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OLEAGINOUS MICROORGANISMS AS SOURCE OF GAMMA LINOLENIC ACID

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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. Bacterial and fungal cultures were screened for production of GLA.

KEYWORDS: ***M.ramanniana*, linoleic acid,oleaginous microorganisms,bacteria,fungi.**

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INTRODUCTION

The concept of single cell oil produced by oleaginous microorganisms as the supplementary sources of conventional oils and fats is not new. It has been attempted since 1980s from bacteria, fungi and yeast and was also reported to be the convenient substitutes for conventional oils because of their ability to accumulate more than 60% lipids resembling the plant and animal oils. Although it was always evident that microbial oils could never compete commercially with the major commodity plant oils, there were commercial opportunities for the production of the higher valued oils. If microorganisms could be isolated that could produce some of the highest valued oils, then commercial development of them would still be a reality. Another major consideration in evaluating the potential of a microorganism for the oil production is the amount of oil it can produce. Those organisms that could accumulate lipid to more than about 20% of their biomass were termed as oleaginous species (Thorpe and Ratledge, 1972; Moon *et al*., 1978; Kyle, 1997). In the present study, we are interested in GLA; a precursor to other EFAs as well as to eicosanoids, a biologically important class of molecules (Erasmus, 1993). The fungal genus *Mortierella* has two subgenera: *Mortierella* and *Micromucor* which are both possible alternate sources of GLA (Dyal & Narine, 2005)**.** The *Micromucor* subgenus species can produce fatty acids containing a maximum of 18 carbons (C18) and the species in the *Mortierella* subgenus can produce fatty acids up to twenty carbons C20 (Amano *et al*., 1992). The deficiency in C20 fatty acid production of *Micromucor* is presumably a result of a mutation or other type of selection that inactivated or deleted the action of elongases; the elongation enzymes responsible for the production of longer chain fatty acids (Weete, 1980)**.** The difference in the biochemistry suggests that species found in the subgenus *Micromucor* would be more viable sources of GLA since their fatty acid pathways do not allow for further elongation than C18.. The lipid accumulation and yields however, could be significantly affected by growth media variables as seen in other species. Several studies on *Mortierella* and other fungi have shown that media variables affect the growth and lipid accumulation that occur within various species (Aki *et al*., 2001, Bajpai *et al*., 1991a, Funtikova and Mysyakina, 1997, Hansson and Dostalek 1988,Kavadia *et al*., 2001, Leman, 1997, Leman and Brakonieecka-Sikorska, 1996, Nakahara *et al*., 1992, Sajbidor *et al*., 1988, Weinstein *et al*., 2000,Xian *et al*., 2001 and Yokochi *et al*., 1992) To date, there have been studies that have looked at the fatty acid profiles of some *M.rammanniana var.rammanniana* isolates (Amano *et al*., 1992). The growth manipulation studies involving *M. rammanniana* focused on either unspecified varieties or on the *var. angulispora* (Xian *et al*., 2001 and Yokochi *et al*., 1992) but there are no specific studies on *M.rammanniana* var.*ramannian*The occurrence of gamma linolenic acid, in fungi was first reported by Bernhard *et al*. (1957) and is characteristic of the class phycomycetes.Shaw (1965)**,** studied the fatty acid composition of 31 species of fungi artificially cultured on fat free media and analyzed through gas liquid chromatography. All nine species of phycomycetes studied, including six species of the order Mucorales, contained gamma linolenic acid but no alpha linolenic acid. No phycomycete was found to contain alpha linolenic acid. Gamma linolenic acid has not been found in ascomycetes or basidiomycetes. This is probably the most fundamental biochemical difference between the phycomycetes and the other fungi yet observed. This difference is so marked that, bearing in mind published data on the fatty acid composition of other primitive organisms as summarized by Erwin and Bloch it would seem that the evolutionary pathway leading to the phycomycetes was quite different from that the higher fungi. It is widely assumed that fungi were collected from algae like forms. The composition of the fatty acids of the ascomycetes, basidiomycetes and a few algae studied so far, does not contradict this theory. However, the common occurrence of gamma

