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preliminary communication

Effects of Various Process Parameters on the Production of γ-Linolenic Acid in Submerged Fermentation

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Summary

Studies were carried out on the fermentative production of γ-linolenic acid (GLA) using seven strains belonging to Mucorales. An oleaginous fungal strain, isolated from the Western Ghats of Kerala produced GLA at a level of 8 % (by mass), when grown in a complex medium containing glucose as the sole carbon source. Effects of different culture conditions were investigated in shake flasks. Maximum dry biomass and total GLA obtained were 48.4 g/L and 636 mg/L, respectively, in the culture cultivated at 30 °C and 200 rpm for 7 days. Among the organic nitrogen sources investigated, yeast extract, and combination of corn steep liquor and baker's yeast in 1:1 ratio were useful for enhancing the GLA production and the effects were comparable.

Key words: γ-linolenic acid, submerged fermentation, *Mucor* sp., process parameters

Introduction

There are two different groups of polyunsaturated fatty acid families, namely omega-3 and omega-6 PUFAs. The γ-linolenic acid (GLA, 2-octadecatrienoic acid) belongs to omega-6 PUFA series. It consists of 18C atoms and three double bonds, all located in *cis* position. GLA is an important intermediate in the biosynthesis of prostaglandins. Lately, many food products are also being enriched with GLA, and there is rapid increase in its demand. Conventionally, it is produced from plant sources such as seeds of evening primrose, borage and blackcurrant. However, to meet the increasing demand, extensive research is being carried out for its production from microbial sources so as not to rely only on plant sources.

Production of GLA from fungal sources is an alternative to production from plant seeds. Many reports have shown the ability of a few fungal species of storing high lipid profile composed of unusual fatty acids. Recent research suggests that γ-linolenic acid is unique among the n-6 polyunsaturated fatty acid (PUFA) family members as it ameliorates many health problems (*1*). There are many evidences to suggest GLA for pharmacological and dietetic purposes. GLA administered orally is capable of suppressing human T-cell proliferation (*2*). It suppresses activation of T-cell (*3*), it is effective against rheumatoid arthritis (*4*), decreases cardiovascular risk factors (*5*), normalizes nerve conduction velocity and sciatic endoneurial blood flow (*6*).

Over the past several years extensive research has been done for microbial production of PUFA (*7–13*). The focus, of late, has been to maximize PUFA yield by novel mutations, genetic manipulations, isolating new strains as well as optimizing the media for cultivating more efficient strains. The aim of this work was to isolate and

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screen GLA-producing filamentous fungi, and to optimize various process parameters for GLA production.

Materials and Methods

Chemicals

Media components in the experiment were procured from Hi-media (Mumbai, India); solvents used were of reagent grade (RG), boron trifluoride used in the preparation of methyl esters was procured from Sisco Research Laboratories (Mumbai).

Fungal strains obtained

The cultures of *Cunninghamella echinulata* MTCC 552, *Mucor hiemalis* MTCC 1277, *Mucor hiemalis* MTCC 157, *Rhizopus stolonifer* MTCC 162, *Rhizopus stolonifer* MTCC 2591, *Rhizopus stolonifer* MTCC 2198, and *Zygorhynchus moelleri* MTCC 887 were obtained from Microbial Type Culture Collection and Gene Bank (MTCC) of Institute of Microbial Technology, Chandigarh, India and were used in this study.

Isolation and identification of oleaginous fungi

Soil samples were collected from the Western Ghats of Kerala, 1 g of each soil sample was individually suspended in 1 mL of sterile distilled water, serially diluted to 10^{-3}-fold and plated on potato dextrose agar (PDA) plates containing kanamycin to a final concentration of 10^{-6}. The plates were incubated at 30 °C for 3 days in an environmental chamber under controlled conditions. Single colony of fungus was isolated and transferred repeatedly to a new PDA plate until pure cultures were obtained. These were grown on PDA slants as above and stored at 4 °C.

