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Isolation and Characterization of Gene for Expression of Delta 9 acyl-lipid Desaturase from *Nostoc sp.* Involved in Lipid Production

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Algae have received global attention as a renewable resource of biodiesel and may play an important role as a component contributing to the economic growth of the country. A variety of blue-green and green algae are found in the wetlands of various regions of India, however, they have hitherto remained untapped. Present research work deals with the isolation of gene responsible for expression of delta 9 acyl-lipid desaturase from *Nostoc sp.* which is a potent source of lipids that can be further utilized in biodiesel production. Due to the ever increasing fuel need it is of great importance to find an alternative feedstock for energy source. The above mentioned gene was then sequence characterized. The ORF sequence was identified in the gene sequence of Acyl lipid desaturases from *Nostoc sp.* Eight similar sequences were also identified which are present in different organism. The ORF sequence belongs to the Functional domain (Membrane_FADS) –like superfamily. Four different motifs of different length were also identified.

Keywords: 9 acyl-lipid desaturase, *Nostoc* sp., biodiesel.

The world is about to face the predicament in energy sources. The consumption of fossil fuels and the available energy sources are the main problems needed to be solved. This critical situation that the world is about to face is a threat to modern human existence. Energy crisis will arise when there will not be enough energy sources to survive the demands of the world as the demand for energy increases. The focus is on the biomass energy and there are biochemical processes for conversion of biomass into biological hydrogen gas, biodiesel and ethanol which are the possible biofuels of future generation. Algae are simple autotrophic organisms and from simple inorganic molecules such as carbon dioxide they produce complex organic compounds using energy from light or inorganic chemical reactions. These complex organic compounds include a significant amount of triacylglycerol which can be utilized to produce biodiesel using the process known as Transesterification. Thousands of forms of algae have been identified, but some algae strains are more suitable for biofuel production because of their high oil yield. Genetically modified strains of algae are being developed for algae biofuels for yielding high lipid-content algae. Some companies have developed algae strains with unique characteristics for example, strains of algae that produces ethanol at a rate of over 6,000 gallons per acre per year and genetically modified algae that grows in the dark. This is only possible if we identify the genes responsible for lipid production in algae. Oil producing(Oleaginous) microalgae like *Nostoc sp.* are good sources of lipids for the production of biodiesel. Long-chain polyunsaturated fatty acids (PUFA) synthesis pathways are not very well known in *Nostoc sp*

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and they affect the production of biodiesel. Acyl lipid desaturases are fundamental enzymes in PUFA synthesis pathway. These enzymes convert a saturated bond into an unsaturated bond between carbon molecules in fatty acids and they are further esterified to glycerolipids. (Sing *et al.,* 2011).

In the present research work gene responsible for the expression of delta 9 acyl-lipid desaturase of *Nostoc* sp. was isolated and its sequence characterization was performed. Delta 9 acyl-lipid desaturase is known for converting saturated fatty acids into unsaturated fatty acids that is there are involved in the production of unsaturated fatty acids.The ORF sequence was identified in the gene sequence of Acyl lipid desaturases from *Nostoc* Sp. Eight similar sequences were also identified which are present in different organisms. The ORF sequence belongs to the Functional domain (Membrane_FADS) – like superfamily. Four different motifs of different length were also identified.

MATERIALS AND METHODS

The algal strains namely *Nostoc* sp., *Vaucheria* sp., *Diatom* sp., *Microcystis* sp. and *Oedogonium* sp. were screened for fast growth and high lipid content. Algal sp. which was the highest in lipid production was used for sequencing and genome analysis.

