Differential temperature effect on the production of enhanced gamma linolenic acid in *Mucor rouxii* CFR-G15

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Abstract *Mucor rouxii* CFR-G15, a locally isolated phycomycetous fungus, on cultivation at room temperature produced more than 30% (w/w) lipid in their dry cell weight, in which 14.2% accounted to be GLA content of the total fatty acids. It was observed that when incubation temperature lowered at 14°C, GLA content of the mycelium increased significantly ($P < 0.05$) from 14.2% to 21.97%. In order to optimize the cultural conditions for high biomass and lipid production with high GLA content, the fungus was grown in association of two different temperatures and supply of additional glucose in culture medium. Maximum lipid and GLA were obtained 23.56 and 19.5% respectively, when the culture was grown at 28°C for four days and followed by addition of glucose (5%), and lowered the incubation temperature to 14°C for another four days. The presence of GLA in the oil obtained from *M. rouxii* CFR-G15 was confirmed by the gas chromatography-mass spectrometry. Gamma linolenic acid (GLA, n-6) is gaining importance in pharmaceutical and nutraceutical industries because of clinical evidence demonstrated that it has various beneficial effects in human health. In this paper temperature played a major role in enhancing the GLA content which has been described.

Keywords Biomass · Gamma linolenic acid · Microbial lipids · *Mucor rouxii* · Polyunsaturated fatty acids

Introduction

Omega-6 and omega -3 series of polyunsaturated fatty acids (PUFAs) are identified as potential food additives and pharmaceuticals for various biological activities. The PUFAs are used for treating many major ailments [1–4]. Gamma linolenic acid (GLA, 6, 9, 12-cis, cis, cis- octadecatrienoic acid) is an omega-6 PUFA and has beneficial effects on the human health and development. It acts as a precursor for the production of long chain fatty acids like dihomogamma linolenic acid (C20:3n6) and arachidonic acid (C20:4n6) in n-6 pathway. These long chain fatty acids are converted into eicosanoids like prostaglandins series 1, 2 and 3, leukotrienes and thromboxanes. Nutritional and therapeutic studies suggest that GLA is involved in the blood lipid profile, inflammation, anti-inflammatory effect, atherosclerosis, osteoporosis and also used mainly in the treatment for premenstrual disorder, eczema and skin problems. Thus GLA has potential application in food, pharmaceutical and nutraceutical industries. The current sources for GLA are from plant seed oils like evening primrose, black currant and borage. GLA is also obtained from the microorganisms like *Mucor* spp, *Mortierella* spp, *Cunninghamella* spp, *Rhizopus*, *Entomophthora* spp, *Thamnidium* and *Absidia* [5–7]. These microorganisms are promising lipid sources because of their extremely high growth rates on cheap substrates and simplicity of manipulation of cellular lipid contents by environmental and nutritional conditions over the plant sources.

Biomass build up and lipid synthesis in any oleaginous fungus is highly influenced by number of environmental factors like pH, temperature, light and aeration. Among these, temperature is one of the most critical factors that

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affect all species of living organisms and controlling the growth rate, lipid synthesis and alters the composition of fatty acids in cellular level. Earlier studies showed that temperature has pronounced effect on growth and biosynthesis of unsaturated fatty acids in certain oleaginous microorganisms [7–10]. Fermentation at high temperature produce more saturated than unsaturated fatty acid and vice versa when grown at low temperature. This phenomenon is a part of the adaptive response to the cold environment. Our focus in this study was to optimize the cultivation temperature for *M. rouxii* CFR-G15 an oleaginous fungi at different levels ranging from low temperature (5–15°C) to the high temperature (40–45°C) for the enhanced production of biomass, lipid accumulation and their fatty acid profiles with special reference to GLA in association of these two varied temperatures and supply of additional glucose in the culture medium.

Materials and Methods

Strain: *M. rouxii* CFR-G15, an oleaginous fungus was isolated from soil sample. This isolate was deposited in CFTRI microbial culture collection centre and the strain was maintained on potato dextrose agar slants with repeated sub-culturing for its viability.

Chemicals: The chemicals and solvents used in this study were of analytical grade and procured from Merck chemicals, Mumbai, India. Fatty acid standards were obtained from Sigma chemicals Co. (St. Louis' Mo, USA).