Media used and cultivation conditions

Screening medium was composed of (in g/L): glucose 30 and yeast extract 5, with the initial pH=5.4. The production medium was composed of (in g/L): glucose 100 and yeast extract 10, with pH adjusted to pH=5.4. The 10 % (by volume) mycelial suspension of isolated culture was inoculated in 250-mL flask containing 50 mL of broth and incubated in an incubator shaker (Innova 4230, New Brunswick, USA) at 30 °C and 200 rpm for 7 days.

Cell dry mass determination and lipid extraction

Flasks were periodically removed and mycelia were harvested from the fermented medium by suction filtration through Whatman No. 1 filter paper and thoroughly washed with distilled water, then freeze dried at –50 °C. Lipid was extracted from the dried mycelia with volume ratio of chloroform:methanol=2:1 (*14*). Residual moisture in the extracted lipid was removed by adding anhydrous sodium sulphate, filtered with Whatman filter paper and then the lipid was concentrated under vacuum drying (Buchi Rotavapor, Germany).

Methyl ester preparation and analysis of fatty acid composition

A portion of the extracted lipid was taken and fatty acid methyl ester (FAME) was prepared by refluxing with boron trifluoride and dried methanol (*15*). Methyl esters were extracted twice by adding 2 volumes of hexane and washed with 1 volume of distilled water. After mixing (shaking) briefly in a separating funnel, hexane layer was separated. Residual moisture was removed from FAME by adding anhydrous sodium sulphate and then filtered using Whatman No. 1 filter paper. The FAME was concentrated by evaporating the hexane on a boiling water bath and methyl esters were analyzed by GC-MS.

Gas chromatographic conditions

GC was performed on Agilent 6890 Series Gas Chromatograph equipped with a FID and the capillary column DB-23 (30 m × 0.25 mm i.d., 0.5 mm film thickness; J & W Scientific, USA). Injector and detector temperatures were maintained at 230 and 250 °C, respectively. The oven was programmed for 2 min at 160 °C, then increased to 180 °C at 6 °C/min, maintained for 2 min at 180 °C, increased further to 230 °C at 4 °C/min and finally maintained for 10 min at 230 °C. The carrier gas, nitrogen, was used at a flow rate of 1.5 mL/min. The injection volume was 1 μL, with a split ratio of 50:1.

GC-MS conditions

Identification of fatty acids was made using GC-MS performed with Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50–550; source at 230 °C and quadruple at 150 °C) in the EI mode with an HP-5ms capillary column (30 m × 0.25 mm i.d., 0.25 mm film thickness; J & W Scientific, USA). The carrier gas, helium, was maintained at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300 °C and the oven was programmed for 2 min at 150 °C, then increased to 300 °C at 4 °C/min, and maintained for 20 min at 300 °C. The injection volume was 1 μL, with a split ratio of 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

Results and Discussion

Screening of filamentous fungi for GLA

The present study represents isolation and screening of fungal strains for the production of γ-linolenic acid. After primary screening of soil samples, several colonies were obtained and six of these, showing good mycelial growth on plates, were evaluated for GLA production. The isolate RRL001, identified as *Mucor* sp. and showing high GLA production (results not presented), was selected for further study along with seven isolates obtained from MTCC, India.

In all the eight fungal isolates (including *Mucor* sp. RRL001) investigated, there was increase in biomass with time and maximum biomass obtained was at 168th hour of incubation. Table 1 shows the dry biomass (DBM)

and total lipid obtained from all the fungal strains used. Table 2 shows the fatty acid profile of these cultures. All the seven MTCC strains showed similar fatty acid profile, having maximum percentage (by mass) of oleic acid, followed by linolenic acid. However, *Mucor* sp. RRL001 showed maximum concentration of oleic acid, followed by palmitic acid. Although the maximum percentage of GLA, compared to that of total lipid (by mass), was observed in *M. hiemalis* MTCC 157, the yield of total lipid to biomass was the highest in *Mucor* sp. RRL001. It has been observed that higher GLA content with reference to total fungal lipid was in strains with low total lipid and vice versa (*16*), hence to obtain high overall yield of GLA, a mould with high total lipid content was selected for further studies.