Isolation of algae DNA and Sequencing of lipid producing gene

Genomic DNA extraction from the algal sample was performed using the method given by Huang *et al.* (2000). For the DNA extraction the algae cells were harvested by centrifugation at 5000 rpm for 10 minutes. The pellet cells were frozen in liquid nitrogen prior to grinding in pre-chilled pestle and mortar. Powdered microalgal mass was immediately transferred to falcon tube containing 3ml CTAB buffer (pre-incubated at 60^0C) and was incubated in water bath at 60^0C for 1 hour with intermittent shaking of the tube to allow the DNA to be released from the cells. Then, an equal volume (3ml) of chloroform:isoamylalcohol (24:1) was added to the tube. The tube was slightly shaken and centrifuged at 10,000 rpm for 10 minutes at 40^0C. The supernatant was collected in a fresh tube and double the volume of chilled isopropanol was added to the tube. The tube was again centrifuged at 14,000 rpm for 10 minutes at 4^0C. The supernatant was carefully removed and equal amount of chilled 70% ethanol was added to the tube and centrifuged at 14,000 rpm for 5 minutes. Finally, ethanol was carefully removed and the pellet was air dried in the laminar flow hood. The pellets were suspended in 100µl TE buffer (10mM Tric-HCl, pH 8.0; 1mM EDTA, pH 8.0) and stored at 4^0C.

Quantification of Isolated DNA

he quantity of the isolated DNA was checked in UV-VIS spectrophotomer (Vivaspec Biophotometer, Germany). From the stock 1¼l DNA was mixed with 49-¼l sterile distilled water to get 50 times dilution. DNA concentration was determined by the equation:

1 OD260 unit = 50 µg/ml (1 ng/µl =1 µg/ml).

The A260/A280 ratio was recorded to check the purity of DNA preparation. (Desjardins and Conklin, 2010)

Primer Designing and synthesis

The gene selected for the primer designing was delta 9 acyl-lipid desaturase with an amplicon size of 1324 bp. The specific primers were designed using Primer Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and the designed oligonucleotides were synthesized in Sigma Corporation USA.

PCR Amplification

For the amplification of DNA, PCR was used. Table 1 shows the reagents used in the PCR reaction mixture and Table 2 shows the temperature profile used for the PCR.

Genomics of the selected algae sample

Gene named, delta 9 acyl-lipid desaturase was nucleotide sequenced from the selected algal sample which was a good source of lipid for the production of biodiesel. Acyl lipid desaturases are fundamental enzymes in PUFA synthesis pathway. These enzymes convert a saturated bond into an unsaturated bond between carbon molecules in fatty acids and they are further esterified to glycerolipids.

Sequence analysis

Sequencing of delta 9 acyl-lipid desaturase gene was carried out by Sanger sequencing method (Sanger and Coulson, 1975) using ABI automated DNA sequencer. Following sequence analysis tools were used:

1. Open reading frame (ORF) was predicted by using ORF finder (http://www.ncbi.nlm.nih.gov/

gorf/gorf.html).

2. The identification of predicted ORF protein is carried out by using BLASTP server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at National Center for Biotechnology Information (NCBI). Five most identical sequences were identified.

3. Sequence alignment and phylogenetic analysis was carried out by using ClustalW server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) at European Bioinformatics Institute (EBI).

4. The amino acid sequence of the selected enzymes were further used to identify the presence of conserve domain with the help of the conserve domain identification tool. The NCBI conserved domain database search was performed with NCBI conserved domain search program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer *et al*).

5. The search was performed at E-Value 0.01 and the database used for conserve domain is CDS search with tool cddv2.25. The motifs were predicted using motif search server at http://www.genome.jp/tools/motif/

RESULTS AND DISCUSSION

The algal strains namely *Nostoc* sp., *Vaucheria* sp., *Diatom* sp., *Microcystis* sp. and *Oedogonium* sp. were screened to find the best candidate for lipid production and among the above five strains *Nostoc* sp. was the most potent strain for lipid production. Therefore *Nostoc* sp. was used for sequencing and further analysis.

