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Research article

SCREENING OF FUNGI FOR PRODUCTION AND PURIFICATION OF OMEGA-3 FATTYACID

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ABSTRACT

Omega fatty acids, major importance in the prevention or treatment of a range of human diseases or disorders related with inflammation. These fatty acids are found in transgenic plants, fungi, and animals and even in microorganisms but in major amounts can be extracted from fatty fish. However, due to bioaccumulation of fat-soluble vitamins and high levels of saturated and omega-6 fatty acids, they may have deleterious health effects. It becomes necessary to search for novel and rich sources containing omega-3 fatty acids and one of the alternatives include fungi. The present study deals with production and purification of omega-3 fatty acids from Trichoderma viride and Aspergillus niger. In the present study, the main objective was to explore the beneficial effects of fungi for the maximum lipid production through optimized conditions and the results clearly showed that Trichoderma viride was the significantly highest lipid producer, with lipid production at initial pH 6.0 and incubation temperature 40°C.

Keywords: Fungi, fatty acids, pH, PUFA, temperature

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INTRODUCTION

Omega-3 (ω-3) fatty acids essential for cardiovascular health are usually polyunsaturated fatty acids (PUFAs) and are recognized as essential dietary components for the human health. [1] Omega-3 fatty acids with three essential fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA) have significant health benefits in preventing arteriosclerosis and coronary heart disease, and for reducing arthritis by preventing certain inflammation.[2] They are considered as essential nutrients since human body cannot synthesize them, they have to be provided through food. Even tough, these essential fatty acids can be synthesized in the body using alpha linolenic acid (ALA) but only in meagre amount. Such ALA which also an 18-carbon omega-3 fatty acid are found in plants such as flaxseed, soybeans and walnuts.[3] Omega fatty acids are rich in salmon, halibut, tuna and other sea foods include algae and krill. [4] Consuming omega-3 PUFA may be the one among therapeutic strategies to prevent the "cytokine storm" in cardiovascular complications associated to COVID-19. [5] Generally, omega fatty acids are structure with repeated double bonds. Such double bond occurs first between the third and fourth carbon counting from the methyl end (omega carbon) of the chain. [6]

Omega fatty acids can change the rigidity property of the cell membrane by modulating the membrane channel proteins with altered cellular function.[3] They can bind to transcription factors such as PPAR-α, HNF-4α and SREBP-1c in order to regulate gene expression that has direct impact on inflammatory pathways. Even they regulates proliferator-activated receptor of peroxisome and helps in the healing of intestinal mucosa. [7] By incorporating in membrane phospholipids, omega fatty acids are increasing systemic arterial compliance. [8] In endothelial cells, omega fatty acids are involved in the release of nitric oxide for improved endothelial function. Omega fatty acids can decrease serum levels of triglycerides through fatty acid degradation. [9] Furthermore, they are anti-thrombotic, when taken in high doses.[10] DHA is the fatty acids found rich in retinal phospholipids and they involved in maintaining the functional integrity of retina. [11]

Current dietary sources of omega-3 PUFA is from certain fish oils which contain up to 20-30% of these fatty acids. However, consuming fish oils have some significant problems such as bioaccumulation of fat-soluble vitamins and high levels of saturated and omega-6 fatty acids which have negative impact on human health.[12] In addition, fish oil application also have a chance of environmental pollution with heavy metal contamination, fishy smell and unfavorable taste. [13] In another way, plant sources are also found to be containing alpha-linolenic acid (ALA) and by consuming the plant sources, human body can converts alpha-linolenic acid rapidly into both docosahexaenoic acid and eicosapentaenoic acid at a very slow rate.[14] Oil processing from natural sources such as fish are cost-effective and time-consuming. Therefore, it becomes necessary to search for other natural sources containing omega fatty acids. Some alternative sources may be microorganisms like bacteria, microalgae fungi or plant sources and are currently exploring for commercial production. [15] Oils when extracted from microorganisms can be easy to produce in large scale as they have shorter life cycle and required cheap raw materials.[16] Among microorganisms, fungi is the foremost and recently found microorganism for the industrial production of omega fatty acids. [14] This study focuses to explore the potential of two fungal strains *Aspergillus niger* and *Trichoderma viride.* Therefore, in the present study, the main aim was to enhance oil biosynthesis by the selected two fungal strains with optimized culture condition.