linolenic acid in phycomycetes, as in protozoa, means that there may be some phylogenetic relation. Evidence is available, which suggests specific functions for alpha linolenic acid in algae and gamma linolenic acid in protozoa (Erwin and Bloch 1964). But no role for either isomer in the fungi has been demonstrated. It has been suggested that synthesis of gamma linolenic acid was utilized as a means of adaptively decreasing the saturation of Phycomycetes fat (Shaw, 1965). Adaptive desaturation of mycelia fat with decreasing culture temperature has been demonstrated in some fungi. Bernhard *et al* (1957, 1958) suggested that gamma linolenic acid might have a more specific role as a growth-promoting factor. It is suggested that gamma linolenic acid may be vestigial in phycomycetes relating these fungi phylogenetically to certain fungi (Shaw 1965).A number of microbes from different sources have been isolated and studied for the production of SCO and PUFA (Bowles *et al* 1999; Papanikolaou *et al* 2002; Zhu *et al* 2002.) Various fungal strains have been screened in order to find the best producer. Filamentous fungi belonging to the genus *Mortierella* have been identified as a promising producer of GLA (Kendrick and Rat ledge 1992; Botha *et al* 1999) some screening methods were described. Eroshin *et al* (1996) utilized aspirin to distinguish PUFA producing *Mortierella* strains from other members of this genus that do not produce PUFAS**.** Botha *et al* (1999) developed an isolation procedure at low culture temperature. Zhu *et al* (2002) developed a fast and effective method to isolate strains of high AA yield according to the finding that TTC staining degree of mycelia of *M.alpina* was positively correlated with fatty acid content in mycelia lipid. Ahmed *et al* (2006) carried out studies on the fermentative production of gamma linolenic acid using seven strains belonging to Mucorales. An oleaginous fungal strains isolated from the western ghats of kerela produced 8% gamma linolenic acid (by mass), when grown in a complex medium containing glucose as the sole carbon source. Effect of different culture conditions was investigated in shake flasks.The first report of bacterial screening associates of marine sponges for SCO production was done by Patnayak and Shree (2005). They studied the effect of C: N ratio on the two isolates; *B.subtilis* and *Pseudomonas s*p. Both the isolates produced gamma linolenic acid 4.5 and 1.12 %respectively, where as *B.subtilis* showed 3.8% of EPA. Advantage with bacterial oils only PUFA is produced, rather than the complex mixture yielded from fish or algal sources (Nicholos *et al*., 1995,1997).

MATERIALS AND METHODS

Chemicals

The media ingredients were obtained from Hi media (India). All chemicals obtained from Ranbaxy (India) and Qualigens (India) were of analytical grade. Standards of Docosahexaenoic acid and its methyl ester were obtained from Sigma–Aldrich Co. (India).

Sample collection and isolation

Samples were collected, from the virgin areas of Northwestern Himalayas including leh and ladakh between July and August 2012.The mean annual temperature is approximately 10°C. About 4 to 6 (deep soil was collected and used for studies. The soil was properly cleaned, dried and was passed through 1mm sieve. At the time of sampling the soil temperature was 5 (C. The moisture content was 28.79 (0.18% w/w, determined by drying the soil in an electric oven at 105 (C for 12 h (Eicher, 1970). The pH of the soil was 4.96 determined according to the method of Spots and Cervantes (1986). The organic matter content of the soil was determined by ignition (Eicker, 1970). It was made sure that no fungicidal or manorial treatment has been applied to it previously. Grasses and other weeds were carefully removed from this spot and surface was cleared. Samples were air-dried and 1 g of soil was added to 10 ml of sterile distilled water and then serially diluted. Isolation of bacteria 0.1 ml of the sample from 10^{-2} and 10^{-3} dilutions was pour plated on nutrient agar and incubated for 24-48 h at 30°C. Single colonies were picked and sub cultured at least three times on plates for purity and then maintained on nutrient agar slants. Standard biochemical methods and

Bergeys manual of determinative bacteriology (Holt *et al.,*2004) were used for identification of bacteria.