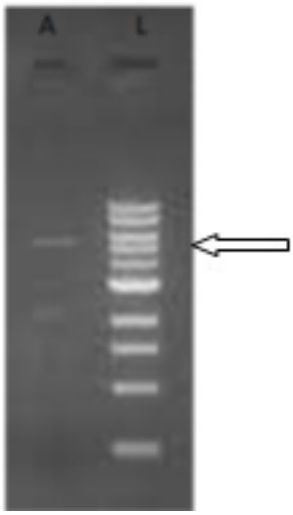
Isolation of algae DNA and Sequencing of lipid producing gene

Genomic DNA isolated from the purified cultures of microalgae was run in 0.8% Agarose gel to verify the quality of the DNA. Figure 1 shows the PCR gel picture of the DNA sample with the ladder. Arrow in the figure signifies the size of the gene in comparison to the ladder i.e. 1300bp. comparison to the ladder i.e. 1300bp) Jin *et al.* (1997) tested 70 species of brown, red, and green algae for DNA quality using PCR. Hong *et al.* (1997) found that DNA extracted from most green algae by the CTAB method were of sufficient quality to be used as a template for PCR amplification.

Quantification of Isolated DNA

The concentration of the isolated DNA was measured to be 325ng/ ¼l and OD ratio (A260/280) was estimated to be 1.82. This signified DNA to be pure with very minor RNA contamination.

Assessment of purity of DNA is an important step. The most commonly used assay is the A260/A280 ratio. DNA absorbs almost twice



PCR GEL PIC with laddder (L-1.5KB and sample (A))

Fig. 1. PCR gel picture of algal DNA (arrow signifies the size of the gene in comparison to the ladder i.e. 1300bp)

Table 1. Reagents and the optimal PCR reaction mixture

PCR components	Volume (µl)
Nuclease free water	10.75
10X reaction buffer with $MgCl_2$ (1.5mM)	2.00
dNTP mix (2.5mM)	2.00
Primer I (10picomoles/ ¼l)	2.00
Primer II (10picomoles/ ¼l)	2.00
Taq DNA polymerase (5U)	0.25
Template DNA (50ng/ ¼l)	1.00
Total volume	20.0

Table 2. PCR temperature profile

Initial denaturation	94°C for 2 min	
Denaturation	94°C for 1min	
Annealing	48°C for 30 s	30 cycles
Extension	72°C for 2min	
Final extension	72°C for 6 min	

as much UV at 260 nm as it does at 280 nm. If there is no protein present, the target ratio is 1.8. However, because 280 nm is a peak for protein absorption, protein contamination will increase the A280 reading but have little effect on the A260 reading. Thus the A260/A280 ratio will be lower than 1.8. (Desjardins and Conklin, 2010).

Primers used

To isolate the gene delta 9 acyl-lipid desaturase PCR was run with the following primers:

Forward
(5' CCTGAGAGA GGCAATT CA CC3') and
Reverse primer
(5'TGAGGGGGACTTGAAACAAC 3').

Sequencing

Gene obtained as PCR product was sequenced. Sequence was 1324 bp long. The isolated delta 9 acyl-lipid desaturase nucleotide sequence in FASTA format is given below.

>gi Nostoc sp. 36 desC2 gene for delta 9 acyl-lipid desaturase, strain 36 C C TGAGAGAG GCAATTCACCTCAACTCAGGTGGATAAATG T G G T A T T T T T T G G T G T A T T C C A T G C C T T A G C A C T T C T G T C T C C T T G G T T T T T C T C T T G G T C A G C A T T A G G T T T A C T A G T G T T T C T G C A C T G G T T A T T C G G G A G C A T T G G T A T T T G C T T G G G A T A T C A C G A C T A C T G A G C C A T A A G A G T T T C C A A G T T C C T A A G T G G T T A G A G T A T G C G A T C G C C C T T A T T G G A G C G C T G G C T T T G C A A G G T G G G C C A A T T T T T T G G G T A G G T G G A C A C C G C C A G C A T C A C G C C C A C A C G G A A G A T A T T G A C C T A G A T C C C T A T T C C G C C C A A A A A G G A