MATERIALS AND METHODS

Preparation of microorganism

Aspergillus Niger and Trichoderma viride were obtained from CBNR - Centre for Bioscience and Nano science Research Laboratory, India. The fungal cultures were allowed to grow in potato dextrose agar medium and maintained at 300C and were sub cultured for fatty acid screening. [17]

Cultural conditions

Maltose, glucose, yeast, peptone medium (MGYP) was the culture medium to observe omega 3 fatty acids production. After sterilization, MGYP medium was set cool for about 30 minutes. Then Aspergillus Niger and Trichoderma viride were inoculated (25 ml each) in separate conical flask. After inoculation, the samples were allowed to grow 370C for 3-5 days at room temperature.

Determination of biomass

For the dry weight biomass determination, 1ml sample of Aspergillus niger and Trichoderma viride were taken separately in a tube and transferred to a dry weight centrifuge tube. It was allowed to centrifuge for 5 minutes at 5000rpm. The pellet with mycelial mats was collected after washed with sterile distilled water whereas the supernatant was discarded. The biomass was then dried at 60 °C and the determined biomass were expressed in micro gram per 50ml of MGYP broth medium. Fungal growth was usually weighed as dry weight of the biomass in one liter of culture medium. [18]

Optimum pH and temperature for highest omega-3 fatty acid production

This was carried out to evaluate the effect of initial pH and temperature of the medium on the biomass dry weight and lipid production. Here, the MGYP medium was prepared, inoculated with Aspergillus Niger and Trichoderma viride for incubation period of 10 days at normal room temperature. Each day the samples were withdrawn and was centrifuge for 15 min at about 5000 rpm. The production medium was prepared with various pH (5, 6, 7, 8) and was incubated at different incubation temperatures (300C, 400C, 500C) for five days in a static condition.

Lipid Estimation

To analyze the lipid content present in the fungal culture, a measurable amount of biomass in 100µl water was used. For routine assay, the biomass sample was heated at 100°C continuously for 10 min after added to 2.0 ml of 98% concentrated sulfuric acid. It was allowed to cool in an ice bath for about 5 min. To this cooled biomass solution, 5ml of sulfo-phospho-vanillin reagent (SPV) was added and incubated at 37°C for 15 min at 200 rpm, and the chromogen formed at 530 nm was measured with UV-Visible spectrophotometer. [19]

Purification of fungal samples

The overnight incubated sample of Aspergillus Niger and Trichodrema viride were filtered through whatmann no 1 filter paper. To this sample volume, acetone was added equally. After the addition of acetone, a color change was obtained. Then added phosphate buffer solution and kept for 24hrs in pre-chilled condition. The precipitate was undergone dialysis for desalting procedure following the standard protocol. The dialysis bag was then rinsed using double distilled water. After proper rinsing, the dialysis bag was tied at one end in order to prevent leakage and the precipitate was added to the dialysis bag. Dialysis bag was then suspended with tris buffer (pH 7). After 24 hours, TLC analysis was carried out using TLC plate. [20], [21]

Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared and were used for gas chromatographic analysis. In general, to lipid sample, toluene and 1% sulfuric acid dissolved in methanol were added before refluxing the mixture. After that, 5% sodium chloride in water was added. Hexane was used to extract the esters and later the hexane layer formed was treated with potassium bicarbonate in order to wash the layer. [22]

Gas chromatographic conditions

The GC-MS analysis was performed using Agilent Technologies 6890 N (Net Work GC system) USA. Oven was held at

initial temperature 50ºC and maintained for 2 min, at rate 10, 8, 5, 60C/ min, raised to 70, 170, 200 and 2400C, at the rate of 2, 9, 5, 10 min and run time 55 min. Fused silica (Rtx1 fused silica) capillary column (30 m x 0.25 mm ID) was used with nitrogen at a flow rate of 1.84 mL/min. The column temperature was 180o C and the detector temperature was 250o C. The injection was performed in split mode 50:0. [23]