Growth profile of *Mucor* sp. RRL001 is illustrated in Fig. 1. DBM increased with time and at 168th hour, 27.78 g/L of DBM was obtained with 5.8 % (by mass) of GLA and 7.4 g/L of total lipid. Decrease in lipid and GLA percentage (by mass) has been reported after prolonged incubation of 168 hour due to cell lysis (*13,17,18*).

Effect of initial glucose concentration and different carbon sources

DBM and total lipid increased with increase in glucose concentration in the medium and highest DBM was obtained in the culture grown in 10 % initial glucose concentration (Table 3). With further increase in initial glucose to 12.5 % (mass/volume), DBM decreased, which might be due to intolerance of the cells to high concentration of glucose as higher concentration of glucose increases the osmotic potential of the medium. Lower glu-

Table 1. Total dry biomass (DBM), lipid and GLA yield by different moulds of Mucorales

Mould	γ(DBM)/(g/L)	γ(TL)/(g/L)	w(GLA)/%	η(GLA)/(mg/L)
C. echinulata MTCC 552	9.21±0.73	0.63±0.14	12.9±0.91	58.7
Z. moelleri MTCC 887	5.08±1.03	1.02±0.10	14.4±1.11	122.2
R. stolonifer MTCC 2198	7.58±0.44	0.98±0.16	9.0±1.18	64.1
R. stolonifer MTCC 2591	9.22±0.58	1.18±0.32	14.8±1.10	117.8
R. stolonifer MTCC 162	6.89±0.15	0.44±0.12	13.1±0.81	39.3
M. hiemalis MTCC 1277	9.83±0.71	1.22±0.06	20.2±0.65	226.7
M. hiemalis MTCC 157	7.08±0.46	1.32±0.13	23.4±0.14	276.7
Mucor sp. RRL001	13.38±1.04	5.84±0.96	9.1±1.26	431.2

Table 2. Relative percentages of fatty acid profile obtained from different moulds belonging to the order Mucorales

Mould	PA (16:0)	SA (18:1)	OA (18:1)	LA (18:2)	GLA (18:3)
C. echinulata MTCC 552	23.6±1.12	7.2±0.98	36.9±1.61	19.4±1.10	12.9±0.91
Z. moelleri MTCC 887	28.4±1.10	15.5±1.12	24.7±1.21	13.5±0.68	14.4±1.11
R. stolonifer MTCC 2198	29.1±0.96	9.9±1.38	31.0±1.48	21.0±1.31	9.0±0.18
R. stolonifer MTCC 2591	28.5±1.11	6.7±1.41	28.4±1.12	21.6±1.62	14.8±1.10
R. stolonifer MTCC 162	17.8±1.21	2.6±0.37	37.8±0.93	28.7±1.88	13.1±0.81
M. hiemalis MTCC 1277	23.3±1.11	3.1±0.41	28.4±1.12	22.9±1.92	20.2±0.65
M. hiemalis MTCC 157	21.7±1.74	7.4±1.01	24.4±1.16	17.4±1.39	23.4±0.14
Mucor sp. RRL001	28.1±0.61	1.4±0.18	43.7±1.92	8.6±1.03	9.1±1.26

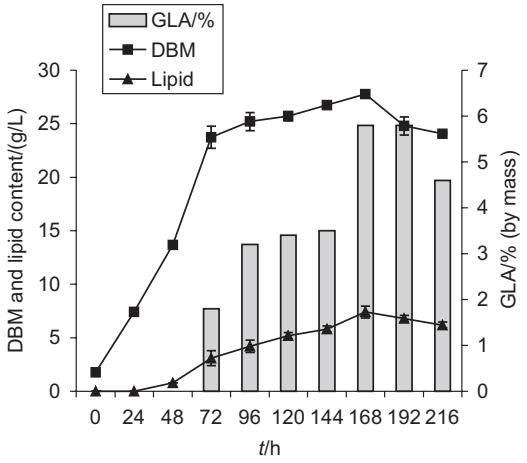

Fig. 1. Time course of dry biomass (DBM), lipid content and GLA in *Mucor* sp. RRL001