Methodology adapted for performing biochemical tests

All the biochemical tests for identifying isolated bacteria and fungi were performed according to the standard protocols available (Cappiccino and Sherman, 1996).

Nitrate reduction test

Using sterile technique, inoculated each experimental organism into its appropriately labeled tube containing nitrate broth medium, by means of a loop inoculation. The last tube will be served as control. Incubate cultures for 24 to 48 h at 37°C. Observations: On addition of reagents solution A and solution B a cherry red coloration indicates: Nitrate not reduced by microorganisms.Presence of potent nitrate reductase, which converts nitrates beyond nitrites to ammonia or even molecular nitrogen. Zinc powder on addition to colorless cultures results in development of red color. Confirming nitrates in the medium were reduced beyond nitrites to ammonia or nitrogen gas.

Lipase production

In the experimental procedure Tributyrin agar is used as a substrate. Following Inoculation and incubation of the agar plate cultures, organisms excreting lipase will show a zone of lipolytic enzymes the medium retains its opacity. This is a negative reaction.

Carbohydrate fermentation

Most microorganisms obtain their energy through a series of orderly and integrated enzymatic reactions. Organisms use carbohydrates differently depending on their enzyme complement. Fermentative degradation under anaerobic conditions is carried out in fermentation broth containing Durham tube. A typical carbohydrate fermentation medium contains. Nutrient broth, a specific carbohydrate that serves as the substrate. The pH indicator is phenol red. Inoculate experimental organism. Incubate for 24 to 48 h at 37 (C. Examine carbohydrate broth cultures for color and the presence or absence of a gas bubble.

Catalase test

During aerobic respiration microorganisms produce hydrogen peroxide. Accumulation of these substances will result in the death of the organism unless they can be enzymatically degraded. Streak culture on fresh tube. Incubate all cultures for 24 to 48 h at 37 (C.

Methyl Red test

Inoculate each experimental organism. Incubate for 24 to 48 h at 37 (C Into the culture tube add five drops of methyl red indicator.

Voges Proskauer test

To the culture add 10 drops of Barritts reagent A and shake. Immediately add 10 drops of Barritts reagent B and shake. Examine and record the color of color of cultures 15 min after the addition of barritts reagent.

Fungal isolation

The soil plate technique of warcup (1950) utilizing MEA was then used to isolate the fungi from free sample. Soil samples were prepared by transferring 0.005 g of soil from the samples to each of five sterile petri dishes. The cultures were incubated at 5 (C in the dark and observed for growth. After 8 days of incubation, growth from each of the developing colonies were transferred to fresh PDA and further purified. Isolates were identified according to the keys and descriptions given by Siepmann (1969) Gams (1976, 1977), Schipper (1978), Domsch *et al*. (1980) and Sultan (1980).

Fungal cultures obtained

The cultures of *Mucor Circinelloides* MC12, *Mucor Javanicus* NRRL 1406, *Mucor javanicus* 1423, *Cunninghamella* NRRL 3655,*Mortierella rammanniana* 5843 and *Mortierella rammanniana* 5844 were obtained from National Center for Agricultural Utilization Research University Street, Peoria.

