous fungi such as species of *Mortierella*, *Cunninghamella*, *Mucor*, *Rhizopus* and *Syzygites* have been reported to accumulate lipids and GLA in their mycelia [7–11]. Thus, these lower filamentous fungi are potential candidates for the production of single cell oil containing Gamma-linolenic acid. Of late, the focus has been to maximize PUFA production by genetic manipulation, mutagenesis, response surface methodology, as well as optimizing the media conditions of the selected strain. The aim of this work was to isolate soil fungi from tropical areas and identify GLA producing fungi. Subsequently, optimizing the physiological conditions for suitable growth and maximal GLA production which would serve as models for bioprocess development.

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Materials and Methods

Chemicals

Media components used in the experiment were obtained from Merck (Bangalore, India). The carbohydrates ($\geq 99\%$ purity) were procured from Sigma (USA). In all our experiments, soluble starch from potato was used as the biological source. Solvents were of reagent grade. 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) [12] and incubated at 28 °C for 72 h. Individual fungal colonies were transferred to PDA slants, incubated at 28 °C, assessed for purity after 4 days and stored at 4 °C, till further use.

Identification of Fungi

The isolates were identified initially by observing their morphological and microscopic characteristics [12] and molecular identification was done by following the method of White et al. [13].

Culture Media and Conditions

Cunninghamella blakesleeana-JSK2 (JSK2 is the code given to the isolate chosen for further study) was grown on PDA for 24 h under static conditions at 28 °C before being transferred to a nitrogen limiting medium of the following composition (g/l): KH_2PO_4 2.5; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.002; MnSO_4 0.01; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02; CaCl_2 0.1; yeast extract 5.0; KNO_3 1.0 and glucose 30.0 [14]. The effects of various nitrogen and carbon sources were tested by replacing potassium nitrate and glucose, respectively of the above medium with other nitrogen and carbon sources at the same concentration. The cultures were incubated at 28 °C under shaken condition (120 rpm) for 6 days.

Growth Condition of Fungi

Growth Studies

The time courses of lipid and GLA production were studied by growing the culture in 50-ml aliquots (but results expressed as g/l) at 28 °C for 2, 4, 6 and 8 days on a rotary

shaker (120 rpm). Cultures were harvested on respective days and washed with 100 ml distilled water.

Effect of pH

The effects of pH ranging from 4.0 to 8.0 (by adjusting the pH of the medium before autoclaving using 0.1 M HCl/NaOH) with one pH increment were used to study their effect on biomass, lipid and GLA production.

Effect of Temperature

The effects of temperature on biomass, lipid and GLA production were studied by incubating the cultures at different temperatures ranging from 24 to 32 °C.

Effect of Carbon Source

The effects of various carbon sources on biomass, lipid and GLA production were studied. To find out the suitable carbon source, glucose was replaced by other carbon sources such as sucrose, starch (plant origin), maltose, D-mannitol and fructose at 30 g/l in the basal medium.

Effect of Nitrogen Source

The effects of various nitrogen sources such as sodium nitrate, potassium nitrate, ammonium chloride, ammonium nitrate, ammonium sulfate and urea were incorporated at 1 g/l separately in the basal medium to study their effects on biomass, lipid and GLA production.

Biomass and Lipid Analysis

Cell Dry Biomass Mass Determination and Lipid Extraction

After 6 days (144 h), cultures were harvested by filtering through muslin cloth and washed repeatedly with distilled water and lyophilized. The dried mycelial mat weight was recorded. The dried mat was made into fine powder using a mortar and pestle. The extraction of total lipids was done by following the method of Folch et al. [15]. A known amount of powdered mycelial mat was mixed with the actual volume/mass ratio of chloroform:methanol (2:1; v/v) and the lipids were extracted using a separating funnel. The lower fraction was collected in a beaker and allowed to evaporate to dryness. The residue containing lipids was dissolved in diethyl ether and later was transferred to pre-weighed sterilized vials, allowed to evaporate to dryness and the weights of the vials were recorded again [16].

Fatty Acid Analysis

The fatty acids of total lipid were analyzed as their methyl esters by gas chromatography according to Certík et al. [17]. The gas chromatograph (GC-6890 N, Agilent Technologies) was equipped with a capillary column DB-23 (60 m × 0.25 mm, the film thickness was 0.25 µm, Agilent Technologies) and an FID detector (constant flow, hydrogen 35 ml/min, air 350 ml/min, 250 °C). Analysis was carried out with a temperature gradient (130 °C held for 1 min; raised from 130 to 170 °C at a rate of 6.5 °C/min; then from 170 to 215 °C at a rate of 2.7 °C/min; 215 °C for 7 min; from 220 to 240 °C at a rate of 2 °C/min; then held at 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). We are expressing GLA mass on the basis of the GC response data. All values were means of triplicate determinations.

