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BIOREACTOR STUDIES ON THE OLEAGINOUS FUNGUS *MORTIERELLA RAMANNIANA* FOR THE PRODUCTION OF PROSTAGLANDIN PRECURSOR GAMMA LINOLENIC ACID.

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Running Head: Gamma linolenic acid from fungi

ABSTRACT

The valuable polyunsaturated gamma linolenic acid (fatty acid), having applications in foods and pharmaceuticals, can be produced at a high overall volumetric rate by cultivation of *M.ramanniana*. GLA obtained was identified by gas liquid chromatography and spectroscopic studies. No GLA was detected at zero time cultures broth. The maximum yield (791 and 960 mg/L) of GLA was recorded during 144 and 120 h in shake flask and 13 L bioreactor, respectively.

KEYWORDS: polyunsaturated, gamma linolenic, *M.ramanniana*, bioreactor.

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INTRODUCTION

Linoleic (18:2), Arachidonic (20:4) and Gamma linolenic acid (18:3) belonging to ω-6 family of fatty acids has attracted interest of researchers in recent years. These long chain polyunsaturated fatty acids (PUFAs) have increased advantages significantly due to their recognition for human health as they have been reported to play an important role in prevention or treatment of a variety of diseases, such as arteriosclerosis [1,2] thrombosis [3], arthritis [4] and several types of cancer [5,6]. Gamma linolenic acid (GLA) is also known as a precursor of number of biologically active compounds,like prostaglandins thrombozanes and leukotrienes [7]. Production of GLA from fungi is regarded as an alternative way [8] from plant seeds (*Oenothera spp*. or *Borago officinalis*). *Mortierella ramanniana* (oleaginous fungi) is an interesting source of GLA production and for research on GLA biosynthesis [9-16]. Due to its unique fatty acid composition *Mortierella ramanniana* can accumulate a relatively high amount of lipid with 5-37% GLA in the total fatty acids produced [17]. As not much work has been reported on the production of GLA at higher level, hence this paper deals with the optimization of fermentation parameters for the production of GLA in the shake flasks and in 13 L bioreactor..

MATERIALS AND METHODS

Microorganism

M. ramanniana obtained from the culture repository of Regional Research Laboratory, Jammu, India, was maintained (28±1°C) on potato dextrose agar slants. Spores from this experimental organism were harvested after 7 d of incubation, when profuse sporulation used to occur. The spores were harvested using Tween 80 (0.01% v/v aqueous solution) to dislodge from the mycelial 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, Nuebaur, Germany)

Scanning electron microscopy

Recovered spores were fixed and processed using the modified method of Millionig [18]. The spores were recovered on a clean cover slip and fixed with 2.5% (v/v) glutaraldehyde in 0.1-mol/L-phosphate buffer (pH = 7.2) for 2 h. The material was again fixed with 1% (v/v) osmium tetra oxide in the same buffer for 3 h, dehydrated in a graded ascending acetone gradient (10%-100%) and dried using carbon dioxide. The samples were then mounted on stubs, coated with carbon in a JEOL-JEE 4X vacuum evaporator, and then again coated with gold in a polar on sputter coater. Finally, the samples were observed in a JEOL –100 CXII electron microscope with ASID operating at 40 Kv. The mycelia and spores were observed under light and phase contrast microscope (Olympus Research Microscope VANOX, USA).