Identification

Microscopic slides were prepared, stained using lacto phenol cotton blue (Vainio *et al*

1998) and were examined under light microscope (Olympus, USA). Photographs were taken by using digital camera (Camedia camera, C-2100 ultra zoom, Tokyo, Japan). The total genomic fungal DNA was extracted by CTAB method (Cappuccino and Sherman, 1996) for DNA extraction, the fungus was grown in 100ml Potato Dextrose Agar at 28°C with constant shaking for 3 days. One hundred milligram mycelium's biomass was taken following washing with sterile distilled water; 6ml of CTAB extraction buffer and 60µl of β mercaptoethanol were added. After mixing the mixture was incubated at 65°C for 45 min, and the contents cooled to room temperature. This was followed by extraction with equal volume of chloroform and centrifugation at 10,000-x g for 10 min. Equal volume of isopropanol was added to the supernatant and mixed gently. The DNA pellet was vacuum dried and dissolved in 100 µl of TE (pH 8.0). The fungus was identified by analysis of the large and small subunit of ribosomal genes. The large subunit of ribosomal gene was amplified and sequenced using MICROSEQ D2, Large subunit (LSU~300 bp) fungal rDNA sequencing kit (Applied Biosystems, USA). The small subunit ribosomal gene was amplified with primers 5'TCCGTAGGTGAACCTGCGG3'and 5'TCCTCCGCTTATTGATATGC3'. The amplified products were purified utilizing Microcon columns (Millipore, USA), and sequenced using AB1 prism 310 genetic analyzer (AB1, USA) as per the manufacturers instructions. The DNA sequences~300 and 500 bases, thus obtained were submitted to gene bank for homology studies by BLASTIN program (Altschul *et al*., 1997). The ribosomal gene database (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov) was accessed and sequence alignment was used as an underlying basis to identify the fungus.

Harvesting of spores

Spores from this experimental organism were harvested using Tween 80 (0.01% v/v aq. Solution) to dislouge 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).

Scanning electron microscopy

Recovered spores were fixed and processed using the modified method of Milliong (1961). Spores were recovered on a clean cover slip and fixed with 2.5% (v/v) glutaraldehyde in 0.1 $molL^{-1}$-phosphate buffer (pH-7.2) for 2h. The material was again fixed with 1 %(v/v) of 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 states, 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.).

Biomass production for fatty acid screening

To screen isolates for growth and the fatty acid production, liquid medium was prepared using nutrient broth for bacteria and control media for fungi. A 50 ml volume culture was inoculated by loop from an agar slant and grown for 5 days to 30°C on a shaker at 200 rpm bacterial cells were harvested by centrifugation and fungal cells by filtration. Cultures were dried lipid extracted and then converted to fatty acid methyl esters for GC analysis.

Cytotoxic activity

Fungi was grown on growth medium i.e. control media and incubated at 30°C for 10 days. After that cultures was homogenized and kept for drying. Dried sample was tested for the anticancer activity. Cell line (100 µl) maintained in RPMI 1640-growth medium were seeded in 96 wells tissue culture plates. The test materials (100 µl) in each well were added after 24 h to the wells containing cell suspension and blank wells. The cells were allowed to grow in presence of test material by further incubating the plates for 48 h. At the end of incubation period the cell growth was

stopped by gently layering trichloroacetic acid (50% TCA, 50µl/well) on top of the medium in all the wells. The plates were incubated at 4°C for 1 h. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc. and air-dried.The cell growth was, measured by staining with Sulphorhodamine B[6]. Sulphorhodamine B (SRB, 0.4% in 1% acetic acid, 100 µl/well) was added to each well and plates were allowed to stand at room temperature for 30 mins. The plates were washed with 1% acetic acid four times and then dried. Tris-HCl buffer (0.01 M, pH 10.5 100 µl/well) was added to each well to solublise the dye. The plates were shaken gently for 10 mins on a shaker and the optical density was recorded on ELISA reader at 540 nm. The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated. All the experiments were carried out in quadruplicate.