Medium and culture conditions: The culture was grown aerobically in 500 ml capacity Erlenmeyer conical flask for 6 days at 28±2°C on a controlled rotary shaker at 180 rpm (Innova 4230, New Brunswick, USA). The basal medium consists of (g/l): glucose 40, yeast extract 1.5, ammonium nitrate 0.286, KH₂PO₄ 0.75, MgSO₄·7H₂O 0.4 and CaCl₂ 0.4. pH 5.8. The pH of the medium was adjusted before sterilization by adding 1N NaOH or 1N HCl. The flasks were inoculated with suspension containing 1×10⁷ spores per ml. The fungus was grown at different temperatures ranging between 5 and 40°C to estimate the optimal growth temperature and study the effect of temperature on the lipid contents. The rpm level in the shaker and aeration of the medium during cultivation of *M. rouxii* CFR-G15 were maintained all through the experiments

Analytical Methods

Dry biomass and total lipid content: Biomass was separated from culture broth through Whatmann No.1 filter paper, washed twice with distilled water, dried at 50–55°C for overnight or until the constant weight was obtained.

Dry biomass (1.0 g) was powdered with neutral sand and then macerated with 0.5 N HCl followed by thorough washing and dried at 55°C for overnight. Lipid was extracted from the dry powder for 6–8 h by soxhlet method and total lipid content was determined [11].

Preparation of fatty acid methyl esters (FAMES): FAMES from the extracted lipid samples were prepared by Kates method [12]. Known amount of lipid obtained from the culture, dissolved in chloroform and methanol (2:1) and saponified with 0.88% KOH. The upper layer containing lipid was taken and passed through an anhydrous sodium sulphate to remove the residual moisture content and the solvent evaporated through nitrogen reflection. The samples were then methylated by adding methanolic –HCl (methanol: acetyl chloride, 95:5 v/v) and kept for reflection on water bath for 2 h at 80°C. After cooling to room temperature, FAMES were extracted with hexane for two to three times and sample reflected with nitrogen gas and stored in freezing temperature for further use.

Fatty acid Analysis: The FAMES were dissolved in known amount of chloroform and analyzed by gas chromatography (Shimadzu 15A, Tokyo, Japan) equipped with flame ionizing detector (FID). The separation was carried out on 3 meter column packed with 12.5% diethyl glycol succinate (DEGS, 60/80 mesh). The operative conditions were as follows, column temperature (180°C), injector (220°C) and detector temperature (230°C). Nitrogen was used as the carrier gas at a flow rate of 40 ml/min. The individual fatty acids were identified with the retention time of authentic standards of Sigma (Sigma Chemical Co, USA). The degree of unsaturation (Δ /mole) in the lipid fraction was calculated using the formula Δ /mole = 1 (% of monoene) + 2(percentage of diene) + 3(percentage of trienes) / 100. Analysis of residual sugar in the medium was performed using Dinitro-salicylic acid (DNS) method [13].

Statistical Analysis: All the experiment were done in triplicate, and experimental data was subjected to analysis of variance and Duncan's multiple range test ($p = 0.05$) using the Statistical Analysis System [14].

Results and Discussion

PUFAs are the primary metabolites produced in *M. rouxii* CFR- G15, their yield was associated with the growth of the fungus, lipid accumulation and temperature of the media. This fungus was grown at different temperatures to optimize GLA yield. Our results (Fig. 1) indicate that this strain did not show significant ($p > 0.05$) growth and lipid yield both in low temperature (5°C) and high temperature (40°C) respectively. Optimal growth with reference to biomass and total lipid was observed at 30°C under normal cultural

cultivation. Further, to study the biomass build up, lipid accumulation and pattern of lipid profile, 14°C, 21°C, 28°C and 35°C temperatures were selected. During growth study of 10 days incubation period, the cultures were removed periodically every 48 h. Optimal growth with reference to biomass and total lipid yield observed significantly ($p < 0.05$) at 28°C under normal cultural condition in the 6th day (Table 1). Maximum GLA production, was obtained when the fungus was grown at 14°C for 6 days incubation resulted 21.97% of the total fatty acids. This clearly indicates that the low temperature favors PUFAs formation specially GLA in the cell.

