Chapter 5

BIOREACTOR STUDIES ON THE OLEAGINOUS FUNGUS MORTIERELLA RAMMANNIANA FOR THE PRODUCTION OF GAMMA LINOLENIC ACID
Introduction

The oleaginous fungus, *Mortierella rammanniana* is an interesting source for GLA biosynthesis (Certik et al., 1997; Dyal et al., 2005; Kendrick and Ratledge, 1992; Ratledge, 2002; Ratledge and Wynn, 2002; Preez, 1993; Yokochi and Suzuki, 1989). Due to its unique fatty acid composition *M. rammanniana* can accumulate relatively high amount of lipid with 5-37% GLA in the total fatty acids produced (Amano et al., 1992). Yokochi and Suzuki (1989) cultivated nine species of genus *Mortierella* batch wise in a 30L fermenter with glucose (100-200 gL⁻¹). Four strains attained a GLA productivity exceeding 2gL⁻¹. Maximum lipid productivity (13.9 gL⁻¹d⁻¹) was obtained in the fermenter with a double stage repeated batch culture. The lipid productivity by six species of Mucorales has been studied by Suzuki et al., (1981). The lipid content of *Mortierella isabellina* (IFO No. 7884) was 86.1% of the dry fungus cells cultivated at 20°C. Hansson et al., (1989) studied the production of GLA in *Mucor rouxii* (CBS 416.77). In fed batch culture, the production of GLA was 99 mgL⁻¹h⁻¹ whereas in continuous culture it was 37 mgL⁻¹h⁻¹.

The production of GLA by *Mucor rouxii* (CBS 416.77) was studied on low budget nitrogen and carbon sources. Difco yeast extract gave the high productivity (14 mgL⁻¹h⁻¹) of GLA (Lindberg and Hansson, 1999). The yield of GLA increased in order of yeast extract < rape meal < cocos expeller that ranged from 0.15 to 0.33 gL⁻¹. Starch and starch hydrolysate resulted in better productivity of GLA (4.7 and 4.9 mgL⁻¹h⁻¹) as compared to glucose (3.4 mgL⁻¹h⁻¹).

So far, a limited number of studies on the production of GLA in bioreactors have been reported. In the present study, we have analyzed the profiles of GLA-producing

H S Gour University, Sagar and Indian Institute Of Integrative Medicine, Jammu.2008.
Mortierella rammanniana. In order to enhance the GLA production, the optimization studies have been carried out in shake flask and in bioreactor.
Materials and Methods

Microorganism and cultural conditions:

*M. rammanniana* used in this study, was obtained from the culture repository of Indian Institute of Integrative Medicine (formerly known as Regional Research Laboratory), Jammu, India. Seven different media were tested for GLA production efficiency, out of which only two media (medium 1 and medium 2) turned out to be productive. The organism in the present study was grown in presterilized medium 1 containing (g l⁻¹): glucose 100, peptone 10, yeast extract 1 and distilled water 1. The shake flask experiments (220 rpm/ 28±1°C) were carried out in 500 ml Erlenmeyer flasks containing 100 ml of medium. Large scale cultivations of fungus was performed in a presterilized medium 2 containing (g l⁻¹): glucose 100, NH₄SO₄ 0.97, KH₂PO₄ 9.0, MgSO₄.7H₂O 1.0, NaCl 0.3, malt extract 0.6, yeast extract 0.6, peptone 0.3, distilled water 1 and minerals 3 ml: Mineral solution (mg/ml): FeSO₄.7H₂O 10, CaCl₂.2H₂O 1.2, CuSO₄ 5 H₂O 1.0, ZnSO₄.7 H₂O 1.0, MnCl₂.7H₂O 1.0) in 13 L bioreactor (Biostat E B.Braun, Germany). The fermentation was done (1.25 vvm/0.2 kg/cm² 28±1°C/ 500 rpm) using 7L media. In all the experiments the initial spore count in the broth was 1×10⁴ spores/ml. The spores obtained from this experimental organism were harvested using tween 80 (0.015 v/v aq. solution) to dislodge from the mycelia mass. The suspension was suctioned through filter paper (Whatman no.1) using a Millipore assembly (Millipore corp. Bedford, Massachusetts, USA) and later all the filtrates were pooled. After appropriate dilution, the spores were counted using a haemocytometer (Counting chamber, Nuebour, Germany). The samples were drawn 2 h after inoculation (0 h) and every 24 h thereafter, until either 144 h (shake flask experiments)/120 h (bioreactor

---

H S Gour University, Sagar and Indian Institute Of Integrative Medicine, Jammu.2008.
experiments). The residual sugar, lipid, biomass and the GLA production were
determined for all the samples withdrawn. The effect of pH (2.0 to 9.0) was studied,
which was adjusted with 1M HCL or NaOH using pH meter (Genei, India).

