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Rbcl Marker Based Approach for Molecular Identification of Arthrospira and Dunaliella Isolates from Non-Axenic Cultures

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ABSTRACT

Population escalation and energy crises have increased the urge to find renewable energy sources with higher sustainability index. Microalgae are potential candidates for biofuel feedstock production without utilizing fertile land and drinking water owing to their efficient oil production pathways and high value bio molecules. Morphological identification of potential microalgae species needs accurate molecular validation due to the versatility in nature, however PCR based molecular identification requires pure and axenic culture of isolates in universal primers based gene targets. In present study, monospecies algal culture were directly used to extract DNA followed by PCR amplification of 18S rDNA gene target using universal primers ITS1F-4R and rbcL gene target using primers designed for species specific gene. rbcL primers successfully amplified specific microalgae gene. Resulting sequences were annotated using multiple sequence alignment with Genebank databse and phylogenetic relationship study. These rbcL gene primers and validated PCR conditions can be used for non-axenic monospecies green microalgae isolates for easy and efficient molecular identification.

Keywords: *Arthrospira ; Dunaliella; Microalgae; Molecular identification; non-axenic culture; rbcL gene marker.*

INTRODUCTION

Fossil fuel sources are finite and exhaustible, unable to meet the increasing demand of energy [1]. Photosynthetic microscopic microalgae have potential for biofuel feedstock production with higher rate of CO2 sequestration utilizing high salt water from industrial effluents as well as coastal water in diverse climatic conditions [2]. Such high sustainability index of microalgae is favorable for its industrial application [3]. Identification of these locally adapted microalgae is essential for industrial application because value chain of microalgae varies from species to species [4]. Key challenge lies in the methodologies used to identify the algae species [5].

Microscopic observation of microalgae cell structure, color, size and arrangement of organelles is a base to morphological identification. However, species level identification requires genetic approach wherein morphologically similar species may have different genetic makeup and therefore have different protein expressions [6]. Algal industries invest in technology based on the end product extraction from algae. Therefore species identification is important function of product portfolio extraction from algae species by understanding species level identification through genetic approach. Different molecular techniques have been developed and widely utilized for identification of several algae taxa [7–9]. Some nuclear genes like 18S rDNA, nuITS1 and nuITS2 and chloroplast gene like rbcl, tufA and 23S have also been used for molecular identification of green algae [10][11]. Among all methods 18S rDNA marker has been used successfully with higher accuracy [12]. However, this method can only apply when DNA is extracted from isolated axenic culture of microorganism. Isolation and maintenance of axenic culture of algae species require lots of efforts and requires significant time, manual

skill and facility [13]. The ribulose biphosphate carboxylase (rbcL) sequence method targeting chloroplast gene has also been used in phylogeny study as it can be easily amplified and restricted to photosynthetic organisms [14].

The key objective of the present study was to isolate native potential microalgae species from natural water bodies as well as longest coastal line of industrialized state of Gujarat in India for its sustainable application in value added industrial products. The isolated algae were further identified using morphological as well as molecular approaches for genus and species level identification. The rbcL marker based method was adopted to identify green isolates from monospecies but non-axenic cultures of microalgae with 18S universal markers as control.

In present study, authors have developed and validated a novel method for molecular identification of microalgae even from non-axenic cultures. This method uses rbcL gene specific primers.

METHODOLOGY

Sample Collection

Water samples were collected from Gujarat, on the basis of visible microalgae population. Total 10 samples were collected, 5 from Gujarat coastline at Dwarka (Samples S1 to S5) and 5 from Sabarmati riverfront, Ahmedabad (Samples S7 to S10). Collections were conducted with a goal of isolating of maximum microalgae species from each site. All the samples were collected in 500 ml sterile plastic bottles and maintained refrigerated during transferring to lab.

Isolation Of Pure Culture

To obtain pure culture from collected water samples, standard plating technique was used to dissociate algal population. Two basic nutrient media Zarrouk's medium [15] and Artificial Saline Water (ASW) [16] were used at isolation stage for fresh water and marine water habitants respectively. Collected samples were serially diluted up to 10-5 dilutions. Sterilized glass petri plates containing 25 ml of agarized media (Zarrouk's media and ASW media respectively) were used for plating the dilutions. From every dilutions, 0.1 ml samples were transferred on media plate and spreaded over the surface of media and plates were incubated at 25˚C with approximately 12-12 h light-dark periods. Microalgal culture colonies were allowed to grow for three week. After visible growth, isolated cultures were streaked on sterile nutrient media plates through standard streak plate technique. Streaked plates were incubated at 37 ˚C till the visible growth of colonies observed. Streaking method followed by incubation were repeated until mono species culture of microalgae was isolated. Uni-algal colonies were transferred on respective nutrient media plates based on colony morphology and microscopic colony characteristic.