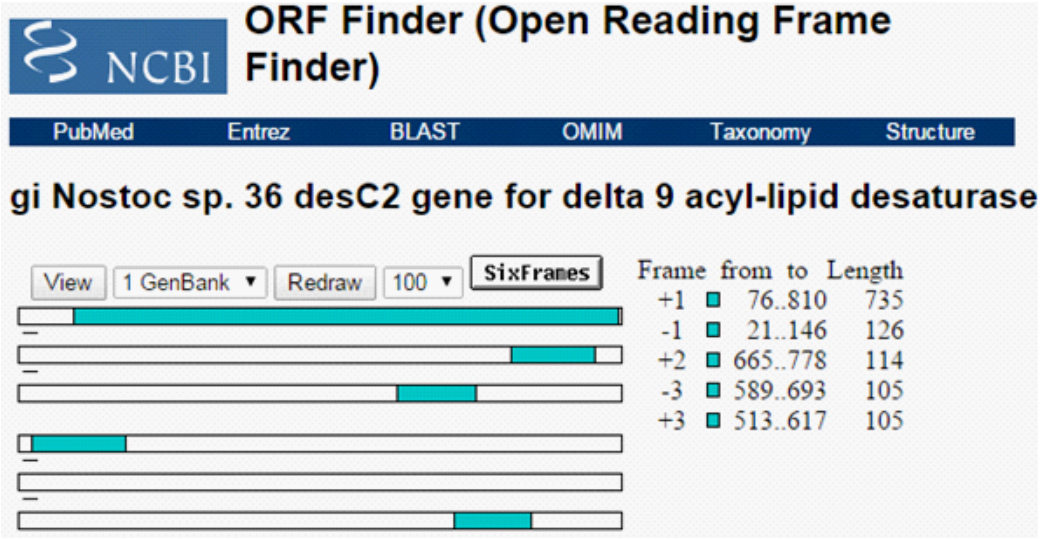


Fig. 2. ORF predicted for the isolated gene sequence

Table 3. List of enzymes selected for sequence similarity among species known for lipid production

S.no	Accession No.	Enzyme	Organism
1.	WP_012410332.1	delta-9 desaturase	*Nostoc punctiforme*
2.	WP_015139216.1	fatty-acid desaturase	*Nostoc* sp. PCC 7524
3.	WP_010999117.1	delta-9 desaturase	*Nostoc* sp. PCC 7120
4.	WP_016949240.1	delta-9 desaturase	*Anabaena* sp. PCC 7108
5.	WP_015151947.1	fatty-acid desaturase	*Oscillatoria acuminata*
6.	WP_009555370.1	fatty-acid desaturase	*Oscillatoriales cyanobacterium* JSC-*12*
7.	WP_015175264.1	Delta-9 acyl-phospholipid desaturase	*Oscillatoria nigro-viridis*
8.	WP_015177399.1	Stearoyl-CoA 9-desaturase	*Oscillatoria nigro-viridis*