RESULTS AND DISCUSSION

Screening for the production of omega-3 fatty acid

Aspergillus Niger and Trichoderma viride were showed to accumulate lipids in different amounts when they were assessed for the production of omega-3 fatty acid. Interestingly, Trichoderma viride revealed higher concentrations of EPA than DHA such as 3mg/ g of EPA and 2mg/g of DHA (Table 1). The low levels of DHA was reported and such lower levels may be due to the enzyme inactivation in DHA synthesis at room temperature. We also observed the maximum production of EPA in Trichoderma viride than Aspergillus Niger where they produce 2mg/g of EPA and 1.25mg/g of DHA.

Table 1: Screening of *Aspergillus niger*and *Trichoderma viride* to produce lipid

Microorganism	Biomass (g/l)	EPA mg/g	DHA mg/g
Trichoderma viride	13.3	3	2
Aspergillus niger	10.5	2	1.25

Studies reported that several algae are able to produce EPA and DHA, even in large quantities. It was known that each group of organisms have their distinctive fatty acid profile with individual specific biologically important fatty acids which can be act as biomarkers for that typical class of organisms. [24] It was also found that several filamentous fungi may also secrete large quantities of EPA and DHA. [25]

Purification of fungal sample

Acetone purification done in dialysis membrane and this purified sample were taken after 24hour incubation as shown in Figure 1.

Effect of pH on lipid production

Medium pH was the important factor for biomass formation with lipid accumulation. In the present study, optimum pH for *T. viride* was reported with pH 6.0 as determined by Rf value using paper chromatography as shown in Figure 1a and 1b. Moreover, EPA productivity was significantly inhibited when pH was at 7 or above. This result was supported by previous studies. The hydrogen ion concentration (pH) in the medium is essential for fungal growth, sporulation and the plasma membrane permeable property which depends on the pH of the medium. [26, 27]

Figure 1a: Acetone purification through dialysis membrane

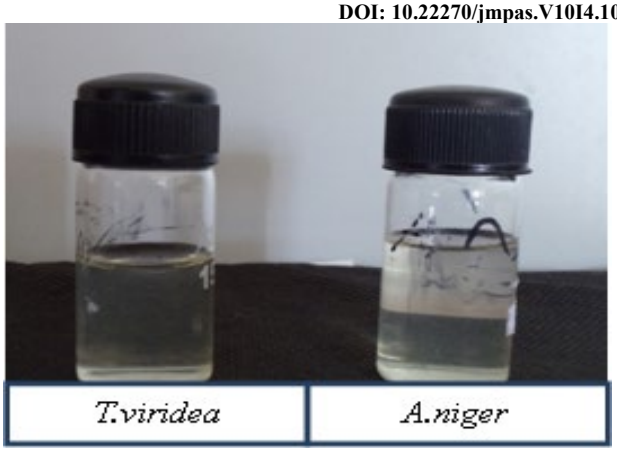


Figure1b: Purified sample of *Trichoderma.viride* and *Aspergillus.niger*

Effect of temperature on lipid production

In the present study, maximum lipid accumulation by *T. viride* was at temperature 40^{0}C and was determined byRf value using paper chromatography as shown in Figure 2.Increase in temperature causes lower level lipid accumulation. Basically, fungi are exposed to a wide range of temperatures and all the fugal enzymes show higher activity at the temperature from 30 to 40^{0}C. [28]

