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EVALUATION OF POLYUNSATURATED FATTY ACID (PUFA) PROFILE OF *TRICHODERMA VIRIDE* ISOLATED FROM *LINUM USITATISSIMUM* L.

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Abstract

The Latin name of the flaxseed is *Linum usitatissimum*, which means "very useful". Flaxseed is emerging as an important functional food ingredient because of its rich contents of α-linolenic acid (ALA, omega-3 fatty acid), lignans, and fiber. Flaxseed oil, fibers and flax lignans have potential health benefits such as in reduction of cardiovascular disease, atherosclerosis, diabetes, cancer, arthritis, osteoporosis, autoimmune and neurological disorders. Fungal endophytes are extremely common and highly diverse microorganisms that live within plant tissues, but usually remain asymptomatic. Fungal endophytes continue to be a source of novel drug; they produce an array of metabolites having antibacterial, anti-viral, antifungal and anticancer properties. A very few studies are available on fattyacid composition of endophytic fungi. In this study nine endophytic fungi were isolated from various plant parts (stem, leaf and root) of *Linum usitatisumum*. Polyunsaturated fatty acid profile of *Trichoderma viride* which was isolated from *Linum usitatissimum* was discussed in this research article. Identification and determination of fatty acids by GC/MS (BPX70) revealed the presence of long chain fatty acids in *Trichoderma viride*. Among the 8 FAME compounds obtained from *Trichoderma viride*, the higher percentage area was registered for Diethyl phthalate (10.15), followed by Hexadecanoic acid (17.71), Methyl linoleate (13.13), 9-Octadecenoic acid (21.86), Stearic acid (11.56), Ethyl 5,8,11,14,17-icosapentaenoate (7.56), Eicosanoic acid (2.22), and Methyl cis-4,7,10,133,16,19-Docosahexaenoate (15.81). Further, the retention time of FAME compound which was obtained from fungal source was similar to that of the standard fatty acid compounds. In conclusion, this work revealed the possibility of using the promising fungal isolate *Trichoderma viride* in PUFA production.

Keywords Lipids, Polyunsaturated fatty acids, GC-MS, *Linum usitatissumum*, Endophytic fungi, *Trichoderma viride* and Flax plant.

INTRODUCTION

Endophytic fungi are a group of fungi living inside the host plant tissues for all or part of their life cycle, cause no apparent infections and are known to occur ubiquitously in plants [1]. Endophytic fungi have the ability to adapt to its host and external environment during stress and unfavorable conditions. They are considered as treasure house of secondary metabolites when it is grown *in vitro* under controlled conditions [2]. Thus endophytic fungi have been recognized as an alternative source for various secondary metabolites in nutraceutical and pharmaceutical industry. They are also capable of producing primary metabolites such as gibberellins, enzymes like lipase, laccase, amylase, pigment, chitoson, biodiesel etc., [3,4]. A very few evidences are available on lipid production in general and Polyunsaturated fatty acids (PUFA) in particular.

Polyunsaturated fatty acids are the important components of human nutrition. These fatty acids are characterized by at least two carbon-carbon double bonds in a hydrophobic hydrocarbon chain. They cannot be synthesized by our body and hence have to be obtained through diet and therefore they are referred as essential fatty acids (EFA). There are two families of EFA namely ω -3series, derived from alpha-linolenic acid (ALA 18:3) and ω -6 series, from linoleic acid (LA).

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Polyunsaturated fatty acids regulate a wide range of functions in human body including blood pressure [5], vasoconstriction [6], vasodilation [7], vision [8] and normal development, maintenance and functioning of the brain [9].

The therapeutic significance of ω-3 polyunsaturated fatty acids such as EPA and DHA has been clearly indicated by recent clinical and epidemiological studies [10,11]. Long-chain polyunsaturated fatty acids with the first double bond at the third position from the methyl terminal (so called n-3 FAs or omega-3 FAs) can be found in plants and fish. The essential omega-3 FA is alpha-linolenic acid (ALA) that occurs in plants (walnuts, soybean, flaxseed and their oils). Currently, the most common source of EPA and DHA is fish oil. Unfortunately, there are several limitations with fish oil as an omega-3 source such as its undesirable taste and odor [12]. Possible alternate sources of the ω -3 fatty acids include certain algae which are actually the dietary origin of such fatty acids in fish. Although restricted in occurrence, EPA appears to be more widespread in nature than DHA. It has been reported in certain marine bacteria [13] and some of the more primitive fungi such as the Oomycetes, including the *Pythiaceous* species (*Pythium* and *Phytophthora*) [14], and the Zygomycete genus *Mortierella* [15].