Table 3. Effect of initial concentration of glucose on dry biomass (DBM), total lipid and GLA in *Mucor* sp. RRL001

γ(glucose)/% (mass per volume)	γ(DBM)/(g/L)	γ(lipid)/(g/L)	w(GLA)/% (by mass)
2.5	15.38±1.12	4.1±0.8	8.8
5.0	27.00±2.10	4.8±0.2	5.8
7.5	30.00±1.20	6.0±0.1	6.0
10.0	35.46±0.50	6.3±0.5	6.3
12.5	34.13±0.80	5.5±0.2	5.5
15.0	28.00±1.60	5.4±0.3	5.4

cose concentrations were not so effective. Cell lysis was seen in cultures grown in 15 % initial glucose concentration due to intolerance to high osmotic potential (data not shown).

Different carbon sources, namely sucrose, lactose, soluble starch and tapioca starch were investigated for lipid production. For this, glucose in the medium was replaced by these substrates, individually at 10 % concentration. Glucose was found to be the best carbon source with a DBM production of 34.6 g/L and 5.8 % GLA (by mass) (Fig. 2). However, highest GLA percentage (by mass) was seen in the culture grown in tapioca starch (Fig. 2), but total lipid and DBM were lower, when compared to glucose. This may be because, generally, fungi belonging to order Mucorales are saprophytes, which prefer to grow rapidly and proliferate extensively on simple sugars compared to complex molecules. Lactose was the poorest growth supporter with 12.3 g/L of DBM and 1.9 g/L of total lipid. Similar results on lactose have also been reported earlier (*19*).

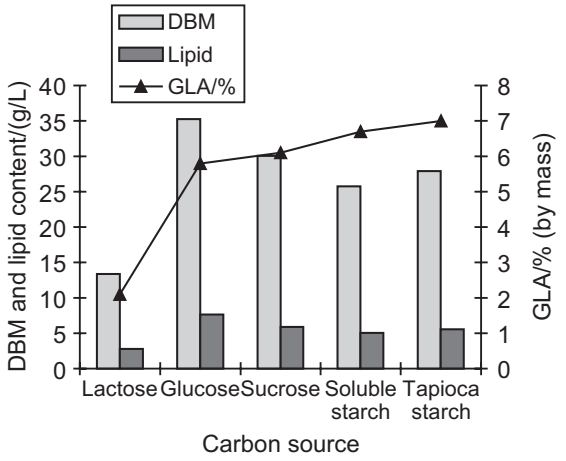

Fig. 2. Effect of carbon source (10 %) on dry biomass (DBM), lipid content and GLA in *Mucor* sp. RRL001

Effect of temperature and pH

It has already been reported that the degree of unsaturation is higher in the microorganisms cultivated at lower temperature than in microorganisms cultivated at higher temperature (*20*). Variation in lipid composition due to change in cultivation temperature has been reported in the order Mucorales (*21*). Table 4 shows the effect of temperatures ranging between 20–35 °C on DBM as well as lipid yield of *Mucor* sp. RRL001. Maximum DBM of 48 g/L was seen when the mould was cultivated at 35 °C but had the least amount of lipid as well as GLA percentage (by mass); on the contrary, when the mould was cultivated at 20 °C, 19 g/L of DBM was obtained with the highest 8.2 % GLA (by mass). However, the optimum cultivation temperature was 30 °C as total lipid was 8.1 g/L and overall GLA yield was 542.7 mg/L, which was the maximum when compared to other cultivation temperatures.