RESULTS AND DISCUSSION

A collection and screening process was developed whereby oleaginous microorganisms producing polyunsaturated fatty acid were isolated using standerdised protocols for fungi like warcup 1950 method with MEA and bacteria nutrient agar media. This study covering 10 unique collection sites dispersed throughout northwestern Himalayas as mentioned (Table 1),and produced pure strains identified microscopically (Table 2 and Table 3). The isolated microorganisms were deposited in the culture repository of Lovely Professional University, Jalandhar. All the pure bacteria were given the accession numbers from LPU 451 to LPU 526 and the fungi were given the numbers from LPUF1 to LPUF30. The selection of the oleaginous strains with more than 20% of their cell dry weight being fatty acids was based on the results of GC PUFA profiling, biomass productivity, maximal total fatty acid and GLA. Values of biomass ranged from 2.14 to 26.75 gL^{-1} and values for total fatty acid and subsequent productivities of GLA ranged from 0.206 to 6.539 gL^{-1} to 2 to 250 mgL^{-1} respectively.All isolates that grew in liquid medium produced major amount of unsaturated PUFA, particularly GLA that comprised between 0.7 to 12.9% of total fatty acids.The isolate LPU F30 identified as *Mortierella rammanniana* and showing the high GLA production was selected for further studies. Growth profile of *M. rammanniana* is illustrated. Microsopic photographs of *Mortierella rammanniana* are provided. Gel Electrophoresis of DNA of *Mortierella rammanniana* is provided (Figure 1,2,3).

Table 1
Collection sites for bacterial and fungal isolates

S. No.	Locations
1.	Takesy monument soil.
2.	Indus river soil surface
3.	Indus river soil basin
4.	Zanskar soil
5.	Chumathang hot water spring
6.	Pugga hot water spring
7.	Shashu nala kistwar
8.	Paddar hot water spring
9.	Chenab basin soil
10.	Tatapani kalakot.

Table 2
Screening of bacterial isolates for PUFA production

Isolate no. LPU	Microscopic observation	Mannitol	Glucose	Lactose	Catalase	Nitrate	MRVP	Growth 30°C	PUFA Production
476	*B.cereus*	-	-	-	-	-	-	+	-
456	rods	+	-	-	+	-	+	+	-
497		-	-	-	-	-	-	+	-
451	Cocci	-	-	-	-	-	-	+	-
462	*B.cereus*	-	-	-	-	-	-	+	-
485	Cocci	-	-	-	-	-	-	+	-
477	*B.cereus*	-	-	-	-	-	-	+	-
495	Cocci	-	-	-	-	+	-	+	-
453	*B.cereus*	-	-	-	-	+	-	+	-
480	*B.cereus*	-	-	-	+	-	-	+	-
455	*B.cereus*	-	-	-	+	-	-	+	-
473	*B.cereus*	-	-	-	-	+	-	+	-
458	*B.cereus*	-	-	-	+	-	-	+	-
490	*B.cereus*	-	-	-	-	+	-	+	-
483	Cocci	-	-	-	-	-	-	+	-
503	Cocci	-	-	-	-	-	-	+	-
463	*B.cereus*	-	-	-	+	-	-	+	-
471	*B.cereus*	-	-	-	-	-	-	+	-
530	*B.cereus*	-	-	-	+	-	-	+	-
531	*B.cereus*	-	-	-	-	+	-	+	-
526	*B.cereus*	-	-	-	+	+	-	+	-
493	*B.cereus*	-	-	-	-	+	-	+	-
452	*B.cereus*	-	-	-	+	+	-	+	-
461	Cocci	-	+	-	+	-	-	+	-

Table 3
Screening of fungal isolates for PUFA production.

