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Fermentation-Based Biosynthesis of Polyunsaturated Fatty Acids by Endophytic Microorganisms of *Portulaca oleracea*

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ABSTRACT

As the human body is incapable of synthesizing polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), they must be obtained from the diet. Heavy metal contamination of PUFA from fish oils and production limitations of PUFA-producing plants demand the investigation of alternative sources. PUFA produced from microbes has recently attracted great interest, due to the efficiency of synthesis and scalability, and hence could be an alternative source of PUFA. This research used a culture-dependent approach for enumerating microorganisms from distinct plant sections of the plant Portulaca oleracea. Cultures were isolated and evaluated for their potential for producing PUFA using the Bligh and Dyer technique and gas chromatography. This is the first time PUFA-producing microorganisms have been identified from the Portulaca oleracea. Four out of 31 endophytes isolated were found capable of synthesizing PUFAs. Of the selected isolates, RSMAB-1 and MD cultures produce 8.8 and 10.83 mg of PUFA, 3.2 and 2.63 mg of EPA, and 0.12 and 0.25 mg of DHA per 100 mg of fatty acids, respectively. These cultures were identified as Bacillus subtilis and Saccharomyces cerevisiae, respectively. The current primary source of PUFA has limited production capacity, making microorganisms an excellent option for PUFA synthesis. In the current investigation, it was determined that four cultures create distinct forms of PUFA. Two of the chosen cultures had the highest PUFA output. This work emphasizes the significance of screening endophytes from PUFA-producing plants for their capacity to produce PUFA.

INTRODUCTION

Polyunsaturated fatty acids (PUFA) are essential macromolecules with beneficial effects on several physiological processes and a positive impact on human health [1]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two essential forms of polyunsaturated fatty acids (PUFA) that fall under the category of essential fatty acids (EFA) [2]. EPA and DHA serve a significant effect in lowering plasma cholesterol levels and reducing the risk of cancer, while DHA is essential for newborns' eyesight and brain development [3]. According to the 2019 WHO estimate, around 31% of the world's population is afflicted by poor PUFA consumption [4]. Although the human body is capable of synthesising PUFA, the creation of PUFA is limited by poor production rate, the inability to form double bonds, and other factors [2]. Thus, an external supply of PUFA is necessary for the normal functioning of the human body. Existing natural sources of PUFA include walnut,

flaxseed, and soybean oil from plants, and salmon, mackerel, sardines, and herring from fish [5]. Fish oil is the primary commercial source of polyunsaturated fatty acids, despite having disadvantages such as the presence of heavy metals and other pollutants. Current research has focused on oleaginous microorganisms such as microalgae and fungi as an alternative source of polyunsaturated fatty acids (PUFA), which gives flexibility with regard to season, climate change, and raw materials [6]. Yet, the exorbitant cost of manufacturing has been a key deterrent for these microbes. Hence, it is necessary to identify PUFA-producing microbes that can grow readily on inexpensive media and are responsive to all microbial modifications in terms of fermentation.

The key to isolating microorganisms for a certain metabolic activity is the selection of the right source. *Portulaca oleracea* is well well-known PUFA-producing plant [7] and are traditionally used as a wild vegetable in various parts of India [8]. Endophytes are the microorganisms that grow in plant tissues without causing damage, and they provide numerous advantages for the plant [9]. These organisms can reside within the plant cells, in intracellular spaces, or in vascular systems [10] and their number can vary between $\log 10^{2-6}$ CFU/grams. These microorganisms thrive on various nutrients or alkaloids produced by the plants, and can also have the ability to produce similar alkaloids [11]. Hence, this study was designed to isolate endophytic microorganisms from *Portulaca oleracea* and screen for their ability to produce PUFA.

MATERIALS AND METHODS

Sample collection

The common purslane (*Portulaca oleracea*) plant was collected from two different locations in Pune. Maharashtra, after obtaining the state biodiversity board approval (No. MSBB/Desk-5/Research/763/2022-23). The root, stem, and leaf were surface sterilized by washing them with 0.1% $HgCl_2$, followed by 70% ethanol and distilled water before isolation of endophytes. All sterilized samples were separately crushed, and 1 mg of the samples was suspended in sterile distilled water [9].

Culture isolation

The sample suspensions were serially diluted using sterile distilled water, and 100µL of the suspension was spread on De Man, Rogosa, and Sharpe (MRS) media (pH 6.5) and incubated at 37°C for 48 hours. [12-14]. Excellent growth of culture was observed on MRS media, hence it continues for all further studies.

