

Effects of Mineral Addition on Production of γ -Linolenic Acid by *Rhizopus nigricans*

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Abstract: The effect of mineral addition on γ -linolenic acid (GLA) production by *Rhizopus nigricans* SSSD-8 was studied. The cultivation was conducted at 30°C for 6 d in a 250 ml flask containing potato-dextrose-yeast medium with or without Na₂SO₄, CaCl₂, MgCl₂, NaCl and/or KCl. Cultivation in potato-dextrose-yeast medium without minerals produced 0.53g/L-culture of GLA, and the addition of 0.4% KCl increased the GLA production to 2.52g/L-culture. The addition of mineral mixture failed to increase the GLA production. The addition of mineral mixture, caused no change in triacylglycerol (TAG) and phospholipid (PL) compositions of the fungus.

Key words: γ -linolenic acid (GLA), *Rhizopus nigricans*, mineral, single-cell oil

1 Introduction

γ -Linolenic acid (GLA) is an essential n-6 polyunsaturated fatty acid (PUFA) and a precursor of di-homo- γ -Linolenic acid (20:3 n-6) and arachidonic acid (20:4 n-6) (1,2). It plays an important role in the treatment of diabetes (3), hypertension (4), thromboembolic disease (5), atopic eczema (6) and in the regulation of inflammatory responses (7).

GLA has been available in small commercial quantities from certain plant seeds (evening primrose, black-current and borage) (8). Microbial production of lipid rich in GLA has been attempted widely and strains of *Mortierella* (9,10), *Mucor* (11), *Cunninghamella* (12), *Rhizopus* (13), *Thamnidium* (14) and *Absidia* (15) were found to be promising producers.

In the previous paper (16) we reported the isolation of *Rhizopus nigricans* SSSD-8 as a potent producer of GLA and the fundamental culture conditions for GLA production. In order to obtain a higher yield, further

optimization of the culture conditions was essential. Several minerals may be effective for the GLA production as reported in production of arachidonic acid-containing oil (17). Therefore, we attempted to enhance GLA productivity by adding minerals to potato-dextrose-yeast medium.

2 Experimental

2.1 Cultivation of Fungus

Rhizopus nigricans SSSD-8 (16) was used in this study. The strain was cultivated at 30°C for 2 d on a potato-dextrose-yeast-agar medium (200 g potato extract, 20 g dextrose, 5 g yeast extract and 20 g agar in 1L water, pH-5.5) and was stored at 5°C.

Liquid culture was performed at 30°C for 6 d in a 250 ml culture flask containing 60 ml medium (200g potato extract, 20 g dextrose, 5 g yeast extract in 1 L water, pH-5.5) with shaking (120 strokes/min) and with or without addition of minerals at different concentra-

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tions.

The minerals (Na_2SO_4 , CaCl_2 , MgCl_2 , NaCl and KCl) were used at the concentrations of 0.025, 0.05, 0.1, 0.2 and 0.4 (% wt/vol).

2.2 Biomass and Lipid Content Determination

Fungal mycelia harvested by filtration were washed three times with 50 ml distilled water, and dried at 30°C in a vacuum oven for 5 h. The dried mycelia were weighed (moisture content of biomass being determined). Then the cell mass was subjected to solvent extraction in a Soxhlet apparatus with chloroform : methanol (1:1, vol/vol). After extraction the solvent was removed under a stream of nitrogen, the extract was dried under vacuum and then weighed to determine the lipid content (16).

2.3 Fatty Acid Analysis

The fatty acids in the lipids were methylated (18) and the composition was then determined by gas-liquid chromatography (GLC) of their methyl esters. Data are averages of three determinations.

The isolated fungal lipid was mainly a mixture of phospholipids and triglycerides, which was confirmed on a TLC plate (Silica gel-G; solvent system, hexane:diethyl ether(7:3, vol/vol) using phosphate staining. The phospholipids and triglycerides were separated on a preparative TLC plate (20 cm \times 20 cm) with the above solvent system, and the two fractions were extracted with diethyl ether.

3 Results and Discussion

3.1 Effect of Mineral Addition on Biomass Yield

Table 2 Effect of Mineral Addition to Potato-Dextrose-Yeast Medium on Biomass of *R. nigricans*.

Mineral Conc. (%)	Biomass (g/L)				
	Na_2SO_4	CaCl_2	MgCl_2	NaCl	KCl
0.025	12.81 \pm 0.74	12.21 \pm 0.34	19.60 \pm 0.26	12.80 \pm 0.54	14.39 \pm 0.34
0.05	12.97 \pm 0.45	17.53 \pm 0.25	13.70 \pm 0.34	28.60 \pm 0.09	13.97 \pm 0.32
0.10	17.48 \pm 0.67	18.97 \pm 0.55	18.51 \pm 0.37	25.40 \pm 0.27	14.54 \pm 0.11
0.20	26.23 \pm 0.62	17.31 \pm 0.38	22.10 \pm 0.69	18.30 \pm 0.24	23.18 \pm 0.24
0.40	25.19 \pm 0.26	14.54 \pm 0.24	20.10 \pm 0.38	19.80 \pm 0.41	28.97 \pm 0.22

Values are mean \pm s.d., n = 3

Table 1 Biomass, Lipid Content, GLA in Lipid and GLA Yield of *Rhizopus nigricans* Grown in Potato-Dextrose-Yeast Medium.