Flax (*Linum usitatissimum*) is a multipurpose crop. Flax crop is a cool season herbaceous annual plant crop with a short tap root system. Mediteranean region is the primary centre of origin of flax and from here it spread to other regions of the World. Its seeds containing about 36 to 40 % of oil have long been used in human and animal diets and in industry as a source of oil and as the basic component or additive of various paints or polymers [16]. Recently there has been growing interest in the probiotic properties of flax and in its beneficial effects on coronary heart disease, some kinds of cancer and neurological and hormonal disorders. The beneficial effects are mostly due to flax lipids. Flax oil is the richest plant source of linoleic (omega-6) and linolenic (omega-3) polyunsaturated fatty acids (PUFA), which are essential for humans since they cannot be synthesized in organism and must be ingested in food [17]. The main aim of the present work was to ensure endophytic fungi isolated from different plant sources of *Linum usitatissimum* to ascertain its ability to produce pharmaceutically important polyunsaturated fattyacids.

MATERIALS AND METHODS

Isolation and identification of fungi

Flax seed were raised, when fresh seeds were sprinkled in a container, covered with soil and allowed to germinate for up to 120 days. Healthy leaves, stem and root of *Linum usitatissimum* were washed in running tap water to remove adhered dust particles. Then, the plant samples were processed under laminar chamber using 75% (v/v) ethanol for 1 min and 2.5% (v/v) sodium hypochlorite for 15 min for the surface sterilization. Later, they were washed three times thoroughly using sterile distilled water. The plant materials were aseptically cut into small pieces and were plated on Potato Dextrose Agar (PDA) containing (200µg/ml) Streptomycin. The plates were incubated at 25 ±3°C for 7 days. The temperature and days of incubation was monitored and optimized. Lacto phenol cotton blue staining was used for the morphological identification. Each fungal culture growth should be checked for purity and sub cultured to another agar plate by the hyphal tip method [18]. Population density was expressed in terms of Colony Forming Unit (CFU). The percent contribution of each isolate was calculated by

$$C\ (\%) = \frac{Total\ No.of\ CFU\ of\ an\ individual\ Sps}{Total\ No.of\ CFU\ of\ all\ sps} X100$$

C – Contribution; CFU – Colony Forming Unit

Bio mass production

The pure culture of entophytic isolate was inoculated into 250 ml conical flask contain medium. Three different type of medium was tested for the production of lipid. Yeast peptone dextrose Agar (composition of Startch-20, Yeast extract 5, Potassium nitrogen 10, Potassium di hydrogen phosphate 1, Magnesium sulphate 0.5

0.5 grams/Litre), Potato Dextrose agar with 2% glucose, PDB with raw potato. The flasks were incubated at temperature between 22°C to 25°C for 7 days in shaker. After 7 days, the culture was passed through four layers of cheese cloth. The mycelial mats were dried and used for the extraction of lipid. The lipid was extracted using soxhlet solvent extraction method. The dried mycelia were extracted using 200 ml of chloroform and methanol in 2:1 (v/v) ration. The extract obtained was then concentrated to 2 ml. The total extracted lipid yield (%w/w) was then quantified gravimetrically by calculating difference between the weights of the empty tube as in the following equation.

$$Y\,(\%) = \frac{Weight\ of\ lipid}{Weight\ of\ fungal\ biomass\ (g)} X100$$

Y- Extraction yield

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 Mohammad and Klein, 2007 [19] and analyzed by gas chromatography fitted with a FID detector.

GC-MS

Microbial oil was extracted as mentioned and FAME was prepared by above procedure. The carrier gas, helium, was maintained at a flow rate of 1.0 ml/min. The inlet temperature was maintained at 300°C and the oven was programmed for 2 min at 150°C, then increased to 300°C for 4 min, and maintained for 20 min at 300°C. The injection volume was 1ml, with a split ratio of 50:1. A structural assignment was based on interpretation of mass spectrometric fragmentation and was 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.

RESULTS AND DISCUSSION

The cultivated flax plant (Linum usitatissimum) grown to a height of 1.2 m tall, with slender stem. The leaves were glaucous green, lender lanceolate, 20-40 mm long and 3 mm broad. The flowers were pale blue, 15–25 mm in diameter, with five petals. The fruit was round, dry capsule 5–9 mm in diameter, containing several glossy brown seeds, 4–7 mm long (Fig 1).

