



Competence evaluation of mycodiesel production by oleaginous fungal strains: *Mucor circinelloides* and *Gliocladium roseum*

Sandip S. Magdum, Gauri P. Minde, Upendra S. Adhyapak, V. Kalyanraman

COE Biotechnology, R.D. Aga Research, Technology and Innovation Centre, Thermax Ltd., Pune 411019, India.

Abstract

Comparing with lesser algal growth rate for biofuel production along with many constraints, fungal route should be analyzed for its capability of biodiesel or mycodiesel production. The two fungal stains namely, *Mucor circinelloides* (MTCC1297) and *Gliocladium roseum* (MTCC6474) were analyzed for laboratory scale mycodiesel production. The *M. circinelloides* and *G. roseum* were able to produce biomass of 0.404 mg VSS/mg sucrose and 0.642 mg VSS/ mg sucrose with the mycodiesel content of 20.69% (w/w) and 11.37% (w/w) respectively. Furthermore, qualitative analysis of the oil contents by GC-MS were identified the presence of Tetradecanoic and Octadecanoic acids.

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Keywords: Biodiesel; Biomass; *Mucor circinelloides*; Mycodiesel; Sucrose.

1. Introduction

Food to fuel pathway always been criticized for sustainability, but the waste to fuel will have the potential for energy recycle and reuse. Sustainable second generation biofuel production with its required potential and economy needs the novel processes having higher fuel yielding reactions. As the petroleum prices are growing and will continue to grow in near future leading to the urgent need of alternate sustainable energy sources [1]. The bio-diesel offers many advantages over other petroleum derived fuel substitutes due to the fact that it is comparatively environmental friendly in addition it is an excellent fuel for existing diesel engines. Nowadays, biodiesel is the only direct substitute for diesel fuel in compression ignition engines and the interest in this biofuel has been growing in recent decades because it may effectively reduce the dependence on imported the fossil oil in the transport sector, in which the security of the energy supply problem is most acute [2]. In addition to oil-producing microalgae, many species of yeast and filamentous fungi have the ability to synthesize lipids intracellularly. The bio-oil produced from fungal cellular assimilation can be called mycodiesel and it contains the properties similar to biodiesel which can be used directly as fuel after its transesterification. The cost of biodiesel depends upon feedstock cost, which also contains edible or vegetable oil source such as palm, soya bean, sunflower, peanut, and olive oil [3]. The non-edible oil sources, such as *Jatropha*, *Karanja*, *Pongamia*, *Linseed*, *Mahua*, *Kusum* etc. were attractive, but not able to replace petroleum diesel due to natural growth limitations. The algal research also has shown the potential to produce biodiesel [4, 5]. But this option of algal biodiesel also has the production dependency on factors such as efficient photosynthesis, CO₂, nutrient and land availability which has addressed for successful commercialization. The waste to

energy pathways are promising nowadays, such as waste edible oil to biodiesel [6], waste biomass to alcohol [7], kitchen waste to biogas [8], wastewater to bio-oil [9] etc. The fungi have an advantage over algae that its growth does not required light, CO₂ and huge area. Various yeast strains also have the ability to degrade tough compounds in waste with high organic content [10] and oil assimilating properties [11-13]. Many fungal species are able to accumulate lipids, including *Aspergillus oryzae*, *Mortierella isabellina*, *Mortierella alliacea*, *Humicola lanuginosa*, *Trichoderma reesei*, *Mortierella vinacea* and *Mucor circinelloides* [9, 14-16]. Isolated strains of *Drechslera nobleae*, *Fusarium solani*, *Fusarium neocosmosporiellum* and *Aspergillus fumigates* also are reported as potential oil accumulators [16]. Some recent studies are searching ways to explore the fungal ability to produce mycodiesel by using waste organic substrates [9, 17]. The objective of the current study was to observe the oil accumulation capacity of *M. circinelloides* and *G. roseum* with oil extraction process optimization for mycodiesel production.