Morphologically distinct colonies were selected and inoculated in MRS broth [15]. After 24 hours of incubation, cultures were centrifuged and suspended in saline to obtain 10^7 CFU/ml. The suspension (1%) was used for all the tests [14,15].

Toxicity assay

Isolates were spot-inoculated on sheep blood agar plates for toxicity assay and incubated at 37°C for 24 hours. The haemolysis pattern on blood agar plates was observed to determine the toxicity of the cultures [16,18,19].

Screening for fatty acid production

Sudan black B staining method

Selected microorganisms were stained with Sudan Black B and microscopically observed for the presence of blue or greyish fat globules inside the cells under oil immersion [20]. Bacterial strains displaying fat globules inside the cells were selected for further studies.

Colorimetric screening for PUFA production

Presence of PUFA in the supernatant was detected by using the method described by Gad et al. (2016). Accordingly, 0.1 % w/v of 2, 3, 5-triphenyl tetrazolium chloride (TTC) dye was added to the culture supernatant and incubated for one hour at 25°C. The red colour formed by the reduction of TTC indicated the presence of FA and can be seen by visual observation [21,22] against fish oil as a control. As a screening test for the production of microbial FAs, TTC has been used. There is a direct correlation between the potential for growth in broth containing TTC, the ability to convert TTC to Triphenyl formazan (TF), indicates the production of fatty acids [22].

H_2O_2 - plate assay method

All the selected cultures were grown in LB agar medium for 24 hours at 37°C. Microbial cultures (1 Optical Density at 600 nm) were spread over an LB agar plate containing 1mM sodium azide. On the surface of the agar, paper discs of diameter 5 mm were placed, and 10 µl aliquots of solution containing different concentrations of H_2O_2 (0.01, 0.5, 1.0 % prepared from 6 % of the stock solution) were added on filter disc. Plates were incubated for 24 hours at 37°C and observed for a zone of inhibition [22].

Extraction of lipids

Lipid extraction from microbial isolates was carried out as per the method given by Bligh and Dyer (1959). Accordingly, 1 ml of culture suspension in distilled water was disrupted for 100 milliseconds by electroporation with 10 pulses of 20 kV/cm. The total lipid was extracted by vigorously vortexing the electroporated cell suspension in the mixture of 1 ml of water and 4 ml of a 2:1 chloroform: methanol

solution containing 15 mg/ml of butylated hydroxytoluene (BHT). The vortexed mixture was then centrifuged for 10 min at 5000 rpm. The lipid-containing chloroform layer (lower layer) and the total lipid content concentration were calculated after drying the content in an Eppendorf tube [22, 23].

Fatty acid methyl ester preparation

The Supernatant and culture pellets were suspended in 1 ml of 0.5 N methanolic HCl and heated in sealed glass tubes for 4 hours at 40°C. The tubes were cooled for 30 minutes at room temperature, then 1 ml of distilled water was added and vortexed the tubes. The chloroform layer (lower layer) was transferred to separate fatty acid methyl esters (FAME), evaporated, and dissolved in 0.05 ml hexane, and these FAME were used for GC analysis [22, 23].

Gas chromatography (GC) analysis

The lipid profile of the samples was analysed using an Agilent GC system equipped with a capillary column (Suplecowax 10, 30 m x 0.25 mm, 0.25 m film thickness) and a flame ionization detector. Helium at a flow rate of 1.5 ml/min was used as carrier gas. The injector was maintained at 250°C, and a 1 L sample with a 50:1 split ratio was injected. From 140°C (5 minutes hold) to 240°C, the oven was programmed at a rate of 4°C/min (10 minutes hold). By comparing the retention period of a sample with the external standards, fatty acid peaks were identified. Using chromatographic instruments and theoretical relative FID response factors, peaks were quantified and corrected by manual integration. [22, 23].

Molecular identification of isolates

Whole genomic DNA was extracted using the genomic DNA isolation kit (Sigma, USA) and used as the PCR template in accordance with the manufacturer's instructions. For each dCTP, dGTP, dATP, and dTTP reaction combination, including 10 ng of DNA, 2.5 mM MgCl2, 1x PCR buffer, and 10 ng of dGTP, dATP, and dTTP-specific DNA; 2.5 mM Mgcl (Bangalore Genei, Bangalore, India), 200 M; 2 per mol ABI Prism Big Dye Terminator Cycle Forward and reverse primers from a sequencing reaction kit were used to sequence the PCR result. A combination of universal primers FDD2-RPP2 (universal primers for 1.5 kb fragment amplification for eubacteria) was used for sequencing the virtually finished genome. The ABI Prism 3100 Genetic Analyzer was processing samples while the DNA sequence analyser software evaluated the sequencing performance. The sequence was compared to National Centre for Biotechnology Information GenBank entries using the BLAST programme [25].