Screening and Selection

Growth cycle parameters, doubling time and productivity analysis were the key parameters considered to study the culture characteristics. Biomolecule profiles of harvested biomass of each isolates like protein [17], lipid [18], Chlorophyll [19] and carbohydrate [20] were estimated. On the basis of growth characteristics and biomolecule profile, all isolates were screened and subjected to shake flask level cultivation, two potential microalgae isolates were selected for further study and scale up. Major criteria for selection was production of specific biomolecules and higher biomass production.

Morphological Identification

Morphological study was carried out using light microscope and digital camera (Olympus CH20iBIMF light microscope; Sony Optical Steady Shot DSC-W730). Culture in exponential phase was used with media for light microscopy, Cell density, cell shape, color and cell arrangement were studied for both selected isolates. Taxonomic identification were performed according to the Classification by A. F. E. Fritsch (1935, 48).

DNA Extraction and Quality Check

Culture suspensions were centrifuged at 3000 g for 15 min. at 4˚C to pellet out the cells from culture media. Pelleted cells were washed with NS solution and freeze dried with liquid nitrogen and powdered with mortar and pestle. DNA was isolated (XcelGen Genomic DNA extraction mini kit-Cat No: XG2611-01) as per manufacturer's instructions. Quality was evaluated by agarose gel electrophoresis and

DNA concentration and purity were determined by Nanodrop8000 [21].

PCR Amplification of 18S Rdna Gene

Based on conserved region of 18s rDNA, pcr amplification of 18s genes were performed with forward primer ITS 1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') [22] and reverse primer ITS 4R (5'-TCCTCCGCTTATTGATATGC-3')[23]. The thermal cycling conditions were Initial denaturation at 96 °C-5 min; 35 cycles of 94 °C, 30 s (denaturation); 57°C, 30 s (annealing); 72 °C, 45s (DNA synthesis, Elongation); Final extension at 72 °C for 10 min; and 4 °C hold.

PCR Amplification of Rbcl Gene

Ribulose-1,5-biphosphate carboxylase / oxygenase large subunit (rbcL) gene for both species were selected as markers for species level identification. Four sets (give primer sequence for all 8 primers) of primers were designed using Primer3plus tool [24] (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). Designed primers were characterized by OligoCalc: Oligonucleotide Properties Calculator [25],

synthesized by Primex services from Xcelris genomics and quality evaluated through Nanodrop8000 spectrophotometer [21] and were validated by PCR amplification of targeted gene using Veriti® 96 well Thermal Cycler (Applied Biosystem Model No. 9902).

Targeted genes for both isolated DNA samples were amplified with rbcL_1A and rbcL_1D specific Primers using Veriti® 96 well Thermal Cycler. The PCR program was as follows: Initial denaturation at 96 °C-5 min; 35 cycles of 94 °C-30s (denaturation); 64°C-45s (annealing); 72°C-45 s (DNA synthesis, Elongation); Final extension at 72°C-10 min; and 4°C hold.

