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Research Article

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Fatty Acid Methyl Esters (fames) profiling from Endophytic Fungi of Eichhornia Crassipes Mart (Solm) by Direct Trans-Esterification Process - a Simplified Method in Lipid Research

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ABSTRACT

'Fatty Acid Methyl Esters (FAMEs) are considered as a valuable and important biologically active compounds based on their many health benefits in addition with radical scavenging properties. FAMEs were obtained from an endophytic fungus of Eichhornia crassipes (a common water hyacinth). The fatty acid composition was determined in the endophytic fungi strains of two genus which includes Aspergillus terrus, and Curvularia species, using direct trans-esterification method. The FAMEs prepared by direct trans-esterification method was analyzed using Gas Chromatography - Mass Spectrometer Detector). Direct trans-esterification of fatty acid extracts from endophytic fungi mycelial mat and GC-MS analysis has made the identification of fatty acid composition in single step procedure. Totally 49 fatty acids in Curvularia species, and 33 fatty acids in Aspergillus terrus, were determined from GC-MS spectrum observation. The present study was focused on FAME preparation and identification of fatty acid composition using GC-MS analysis and further purification of each fatty acids with their biological activity evaluation is required.

Keywords: *Fatty acid methyl esters*; Eichhornia crassipes; Aspergillus terreus; Curvularia species

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INTRODUCTION

Plants are naturally associated with microbes which are beneficial to the host system. Based on this endophyte are the microorganism which living inside the plants as symbionts without developing harm to plants. Endophytic fungi are crucial attention creators to researchers because of their beneficial properties such as bioactive secondary metabolites, growth promoting compounds, stimulation of host system to tolerate biotic and abiotic stresses. From the previous work the endophytic fungi Aspergillus terries isolated from sunflower plants showed disease resistant to Sclerotium rolfsii with increase in biomass yield of sunflower plants. Association between endophytic fungi and their host plants are generally involved in commensalism, mutualism, virulent and latent pathogenic activities . Hence there is high chances of identifying potent endophytic fungi from different plant species which is having high industrial applications in the field of biotechnology, pharmaceutical and many more. The significant biocontrol agent role of endophytic fungi against host pathogens, insects, nematodes and pest is one of major application in agricultural field of pest and plant pathogen control management.

FAMEs of Eichhornia Crassipes

Water hyacinth (Eichhornia crassipes) was a promising high availability and high biomass producing aquatic invasive species for fuel ethanol production. Bio-conversion of biomass to produce bio-ethanol could be wise method in bio-fuel production. Pre-treatment of water hyacinth (10 %, w/v) with dilute sulfuric acid (2 %, v/v) at high temperature, pressure and further enzymatic process was studied. Usage of naturally available water hyacinth biomass relies on the research in lipids. Moreover, water hyacinth was an inexpensive biomass forming plant and there is no special nutrient requirement for their growth. Thus, with results from upto 95% fatty acids were recovered from this work. The water hyacinth Eichhornia crassipes is versatile for its rapid growth in water bodies under eutrophication conditions. The pattern of extract lipid compounds in E. crassipes are significantly for higher in terrestrial plants. In comparison with other submerged and floating aquatic weeds the n-alkane composition was dominant by C-29 to C-33 compounds Hence the main focus of this work is to profile fatty acid methyl esters from fungal isolates of Eichhornia crassipes to study fatty acid compositions [1].

MATERIALS AND METHODS

The healthy whole plants of Eichhornia crassipes (common water hyacinth) was collected at different locations in Chennai, Tamil Nadu, India in the month of August, September 2019. The plant samples were taken in a clean plastic container. The collected samples are transferred to the laboratory within 2 hours.

Surface Sterilization

The collected plant sample was washed thoroughly with tap water to detach the attached epiphytes from the plant parts (root, stem and leaves). Initially the sample was cut into 1 - 2 cm pieces and washed with distilled water for 2 minutes to remove the unwanted contaminants. The distilled washed plant samples are washed with 70 % ethanol for about a minute. Followed by ethanol treatment, the plant samples are washed with 3% Sodium hypochlorite for about 4 minutes. After the treatments, the plant samples were subjected to a wash with 70% ethanol for 30 - 60 seconds. Then the sample was washed with autoclaved demineralised water for about 1 minute to remove the surface sterilized solvents trace. Finally, the surface sterilized samples were dried under a laminar air flow chamber. All the steps of surface sterilization were done inside the Laminar Air Flow Chamber to avoid cross contamination [2].