**Lipid and fatty acid analysis:**

The mycelium was harvested by filtration, through Whatman filter paper No.1,
and washed with distilled water. The biomass thus obtained was disrupted and
homogenized in pestle and mortar using acid washed sand (1:2) followed by acid
hydrolysis with 50 mL (0.25 mol l⁻¹ HCl) by keeping in boiling water bath. The lipid was
extracted from the homogenized fungal biomass with chloroform: methanol (2:1) for 3 h
(Folch et al., 1957). Anhydrous sodium sulphate is added to the extracted lipids to
remove residual moisture. The solvent was removed by evaporating on rotavapour at low
temperature and the total lipid thus obtained was weighed in triplicates. The fatty acid
profile of the mycelium was determined by saponification followed by methylation for
conversion of fatty acids to corresponding methyl esters.

**Methyl ester preparation:**

Fatty acids are normally found in the triacylglycerol forms. Fatty acids can be
separated from triacylglycerides by fatty acid methyl ester (FAME) preparation, as it is
necessary for GC-MS analysis. The extracted lipid (0.5 g) was used for preparation of
FAME. Refluxing was done with 25 ml potassium hydroxide for approx. 2 h. After
cooling 75 ml of chilled double distilled water and 30 ml diethyl ether (DEE) was added.
Washing of FAME was done for two to three times and later the upper layer of DEE was
discarded. The pH of the lower layer was set to 2.5 – 3.0. The hexane (20 ml) was added
to this solution and upper layer (hexane) was pooled for further use. This step was
repeated for three times. Anhydrous sodium sulphate was added to the hexane to remove excess moisture. After filtration with Whatman No.1 filter paper, the hexane was evaporated on rotary evaporator until a drop or two are left. To this, 10 ml of methanol and 0.2 ml of conc. H₂SO₄ was added and refluxed for 4 h. After reflux, evaporation was done to reduce the volume to the half of initial. To this, 20 – 30 ml chilled distilled water was added. The extraction was done with 20 ml hexane and thus the upper layer of hexane was pooled. The sodium carbonate (5%) was mixed with 50 ml of hexane layer and later the layers were allowed to separate, using separating funnel. Lower layer of sodium carbonate was discarded and the 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 rotary-evaporator and the methyl esters were analyzed by GC-MS. The fatty acid methyl esters (FAME) were stored at 4 °C prior to gas chromatographic analysis (Christopherson and Glass, 1969). TLC, followed by gas chromatograph fitted with a FID detector, first analyzed them. All comparative TLC analysis was carried on Merck 0.25mm silica gel plates developed in solvents hexane/ethyl acetate (9:1). The GLA methyl ester was detected with 1% cerric ammonium sulphate reagent after gentle heating, which appeared as colored spots. (Yokochi et al., 1990). Fatty acids were analyzed as methyl esters on Gas chromatograph (NUKON 5765) using Agilent (India) fused capillary column DB-23 (30M×0.25mmID×0.25μmT) containing 70% cyanopropyl (equi) polysil phenylene. The operating conditions were: column temperature 150 °C, injection temperature 230 °C, and detector temperature 250 °C. The column temperature was programmed (5°C /min⁻¹ ) till the final temperature (230 °C). Nitrogen was used as a carrier at a flow rate of 1 ml
min⁻¹. The injection volume was 1 μl. The product was identified on DB-225 column (Agilent) with similar conditions as described above using coupled gas chromatography and mass spectroscopy. The individual fatty acids were identified by comparing retention times and mass of GLA methyl ester with authentic fatty acid standards obtained from Sigma.
Results and Discussion

Shake flask experiments:

Shake flask experiments were conducted to evaluate the optimal conditions for growth and its capacity to produce polyunsaturated fatty acids, in particular GLA. In shake flasks, the growth period of the culture could be extended to 6 d. The fungal biomass was calculated (g l⁻¹) and was found to be 0.38/24 h, and 29.65/144 h with 8.717 g l⁻¹ of lipid. The GLA content (%) in total lipid was 5.8/24 h, and 9.1/144 h. The high GLA content was found to be 9.1%, which corresponds to 791 mg l⁻¹. The spectroscopic data of pure fungal GLA was obtained by repeated column chromatography of the fatty acid methyl ester of fungal lipid. All the experiments were done in triplicates (Table 21).