Statistical analysis

All experiments were performed in triplicate. One-way ANOVA and the PRISM software were used to analyse the data, and the results were expressed as the mean and standard error. Statistical significance was determined when p 0.05 (p > 0.05 = ns, p 0.05 = *, p 0.01 = **, p 0.001= ***).

OBSERVATION AND RESULTS

Plant sample

The plant *Portulaca oleracea* (Fig. 1), together with root, leaf, and stem, was collected from two different locations in Pune, India. Location 1 Supe, Saswad Pune (18.303339,74.011310) and location 2 Hadapsar, Pune (18.489515, 73.992467). In all, 31 cultures were isolated from the samples, including 19 cultures from location 1 and 12 from location 2 (Table 1). Morphologically distinct colonies were selected for further characterization.

Table 1: Number of cultures isolated from different locations

Samples *Portulaca oleracea*	Root	Stem	Leaves	Total
Location 1 Saswad, Pune	8	6	5	19
Location 2 Hadapsar, Pune.	6	4	2	12

Toxicity (Hemolytic) assay

A total of 31 cultures were used for the toxicity test using sheep blood agar, in which 23 cultures were found to be non-haemolytic and showed γ lysis on the sheep blood agar plate.

Screening of fatty acid production: Sudan Black B

Those cultures that showed pink exterior and bluish black intracellular granules were considered positive for lipid production. Cultures with bluish black lipid granules, when observed under microscopy, were selected for further studies (Fig. 2A and 2B). The cultures that showed more than half of the cells with lipid granules under 100X magnification were identified as strongly positive cultures, while fully pink cells were considered negative. A total of 23 cultures were screened, and only 12 isolates were found to be strongly positive for lipid accumulation.

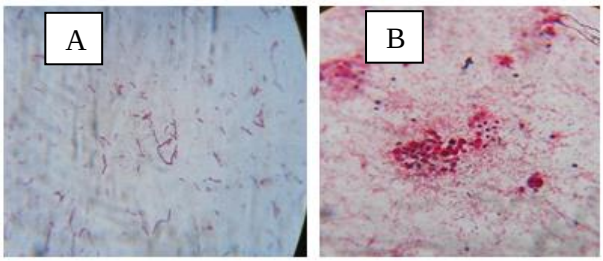

Figure 2: Sudan Black B staining (Microscopic image) A. RSMAB1 and B. MD

H_2O_2 Plate assay

The hydrogen peroxide plate assay is based on the ability of FAs to reduce the inhibitory effect of hydrogen peroxide added externally to the plate. Normal cells are susceptible to externally added hydrogen peroxide, resulting in a zone of inhibition around the wells in the plate. As given in the figure 3, there was no inhibition zone around the cultures designated as MD, PP6C, RSMAB-1, and BPRC32. A total of 4 isolates could grow even after the addition of hydrogen peroxide formed no inhibition zone, indicating the ability to produce FAs.

Sr. no.	Cultures	Total PUFA	DGLA	AA	EPA	NA	DHA
1	BPRC32	7.5	4.28	0.28	2.31	0.44	0.19
2	RSMAB-1	8.8	4.32	0.45	3.2	0.71	0.12
3	MD	10.93	4.94	2.69	2.63	0.42	0.25
4	PP6C1	7.69	3.7	0.05	2.28	1.55	0.11

Figure 3: H_2O_2 Plate assay

Table. 2 Fatty acid profile of cultures by GC analysis

Colorimetric assay

All 4 cultures showed development of various shades of red colour, indicating the presence of FA. Based on all *in-vitro* screening assays, 4 isolates were chosen for GC analysis, which indicated the presence of various FAs in differing amounts.

Gas chromatography (GC)

Cultures were lysed in an electroporator, and methyl esters were prepared by the chloroform-methanol extraction method. All 4 cultures showed various forms of PUFAs in GC spectra (Table 2 and Figure 4).