Growth of organism

The organism in the present study was grown in medium 1 containing (g/L: glucose 100, peptone 10 and yeast extract 1.Shake flask experiments were carried (28±1°C /220 rpm/min) out in 500 ml Erlenmeyer flasks containing 100 ml of medium. Large scale cultivations of fungus was performed in medium 2 containing (g/L): glucose 100, NH_4SO 0.97, KH_2PO_4 9.0,$MgSO_4.7H_20$ 1.0,NaCl 0.3, Malt extract 0.6, yeast extract 0.6, peptone 0.3, minerals 3 ml. (Mineral solution (mg/ml) $FeSO_4.7H_2O$ 10,$CaCL_2.2H_2O$ 1.2,$CuSO_4$ 5 H_2O 1.0,$ZnSO_4.7$ H_2O 1.0, $MnCL_3.7$ H_2O 1.0), in 13 L bioreactor (B.Braun Biostat Germany).The working volume was kept at 7 L with an aeration rate of 1.25 vvm, a pressure of 0.2 kg/cm, a temp of 28±1°C and an agitation rate of 500 rpm/min. In all the experiments the initial spore count ($1*10^4$spores/ml) was maintained in the broth, and the samples were drawn 2 h after inoculation (0 hr) and in every 24 h thereafter till 144 h (shake flask) and 120 h (bioreactor). The residual sugar, pH, dissolved

oxygen and GLA production was determined at each point.

Lipid analysis

The mycelium was harvested by filtration using Whatman No.1 filter paper and washed with distilled water. The biomass thus obtained was disrupted and homogenized in a pestle and mortar using acid washed sand (1:2) and then acid hydrolyzed for 45 min with 50 ml of 0.25 mol/L HCl. Lipid was extracted from the fungal biomass after treatment with chloroform: methanol (2:1) for 3 h [19]. Anhydrous sodium sulphate is added to the extracted lipids in order to remove any residual moisture. The solvent was removed by evaporating on rotavapour and the total lipid was estimated. All values were the means of triplicate determination.

Fatty acid determination

The fatty acid profile of mycelium was determined by saponification followed by methylation for conversion of fatty acids to corresponding methyl esters. Fatty acid methyl esters. (FAMES) were prepared [20] and analyzed by TLC followed by gas chromatography fitted with a flame ionization detector.

Thin Layer Chromatography

All comparative TLC analysis was carried out on Merck 0.25mm silica gel plates developed in solvents (hexane/ethyl acetate, 9:1). GLA methyl ester was detected with 1% cerric ammonium sulphate reagent after gentle heating, giving GLA as colored spots [21].

Gas Liquid Chromatography

Fatty acids were analysed 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. Column temperature was programmed to rise 5°C/min and by maintaining the final temperature as 230°C. Nitrogen was used as carrier at a flow rate of 1 ml/min Individual fatty acids were identified by comparing with the retention times of authentic fatty acid standards obtained from Sigma Co. USA.

Gas Liquid Chromatography –Mass Spectroscopy

The product was identified on DB-225 column (Agilent) with similar conditions as described above using coupled gas chromatography and mass spectroscopy. Individual fatty acids were identified by comparing retention times and mass of GLA methyl ester with authentic fatty acid standards obtained from Sigma Co. USA.

RESULTS AND DISCUSSION

Organism characteristics

The fungus with septate mycelium having numerous chlamydospores (10-12μ in diam) could grow on many common media in 6-7 d. Sporangiophores (3.7-4μ in diam), were sympodially branched, bearing spherical sporangia (14-16μ in diam) , columella globose, (8-11μ in diam), spores globose to angular, 2.9μ in diam (Fig 1 A, 2B).

Shake flask experiments for the production of GLA.

Shake flask experiments with the fungal culture were conducted to evaluate the optimal conditions for growth and its capacity to produce polyunsaturated fatty acids. Data presented are the mean of triplicate determinations(Fig 2). The inoculation conditions and the fungal culture in 500 ml Erlenmeyer flasks are summarized in table 1. In shake flasks, the growth period of the culture extended to 6 days. The fungal biomass of 0.38 g/Lwas measured at 24 h, which reached to 29.65 g/L on day 6 with 8.717g/L of lipid. The GLA in total lipid was 5.8% at initial level, which reaches 9.1% at 6 days. The highest GLA content was found to be 9.1% corresponding to 791mg/L. The spectroscopic data of pure fungal GLA was obtained by repeated column chromatography of the fatty acid methyl ester of fungal lipid.