2. Materials and methods

Two strains namely *Mucor circinelloides* (MTCC1297) and *Gliocladium roseum* (MTCC6474) were procured from Institute of Microbial Technology, India in lyophilized forms and stored at - 80° C. The potato sucrose broth (PSB) was selected as inoculum preparation medium for *G. roseum*, which contains potatoes (scrubbed and diced) 200 g/l and sucrose 20 g/l. The potatoes were boiled in water for 1 hour. The pulp was squeezed through cheese cloth. Sucrose was added and boiled until it was dissolved and the pH was adjusted to 6. For *M. circinelloides*; malt extract broth (MEB) was used with malt extract concentration 20 g/l and the pH was adjusted to 6.5. The pH recorded by a Jenco 6230M pH meter (Jenco Instruments, San Diego, CA). Czapek Dox broth (CDB) was used for biomass enrichment having composition of sucrose 30 g/l, sodium nitrate 3 g/l, potassium chloride 0.5 g/l, magnesium sulfate heptahydrate 0.5 g/l, iron (II) sulfate heptahydrate 0.01 g/l, di-potassium hydrogen phosphate 1 g/l with final pH 7.3. Solubilization and sterilization was done by autoclaving the respective media at 121° C at 15 lbs for 20 minutes. *M. circinelloides* and *G. roseum* were inoculated in 250ml conical flask containing 100 ml of PSB and MEB respectively. These inoculated media were kept on a shaker at 200 rpm for 48 hrs at room temperature. The grown cultures were used as inoculum for further 250 ml of CDB for further biomass growth enrichment. The inoculum volume (5% v/v) was used and inoculated flasks were kept on a shaker at 200 rpm for 5 days incubation. The pH was adjusted to 6 and each day pH was observed for change and adjusted back to 6 using 1N HCL. After 5 days incubation in CDB, grown biomass from both the flasks was separated by filtration using Whatman filter papers (40, Ashless, 125 mm). The harvested fungal biomass was washed twice with distilled water and then dried at 90° C to constant weight. The wet weights and dry weights were recorded using weighing balance AB204 (Mettler Toledo Inc. US) and further the fungal biomass growth was quantified. The biomass (mg/l) was determined gravimetrically. The lipids were extracted from dried biomass using chloroform, methanol and water solution system [2:1:1 (v/v) chloroform: methanol: water]. Both dried samples of *M. circinelloides* and *G. roseum* were crushed with a mortar and pestle by simultaneously adding 2 ml of methanol, 2 ml of chloroform with 0.5 ml of water. This mixture was vortexed for 30 seconds to which 2 ml of chloroform and 2 ml of water was added to each tube following vortexing. Then sample tubes were centrifuged at 3000 rpm for 15 minutes and the upper layer of methanol and water was removed. The separated samples were passed through a layer of anhydrous sodium sulfate Whatman 40 filter paper. The samples were evaporated by vacuum evaporation to remove solvent at 45° C. Extracted oil was analyzed for its weight. The samples collected from two sets of flasks were centrifuged at 2000 rpm with Remi make centrifuge and supernatants were subjected to reducing sugar analysis to estimated sucrose utilization by biomass. Concentration of sucrose was analyzed after acid hydrolysis to form reducing sugars and then reducing sugars quantified by 3, 5-dinitrosalicylic acid reagent (DNS) assay [18]. Added a drop, or 20 µl, of concentrate HCL solution to 1 ml of the sample solution and kept it at 90° C for 5 min. Then solution was neutralized by adding KOH. The hydrolyzed sample was mixed with 2.5 ml of DNS reagent. The mixture was heated at 90° C for 5 min to develop red-brown color. After cooling to room temperature, the total volume was adjusted to 10 ml with distilled water. The absorbance with a spectrophotometer (WTW, 6600 UV-VIS) at 540 nm was measured. A standard curve was prepared using different known glucose concentrations. All absorbance were taken with the same square quartz UV-VIS cuvettes (path length, 1cm). Extracted oil samples were also analyzed on a gas chromatograph (GC) (CHEMITO, CERES 800 Plus, Thermo Scientific, MA, USA) fitted with a TG-WAXMS column (30m X 0.25mm X 0.25 µm) and a mass spectrometry (MS) (DSQ II). 1 µl of the final extract was

injected using pulsed splitless mode and GC operated at oven temperature program 40° C for 2 min increased to 250° C at 5° C/min with helium as a carrier gas (99.998 %) with 1 ml/min flow rate. MS data was collected in a scan mode (50–650 m/z) and evaluated using the Xcalibur MS library for confirmative identification of lipid molecules.