Figure 2: Optimum pH for omega-3 fatty acid production

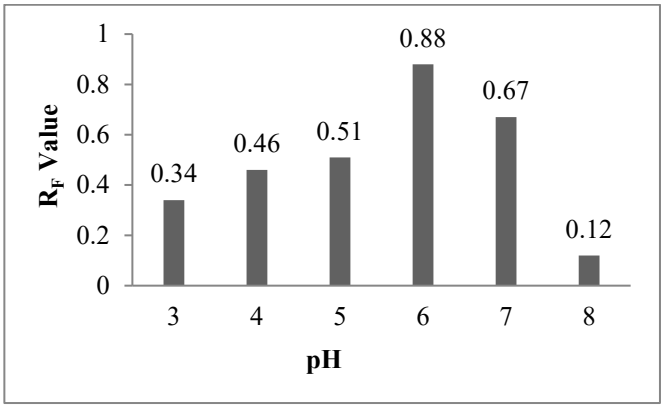
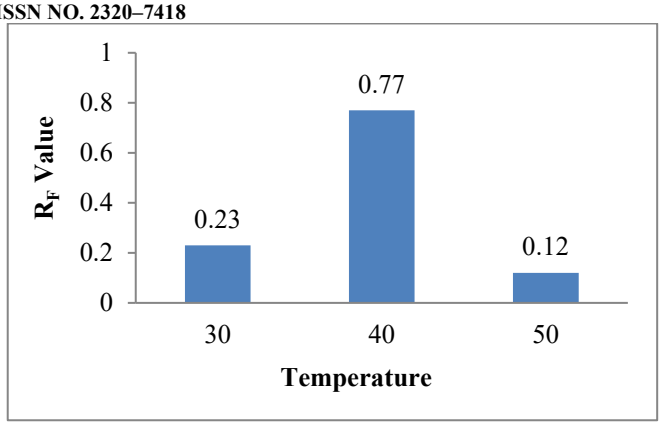


Figure 3: Omega-3 fatty acid production at various temperatures



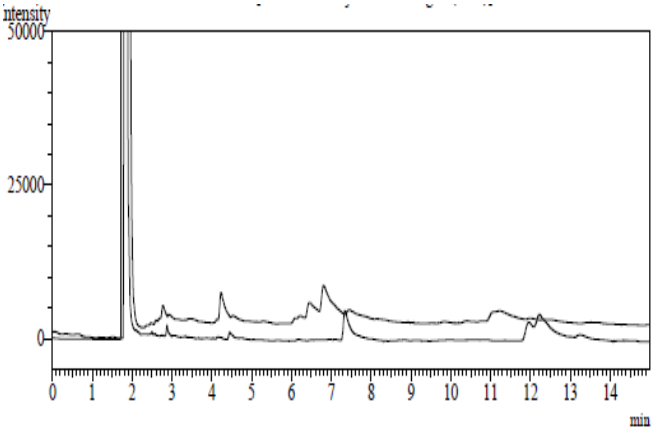
Identification of omega-3 fatty acid concentration

T. viride were found to contain a high fraction of mono and polyunsaturated fatty acids as shown in Table 2. The peak corresponding to retention time 1.49 minute showed mass spectra with molecular mass 256 gram/mol., which is same as the molecular mass of methyl tetradecanoate. Comparison ofFragmentation pattern with general fragmentation ruleof fatty acid confirmed the peak pattern. Fatty acids in the analyzed samples were checked for their observed peak by comparing both the retention time and molecular mass of mass spectra of standard. Such standard was obtained from library (Wiley & NIST) of the GC-MS instrument. The fatty acid concentration by GC-MS analysis was shown in Figure 4. Cardiovascular diseases are the global health threat [29] and identifying the fungal PUFA rich sources is one of efficient way in reducing such disorders.

Table 2: Identification of omega-3 fatty acid concentration from *T. viride*

Peak Value	RT	Fatty acids	Carbon number
1	1.35	Decanoic acid	C10
3	1.49	Tetradecanoic acid	C14
4	6.58	7trans-Hexadecenoic acid	C16
7	7.50	cis-Hexadecenoic acid	C16

Figure 4: Fatty acid composition from *T. viride by GC MS analysis*



CONCLUSION

Omega-3 fatty acids have beneficial effects on cardiovascular health with more anti-inflammatory effects. Fungal species as single-cell oils is a sustainable alternative for PUFA-rich lipids. Such microorganism-based production of fatty acids is reliable and economically attractive. Fungi can be the most promising approach for essential fatty acid production to overcome the global deficits and provide an effective pathway in large-scale production. Research on fungi that produces lipids should be improved in order to supply the fatty acids demand.

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