Growth and GLA production in bioreactors

Different batches of bioreactors with a working volume of 5 to 7 L capacity were run for the optimization of fermentation conditions for the maximum production of biomass, lipid and GLA. The conditions found to be optimal for the growth of *M.ramanniana* and production of GLA are summarized in table 1. Figure (3a,b, c, d) shows that biomass production was maximum at 96 hrs in the bioreactor, after which a gradual fall in biomass production was observed, (fig 3c) which might be owing to partial lysis of cells after attaining stationary phase. A temperature of 28±1°C was found to be optimal for the growth of mycelium and GLA production. The effect of pH was studied and the optimal pH was found to be in 4.0-4.4. The pH of the medium in the bioreactor was controlled online at pH 4.0. At the start of the stationary phase (72hrs), 28 g/L biomass (dry wt) was obtained. The residual sugar concentration of 0.23%(fig 4d) was observed at the time of maximum production of GLA. There was a steady increase in biomass from days 2 in the bioreactor, but maximum GLA productivity was recorded at 120 h of fermentation, which indicates that Lipid accumulation started after 72 h when sugar percentage started declining. Maximum lipid of 9.6g/L was recorded at 120 hrs. In the shake flasks the maximum GLA was recorded at 144 h of fermentation. The production of GLA by the culture started declining after day 6 and there was almost a stationary phase of GLA production after 96 h of fermentation in shake flask. The highest content of GLA in the bioreactor culture was 900-960 mg/L

Table 1
Cultivation parameters for M.ramanniana.

Parameter	Shake Flask (500 mL))	Bioreactor (13L)
Inoculum	Spores (10^4 spores/ml)	Spores (10^4 spores/ml)
Medium	Medium 1	Medium 2
pH	6.5	4.4
Cultivation time	144 hrs	120 hrs
Working Volume	100ml	5-7 L
Temperature	28±1°C	28±1°C
Vessel Pressure	-	2lbs
Aeration rate	-	1.25 vvm
Antifoam	Silicon SE 2	Silicon SE 2
Agitation(r/min)	200-220	500-510

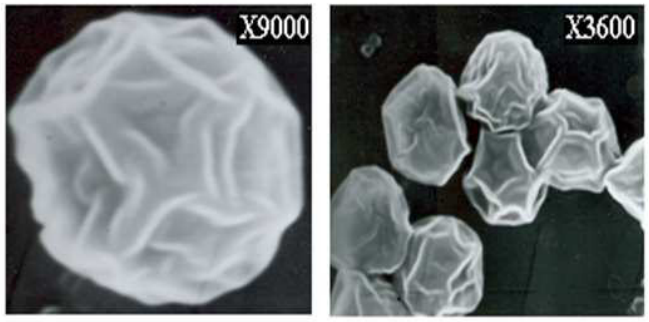

Figure 1
Scanning electron micrographs depicting detailed surface ultrastructural characteristics of spores of M.ramanniana

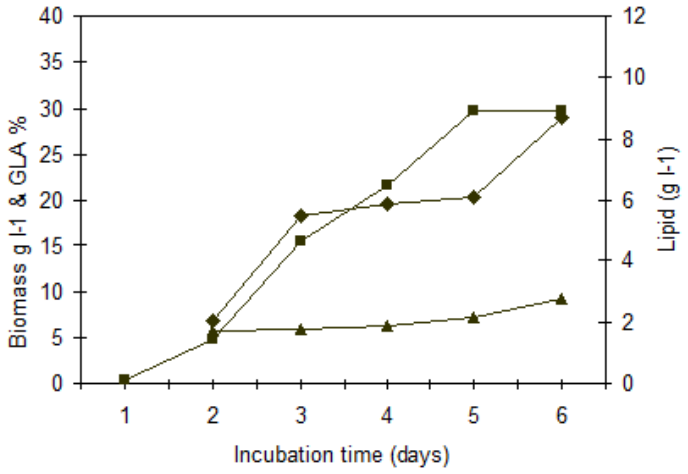

Figure 2.Growth and production profile of *M.ramanniana.*
Biomass (■); Lipid (◆);GLA (▲)