Statistical Analysis

All the experiments were done in triplicate and statistical analysis was performed using SPSS software (version 11.5). Multi-variant ANOVA (*post-hoc* test and Scheffe test) was used to assess statistical differences. The values are given as means ± SD (standard deviations). Levels of significance were considered at $p \leq 0.05$ unless otherwise stated.

Table 1 Production of Dry biomass (DBM, g/l), Total lipids (TL, %) and Gamma-linolenic acid (GLA, %) content in selected Zygomycetous fungal isolates under shaking for 6 days and other conditions are the same as given under “Materials and Methods”

Values are means ± SD, $n = 3$

Sl no	Fungal isolates	DBM (g/l)	TL (%)	GLA (%)	GLA (%) in DBM (%)
1	<i>C.nodosa</i> -JSK1	2.58 ± 0.50	16 ± 0.12	18.25 ± 0.28	2.48 ± 0.24
2	<i>C.blakesleeana</i> -JSK2	3.00 ± 0.28	41 ± 0.46	21.09 ± 0.59	7.34 ± 0.36
3	<i>Rhizopus oryzae</i> -JSK3	1.59 ± 0.36	17 ± 0.18	14.54 ± 0.61	2.10 ± 0.17
4	<i>Mucor</i> spp.-JSK4	0.89 ± 0.68	10 ± 0.85	5.48 ± 0.95	0.46 ± 0.22
5	<i>Mucor</i> spp.-JSK5	5.95 ± 0.47	13 ± 0.46	6.75 ± 0.18	0.74 ± 0.19
6	<i>Rhizopus</i> spp.-JSK6	6.73 ± 0.79	15 ± 0.69	8.19 ± 0.14	1.04 ± 0.09
7	<i>Mucor</i> spp.-JSK7	4.81 ± 0.25	11 ± 0.46	6.48 ± 0.43	0.60 ± 0.31
8	<i>Rhizopus</i> spp.-JSK8	1.25 ± 0.61	10 ± 0.35	4.35 ± 0.34	0.36 ± 0.25
9	<i>Rhizopus</i> spp.-JSK9	2.56 ± 0.94	12 ± 0.36	8.24 ± 0.63	0.84 ± 0.17
10	<i>Mucor</i> spp.-JSK10	4.94 ± 0.27	13 ± 0.26	4.84 ± 0.17	0.53 ± 0.22

Results and Discussion

Screening of Soil Fungi for GLA Production

A total number of two hundred 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 GLA in particular (Table 1). The isolate JSK2, identified as *Cunninghamella blakesleeana* accumulated 41 % total lipid, 21 % of GLA in total fatty acids, the highest producer of GLA 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 National Center for Biological Information (NCBI) to find out the homology with the existing species of *Cunninghamella* and showed 96 % similarity with *Cunninghamella blakesleeana* and subsequently coded as JSK2.

Growth Kinetics of *Cunninghamella blakesleeana*-JSK2 and GLA Production

The maximum biomass was observed on the sixth day under static conditions and on eighth day under shaking (Fig. 2a, b). The growth rate, as measured by mycelial dry weight was five times greater under shaken conditions than that was observed under static culture conditions. *Cunninghamella blakesleeana*-JSK2 grown under static conditions produced biomass in the form of a mycelial mat whereas the culture grown under shaking produced a mycelial mass in the form of pellets.

Fig. 1 Molecular phylogenetic analysis of *Cunninghamella blakesleeana*-JSK2 by Internal Transcribed Spacer (ITS) 18 s rRNA sequence analysis

Fig. 2 **a** Kinetics of growth, dry biomass (DBM, g/l), total lipid (TL, %) and Gamma-linolenic acid (GLA, %) production in *Cunninghamella blakesleeana*-JSK2 under static condition for 8 days and other growth conditions are the same as given under “Materials and Methods”. Values are means $\pm$ SD, $n = 3$. **b** Kinetics of growth, dry biomass (DBM, g/l), total lipid (TL, %) and Gamma-linolenic acid (GLA, %) production in *c. blakesleeana*-JSK2 under shaken condition for 8 days and other growth conditions are the same as given under “Materials and Methods”. Values are means $\pm$ SD, $n = 3$

Differences in fatty acid composition of *C. blakesleeana*-JSK2 is seen under static and shaken condition (Table 2a, b). Lipid accumulation and GLA production under shaken condition was at a maximum on the sixth day producing 41 % of total lipids and 21 % of Gamma-linolenic acid (Fig. 2b). Lipid accumulation under static conditions was at a maximum on the sixth day producing 28 % whereas the GLA production was found to be at a maximum on the fourth day yielding 12 % (Fig. 2a, b). It has been reported that lipid accumulation and GLA production decreases after 8 days due to cell disintegration [18–20].