3. Results and discussions

This study revealed that sucrose consumption by *M. circinelloides* and *G. roseum* were 24.3 g/l and 15.3 g/l respectively (Table 1). The wet biomasses produced were 169.1g/l and 191.6 g/l (Figure 1), dry biomass were 9.83 g/l and 9.831 g/l with moisture content of 94.18 % and 94.87% by *M. circinelloides* and *G. roseum* respectively. The observed biomass yield was 0.404 mg VSS/mg sucrose for *M. circinelloides*, whereas it was 0.642 mg VSS/ mg sucrose for *G. roseum* after 5 days of incubation period, which was higher than reported biomass yield of *Mucor indicus* of 0.2 mg VSS/ mg substrate [19] and *R. oligosporus* of 0.43 mg /mg SCOD removed by using clarified thin stillage as substrate [20]. Another research reported that, the biomass yields were 0.21 mg VSS/mg COD for *M. circinelloides* reactor and 0.22 mg VSS/mg COD for *T. reesei* reactor [9]. After comparing these results it can be concluded that the sucrose can be used for higher fungal biomass yields.

Table 1. Experimental results of biomass and oil yield comparisons

Parameters	<i>M. circinelloides</i>	<i>G. roseum</i>
Wet Weight (g/l)	169.10	191.61
Dry Weight (g/l)	9.83	9.83
Extracted Oil Weight (g/l)	2.03	1.12
Oil Content (%)	20.69	11.37
Sucrose Consumption (%)	81.00	51.00
Biomass Yield (mg VSS/ mg Sucrose)	0.40	0.64
Oil Yield (mg oil/ mg Sucrose)	0.08	0.07

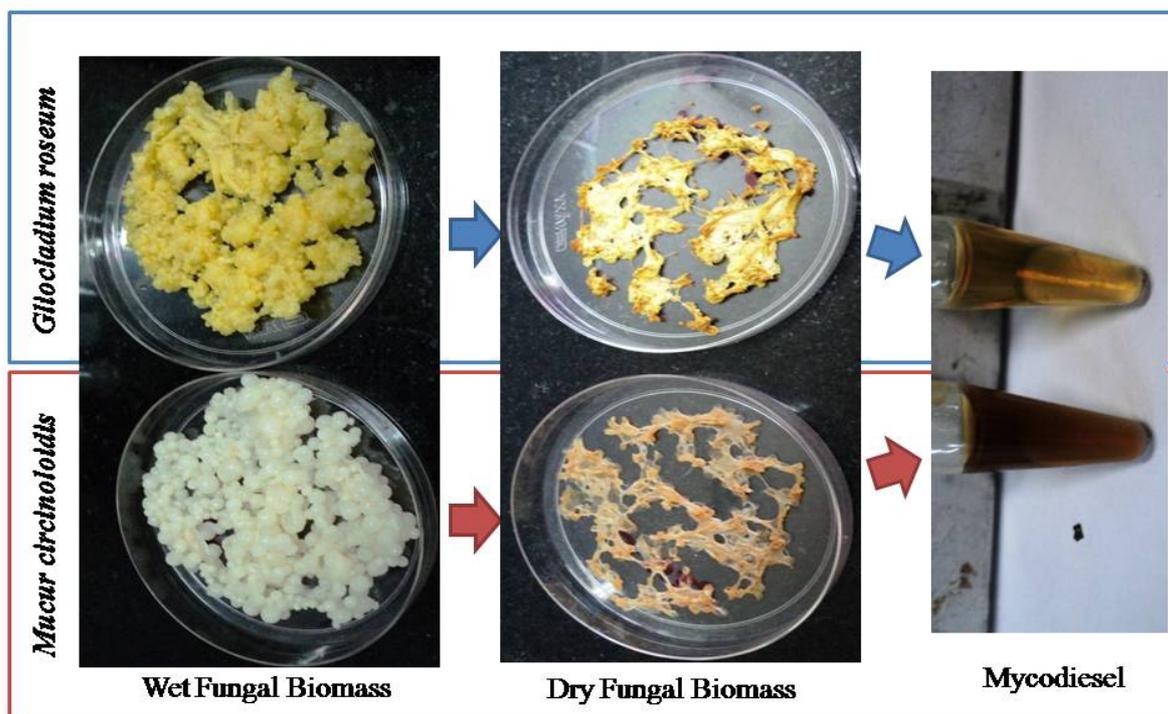


Figure 1. Wet and dry fungal biomasses of *M. circinelloides* and *G. roseum* were shown with the extracted mycodiesel from respective fungi