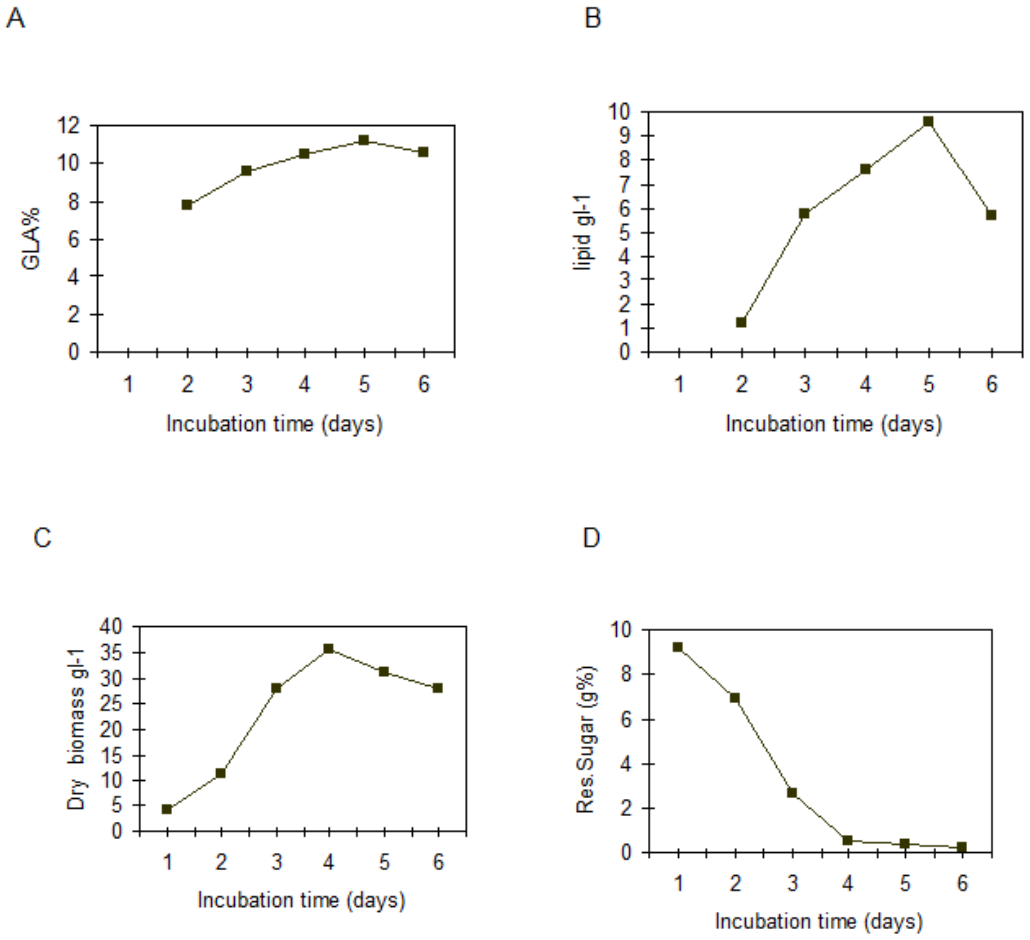

Figure 3
Effect of incubation period on (A) GLA (B) Lipid (C) Dry Biomass and (D) Residual sugar by M. ramanniana in the 13 L bioreactor.

CONCLUSION

The GLA production in shake flasks and in bioreactors after 120 h of incubation is 480 and 960 mg/L respectively. It can be concluded that by optimization of media, pH, aeration and agitation in bioreactors it is possible to increase the yield of GLA.As it is generally possible to achieve an adequate aeration and mixing in bioreactor culture, the higher GLA production yields 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, which can further be used for medicinal development.[22,23.]

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