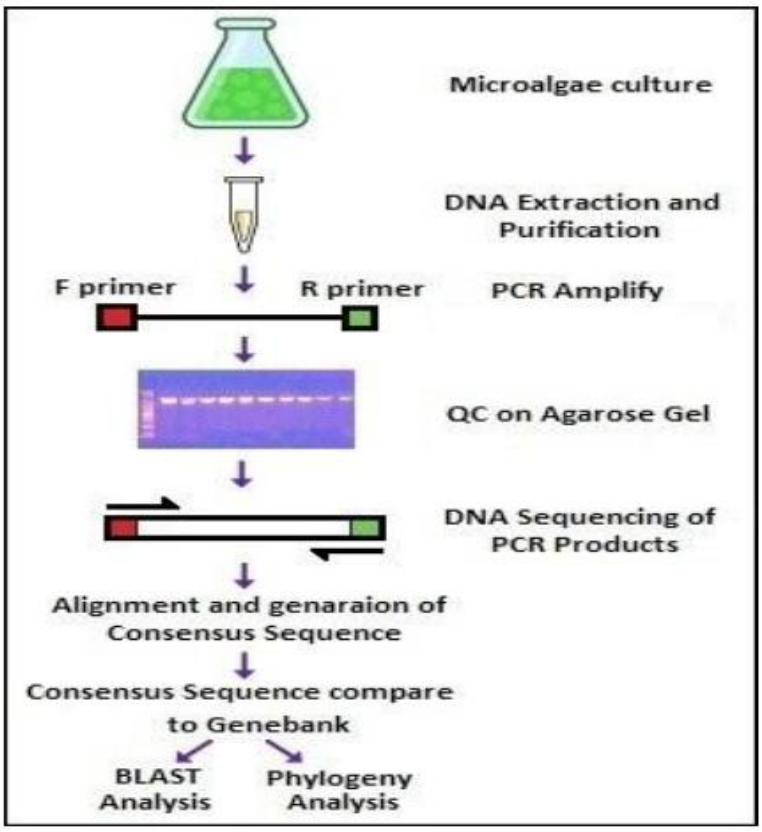

Fig1. *PCR amplification based molecular identification*

Sequencing of Pcr Products and Analysis

After quality check of PCR products, amplicons were bead purified [26] and subjected to Sanger Sequencing [27]. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with F and R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence for both amplicons were generated from forward and reverse sequence data using CodonCode aligner software.

Blast and Evolutionary Relationship Analysis

As described in Figure-1, the consensus sequences generated from Codon code aligner

were used to carry out local alignment search with NCBI Genbank database through BLAST (Basic Local Alignment Search Tool)[28]. First fifteen sequences were selected based on maximum score of identity and aligned using multiple alignment software program ClustalW [29]. The Phylogenetic tree was constructed using MEGA5 [30]. The evolutionary study was done using the Neighbor-Joining method [31]. The evolutionary distances were computed using the Kimura 2-parameter method [32] and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated.

Results

Sample Collection, Isolation and Selection of Microalgae Isolates

Collected microalgae samples were categorized on the basis of source of water, their natural environment, pigment production, basic structure and physicochemical parameters of cultures. Microscopic observation showed that collected samples contained mix microalgae species, bacteria, planktons, parasites and ciliated protozoans. Isolated colonies of microalgae were obtained by serial dilution and spread plate technique with microalgae specific agarized salt media and screened on the basis of their primary product and its commercial applicability. Finally two potential isolates A8 and D2 were selected on the basis of higher biomass productivity as well as significant production of bio molecules productions. Isolate A8 showed highest protein production whereas isolate D2 showed maximum lipid content among all microalgae isolates. Selected isolates were subjected to further study and scale up process for protein and oil production respectively. Selected microalgae cultures were further isolated on agar plates with respective nutrient media salt to reduce the contamination of other microalgae species.

Table1. *Taxonomic classification of selected microalgae isolates A8 and D2*

	A8	D2
Empire	*Prokaryota*	*Eukaryota*
Kingdom	*Eubacteria*	*Plantae*
Subkingdom	*Negibacteria*	*Viridiplantae*
Phylum	*Cyanobacteria*	*Chlorophyta*
Class	*Cyanophyceae*	*Chlorophyceae*
Order	*Oscillatoriales*	*Chlamydomonadales*
Family	*Microcoleaceae*	*Dunaliellaceae*
Genus	*Arthrospira*	*Dunaliella*
Species	*Arthrospira platensis*	*Dunaliella tertiolecta*