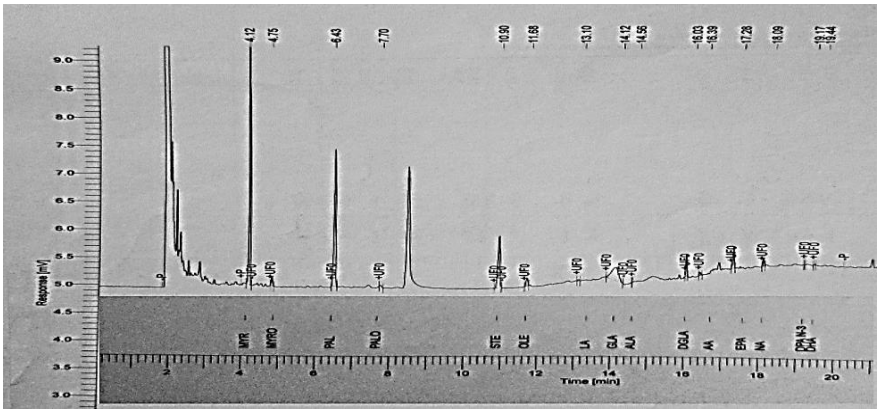

Figure 4-A: GC Chromatogram of culture BPRC32

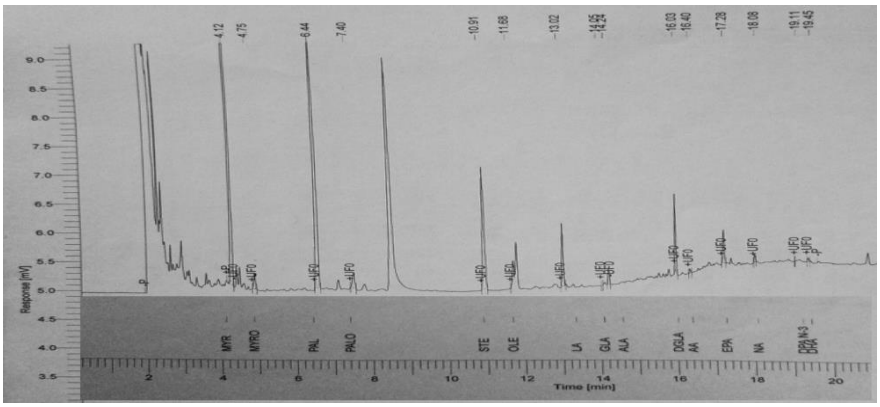

Figure 4-B: GC Chromatogram of culture RSMAB1

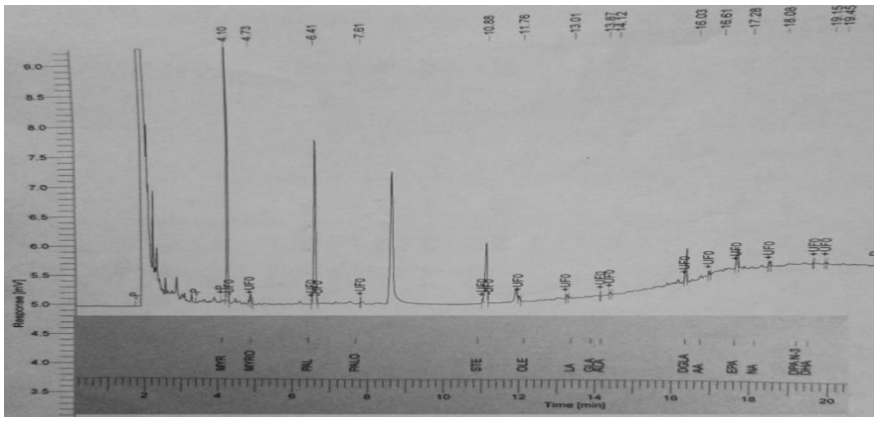

Figure 4-C: GC Chromatogram of culture MD

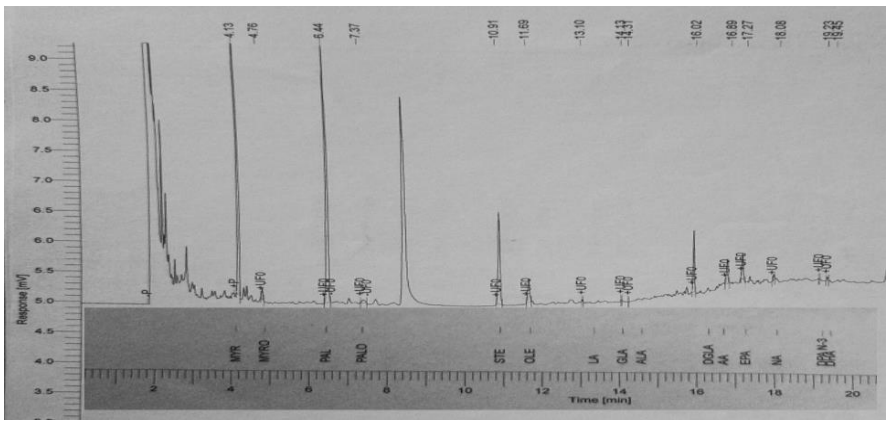

Figure 4-D: GC Chromatogram of culture PP6C1

Molecular Identification

The best two cultures, as per the gas chromatography data, were selected for molecular identification. Culture, RSMAB-1, and MD were identified as *Bacillus subtilis* and *Saccharomyces cerevisiae*, respectively. The sequence was then submitted to the NCBI and DDBJ databases with accession no. MW368768 and LC528142 (Table 3).

Sr.	Strain	Identified as	Similarity in %	Submitted in	Accession no.
1	RSMAB-1	*Bacillus subtilis*	99	NCBI	MW368768
2	MD	*Saccharomyces cerevisiae*	99	DDBJ	LC528142

Table. 3 Molecular identifications of isolates and accession numbers

DISCUSSION

The PUFA production by *Portulaca oleracea* has been well studied by various groups, and it has been identified as an important source of plant-based PUFA [26]. The plant produces various types of fatty acids, such as oleic acid, linoleic acid, and alpha-linolenic through pathways that are well characterized. It has been well documented that there is a strong metabolic connection between the plants and their endophytes; moreover, various phytogenic analyses have proven that metabolic activities or genes can be transferred from plants to endophytes [11]. Hence, endophytes from PUFA-producing plants are an ideal candidate for screening for PUFA production.

A total 31 endophytes isolated from *Portulaca* plants were subjected to various screening, such as toxicity assay using sheep blood agar, presence of fatty acid by Sudan black B staining, and hydrogen peroxide assay, and finally GC analysis. Among the screened cultures, 23 were found to be non-hemolytic and further screened for fatty acid production by the Sudan black B staining method [20]. Strongly positive, 12 cultures were screened for the H_2O_2 plate assay. Five cultures showed no zone of inhibition, indicating the presence of FA. The H_2O_2 plate assay was used to select PUFA-producing bacteria through direct visualization as a reliable method for screening a large number of strains in a short amount of time [22]. Through direct visualization, the oxidative stability of PUFAs in growing bacteria in response to added H_2O_2 is a distinguishing characteristic between PUFAs producers (no zone of inhibition) and non-PUFAs