Growth and GLA production in bioreactors:

Different batches of bioreactors (5 to 7 L capacity) were run for the optimization of fermentation conditions to obtain maximum production of biomass, lipid and GLA. The shake flask medium was not suitable for growth at the bioreactor level. Therefore, Medium 2 was selected for bioreactors studies. The optimal conditions found for the growth of *M. ramanniana* and the production of GLA are described in table 21. The biomass was analyzed for residual sugar, lipid and GLA at an interval of 24 h. The biomass production was high at 96 h in the bioreactor, after which a gradual fall in the biomass production was observed, which might be owing to partial lyses of cells after attaining stationary phase. An optimum temperature (28±1°C) for growth of mycelium and the GLA production was noticed. The optimal pH ranged between 4.0-4.4. The pH of the medium in the bioreactor was controlled online at pH 4.0 (Figure 13 a, b, c, d).
At the start of the stationary phase (72h), 28 g l⁻¹ dry biomass was obtained. The residual sugar (0.23%) was observed at the time of the maximum production of GLA (Figure 13d). There was a steady increase in biomass from 48 h in the bioreactor; however, the maximum GLA productivity was recorded at 120 h of fermentation, which indicates that lipid accumulation could start after 72 h when sugar percentage starts declining. The maximum lipid (9.6g l⁻¹ /120 h) was observed in the present investigation. In the shake flasks, the maximum GLA was recorded at 144 h of fermentation. The production of GLA by the test culture started declining after 144 h and there was almost a stationary phase of the GLA production after 96h of fermentation in shake flask. The high content of GLA in the bioreactor culture was 900-960 mg l⁻¹.
**Table 21:** Optimized cultivation parameters for *M. ramanniana*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Shake Flask</th>
<th>Bioreactor (13L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td>Spores (10^4 spores/ml)</td>
<td>Spores (10^4 spores/ml)</td>
</tr>
<tr>
<td>Medium</td>
<td>Medium 1</td>
<td>Medium 2</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Cultivation time</td>
<td>144 h</td>
<td>120 h</td>
</tr>
<tr>
<td>Working Volume</td>
<td>100ml</td>
<td>5-7 L</td>
</tr>
<tr>
<td>Temperature</td>
<td>28±1°C</td>
<td>28 ±1°C</td>
</tr>
<tr>
<td>Vessel Pressure</td>
<td>-</td>
<td>2lbs</td>
</tr>
<tr>
<td>Aeration rate</td>
<td>-</td>
<td>1.25 vvm</td>
</tr>
<tr>
<td>Antifoam</td>
<td>-</td>
<td>Silicon oil</td>
</tr>
<tr>
<td>Agitation(р/min)</td>
<td>200-220</td>
<td>500-510</td>
</tr>
</tbody>
</table>
Figure 13: Effect of incubation period on the production of GLA by *M. rammanniana* in the bioreactor.

H S Gour University, Sagar and Indian Institute Of Integrative Medicine, Jammu.2008.
Figure 14: Effect of incubation period on lipid production by *M. rammanniana* in the bioreactor.
Figure 15: Effect of incubation period on dry biomass produced by *M. rammanniana* in the bioreactor.
**Figure 16:** Effect of incubation period on residual sugar produced by *M. rammanniana* in the bioreactor.
Figure 17: Effect of incubation time on biomass, lipid and GLA production by *M. rammanniana*
In the present study, the GLA production in shake flasks and bioreactors after 120 h of incubation was found to be 480 and 960 mg/l respectively. The test organism proved to be of intermediate capacity at industrial scale when compared with the previous work (Hooch and Suzuki (1989), who reported the production (2 g/l) of GLA using M. ramanniana var. angulispora (IFO 8187), while Mucor rouxii (CBS 416.77) produced 99 mg/l of GLA (Hansson et al., 1989). It can be concluded that by optimization of media, pH, aeration and agitation in bioreactors, it is possible to increase the yield of GLA. It is generally possible to achieve an adequate aeration and mixing in bioreactor culture and the higher GLA production could be the result of an appropriate combination of all the above given factors. The study indicates that the oleaginous fungus, M. ramanniana may be a potential organism for further development and optimization of a fermentation process as an alternative source for the production of GLA.