The total mycodiesel produced was 2.034 g/l and 1.118 g/l by *M. circinelloides* and *G. roseum* respectively. The *M. circinelloides* produced biomass with mycodiesel content of 20.69% (w/w) and *G.*

roseum produced biomass with mycodiesel content of 11.37% (w/w). The observed mycodiesel yield was 0.084 mg oil/ mg sucrose for *M. circinelloides* and it was 0.073 mg oil/ mg sucrose for *G. roseum*. *Mortierella isabellina* and *Aspergillus oryzae* were reported to produce 11% (w/w) and 18.15% (w/w) of oil on a semi-solid state fermentation of sweet sorghum and wheat straw respectively [21, 22]. A recent study showed that, the *M. circinelloides* and *T. reesei* biomasses containing bio-oil contents were 22.11 % and 9.82 % by using wastewater as substrate [9]. The grown biomasses of both *M. circinelloides* and *G. roseum* in liquid media are shown in Figure 1. The growth conditions can be diverted to nitrogen or pH limiting stress for further enhancement in mycodiesel production. The extracted oil samples processed to produce fatty acid methyl esters (FAME) by using transesterification process explained by Basumatary and Deka [23]. The FAME samples of mycodiesel were analyzed qualitatively with GC-MS for molecular identification. The *M. circinelloides* lipid sample shown the profile peaks dominated by Tetradecanoic acid hits and the *G. roseum* lipid sample profile peaks dominated by Octadecanoic acid hits shown in Figure 2.