Isolation of Endophytic Fungi from Eichhornia Crassipes

Himedia were used throughout the experiments and accurately weighing 5.85 g of PDA in 150 mL of deionized water. The surface sterilized samples are aseptically transferred to freshly prepared PDA containing autoclaved petri plates for 15 minutes at 121℃ under Laminar Flow chamber to avoid aerosol contamination. Addition of Chloramphenicol in PDA plate to suppress the bacterial growth. After incubation the grown endophyte cultures were used to make a pure culture by inoculate in sterilized slants with addition of Chloramphenicol (50 mg/mL) to suppress the bacterial growth. To avoid aerosol cross contamination the inoculated slants were labeled, covered with para film and stored. The last washed water 80 micro litre (µl) was spread on AIA plates and PDA plates separately by spread plate technique. Then incubate the plates at room temperature for 4 to 5 days to validate that no endophytes growth and it is considered as the control sample for reference of endophytes isolation (Figure 1).

Colony Morphology and Microscopic Examination

A small portion of each mycelium samples are focused in 40X magnification microscope to observe colony growth pattern.

Liquid State Fermentation for Lipid Production

The isolated endophytes fungi from Eichhorn crassipes were inoculated in 100 mL of autoclaved PDA culture broths. The inoculated culture broths were incubated at room temperature for 7 to 10 days under a static conditions and mycelial mat dense formation was checked respectively. After the fermentation process, each broth cultures were subjected to filtration process using an autoclaved four layered cheese cloth to separate the mycelial mat by using a separating funnel. The filtered mycelial mats were dried under hot air oven at 60 to 65 ℃ for 3 days.

EFSP09 - *Aspergillus terrus* - Colonies on Potato Dextrose Agar (PDA) are initially white and later at mature stage are beige to cinnamon colour colony. Producing reverse yellow with yellow soluble pigments in broth culture. Moderate to Rapid growth even at room temperature. Colonies are finely granular with conidial spores production.

EFSP1309 - *Curvularia species* - Colonies are fast growing, greyish colour on PDA Agar plate with white reverse colour formation.

Figure 1: Colony Morphology Characteristics of Selected Endophytes

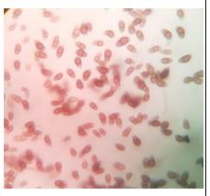
Endophytes	**EFSP09**	**EFSP1309**
Genus	*Aspergillus terrus*	*Curvularia species*
LPCB Staining examination		

Figure 2: Microscopic examination of Selected Endophytes by Lacto Phenol Cotton Blue (LPCB)

Biomass Estimation

The dried mycelial mats were grounded into a powder form using morter and pestle. The powdered mycelial mats were transferred into a fresh eppendorf. The dry biomass weight of powdered mycelial mats was measured by substracting the weights of empty Eppendorf (Figure 2).

Fatty Acid Methyl Esters (FAMEs) preparation by Direct Trans-esterification

- **Drying**: It is important to dry the mycelial mat prior to lipid extraction, because many organic solvents cannot easily extract lipids from dense mycelial mat.
- **Particle size Reduction**: Dried mycelial samples are finely grounded prior to FAMEs preparation to make the samples into more homogenous to increase the surface area exposed to the during direct trans-esterification reaction. Grinding is carried out at room temperature to reduce the lipid oxidation tendency to occur [3].
- **Solvent selection**: The ideal solvent for lipid extraction is Chloroform / Methanol which extract all the bound and free fatty acids in the grounded mycelial sample. The efficiency of solvent selection should meet with an extraction of polar lipids and non-polar lipids. Polar lipids such as glycolipids or phospholipids are more soluble in polar solvents like alcohols and non-polar lipids such as triacyl glycerides are more soluble in non-polar solvents like hexane, chloroform. This principle is the fact that lipids have different polarities and it is not possible to extract both polar and non-polar lipids in a single organic solvent system. Hence Chloroform / Methanol solvents are choosed as an ideal solvent system for lipid extraction. For direct trans-esterification reaction, concentrated sulfuric acid used as a acid catalyst to speed up the reaction. The most common method is used to preserve the extracted FAMEs from samples to prevent degradation in the cold at -20℃ until analysis (Figure 3).

