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Effect of Extraction Methods on Lipid and Fatty Acid Composition by Mortierella Ramanniana

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Abstract- The efficacy of three extraction methods for determining the lipid and fatty acid composition of M.ramanniana was studied. The extraction methods were chloroform:Methanol (2:1),Hexane:Isopropanol (3:2) and ethyl acetate. The total fatty acid composition varied in fungal cultures depending on the extraction conditions of the three methods chloroform:methanol (2:1) was found to be the best for extraction of lipid and fatty acids from M.ramanniana.

Index Terms- Lipid; Gamma; Linolenic acid; Mortierella, Fatty acid; Culture

I. INTRODUCTION

Oils containing dietetically important polyunsaturated fatty acids (PUFAs) such as gamma linolenic acid [18:3 (ω 3)] are considered to be high value [1] A current commercial source of oils containing 18:3 ω 6 PUFAs is plant like evening primrose or borage, but an alternative source which has been extensively studied in fungi belonging to the genus *Mortierella* [2].GLA is important as a precursor of dihomo-γ-linolenic and arachidonic acids which are essential for normal bodily physiological processes, since they are required for cell structures and are also the precursors of the series of 1 and 2 prostaglandins.[3] GLA is also used in the treatment of various illnesses such as rheumatoid arthritis[4], multiple sclerosis[5],schizophrenia [6],atopic ezema[7] and premenstrual syndrome [8].Zygomycetes fungi are known to accumulate GLA in the mycelium[9]. Attempts have been made to improve 18:3 ω 6 production in *Mortierella* sp. by changing the culture conditions [10].Lipid extraction from biomass is an important step in the quantification of lipid from microorganisms.[11]

Rapid and reliable methods of extraction and purification of PUFAs from microbial biomass are required or further development in the area of microbial technology. At the same time, satisfactory treatment must be used to minimize autoxidative degradation and the presence of artifacts. On the other hand, if PUFA are to be used in pharmacological, medical and food applications, solvents should be selected that are acceptable in terms of toxicity, handling, safety and cost [12]. With this as background and the current interest in the production of polyunsaturated fatty acids (PUFAs) by fungi, we report chloroform: methanol to be the most suitable solvent system for extraction purposes.

II. MICROORGANISM AND CULTIVATION

M.ramanniana used in this study, was obtained from culture repository of Regional Research Laboratory, India, and was maintained on potato dextrose agar slants. The culture was grown on a medium comprising of (g l^{-1}) glucose 100, peptone 10, yeast extract 1, pH 6.5 (Control Medium). Fermentation was done in 500 ml Erlenmeyer flasks containing 100 ml medium at 28 ±2^{o}C on a rotary shaker at 220 rpm for 144 h. The mycelium after fermentation was harvested by filtration, washed with distilled water and gently dried at 50^{o}C for 15 h or till the weight is nearly constant. Triplicate standards of each flask were prepared to appraise average.

Lipid extraction

2 Grams of dry biomass was disrupted and homogenized in a pestle and mortar 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^{-1} HCl. Lipid was extracted from the fungal biomass with chloroform: methanol (2:1) Hexane: isopropanol (3:2) and Ethyl acetate for 3 h. 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 estimated. All values were 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. FAMES were prepared according to the methods of Christopherson and Glass [13] and analyzed first by TLC followed by gas chromatography fitted with a FID detector.

Thin Layer Chromatography

All comparative TLC analysis were 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. It appeared as spots.

Gass Liquid chromatography

GC was performed on Agilent 6890 Series Gas Chromatograph equipped with a FID and the capillary column DB-23 (30 m X 0.25 mm i.d. X 0.5 μm film thickness; J & W Scientific, USA). The injector and detector temperatures were

maintained at 230 and 250°C respectively. The oven was programmed for 2 min at 160°C 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.

GC-MS was 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 a HP-5ms capillary column (30 m X 0.25 mm i.d. X 0.25 µM film thickness; J & W Scientific, USA). The carrier gas, Helium was used at a flow rate of 1.0 mL/min. The inlet temp was maintained at 300°C and the oven was programmed for 2 min at 150°C 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.

III. Results and Discussion

In the oleaginous microorganisms, lipids are present in the cell membranes and also in the cytosol. The accumulation of lipid in oleaginous organisms in known to occur when there is depletion of growth nutrient, other than carbon, preventing cell proliferation and allowing accumulation of lipid in the cell [14].
Lipids play an important role in the cells, where they occur in the membranes and cytosol. They are heterogenous compounds with different structures and properties. Due to these attributes, it is difficult to obtain their complete isolation. Pure single lipid classes are soluble in a wide variety of organic solvents are not suitable for lipid extraction from tissues or cells. The used solvent or the solvent system for extracting lipid from cells could be sufficiently polar to remove all lipids from their association with cell membranes or with lipoproteins, but could not react chemically with these lipids. At the same time the solvent should not be so polar that nonpolar lipids do not dissolve. Generally, nonpolar solvents are usually needed for lipid extraction, where most lipids are adequately dissolved. Nevertheless, their combinations with polar solvents are advantageous, mainly because of dehydration, protein denaturation, and degradation of hydrogen bonds between the lipid complex and proteins. The extractants may also have function in preventing enzymatic hydrolysis. Increasingly, attention is being given to the potential toxicity of solvents. Finally, the extractability of cells or tissues is variable and depends both on the nature of the cells and of the lipids.

The data on the amount of lipid content extracted using various solvents are given in the table (1)

As can be seen from this table,chloroform:methanol(2:1) gives the maximum extraction of lipid from the mycelium.The combition of polar and non polar solvents is thus advantages for the extraction of lipids from microorganisms. By the Cholorform:methanol method 37.715% of total lipid was recovered.This solvent system has been employed to extract nonpolar compounds and phospholipids from microorganisms[12]

Hexane:Isopropanol is preffered for extraction of lipids because of there lower toxicity.When compared with chloroform:methanol extraction the yield are lower with hexane:isopropanol,may be due to reduced extraction of less polar lipids.The total lipid yield in this solvent is 20%.The other method chosen for lipid isolation was using ethyl acetate because it is being used as a food grade solvent / because of lower toxicity.But only 15% of the oil was extracted.The extracted oil can be used for various industrial applications as the solvent is considered to be safe.There is no significant effect of various extraction solvents on the fatty acid composition of the total lipid isolated from *M.ramanniana*. The major fatty acids extracted by three methods were palmitic, stearic and oleic acid. Fatty acids profile of *M.ramanniana* with different solvent systems was almost same as shown in (table 2)

The biomass, oil content and fatty acid content of oil are the important parameters for the development of any technology. Ideally all these parameters should be very high, to achieve the desired lipid composition. But practically it is not possible to have all the three parameters high at the same time. Bajpai et al have reported that the DHA content of the mycelial lipid was higher when the biomass contained a low lipid content [10].Ratledge also reported that *M.circinelloides* produces oil containing high GLA, when the lipid content is low [1].

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LIPID COMPOSITION OF *M.ramanniana*.(Table 1)

Solvent System	Lipid content G/l	w/w
1.Chloroform: methanol (2:1)	10	35.725
2.Hexane:Isopropanol (3:2)	5.72	20.445
3.Ethyl acetate	4.242	15.15

Fatty acid profile of *M ramanniana* with different solvent systems.(Table 2)

Solvent System	Palmitic Acid	Palmitoleic Acid	Stearic Acid	Oleic Acid	Linolenic Acid	GLA
C:M	36.36			39.79	5.89	10.68
I:H	36.8		3.9	2.5	41.5	7.0
EA	36.8	5.4	2.5	41.5	5.0	9.0