Figure 1: Selected Medicinal plant *Linum usitatissimum*

In the present study, it was observed that, the selected plant (*Linum ussitatissimum*) parts viz., leaf, stem and root harbor fungal endophytes. A total of 45 fungal colonies were recorded. These colonies were classified in to 9 species belonging to 8 genera (1 Zygomycota, 1 Ascomycota, and 5 Hyphomycetes and 1 Coelomycetes). The isolated endophytic fungi are *Mucor* sp., *Aspergillus niger, Aspergillus flavus*, *Alternaria alternata*, *Curvularia lunata*, *Fusarium oxysporum*, *Helminthosporium* sp, *Trichoderma viride* and *Colletotrichum falcatum*.

The colonization frequencies of individual species were presented in Table 1. The colonization frequency of endophytes in this study was within the range of many host plants studied in the tropics [20-22]. Among the three plant parts tested for the presence of endophytes, colonization frequency was more in leaves (48.8%) followed by stem (35.5%) and roots (15.5%) (Fig.2).

Table 1. Colonization Frequency (CF %) of endophytic fungi isolated from *Linum usitatissimum*

S.NO	Isolated Fungi	Plant parts and CF%					
		Leaf	CF %	Stem	CF %	Root	CF %
	Zygomycetes						
1	*Mucor* sp.	3	13.6	-	-	2	28.5
	Ascomycetes						
2	*Aspergillus niger*	3	13.6	4	25.0	-	-
3	*Aspergillus flavus*	2	9.0	2	12.5	-	-
	Hyphomycetes						
4	*Alternaria alternata*	3	13.6	-	-	1	14.2
5	*Curvularia lunata*	-	-	3	18.7	-	-
6	*Fusarium oxysporum*	-	-	1	6.2	2	28.5
7	*Helminthosporium* sp.	2	9.0	2	12.5	-	-
8	*Trichoderma viride*	6	27.2	4	25.0	2	28.5
	Coelomycetes						
9	*Colletotrichum falcatum*	3	13.6	-	-	-	-
No. of species recorded		**7**		**6**		**4**	
Total no. of isolates observed		**22**		**16**		**7**	

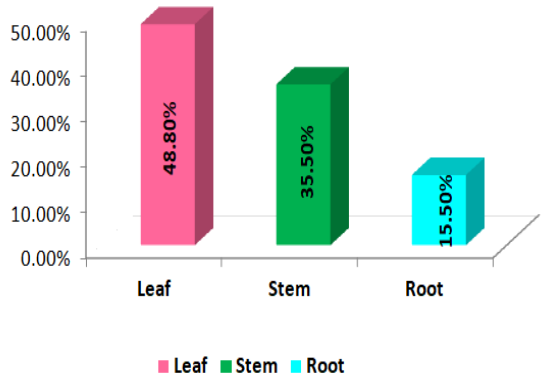

Figure 2. Colonization frequency (CF%) of different plant parts

Relative percentage occurrence of endophytes was maximum in Hyphomycetes (57.7%), followed by Ascomycetes (24.4%), Zygomycetes (11.1%) and Coelomycetes (6.6%) (Fig. 3). The endophytic fungi such as *Mucor* sp. *Aspergillus* sp., *Fusarium oxysporum*, *Trichoderma viride* and *Colletotrichum falcatum* which were isolated frequently in this study, have been reported as endophytes in a wide host range in the tropics [23-27].

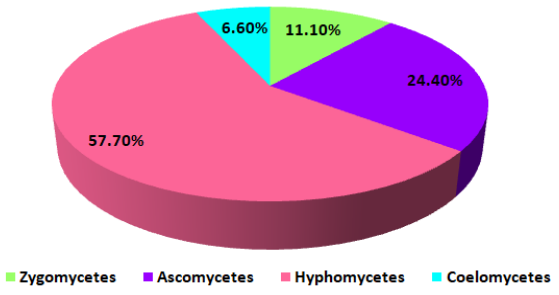

Figure 3. Relative Percentage Occurrence (RPO %) of endophytic fungi isolated from *Limnum usitatissimum*

However, nine species were isolated as endophytes form *Linum usitatissimum*, the PUFA analysis of *Trichoderma viride* was discussed in this research article. The description of the selected fungi was given as follows: *Trichoderma viride* Pers.ex (Fig 4). Colonies grow very rapidly,

Figure 4: Colony growth of *Trichoderma viride* on PDA medium

fast smooth, translucent later becoming floccose, mycelium, hyaline, 12 x 1.5 µm. Numerous terminal chlamydospores, globose, ellipsoidal, smooth walled, hyaline, upto 5 µm diameter; Conidiophores tufted, arise in ring-like zones, main branches of conidiophores 4.5 µm diameter. Phialides, 3.6 – 4.8 x 2.5 – 3 µm, conidia rough, ovoid. The Trichoderma species have been widely studied for their enzyme production such as cellulases [28,29], chitinases [30], xylanases [31]. Furthermore, Trichoderma species dealt with the production of small linear antimicrobial peptides, namely peptaibols. In addition, Trichoderma species have been also studied for the production of flavour compounds such as 6-pentyl-α-pyrone [32,33].