S.No	Isolate No.	Biomass	Lipid	PA	SA	OA	LA	GLA
		(gl^{-1})				(%)		
1.	LPU F1	16.2	2.275	13.0	11.0	11.5	4.4	7.7
2.	LPU F2	20.4	1.788	17.1	9.9	9.9	14.3	8.6
3.	LPU F3	13.9	0.222	0.9	46.6	46.6	6.1	5.7
4.	LPU F4	9.6	0.206	5.8	27.9	27.9	10.8	5.3
5.	LPU F5	14.4	1.452	1.4	26.9	26.9	3.3	1.8
6.	LPU F6	24.75	1.023	1.1	15.1	15.1	1.8	6.0
7.	LPU F7	26.75	6.539	10.2	9.1	9.1	3.2	2.1
8.	LPU F8	10.65	0.688	0.1	59.1	59.1	3.8	4.3
9.	LPU F9	18.9	0.566	1.0	30.0	30.0	2.6	2.8
10.	LPU F10	5.12	0.346	2.4	14.4	14.4	2.0	1.3
11.	LPU F11	7.90	1.683	0.7	27.8	27.8	0.9	4.9
12.	LPU F12	12.246	1.999	1.4	13.132	13.13	3.6	7.7
13.	LPU F13	11.23	0.048	0.8	1.8	1.8	4.0	8.0
14.	LPU F14	11.35	2.278	0.3	56.9	56.9	12.3	1.0
15.	LPU F15	12.65	1.4540	4.4	11.6	11.6	1.9	0.7
16.	LPU F16	5.51	0.7040	3.5	18.1	18.1	1.3	3.4
17.	LPU F17	10.29	0.240	4.1	44.90	44.90	5.42	8.86
18.	LPU F18	9.036	1.818	3.5	40.42	40.42	6.66	6.75
19.	LPU F19	11.708	1.126	34.66	46.73	46.73	5.82	8.25
20.	LPU F20	12.35	2.020	43.06	40.57	40.42	5.04	7.65
21.	LPU F21	14.294	1.786	34.68	41.64	41.64	9.33	9.34
22.	LPU F22	2.14	5.022	36.28	42.47	42.47	6.38	8.96
23.	LPU F23	8.09	0.68	51.12	34.12	34.12	4.19	5.01
24.	LPU F24	24.72	5.24	34.89	46.27	46.27	5.55	2.67
25.	LPU F25	20.21	4.55	37.94	38.72	38.72	8.13	6.03
26.	LPU F26	5.08	0.388	28.4	15.5	24.7	19.9	12.9
27.	LPU F27	12.51	2.29	23.3	3.1	28.4	22.9	2.2
28.	LPU F28	14.30	1.88	29.1	9.9	31.0	21.0	9.0
29.	LPU F29	5.37	1.32	17.8	2.6	37.8	28.7	13.1
30.	LPU F30	12.19	2.72	28.5	6.7	28.4	21.6	8.6

**** Biomass = Dry Biomass, PA = Palmitic acid, SA = Stearic acid, OA = Oleic acid, LA = Linoleic acid, GLA= Gamma Linolenic acid***

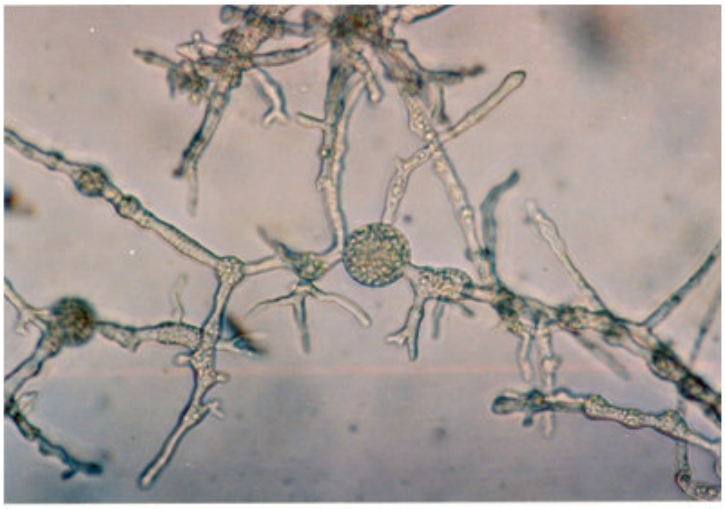


Figure 1(a)
Submerged mycelial cultures of M. rammaniana (6-d old) at (400x)