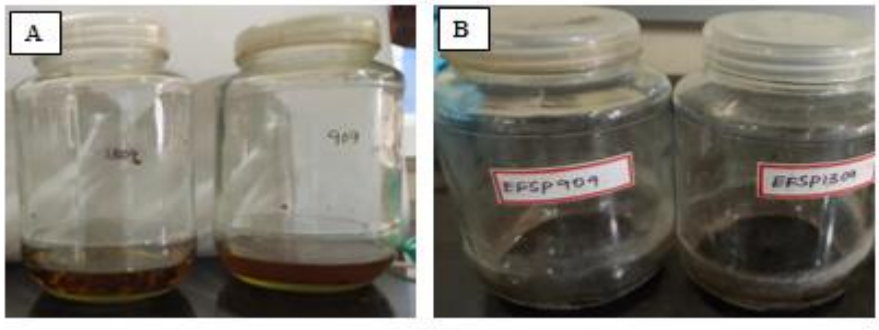


Figure 3: A - Depicts that reaction mixture before proceeds to direct trans-esterification process. B - depicts that reaction mixture after carried out direct trans-esterification process

Direct Trans-Esterification

0.1 g of Grounded mycelial mats were taken per 30 mL of suitable solvents. In direct trans-esterification, the 0.1 g of Grounded mycelial mats were dissolved in 30 mL of methanol: Conc. Sulfuric acid: Chloroform in the ratio to make up to 30 mL. Then the trans-esterification was carried out at 90℃ for 5 hours in a water bath. The esters produced from direct trans-esterification was extracted by the addition of solvents in the ratio to make up to 20 mL volume with mild shaking for phase separation. The upper light phase contains extracted Fatty Acid Methyl Esters. The extracted FAMEs from samples were stored in the cold at -20℃to prevent degradation until analysis[4]. The prepared FAMEs were subjected to GC-MS analysis (Figure 4).

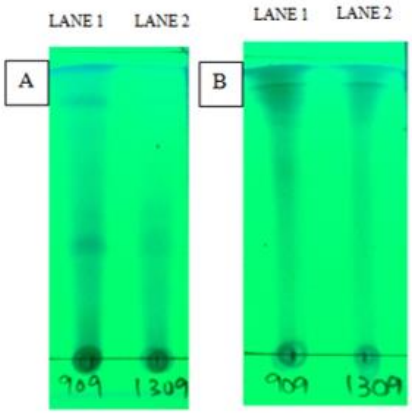


Figure 4: Mobile phase used [A] Hexane : Ethyl Acetate, [B] Hexane: tert butyl methyl ether : acetic acid. In lane 1 represents the sample EFSP09 FAMEs and lane 2 represents EFSP1309 FAMEs. TLC was carried out before GC-MS analysis

Thin Layer Chromatography Analysis of FAMEs

Direct trans-esterified FAMEs samples was run on TLC plate (Merck TLC plate, Silica gel 60 F254) using mobile phase as Hexane: Ethyl Acetate (9: 1), Hexane: tert butyl methyl ether: acetic acid - (9: 1 : 0.05).

Gas Chromatography - Mass Spectrometer (GC-MS) analysis of FAMEs

Gas Chromatography can be used to determine the complete profile of FAMEs are present in a sample. FAMEs in the mixture are separated because of their difference of affinities with the column packed matrix in the Gas Chromatography. The stronger the affinity with the column packed matrix, the retention time will vary [5]. After FAMEs were separated and based on the retention time values the each separated fatty acids were identified. Using Gas chromatography, the concentration of each of the molecules is determined as they pass by a suitable detector (e.g., Mass Spectrometer detector). Chromatography can be used to determine the complete profiling of fatty acid methyl esters in a sample (Figure 5).

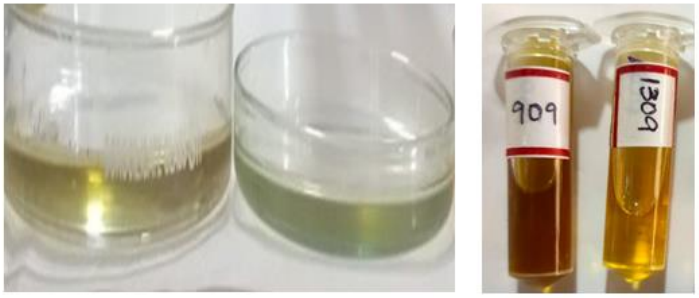


Figure 5: After direct trans-esterification process, the converted FAMEs were labeled and stored in a separate fresh eppendorf at -20°C to avoid degradation until GC-FID analysis