Biomass (g/L)	Lipid content (%w/w)	GLA in Lipid (%w/w)	GLA yield (mg/L)
13.3	25.7	15.5	529.8

Table 1 shows the biomass (13.3 g/L-culture), lipid content (25.7%, w/w), GLA extracted in lipid (15.5%, w/w) and GLA yield (0.53 g/L-culture), when the fungus was cultivated in the medium without any minerals. Minerals (Na_2SO_4 , CaCl_2 , MgCl_2 , NaCl and KCl) at concentrations of 0.025, 0.05, 0.10, 0.20 and 0.40% were examined as additive sources for GLA production. It may be noted from **Table 2**, that Na_2SO_4 , CaCl_2 , MgCl_2 , NaCl and KCl individually at 0.20, 0.10, 0.20, 0.05 and 0.40% concentrations respectively caused maximum cell growth when added to potato-dextrose-yeast medium. A maximum cell mass (28.97 g/L-culture medium) was obtained by adding 0.4% KCl .

3.2 Effect of Mineral Addition on Lipid Content

The effect of mineral addition on lipid composition was investigated. A maximum lipid content in the fungus, when the minerals were considered individually, was obtained at 0.20% Na_2SO_4 , 0.025% CaCl_2 , 0.025% MgCl_2 , 0.05% NaCl and 0.40% KCl when added to the basal medium. Upon addition of 0.20% Na_2SO_4 to the basal potato-dextrose-yeast medium, the lipid content produced by *Rhizopus nigricans* increased to 27.30% (**Table 3**). This was not significantly higher than that attained by cultivation in the basal medium (25.70%, **Table 1**).

Table 3 Effect of Mineral Addition to Potato-Dextrose-Yeast Medium on Lipid Content in Dried Mycelia of *R. nigricans*.

Mineral Conc. (%)	Lipid content in biomass (wt%, dry basis)				
	Na ₂ SO ₄	CaCl ₂	MgCl ₂	NaCl	KCl
0.025	14.20 ± 0.34	18.10 ± 0.31	26.70 ± 0.27	15.30 ± 0.34	18.70 ± 0.34
0.05	19.91 ± 0.21	14.40 ± 0.05	21.50 ± 0.14	25.90 ± 0.37	21.90 ± 0.11
0.10	14.60 ± 0.36	14.50 ± 0.15	19.40 ± 0.21	22.40 ± 0.17	16.90 ± 0.23
0.20	27.30 ± 0.14	12.90 ± 0.21	20.20 ± 0.19	18.30 ± 0.21	19.70 ± 0.11
0.40	20.30 ± 0.52	14.70 ± 0.09	17.40 ± 0.32	15.90 ± 0.12	26.50 ± 0.31

Values are mean ± s.d., n = 3

3.3 Effect of Mineral Addition on GLA Yield

Minerals affected significantly GLA synthesis by *R. nigricans*. The GLA content in lipid was highest at 0.20% Na₂SO₄, 0.025% CaCl₂, 0.025% MgCl₂, 0.05% NaCl and 0.40% KCl. The GLA production of the fungus for the individual minerals was the best (among the several concentrations of the minerals) at the mineral concentrations of 0.20% Na₂SO₄, 0.025% CaCl₂, 0.025% MgCl₂, 0.05% NaCl and 0.40% KCl. When the strain was cultivated in potato-dextrose-yeast medium containing 0.4% KCl, the GLA content in the oil was 32.8% (**Table 4**) and GLA production reached 2.52 g/L-culture medium (**Table 5**). Berkley (19) studied a medium, in which low Mg was desirable for arachidonic acid (AA) production; the effect of addition of phosphorous on AA production has been studied by some groups (20-22). K. Higashiyana *et al.* (17) reported the effect of addition of minerals, such as Na, K, Ca and Mg on arachidonic acid (AA) production by *Mortierella alpina* IS-4. He observed that 1.5% soy flour medium with the addition of 0.3% KH₂PO₄, 0.1% Na₂SO₄,

0.05% CaCl₂·6H₂O and 0.05% MgCl₂·6H₂O enhanced the AA yield 1.7-fold over that without mineral addition. Hansson *et al.* (23) added Na, Ca, Mg and P to medium for GLA production.

Thus, comparing the results obtained by the addition of minerals at their various concentrations it was observed that 0.40% KCl as an additive to the potato-dextrose-yeast medium was most effective for enhancing GLA yield. The degree of GLA production was almost 1.5 fold in comparison to the basal medium alone and these enhancement can be considered to be highly significant.

Individually some electrolytes are very effective in augmenting biomass, lipid content and GLA yield. But when the electrolytes were mixed together at the optimum concentration levels (0.20% Na₂SO₄, 0.025% CaCl₂, 0.025% MgCl₂, 0.40% NaCl and 0.40% KCl) the effects appeared to be quite adverse in terms of GLA yield particularly and also with respect to biomass and lipid content (**Table 6**). The mineral mixture of 0.40% KCl that caused highest cell growth, GLA concentration and GLA yield and 0.20% Na₂SO₄, that

Table 4 Effect of Mineral Addition to Potato-Dextrose-Yeast Medium on GLA Content in TAG Produced by *R. nigricans*.