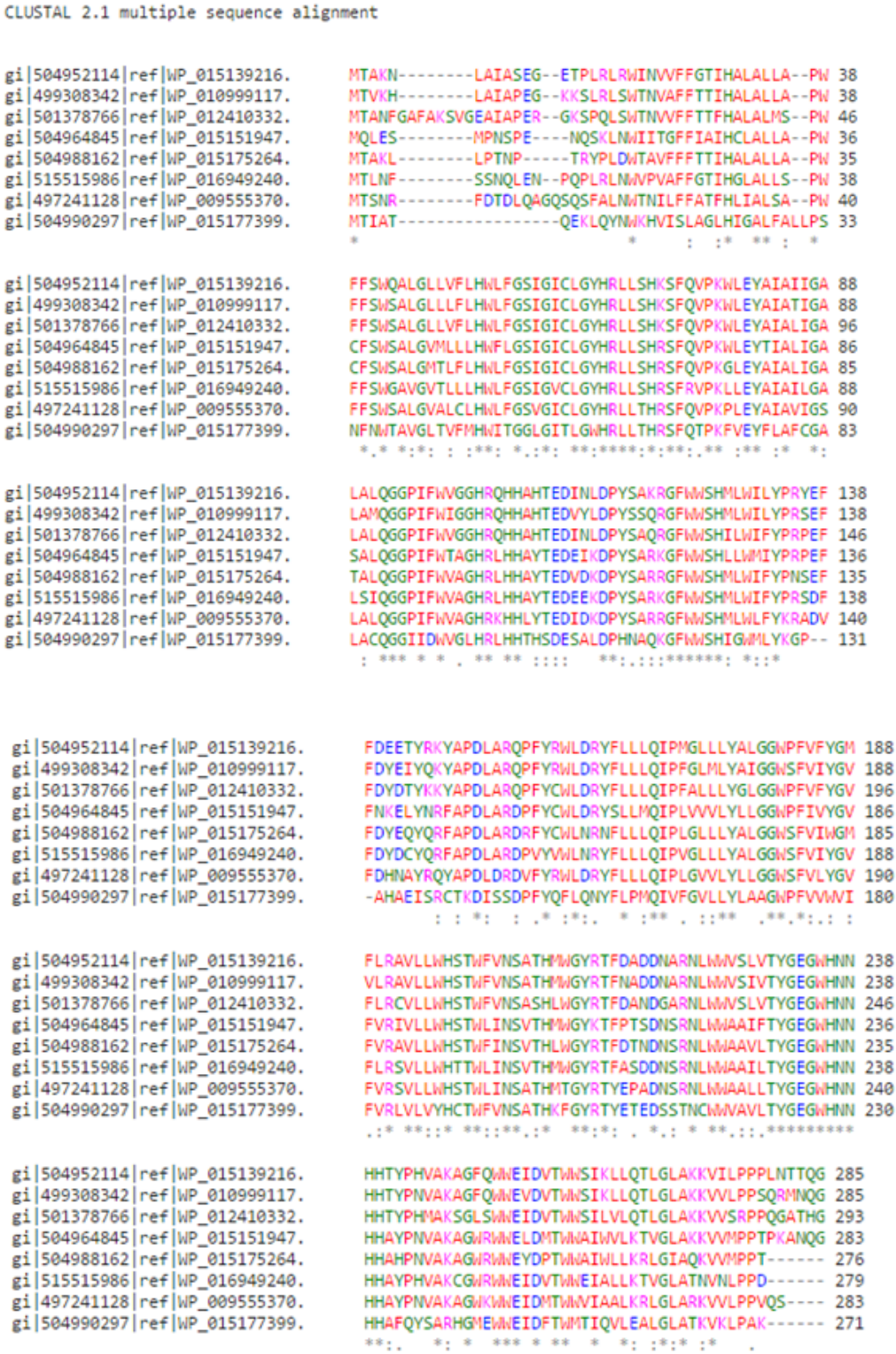


Fig. 3. Sequence alignment of the selected sequences

T T T T G G T G G A G C C A T A T A
C T A T G G A T T T T C T A C
C C G C G C C C A G A A T T T T T T G
ACTATGACACCTATAAAAAATATGCTCCT
GACTTAGCAAGA CAACCCTTTTAT
T G C T G G C T G G A T C G C
TACTTCTTGCTGTTGC AAATACCCTTTGCG
C T G C T G C T A T A C G T C T T A
G G A G G A T G G C C T T T C G T A T T C
TACGGAGTGTTTCTCAGATGTGTAC
T G C T T T G G C A C T C A A C C T G G
T T T G T G A A C T C A G C A T C A C A T C T G
TGGGGTTATCGCACCTTTGATGCTGATGATG
GCGCTCGTA ATCTTTGGTGGGTATC
C A T C G T A A C T T A T G G A
G A A G G A T G G C A C A A T A A C C A T
C A T A C T T A T C C C C A C A T G G C
G A A A T C T G G G T T G T T T T G G T G G
G A G A T T G A T G T T A C T T
G G T G G A G C A T C C A A C T T T T
G C A G A C T T T A G G T T T A G C C A
AAAAAGTTGTTTCAAGTCCCCCTCA