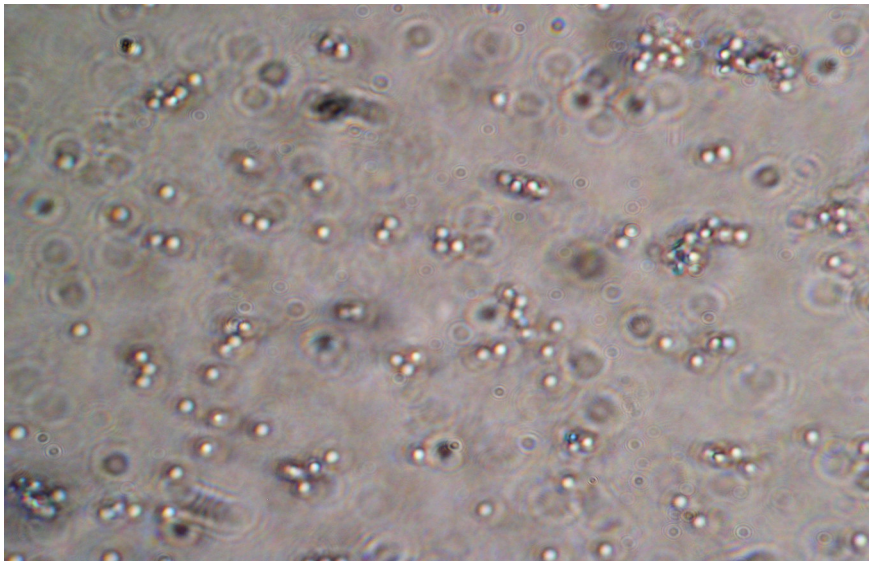
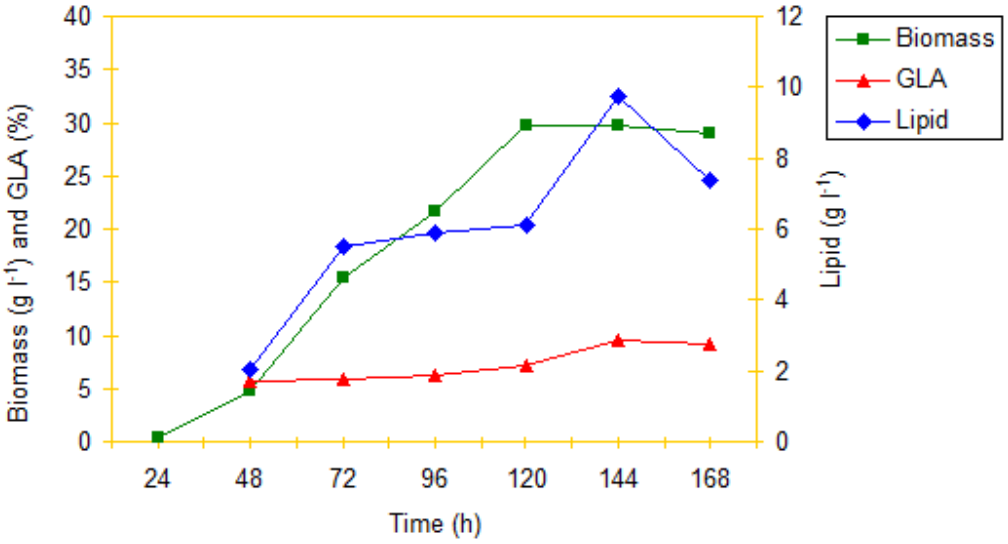


Figure 1(b)
Phase contrast microscopic view of spores of M.rammaniana

Figure 2
Time growth studies of the M. rammanniana grown on control medium.