Lipids are considered to be an important storage compounds in the form of triacylglycerols (TAG) and esters. If the lipid content in the cell exceeds 20%, then microorganism can be called as oleaginous microorganism. 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 [34,35]. The present study revealed that, biomass production was more in Yeast Peptone Dextrose Agar medium when compared to the other medium tested and the lipid accumulation of 32% (W/W) of biomass dry weight was obtained for *Trichoderma viride*. These values are in agreement with previous studies concerning lipid synthesis by *T. viride* [36] and *T. reesei* [37]. Furthermore, the selected fungi were processed for fatty acid extraction and PUFA (Poly Unsaturated Fatty Acid) production. GC-MS results of *Trichoderma viride* showed 8 different FAME compounds such as Diethyl phthalate, Hexadecanoic acid, Methyl linoleate, 9-Octadecenoic acid, Stearic acid, Ethyl 5,8,11,14,17-icosapentaenoate, Eicosanoic acid, and Methyl cis-4,7,10,133,16,19-Docosahexaenoate. Among the FAME compounds obtained from *Trichoderma viride*, the higher percentage area was registered for 9- Octadecanoic acid ethyl ester (21.86 %) followed by Hexadecanoic acid methyl ester (17.71 %) and Stearic acid methyl ester (11.56 %).

Further, the retention time of FAME compound which was obtained from fungal source was similar to that of the standard fatty acid compounds (GC-MS Sargam laboratory report). Earlier reports on fungal fatty acids showed the presence of very long chain fatty acids of 22,23, 24, 26, 28 carbons. The majority of fungal species contain, in order of abundance, oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2) as the major acids, with stearic acid (C18:0), linolenic acid (C18:3) and palmitoleic acid (C16:1) as the minor ones[12].

According to Andrade Domínguez *et al*., (2012) [38] the microorganisms such as bacteria, microalgae and fungi are a viable alternative for the production of polyunsaturated fatty acids. So far, it seems that microbial yields of PUFA are in the order microalgae > fungi> bacteria. A very few studies are available on fattyacid composition of endophytic fungi [1]. Our results suggests that the main fatty acids produced by *Trichoderma viride* are Linoleate, icosa-pentaenoate, Docosa-hexaenoate, Octa-decanoic acid, Hexa-decanoic acid and Stearic acid in the range of 2-22%. (Fig 5) and this data correlates with other studies [2,39]. From biotechnological viewpoint, it is the significant content of the polyunsaturated fatty acids that has singled out fungi for recent attention.

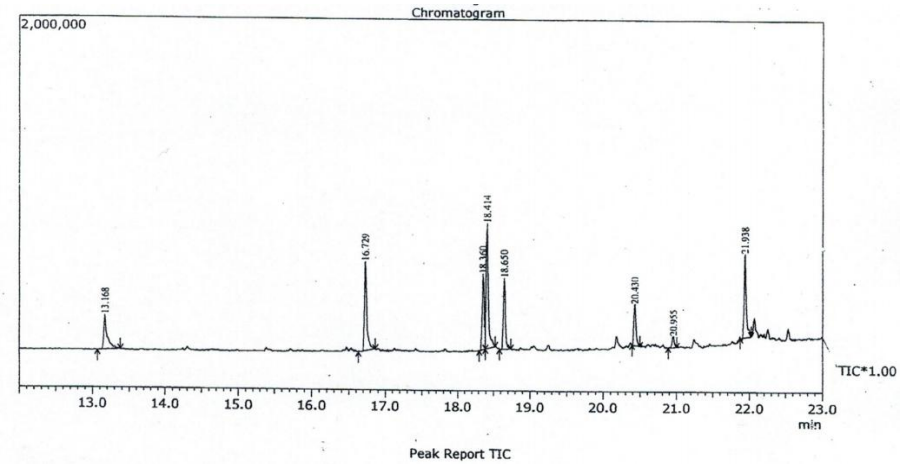

Figure 5: GC-MS report for fungal sample (Trichoderma viride)

Conclusion

PUFAs are important nutrient and structural components in cell membranes and are associated with human health and development. The results of our study revealed that, the endophytic fungi *Trichoderma viride* are capable of producing pharmaceutically important PUFAs. Efficient and cost effective methods of enriching the level of polyunsaturated fatty acids will continue to be needed in order to reduce the cost and to meet the future demand for highly purified lipid products.

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