ORF detection

An ORF is a sequence of DNA that starts with start codon "ATG" (not always) and ends with any of the three termination codons (TAA, TAG, TGA). Depending on the starting point, there are six possible ways (three on forward strand and three on complementary strand) of translating any nucleotide sequence into amino acid sequence according to the genetic code .These are called reading frames. Eukaryotic gene finding is

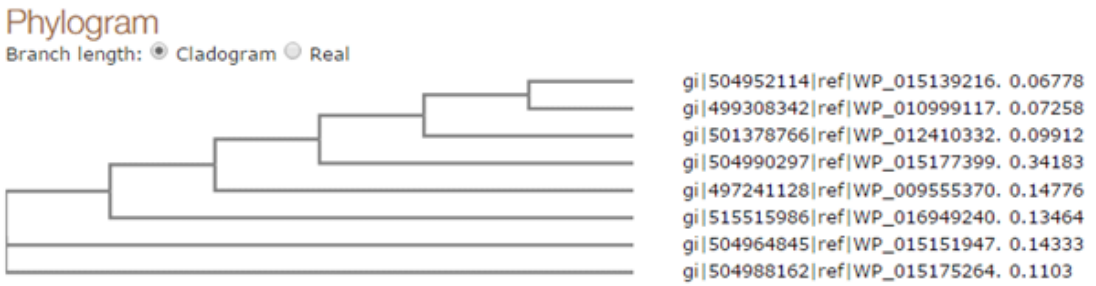


Fig. 4. Phylogenic tree of the selected sequences

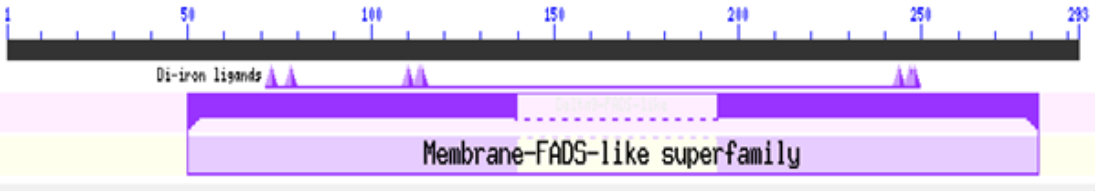


Fig. 5. Predicted domain in the sequence

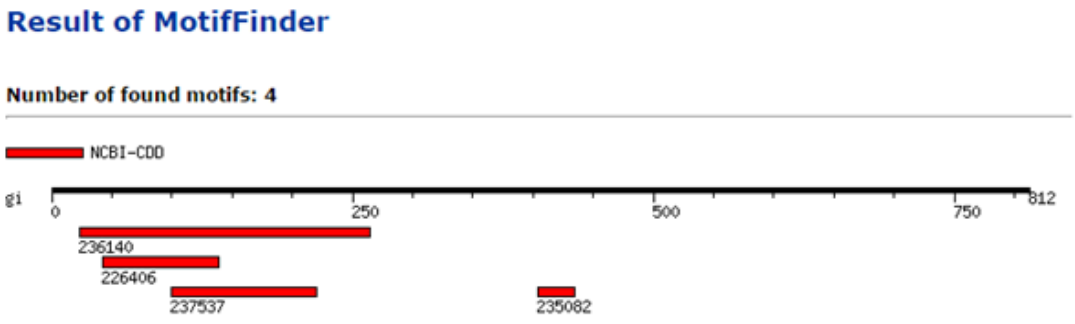


Fig. 6. Predicted motif in the sequence

Table 4. List of motifs predicted in ORF sequence

S. No.	NCBI-CDD I.D.	Position (base pair)	Length (base pair)	Description
1.	236140	24..265	241	PRK08026, PRK08026, flagellin; Validated.
2.	226406	43..139	96	COG3889, COG3889, Predicted solute binding protein [General function prediction only].
3.	237537	99..220	121	PRK13875, PRK13875, conjugal transfer protein TrbL; Provisional.
4.	235082	404..435	31	PRK02888, PRK02888, nitrous-oxide reductase; Validated.

altogether a different task as the eukaryotic genes are not continuous and interrupted by intervening noncoding sequences called 'introns'.