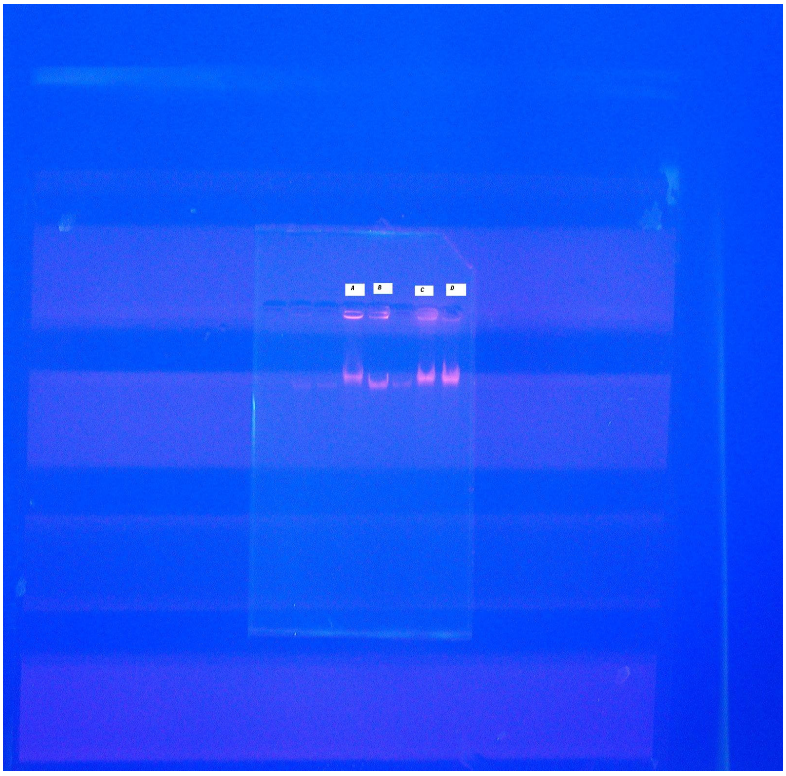


Figure 3
Photograph showing DNA bands of M.rammanniana

Biochemical testing of the culture was done. The culture produced lipase only on tributyrin agar. The zone diameter for lipase production was 10 mm. No lipase was produced on olive oil (Table 4). This indicates that the lipase of this organism is general and not specific so it cannot be commercially exploited. Cytotoxic activity of the culture was checked in vitro system against various human cancer cell lines. The cell lines used were liver, colon, prostate and breast. The culture was not having any cytotoxic activity against the cancer cell lines used (Table 5).

Table 4
Lipase production by M. rammaniana.

Culture		Zone (mm)	
		TBA	Oliveoil
Mortierella rammaniana	Pellet	10	-
	Sonicated	-	-
	Supernatant	-	-

Table 5
Anticancer activities against human cancer cell lines

Culture code	Cell lines				
	Hep-2 (Liver)	MCF-7 (Breast)	Colo-205 (Colon)	DU-145 (Prostate)	PC-3 (Prostate)
	% age growth inhibition				
Fungi-1	13	1	0	25	2
Mito-C	64	78	86	56	43
Adriamycin	51	74	62	56	21

CONCLUSION

Presently the GLA is commercially produced from the seeds of evening primrose (*Oenothera biennis*) and borage (*Borago officinilis*). However, the productivity of GLA from the seed oil is extremely low, since both a long period and a huge area for harvesting seeds are required. Therefore microorganisms have been investigated as an alternative GLA source to overcome these problems. Phycomycetes have been reported to produce GLA. Current interest in the nutritional role of PUFA has stimulated research into their production from a number of fungal sources.In the present investigation, Various bacteria and fungi were isolated and screened for the production of GLA using simple isolation technique. Molecular identification and morphological studies of the selected strain was done.The selected strain out of the isolated lot was identified as *Mortierella rammanniana*.

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