Table 3: GC-MS data of EFSP1309 - *Curvularia species*

S.No.	Fatty Acid Methyl Esters	Retention Time (RT in minutes)
1	Ethanedioic Acid	4.402
2	Phosphoric Acid	6.344
3	Methyl 3-acetylpropanoate	7.353
4	Butanedioic acid	8.952
5	Pentanoic Acid	10.245
6	Octanoic Acid	11.926
7	Methyl 15-acetylhydroxypalmitate	12.265
8	Nonanoic Acid	13.791
9	Cyclopropanedodecanoic Acid	14.277
10	Hexanedioic Acid	14.72
11	7-Oxooctanoic Acid	15.182
12	5-oxo-, methyl ester	15.931
13	Octanedioic Acid	16.583
14	Nonanedioic Acid	18.371
15	2,3-Hexadienoic Acid	19.808
16	Decanedioic Acid	20.039
17	Tetradecanoic Acid	20.219
18	Hexadecanoic Acid	21.229
19	Octanoic Acid	22.163
20	Pentadecanoic Acid	22.745
21	Pentadecanoic Acid	24.283
22	Benzenepropanoic Acid	24.446
23	Hexadecanoic Acid	25.593
24	11,14-Eicosadienoic Acid	26.339
25	9-Octadecenoic Acid (Z)	26.779
26	Tetradecanoic Acid	27.063
27	9-Octadecenoic Acid	27.23
28	Tetracosanoic Acid	28.226
29	Hexadecanoic Acid	28.689
30	Oxiraneundecanoic Acid	29.861
31	17-Octadecenoic Acid	30.042
32	Oleic Acid	31.771
33	9-Hexadecenoic Acid	32.161
34	Octadecanoic Acid	32.936
35	Tetracosanoic Acid	34.363
36	Heptadecanoic Acid	35.396
37	Tetracosanoic Acid	35.731

38	Malonic Acid	36.193
39	Tetracosanoic Acid	36.959
40	Octadecanoic Acid	37.444
41	Tetracosanoic Acid	38.155
42	Octadecanoic Acid	38.679
43	Eicosanoic Acid	39.284
44	Nonahexacontanoic Acid	39.491
45	Octadecanoic Acid	40.1
46	Cyclopropaneoctanoic Acid	40.474
47	DI-N-Propulmalonic Acid	40.644
48	Tetracosanoic Acid	40.939
49	Nonanoic Acid	41.564

Microorganisms and Antibacterial Activity

The microorganisms employed in the current study were obtained from Institute of Microbial Technology, Chandigarh (India) which includes clinical isolates of *Staphylococcus aureus (MTCC 1430), Klebsiella pneumoniaee (MTCC 432), Escherischia coli (MTCC 254), Pseudomonas aeruginosa (MTCC 424), Salmonella typhi (MTCC 733)* and *Bacillus subtilis (MTCC 121).*

Antibacterial Activity

The antibacterial activity of *Vitex negundo* was performed by Kirby-Baeur disc diffusion method. The bacterial strains of *Staphylococcus aureus, Klebsiella pneumoniaee, Escherischia coli, Pseudomonas aeruginosa, Salmonella typhi and Bacillus subtilis* were inoculated on the sterile Mueller-Hinton Agar (MHA) plates with spread plate method using sterile bent glass rod. The sterile discs dipped and soaked with 30 µg concentration of plant extracts were gently placed on the inoculated medium with standard tetracycline disc with the same 30 µg concentration. These plates were incubated at 37°C for 24 hours for the observation of the zone of inhibition. Later the zones of inhibition were measured and compared with the standard antibiotic's zones.

RESULTS AND DISCUSSION

In this current work, totally 2 different endophytic fungi were isolated from Eichhornia crassipes (Aspergillus terreus, Curvularia species). The isolated fungal strains were examined for colony pattern and cellular pattern in 40X magnification. Then the identified strains were proceeded for lipid production under static condition for 7 days. After 7 days of incubation, the broth filtrate was extracted and subjected to FAMEs preparation, TLC analysis and GC-MS for FAMEs profiling. Therefore, that two different species of Genus Aspergillus and Curvularia can be used for industrial Eichhornia crassipes fermentation processes (Figure 6).

Colony Morphology and Microscopic LPCB Staining examination

- In Petroleum ether extracts steroids, flavonoids, tannins and terpenoids were found to be present while amino acids, carbohydrates, alkaloids, cardiac glycosides, saponins and phenols were absent.

- In Chloroform extracts amino acids, carbohydrates, alkaloids, steroids, cardiac glycosides, flavonoids and terpenoids were present and saponins, tannins and phenols were found absent (Figure 7).