Mineral Conc. (%)	GLA in lipid (wt%)				
	Na ₂ SO ₄	CaCl ₂	MgCl ₂	NaCl	KCl
0.025	23.00 ± 0.21	28.30 ± 0.81	27.20 ± 0.34	24.50 ± 0.64	28.80 ± 0.56
0.05	26.10 ± 0.73	27.70 ± 0.06	26.10 ± 0.89	27.20 ± 0.87	30.80 ± 0.31
0.10	25.50 ± 0.90	22.00 ± 0.67	25.50 ± 0.45	23.90 ± 0.56	27.20 ± 0.56
0.20	27.60 ± 0.11	24.90 ± 0.61	26.80 ± 0.19	27.20 ± 0.34	28.90 ± 0.31
0.40	25.20 ± 0.23	23.00 ± 0.33	25.20 ± 0.42	23.30 ± 0.45	32.80 ± 0.32

Values are mean ± s.d., n = 3

Table 5 Effect of Mineral Addition to Potato-Dextrose-Yeast Medium on GLA Yield of *R. nigricans*.

Mineral Conc. (%)	GLA yield (mg/L)				
	Na ₂ SO ₄	CaCl ₂	MgCl ₂	NaCl	KCl
0.025	418.40 ± 0.24	624.90 ± 0.01	1423.40 ± 0.07	479.80 ± 0.74	774.90 ± 0.55
0.05	673.60 ± 0.29	699.20 ± 0.75	768.80 ± 0.05	2014.80 ± 0.27	942.30 ± 0.23
0.10	650.80 ± 0.66	613.40 ± 0.45	915.21 ± 0.11	1359.80 ± 0.97	666.50 ± 0.52
0.20	1976.40 ± 0.10	556.00 ± 0.11	1196.40 ± 0.10	910.90 ± 0.41	1319.71 ± 0.21
0.40	1288.60 ± 0.02	491.60 ± 0.09	881.30 ± 0.12	2117.60 ± 0.52	2518.10 ± 0.67

Values are mean ± s.d., n = 3

Table 6 Effect of Mineral Addition in Potato-Dextrose-Yeast Medium on Biomass, Lipid Content, GLA Content and GLA Yield of *R. nigricans*.

	Potato-dextrose-yeast medium with 0.20% Na ₂ SO ₄ , 0.05% CaCl ₂ , 0.025% MgCl ₂ , 0.40% NaCl and 0.40% KCl	Potato-dextrose-yeast medium with 0.40% KCl and 0.20% Na ₂ SO ₄
Biomass (g/L)	17.70 ± 0.45	21.20 ± 0.67
Lipid content (% w/w)	13.00 ± 0.19	14.80 ± 0.19
GLA content (% w/w)	10.80 ± 0.52	12.80 ± 0.52
GLA yield (mg/L)	248.50 ± 0.09	401.60 ± 0.89

Values are mean ± s.d., n = 3

Table 7 Effect of Mineral Addition on the Composition of *R. nigricans*.

Potato-dextrose-yeast medium		Potato-dextrose-yeast medium+ (Na ₂ SO ₄ , CaCl ₂ , MgCl ₂ , NaCl and KCl added one at a time at all concs)		Potato-dextrose-yeast medium+ (0.20% Na ₂ SO ₄ , 0.05% CaCl ₂ , 0.025% MgCl ₂ , 0.40% NaCl and 0.40% KCl)		Potato-dextrose-yeast medium+ (0.40% KCl and 0.20% Na ₂ SO ₄)	
neutral lipid	polar lipid	neutral lipid	polar lipid	neutral lipid	polar lipid	neutral lipid	polar lipid
75 ± 0.49	25 ± 0.47	76 ± 0.82	24 ± 0.99	76 ± 0.84	24 ± 0.89	75 ± 0.88	25 ± 0.76

Values are mean ± s.d., n = 3

caused maximum lipid concentration also lowered the GLA productivity of *R. nigricans* (Table 6) as compared to the results obtained for basal medium alone. The possible explanation may be that an excess electrolyte concentration, when added in the form of mineral mixture inhibited the microbial growth and consequent lipid production and GLA yield.

3.4 Effect of Mineral Addition on Lipid Composition

Under conditions of basal medium without mineral addition, about 75% of the extracted mycelial oil was

composed of neutral lipids and the rest were polar lipids. Upon addition of the minerals to the basal medium, both as a mixture and individually, the lipid composition was the same (Table 7). The major components of the neutral and polar lipids were triacylglycerols and phospholipids.

4 Conclusion

Lipid with considerably high GLA yield is possible with the fungus *Rhizopus nigricans* SSSD-8 when grown in a Potato-dextrose-yeast culture medium con-

taining 0.40% KCl. This increased production of GLA will be highly beneficial for the dietary, pharmaceutical and medicinal fields.

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