It is observed that, this culture when grown in low temperature, the biomass and lipid production showed variations. While altering the temperature to low level from the optimal, the highest yield of GLA and other PUFAs were recorded. Another interesting observation was, during the exponential growth condition, the lipid profiles were showing more on saturated form than unsaturated whereas in older culture it was more on unsaturated form than saturated form. Hence we concluded that the degree

Fig. 1 All parameters are expressed as Mean $\pm$ values of triplicate. TLC-Total lipid content (%).

Table 1 Effect of incubation temperature on Biomass, Lipid and GLA production in *M. rouxii* CFR-G15 on 6th day of cultivation

Temp °C	Biomass (g/L)	Lipid (% DB w/w)	GLA/Total Fatty acids (% w/w)
14	5.6 $\pm$ 0.6 a	15.75 $\pm$ 0.1a	21.97 c
21	7.218 $\pm$ 1.1b	19.1 $\pm$ 0.15b	15.27 b
28	8.2 $\pm$ 1.8c	30.12 $\pm$ 0.42 c	14.42 b
35	5.9 $\pm$ 0.23a	27.8 $\pm$ 0.24 b	11.17 a

All parameters are expressed as Mean $\pm$ values in the same column with different letters are significant different at $p < 0.05$ according to Duncan's multiple range tests.

DB; Dry biomass.

Lipid; Total lipid in the dry biomass.

of unsaturation in the fatty acid composition is known to influence by low temperature [15]. Further growth in low temperature not only influences the PUFAs production but also alters the cell morphology [16]. In the culture medium, during the first 24 h at 35°C large fluffy pellets (3–5 mm dia) were formed and gradually these pellets transformed into mycelial form whereas in low temperature (14°C) the small pellets were formed and they were stable.

Effect of temperature on fatty acid profiles of total lipid of *M. rouxii* CFR- G15 on 6th day cultivation is given in Fig. 2. Oleic acid (C18:1) was found to be a major fatty acid in all the temperatures tested, followed by palmitic acid (C16:0), GLA (C18:3, n-6), linoleic acid (C18:2) and stearic acid (C18:0). The remaining fatty acids were only fractional. An important observation was made that the increase in GLA and linoleic acid contents inversely proportional to the availability of palmitic acid and oleic acid contents at low temperature level (14°C).

The increased level of GLA at low temperature could be due to presence of desaturase genes that received signals from a specific sector in the cytoplasmic membrane. Influence of carbon in the cystol led to accumulation of acetyl CoA which further led to formation unsaturation of long chain fatty acids from fatty acid synthase [17]. This study also reveals that low temperature brought about higher contents of GLA per unit mass. Lowering the growth temperature led to increase in the degree of unsaturation in membrane lipids. This observation prevails from microorganisms to animals.

The degree of unsaturation was also found to be increased steadily at low temperature in *M. rouxii* CFR-G15 tested here. This modification could be due to the membrane fluid stability of the organisms as an adaptive mechanism to cold environment. This observation is correlates very much with results observed by Robinson [18] and Sumner et al. [19]. Multiple interlocking controls and regulatory mechanisms operate within the cell system necessitate or adjust the cell lipid to alter the lipid profile. Certain key enzymes responsible for fatty acid formation become inactivated due to sudden temperature shock. This temperature shock also alters the structural and functional moiety of the lipid in the cell level [16, 20, 21]. At lower temperature the synthesis of unsaturated fatty acids is less retarded than their degradation, so that under these conditions, more linoleic acid (C18:2) accumulates in the lipids, with consequent increase in the degree of lipid unsaturation. Varying the temperature for growth of the organism modifies its biosynthetic metabolites resulting in an increased or decreased levels of unsaturation (Δ /mole) [22, 23]. Our results indicate that the degree of unsaturation is primarily dependent on the concentration of GLA and oleic acid content in the cell. There

is a correlation prevailing between the degree of unsaturation and concentration of GLA. Further, the results also indicate that, higher the concentration of GLA the higher the degree of unsaturation (Δ /mole) observed in the *M. rouxii* CFR-G15 (Fig. 2).