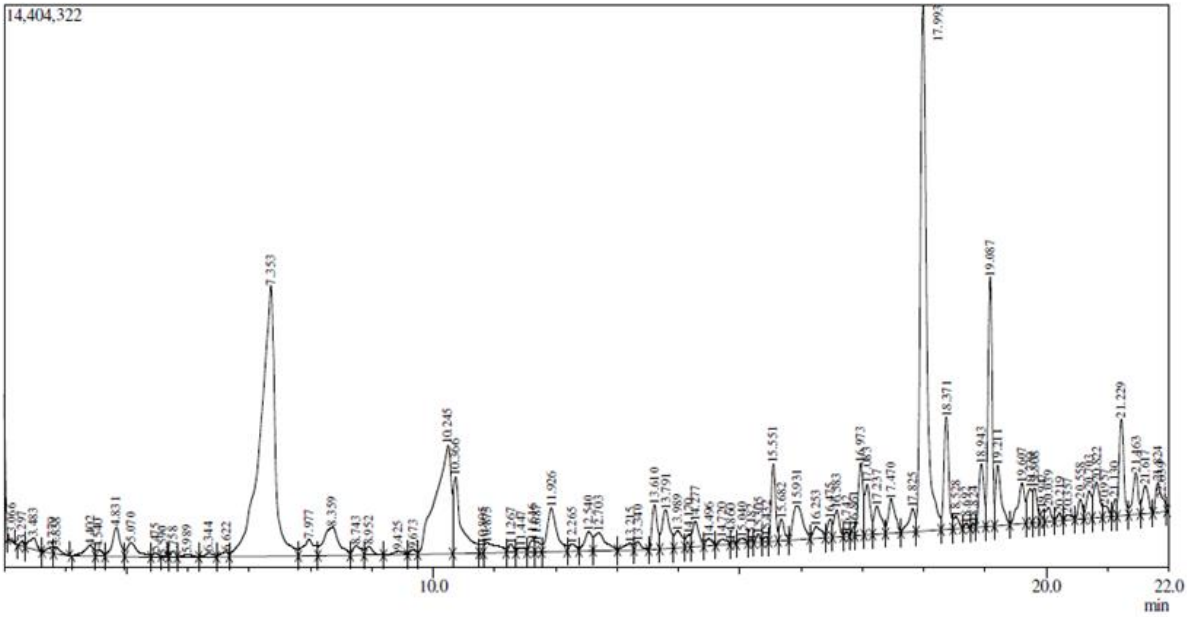


Figure 6: The FAMEs GC-MS spectrum of *Curvularia species* (EFSP1309)