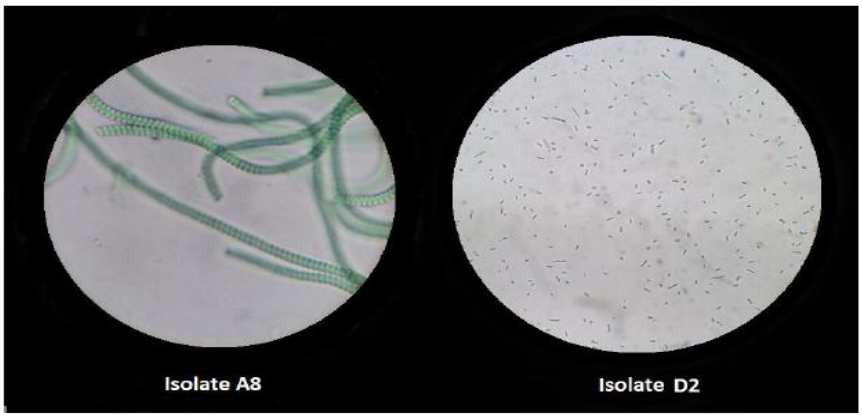

Fig2.*Microscopic observation of isolates A8 and D2*

Morphological Identification of Microalgae

Selected microalgae isolates were identified at genus level on the basis of microscopic study of morphological characteristics according to F.E Fritsch classification. Figure-2 shows microscopic images of both selected isolates. From microscopic observation, cells of isolate A8 were found filamentous blue-green colored with variable size trichomes including tightly coiled to even a straight form. Cells are free floating over or near the surface of the water. On the basis of morphology, isolate A8 showed

highest similarity with Arthrospira platensis [33]. While in case of isolate D2, cells were green, ellipsoidal as well as spherical, oval and apically broader. Cell symmetry was found to be radial and cells lacking rigid cell wall. Absence of cell wall indicates the higher rate of variations in cell shape due to external conditions [34]. Chloroplast located at the basal region of the cell which indicates the isolate belongs to the genus Dunaliella. Basic morphology shares the features of D. salina, D bioculata and D. tertiolecta [35]. However cell shape and chloroplast arrangement highly resembles D. tertiolecta [36]. Table-1 shows taxonomic classification of both isolates A8 and D2.

DNA Extraction and Quality Check

DNA isolated from these two mono spices cultures using mentioned genomic DNA extraction kit followed by quality check on 1 % Agarose Gel showed a single band of high-molecular weight DNA (Fig. 3). Moreover, DNA concentration was determined by Nanodrop8000 in triplicates (Table 2).

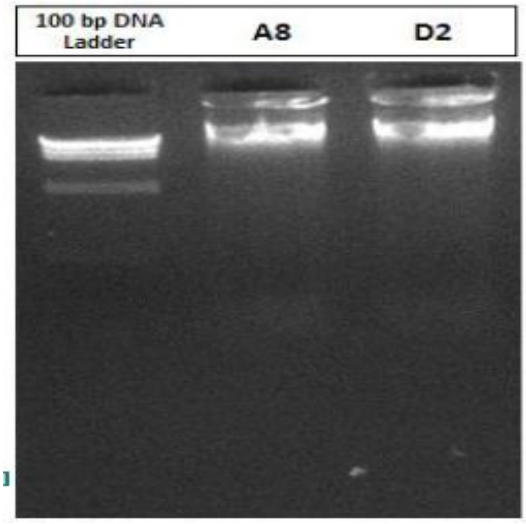

Fig3. *DNA quality check on 1.2% agarose gel*

Table 2. *DNA concentration by Nanodrop (ND8000)*

Sr. No.	Sample ID	A 260	$A_{260/280}$	ηg/µl	ηg /µl (Mean)
1	A8-Algae 1	1.119	1.82	55.95	55.92
2	A8-Algae 2	1.118	1.83	55.90	
3	A8-Algae 3	1.118	1.83	55.90	
1	D2-Algae 1	0.338	1.87	16.91	16.89
2	D2-Algae 2	0.337	1.82	16.85	
3	D2-Algae 3	0.338	1.86	16.90	

PCR Amplification of 18S Rdna Gene and Sequencing

Conserved region of 18S gene were amplified using universal primers ITS 1F and ITS 4R. However, multiple bands with variable size amplicons (~200 bp, ~350bp, ~500bp and ~800bp) were found upon quality evaluation of pcr products on agarose gel. Upon several failed attempts to amplify the amplicon of desired size devoid of any non-specific bands, amplicons of expected size were extracted from gel using gel extraction kit followed by purification and processed for Sanger sequencing. Sequences generated by analyzer revealed mix data from sequencing which shows DNA contamination of microbes other than microalgae. The consensus sequence generated from Sanger sequencing data showed higher similarity with gene region of euplotes and other bacterial rDNA on BLAST study which indicates the amplification of eukaryotic 18S gene regions other than microalgae origin (data not provided).