The ORF present in the isolated sequences was predicted. Total five ORF were identified having sequence length 244, 37, 34, 41 and 34 amino acids Figure 2. The first predicted ORF sequence having amino acid length 244 amino acids is selected based on its length. Based on BLASTP analysis of this predicted sequence was identified as delta 9 acyl-lipid desaturase (*Nostoc* sp. 36).

Based on BLASTP analysis of delta 9 acyl-lipid desaturase [*Nostoc* sp. 36] six similar sequences were identified Table 3.

When two symbolic representations of DNA or protein sequences are arranged next to one another so that their most similar elements are juxtaposed they are said to be aligned. Multiple Sequence alignment of selected sequences was performed. It was found that sequence similarity was between 46 percent to 85 percent Figure 4.

Based on phylogenetic analysis it was found that the Fatty-acid desaturase (WP_015139216.1) is most identical with predicted ORF in the sample sequences . The other aligned sequences were clustered in different groups. Fatty-acid desaturase (WP_015139216.1) was clustered with Delta-9 desaturase (WP_010999117.1). Delta-9 desaturase (WP_012410332.1) was found more similar with WP_015139216.1 and WP_010999117.1. Simillarly Stearoyl-CoA 9-desaturase (WP_015177399.1), Fatty-acid desaturase (WP_009555370.1), Delta-9 desaturase WP_016949240.1 and Fatty-acid desaturase (WP_015151947.1) and Delta-9 acyl-phospholipid desaturase (WP_015175264.1) were also found related with the query sequence and clustered in different groups Figure 5. The analysis of fatty acids in different clade should provide useful information to establish the functional role of the enzyme.

The putative functional domain was also predicted. It was found that the sequence belongs to the (Membrane_FADS) –like superfamily(Figure 6). Membrane FADSs play an important role in the maintenance of the proper structure and functioning of biological membranes as they play key role in are involved in the initial oxidation of inactivated alkanes (Marchler-Bauer *et al.*, 2011).

It was reported that acyl-lipid desaturases contain three histidine clusters, whose structures are unique to individual classes of acyl-lipid desaturases. They are related to the specificity of individual desaturases to the position of carbon atoms in fatty acids at which unsaturated bonds are introduced (Chintalapati *et al.,*2006).

Motif

Sequence motifs are short, recurring patterns in DNA that are presumed to have a biological function. Often they indicate sequence-specific binding sites for proteins such as nucleases and transcription factors (TF). Others are involved in important processes at the RNA level, including ribosome binding, mRNA processing (splicing, editing, polyadenylation) and transcription termination. Four motifs of different length and positions were identified in the predicted ORF sequence Figure 7. It was found that length of motif were between 31 to 241 base pairs Table 4. All motif are involved in different types of biological activity (Marchler-Bauer *et al.*, 2009).

Metabolic engineering with the gene manipulation technique can be a significant approach to improve the function of enzymes playing key role in fatty acid.

CONCLUSION

The gene sequence of Acyl lipid desaturases from *Nostoc Sp.* was analyzed. It's computational characterization revealed that it belongs to Functional domain (Membrane_FADS) –like superfamily. It was also found that Acyl lipid desaturases is involved in the production of lipids. This study provides information that will help in the regulation of the fatty acid profile and functional characterization of Acyl lipid desaturases involved in the lipid production which can further be used for biodiesel production.

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