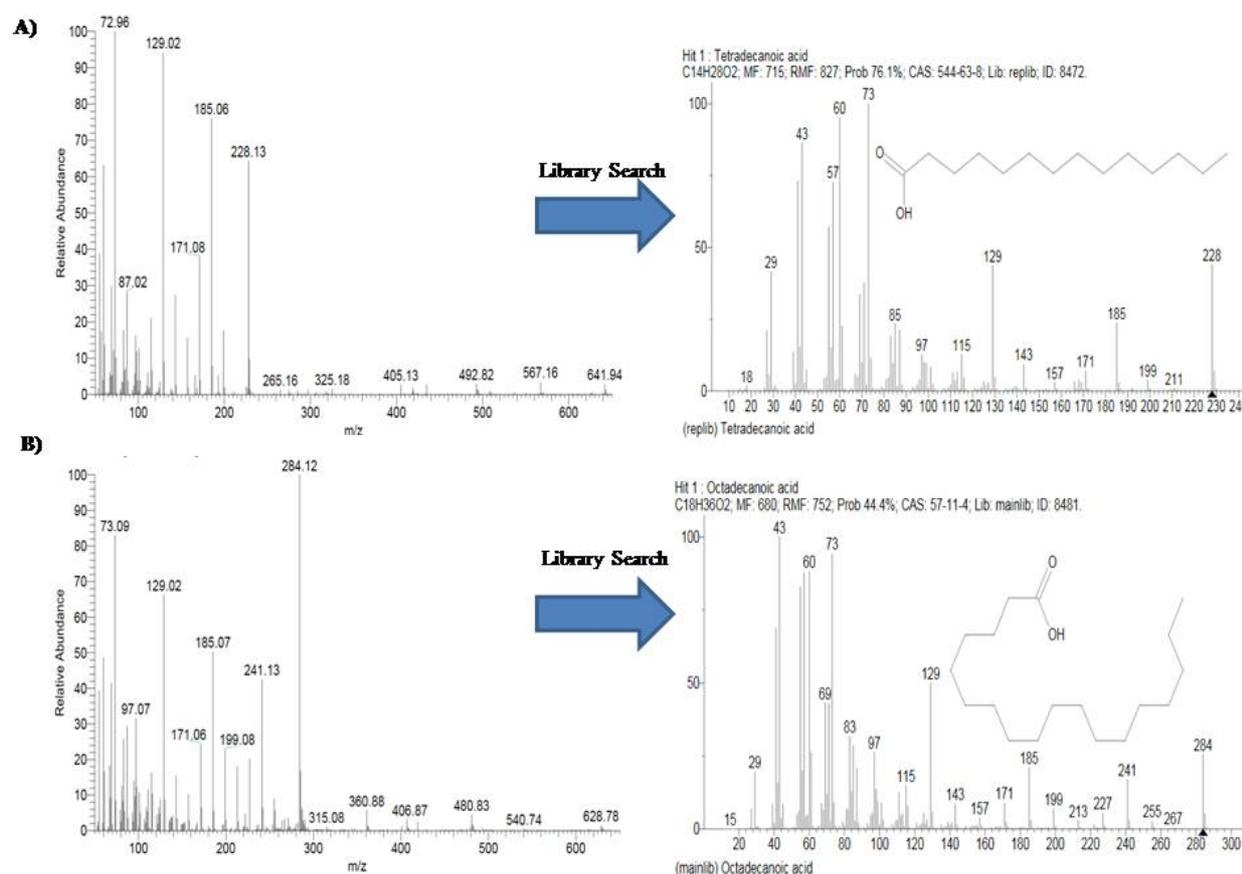


Figure 2. GC- MS analysis of mycodiesel extracted from *M. circinelloides* and *G. roseum*. A) Oil sample of *M. circinelloides* showed peaks (on the left side) and library search of Tetradecanoic acid (C₁₄H₂₈O₂) found maximum similarity at first hit. B) Oil sample of *G. roseum* showed the maximum probability of Octadecanoic acid (C₁₈H₃₆O₂) as a second hit of the molecular similarity search

4. Conclusion

The present comparative study showed that the *M. circinelloides* has lower biomass to substrate yield than *G. roseum*. But the overall lipid content was 20.69 % (w/w) of *M. circinelloides* and 11.37 % (w/w) for *G. roseum* indicated that the *M. circinelloides* has the highest lipid accumulating capacity than *G. roseum*. The hydrocarbon profile of mycodiesel extracted from *M. circinelloides* and *G. roseum* contains number of compounds normally associated with diesel fuel which have the implications in future energy production and utilization. Furthermore, this study can be attempted for many wastes to energy routes for further exploring options of non-edible biodiesel source.

Acknowledgements

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Sandip S. Magdum A Biotechnology researcher from Pune, India; obtained early education at Kolhapur, Maharashtra, India; received Bachelor of Engineering (Biotechnology) in 2007 from Shivaji University, Kolhapur, India; received post graduation degree of Master of Technology (Biotechnology) in 2009 from Amity University, Noida, India; worked as research fellow in Bangalore based Avesthagen company in 2009-2011, India's leading integrated systems biology platform company; working as Assistant Manager in research and development division (RTIC) of Thermax Limited, Pune (2011-till date), on development of water & wastewater treatment technologies, renewable and sustainable technologies etc.
E-mail address: sandip.magdum@thermaxindia.com



Gauri P. Minde She is a microbiologist studied Masters of microbiology from Fergusson College, Pune (Pune University, Maharashtra, India). She has worked on biofuels, water and waste water technologies, biofertilizers, bioinoculants, bacteriophage applications, bacterial cloning and enzyme purification etc. Currently she's working on water-waste water treatment technologies, waste to energy research and environmental biotechnology at a corporate R & D Centre. Gauri is interested in environmental research involving sustainable and green solutions.
E-mail address: gauri.minde@thermaxindia.com



Upendra S. Adhyapak Post graduation in Biochemistry from Shivaji University, Kolhapur in 1993 and Doctorate in Environmental Science from University of Pune, India in Feb 2011. Worked as a Trainee chemist in Sadguru Agro industries LTD, Solapur from August 1993 to May 1994. Worked as a quality controller in Chakan Veg oils LTD from June 1994 to December 1994. Worked as a Quality control Analyst from December 1994 to April 1996 in Tasty bite Eatables LTD, Bhandgaon, Pune. Joined Thermax LTD in April 1996 and presently working as Deputy Manager in Research Technology and Innovation Centre (RTIC).
E-mail address: us.adhyapak@thermaxindia.com



V. Kalyanraman Doctorate in Environmental Biotechnology from National Environmental Engineering Research Institute, Nagpur India and worked as Scientist Fellow from 1990 to 1997. Joined Thermax Limited, Pune India as Technology Manager in 1997. Presently working as Deputy General Manger & Head, Centre of Excellence (Biotechnology) and Water & Wastewater Treatment Technologies in Research, Technology and Innovation Centre (RTIC), Thermax Limited, Pune, India. He has over 25 years of experience in Research and Development on Environmental Biotechnology, Bio-energy, Biofuels, Water treatment and Wastewater treatment systems and responsible for development of various technologies in this domain.
E-mail address: kalyanar@thermaxindia.com