producers (zone of inhibition). Further, these cultures were screened using the 2,3,5-triphenyl tetrazolium chloride (TTC) colorimetric assay for the reduction of TTC to a red-triphenyl formazan by FAs [21]. TTC has been used as a screening test for the production of bacterial FAs. There is a clear connection between the ability to grow on TTC-containing broth, the ability to reduce TTC to TF, and the ability to produce FAs. The enzyme 5-desaturase has been reported [21,22] to be responsible for the reduction of the colorless TTC to the red TF. According to the intensity of the colour, the appearance of a change to red colour has indicated a positive outcome in terms of the number of FAs produced by the culture. Among the screened cultures, only 4 cultures had various red colour intensities. From all the fatty acid screening assays, 4 cultures were selected for further screening of fatty acids by GC. The gas chromatography technique is generally used for the detection of fatty acids. A total of 4 cultures were screened for fatty acid profile, and all of the cultures showed a significant amount of microbial polyunsaturated fatty acids (Figure 4 and Table 2).

The best two cultures which has maximum PUFA-producing ability were selected for molecular identification. RSMAB-1 is identified as *Bacillus subtilis* and submitted to NCBI with accession of MW368768, whereas MD is identified as *Saccharomyces cerevisiae and* submitted to DDBJ with accession number LC528142.

CONCLUSION

Fish such as salmon, mackerel, sardines, and herrings, etc., and plants such as walnut, flaxseed, and soybeans, etc., are the present primary sources of PUFA. Microorganisms are a suitable option for the synthesis of polyunsaturated fatty acids (PUFA) due to environmental contaminants and the limited production capacity of other sources. In the current work, 31 endophytes were isolated from the PUFA-rich Portulaca oleracea plant. Just four of the isolated cultures that were evaluated for toxicity and the potential to create PUFA were found to produce distinct types of PUFA. Two of the chosen cultures had the highest PUFA output. - RSMAB-1 has been recognised as *Bacillus subtilis*, whereas MD has been identified as *Saccharomyces cerevisiae*. This work emphasises the significance of testing endophytes from PUFA-producing plants for their ability to produce PUFA.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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