The conversion of saturated into unsaturated fatty acid is known to be regulated by desaturase enzymes which require oxygen as a cofactor along with acetyl coenzyme A (acetyl Co-A), acyl carrier protein, reduced nicotinamide adenine nucleotide (NADH_2) and reduced nicotinamide adenine dinucleotide phosphate (NADPH_2). Therefore, if the oxygen concentration of the medium falls to a low level where it becomes rate limiting for the desaturation reaction [8, 18, 20].

Results of the present study indicated that, *M. rouxii* CFR-G15 produced more than 30% lipid in their dry cell weight, in which 14.2% accounted as GLA content in an ambient cultivation condition. When the culture was grown at low temperature, as mentioned earlier, the GLA content increased significantly ($p < 0.05$) i.e. 21.97% of the total fatty acid content. Therefore further optimization of temperature as fermentation parameter was needed to produce high percentage of lipid and higher GLA production. Kates and Baxter [24] have reported that the rate of synthesis and degradation of unsaturated fatty acids were temperature dependent and these rates had different coefficients.

Lower temperatures in the medium bring about higher contents of GLA and PUFAs per unit mass, but fungal growth rate, and lipid yield are decreased. Therefore, the highest overall production yields PUFAs and GLA could not be achieved at low temperature levels. In order to combine the beneficial effect of rapid biomass production at its optimal temperature and then shifting it to a lower temperature generate higher yield of lipid, GLA and PUFAs

Fig. 2 All parameters are expressed as Mean $\pm$ values of triplicate.

of *M. rouxii* CFR-G15. It could be concluded through our results that, maximum biomass and lipid production were obtained at 28°C and maximum GLA production at 14°C. Our results also revealed that culture was transferred from room temperature to low temperature, the lipid content of the mycelium decreased. This indicated that the culture utilizes the accumulated lipid in the mycelium as a carbon source when the glucose becomes exhausted in the medium. The fungus in order to adapt to cold environment, utilizes lipids produced by it and protein in the culture medium for mycelial growth [21, 25]. Hence, the combination of two temperatures and also addition of carbon source (glucose) to the culture medium were studied. Fig. 3 shows that the biomass, lipid and GLA content were significantly increased when the fungus was grown at 28°C for four days incubation and the temperature was shifted to low temperature (14°C). Addition of glucose (5%) to medium, and the culture was transferred to low temperature; there was significant ($p < 0.05$) increase in lipid and GLA content (23.56% and 19.5%) of the total fatty acids. Formation of lipid and unsaturated fatty acids tends to increase considerably and these results were in agreement with the observation of Lindberg and Molin [10] and Quoc and Duacq, [22].

Thus our studies concluded that temperature is the principal regulatory factor in the degree of unsaturation in the lipid profile of the organisms. In general, organisms growing under low temperature possess relatively a high degree of unsaturation in their lipid profile and it is a part of the adaptive response to the cold environment. Temperature is also regulate most of the other factors, like pH, water activ-

Fig. 3 All parameters are expressed as Mean $\pm$ values of triplicate. Culture Conditions are as follows: [A] 5 days at 28°C, [B] 5 days at 28°C, 3 days at 12°C, [C] 5 days at 28°C, 3 days at 12°C + 3% Glucose, [D] 5 days at 28°C, 3 days at 12°C + 5% Glucose, and [E] 5 days at 28°C, 3 days at 12°C + 10% Glucose.

ity, aeration and nutrients inside the cell in relation to other cellular functions and environments. Therefore microbes have to adapt to their cellular composition in accord with the demand. The changes in fatty acid composition of a cell by change in the ambient temperature have also altered the growth rate and dissolved oxygen in the growth medium. In this study, it was possible to enhance the lipid and GLA production of *M. rouxii* CFR-G15 from the baseline significantly ($p < 0.05$) 15.75% to 23.5% and 14.2% to 19.5% respectively. The data presented in this paper showed the significant influence of growth temperature and supply of glucose enhanced the production of biomass, lipid, and GLA. These finding suggest that *M. rouxii* CFR-G15 may have a potential for commercial exploitation for the production of GLA by fermentation techniques.

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