Table 4. Effect of temperature on dry biomass (DBM), total lipid and GLA in *Mucor* sp. RRL001

Temperature/°C	γ(DBM)/(g/L)	γ(lipid)/(g/L)	*w*(GLA)/% (by mass)
20	19±0.5	5.0±0.2	8.2
25	22±1.3	6.0±0.3	6.9
30	28±2.4	8.1±0.1	6.7
35	48±2.1	3.0±0.5	4.5

The effect of initial pH was investigated from pH=4.0 to pH=8.0 (by adjusting the pH of the medium before autoclaving using 0.5 M HCl/NaOH). It was found that lipid production was maximum when the mould was cultivated at the pH=6.5. Table 5 shows the effect of pH on lipid production by *Mucor* sp. RRL001. Total lipid drastically decreased at pH=8.0 and at pH=4.0, although the total lipid content was lower but GLA percentage (by mass) was maximum at pH=4.0. There was an increase in total lipid concentration in the pH range of 3.0–6.0, but the GLA percentage (by mass) decreased in this pH range.

Table 5. Effect of pH on dry biomass (DBM), total lipid and GLA in *Mucor* sp. RRL001

pH	γ(DBM)/(g/L)	γ(lipid)/(g/L)	*w*(GLA)/% (by mass)
5.0	28.78±1.1	4.80±0.3	8.3
5.5	30.16±1.4	5.43±0.2	7.2
6.0	31.80±0.5	5.52±0.2	7.2
6.5	36.02±0.5	9.10±0.3	7.0
7.0	35.35±0.5	7.92±0.2	7.7
7.5	35.42±0.4	6.80±0.4	5.7
8.0	29.11±0.8	3.91±1.1	4.5

Effect of different organic nitrogen sources

Yeast extract (YE) in the medium was replaced individually at 1 % (by mass per volume) with corn steep liquor (CSL), peptone, baker's yeast (BY), and a combination of CSL and BY (1:1, mass per volume). The results obtained are presented in Fig. 3. YE and peptone gave 8.4 % (by mass) of GLA. However, total lipid content

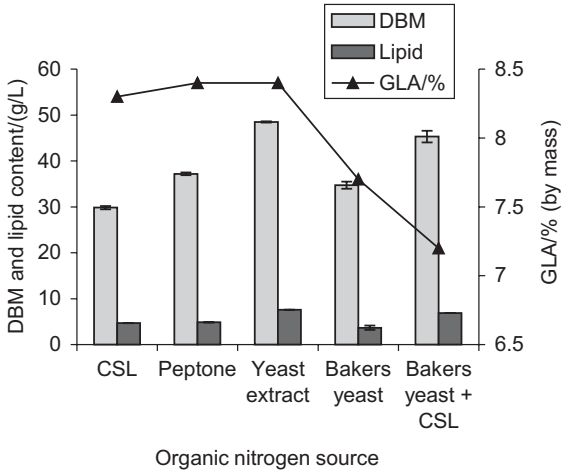

Fig. 3. Effect of different organic nitrogen sources (1 %) (mass/volume) on dry biomass (DBM), lipid content and GLA in *Mucor* sp. RRL001

obtained in the medium containing YE was higher than when obtained from the medium containing peptone, hence, overall GLA yield in culture grown in YE was maximum. By replacing YE with CSL and BY, 4.68 and 3.36 g/L of lipid as well as 8.3 and 7.7 % (by mass) of GLA were obtained, respectively. When a combination of BY and CSL was used as organic nitrogen source, 44.4 g/L of DBM with 6.86 g/L of lipid having 7.2 % (by mass) of GLA was obtained. Findings from the present study suggest YE to be the best nitrogen source for DBM (*13*) and GLA production. They also suggest YE as the best N-source for lipid production. However, combination of CSL and BY was also equally effective, and considering the cost of these materials and commercial application purposes, the use of a combination of CSL and BY could be more attractive as these are much cheaper nitrogen sources than YE and may be explored in future studies.

Conclusions

From the results, it could be concluded that the isolated strain of *Mucor* sp. could be a potential source of GLA. Replacement of glucose with cheaper carbon source such as tapioca starch yielded reasonably good titers of GLA. Similarly, although yeast extract was the best nitrogen source, its replacement by corn steep liquor and baker's yeast exerted more or less similar impact on fungal metabolism to produce GLA, thus indicating an economical alternative to yeast extract.

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