Optimization of Temperature and pH for Growth and GLA Production

Temperature influences growth, lipid content and fatty acid composition in Zygomycetous fungi [21]. The maximum DBM of 6 g/l was reached when the fungus was cultivated at 32 °C and produced the lowest amount of lipid and GLA content (Fig. 3). The optimum temperature for lipid accumulation (26 %) and GLA production (21 %) was 28 °C. While the results of this study are in agreement with previously published results [22], it indicates that in fungi, the optimal temperature for growth may not necessarily be optimal for lipid production. Such parameters should be taken into account during the development of the bio-process for large scale production.

pH has a profound effect on biomass, lipid and GLA production. The effect of initial pH was investigated from pH 4 to 8. Cultivation of the fungus in the range of from pH 5 to 6 yielded no significant difference in dry biomass. Lipid and GLA production was at a maximum when it was cultivated at pH 6. Biomass production and lipid accumulation was drastically reduced at extreme pH values (Fig 4) tested (pH 4 and pH 8). In *Mucor* sp. also, lipid

Table 2 Relative percentage of fatty acid composition of *Cunninghamella blakesleeana*-JSK2 grown at different intervals of time (a) under static condition (b) under shaken for 8 days and other conditions are the same as given under “Materials and Methods”

Days	Percentage of fatty acid composition in total lipid (%)					GLA (%) in DBM (%)
	16:0	18:0	18:1	18:2	18:3 (γ)	
(a)						
2	18.03 $\pm$ 0.12	6.07 $\pm$ 0.14	42.10 $\pm$ 0.07	17.93 $\pm$ 0.05	9.01 $\pm$ 0.11	1.35 $\pm$ 0.37
4	19.71 $\pm$ 0.05	12.11 $\pm$ 0.24	33.69 $\pm$ 0.17	16.21 $\pm$ 0.15	12.19 $\pm$ 0.08	2.55 $\pm$ 0.72
6	18.58 $\pm$ 0.07	4.71 $\pm$ 0.18	39.68 $\pm$ 0.26	20.23 $\pm$ 0.12	10.03 $\pm$ 0.09	2.35 $\pm$ 0.58
8	17.67 $\pm$ 0.16	4.94 $\pm$ 0.06	39.46 $\pm$ 0.16	19.62 $\pm$ 0.24	9.62 $\pm$ 0.14	2.07 $\pm$ 0.86
(b)						
2	16.81 $\pm$ 0.17	9.74 $\pm$ 0.24	34.15 $\pm$ 1.15	17.36 $\pm$ 0.04	9.57 $\pm$ 0.11	1.16 $\pm$ 0.56
4	18.92 $\pm$ 0.14	12.11 $\pm$ 0.08	38.63 $\pm$ 1.07	14.71 $\pm$ 0.19	17.57 $\pm$ 0.07	3.23 $\pm$ 0.62
6	18.36 $\pm$ 0.18	8.37 $\pm$ 0.07	27.7 $\pm$ 1.2	19.0 $\pm$ 0.27	20.48 $\pm$ 0.13	7.06 $\pm$ 0.78
8	20.89 $\pm$ 0.06	6.33 $\pm$ 0.13	36.45 $\pm$ 0.07	18.0 $\pm$ 0.21	11.37 $\pm$ 0.17	1.86 $\pm$ 0.84

[C16:0 (Palmitic acid), C18:0 (Stearic acid), C18:1 (Oleic acid), C18:2 (Linoleic acid), C18:3 (γ) (Gamma-linolenic acid)]. Values are means $\pm$ SD, $n = 3$

Fig. 3 Effect of temperature on dry biomass (DBM, g/l), total lipid (TL, %) and Gamma-linolenic acid production (GLA, %) in *Cunninghamella blakesleeana*-JSK2 under shaken condition for 6 days and other growth conditions are the same as given under “Materials and Methods”. Values are means $\pm$ SD, $n = 3$

production/accumulation was significantly reduced under sub-optimal pH conditions (such as, pH 4 and pH 8). It is possible that interactions between media components and pH reduce the ability of the strain to utilize the available nutrients in the medium [22].