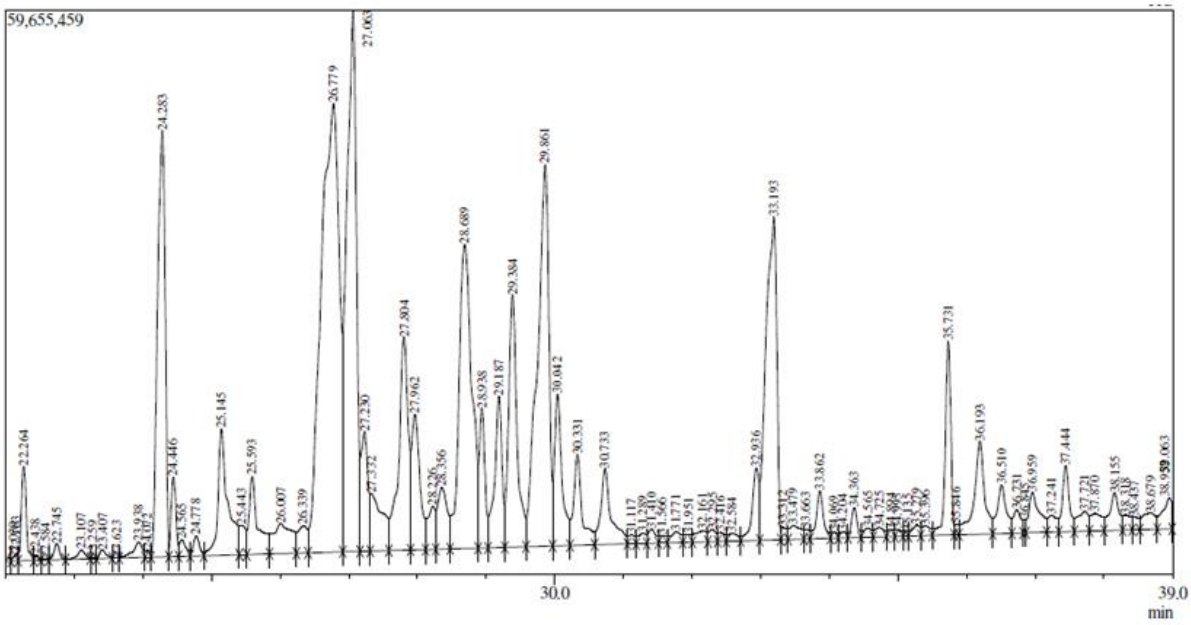


Figure 7: The FAMEs GC-MS spectrum of *Aspergillus terreus* (EFSP09)

GC - MS Data analysis and Interpretation

GC-MS is widely used analytical method to separate, analyze, identify and quantify the fatty acid composition by converting into a FAMEs mixture. The fatty acids are converted into FAMEs mixture can readily used to run and analyze in GC-MS due to its volatility and thermal stability and thus it can give accurate analytical data. In addition with this, a column packing selectivity and separation efficiency of it played a crucial role in GC-MS analysis. The analysis of FAMEs is one of the most rapid method to analyze lipids and fatty acids in lipid research. To improve GC-MS data analysis, the test samples were converted into a FAMEs by acid catalyzed direct trans-esterification process. This process demonstrates FAMEs preparation in a single step with accurate results with even less concentrated fatty acids in a sample in very less time when compared with conventional indirect trans-esterification method. Also, the acid catalyzed direct trans-esterification method improved the good yield for methyl esters production in a FAMEs preparing reaction mixture.

Table 4: GC-MS data of EFS909 - *Aspergillus terrus*

S.No.	Fatty Acid Methyl Esters	Retention Time
		(RT in minutes)
1	Hepatanoic acid	10.125
2	Benzenebutanoic acid	11.503
3	Ethyl-2-Furoic Acid	12.858
4	4-Pentenoic acid	14.998
5	Butyric acid	15.452
6	Carbonic acid	15.867
7	4- Ethylbenzoic acid	16.55
8	Cyclopropanedodecanoic Acid	18.374
9	9-Octadecen-12-ynoic acid	20.561
10	Heptadecanoic Acid	21.464
11	Bromopropionic acid	22.163
12	Pentadecanoic Acid	22.742
13	7-Hexadecenoic acid	23.961
14	Benz16.enepropanoic acid	24.451
15	Hexa17decanoic Acid	25.59
16	Elaidic acid methyl ester	26.627
17	Stearic acid methyl ester	26.958
18	Oleic acid methyl ester	27.17
19	3-Phenylpropanoic Acid	27.425
20	Hexadecanoic acid	28.65
21	2-Methoxypalmitic acid methyl ester	29.355
22	Oxiraneundecanoic acid	29.805
23	Methyl-15-Acetyhydroxypalmitate	30.005
24	Oleic Acid Propyl Ester	31.45

25	Docosanoic Acid	32.568
26	Octadecanoic Acid	32.867
27	15-Tetracosenoic Acid	33.587
28	Tetracosanoic Acid	33.855
29	Tetracosanoic Acid	35.098
30	Tetracosanoic Acid	36.236
31	Octadecanoic Acid	36.699
32	Heptacosanoic Acid	37.393
33	Oxiraneundecanoic Acid	37.598

CONCLUSION

This work is the first attempt was made to isolate endophytic fungi from Ecchornia crassipes (a common water hyacinth) for the identification of fatty acid producer. Most endophytic fungal strains lipids are found to efficient applications in dietary supplements, bio-diesel production and other purposes. More than 150 fatty acids and related compounds are identified in this study. We focused on majorly on fatty acid methyl esters in GC-MS spectrum. Totally 49 fatty acids profiles in EFSP1309 and 33 fatty acids profiles in EFSP09. By following direct trans-esterification solutions used in this study is a convenient reaction mixture for Fatty Acid Methyl Esters (FAMEs) preparation of fungal lipid research. This one step FAMEs preparation reaction mixture can readily not only reduce the time and also can be prepared by common laboratory chemicals. Yields of FAMEs obtained using by our reaction mixture was also significant range. Further lipid production optimization methods are required to yield particular fatty acids on requirement. Hence from this work we conclude that isolated fungal strains Aspergillus terreus and Curvularia species from Eichhornia crassipes have high potential in lipidomics research in future.

CONFLICT OF INTERESTS

All authors declare no conflict of interest regarding about the publication of our research paper.

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