Effect of Carbon and Nitrogen Sources

Among the various carbon sources tested, starch yielded the highest biomass (3.4 g/l, Fig. 5). Similar results on starch were reported earlier in *M. ramanniana* var. *ramanniana* [23]. Total lipid production was highest in the medium supplemented with D-Mannitol (44.25 %), whereas maximum GLA production was achieved in glucose containing

Fig. 4 Effect of initial pH on dry biomass (DBM, g/l), total lipid (TL, %) and Gamma-linolenic acid production (GLA, %) in *Cunninghamella blakesleeana*-JSK2 under shaken condition for 6 days and other growth conditions are the same as given under “Materials and Methods”. Values are means $\pm$ SD, $n = 3$

medium (20.5 %). This is in concordance with *Mucor*. sp.1b and *Mucor* sp. RRL001, respectively [14, 24]. Though glucose gave the highest GLA production, total lipid production was comparatively lower than on maltose, fructose and D-Mannitol (Fig. 5). Sucrose was a poor growth supporter which yielded only 1.3 g/l dry biomass. Like sucrose, lactose have been reported as poor substance for GLA production [14, 24]. The various sugars which supported GLA production are as follows glucose > D-Mannitol = fructose = maltose > starch > sucrose. Reports suggest that a simple sugar such as glucose serves as the best carbon source for GLA production in *Mucor* spp., *Mortierella* spp.

Fig. 5 Effect of different carbon sources on dry biomass (DBM, g/l), total lipid (TL, %) and Gamma-linolenic acid production (GLA, %) in *Cunninghamella blakesleeana*-JSK2 under shaken condition for 6 days and other growth conditions are the same as given under “Materials and Methods”. Values are means $\pm$ SD, $n = 3$

and *Cunninghamella* spp. [14, 23, 25]. This may be because, fungi belonging to the class Zygomycetes are generally saprophytic in nature and prefer to grow and synthesize various metabolites on simple sugars compared to complex sugars.

It has been reported that, accumulation of lipids occurs due to exhaustion of nitrogen in the medium results in cessation of cell proliferation and thus leading to the conversion of carbon into lipid [26]. Studies have indicated that nitrogen source used in the medium plays a vital role in regulating lipogenesis [27]. The influence of various nitrogen sources on biomass, lipid and GLA production was studied. Ammonium sulfate, ammonium chloride and sodium nitrate gave very nearly as much biomass as obtained with potassium nitrate (5.7 g/l, Fig. 6). Maximum lipid production was obtained with ammonium sulfate (36.2 %). Gamma-linolenic acid production was higher in potassium nitrate supplemented medium (21 %). In the present study, least biomass production and GLA content was obtained when urea was used as nitrogen source and similar results were obtained in *M. rouxii* and *M. spp.1b* [14]. However, use of urea as nitrogen source in the growth medium enhanced biomass and GLA production of *C. echinulata* CCRC31,840 [25]. Certik et al.[27] reported maximum GLA production was obtained when sodium nitrate was used as nitrogen source in *Cunninghamella echinulata*. Overall, addition of potassium nitrate as a nitrogen source produced a substantial increase in GLA production over other nitrogen sources examined here.

Fig. 6 Effect of different nitrogen sources on dry biomass (DBM, g/l), total lipid (TL, %) and Gamma-linolenic acid (GLA, %) production in *Cunninghamella blakesleeana*-JSK2 under shaken condition for 6 days and other growth conditions are the same as given under “Materials and Methods”. Values are means $\pm$ SD, $n = 3$

Conclusions

Our investigations uncovered the ability of an oleaginous fungus, *C. blakesleeana*-JSK2 isolated from soil samples to produce polyunsaturated fatty acids in general, omega-6 fatty acid, in particular. This study also optimized physiological growth parameters which would serve as a basis for bioprocess development activities. The differences in fatty acid composition in response to different cultural parameters enable the manipulation of the production of desired polyunsaturated fatty acid. The results indicated that optimum GLA production was obtained when the fungus was grown under shaking at 120 rpm for 6 days with an optimum pH of 6 and a temperature of 28 °C. Glucose and potassium nitrate enhanced the GLA production. Our present study elucidates the various physiological parameters for optimization of GLA production which would serve as a basis for future bioprocess development of therapeutically important Gamma-linolenic acid.

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