Primer Designing For Amplification Of Rbcl Gene

Four primers for rbcL gene were designed to amplify targeted genes by PCR on the basis of standard criteria for primer designing like melting temperature, primer length, hairpin formation etc. [37]. Designed primers were

synthesized at Primex facility of Xcelris labs. Among two sets, one set was selected for each isolate after primer validation and standardization steps. Primer sequence with number of bases, GC content, melting temperature (Tm) and amplicon size in basepairs (bp) are described in table 3 for final primer set selected for each gene.

Table3. *Primers designed for PCR amplification of Rbcl genes in targeted species*

Isolate	Name	Primer sequence	No. of Bases	GC %	Tm (C°)	Product size
A8	*rbcL*_A8F	5'- TTAACCTCCATCGTGGGTAACG	22	50	54.8	769 bp
	*rbcL*_A8R	5'- CAGGCATAGAAGCCCAATCTTG	22	50	54.8	
D2	*rbcL*_D2F	5'- CCATTCATGCGTTGGAGAGACC	22	55	64.2	746 bp
	*rbcL*_D2R	5'- GCAGCTGCTAATTCAGGAGACC	22	55	64.1	

PCR Amplification of Rbcl Gene and Sequencing

Isolated DNA was amplified with rbcL_1S Specific Primer (F and R) using Veriti® 96 well Thermal Cycler (Model No. 9902).

Amplicon specific discrete bands of ~770bp and ~750bp were observed on 1.5% Agarose gel (Fig. 4) for amplicon A8 and D2 respectively. The PCR amplicon were bead purified and further subjected to Sanger Sequencing.

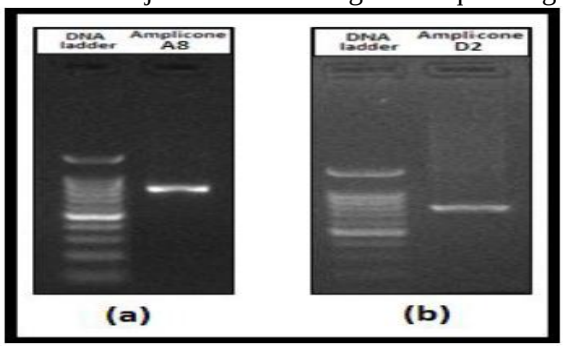

Fig4. *1.5% Agarose gel showing 770bp A8 amplicon in (a) and 750bp of D2 amplicon in (b). Lane 1: 100bp DNA ladder; Lane 2: DNA amplicon*

Consensus Sequence of A8 and D2

Consensus sequence was generated from forward and reverse sequence data using Codon Code aligner software and submitted to NCBI with accession nos. MG 734574 and MG 734575 respectively . The sequence was used to perform nucleotide BLAST on NCBI Genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program ClustalW. The Phylogenetic tree was constructed using MEGA5.

Sequence Alignment and Phylogenetic Tree for Isolate A8

As shown in table 4, consensus sequence shows highest similarity score with Arthrospira platensis chloroplast rbcL gene. Phylogenic tree also shows the evolutionary relationship between amplified rbcL gene of isolate A8 and other highly similar genes (fig.5). Blast results and phylogenic tree validate the presence of rbcL gene of morphologically identified species.

Table4. *Sequence alignment results on nucleotide Blast for isolate A8*

Accession	Description	Max score	Total score	Query coverage	E value	Max Ident.
KF700272.1	Arthrospira platensis HY	1408	1408	100%	0.0	100%
AY147205.1	Arthrospira platensis FACHB439	1408	1408	100%	0.0	100%
FO818640.1	Arthrospira sp. str. PCC 8005 chromosome	1380	1380	100%	0.0	99%
CP013008.1	Arthrospira platensis YZ genome	1286	1286	100%	0.0	97%
KF700270.1	Arthrospira platensis FACHB-350	1286	1286	100%	0.0	97%
AY136285.1	Arthrospira platensis FACHB341	1286	1286	100%	0.0	97%
AY147204.1	Arthrospira maxima OUQDSM	1286	1286	100%	0.0	97%
CP007542.1	Synechocystis sp. PCC 6714	763	763	97%	0.0	85%
CP003614.1	Oscillatoria nigro-viridis PCC 7112	752	752	99%	0.0	85%
CP012832.1	Synechocystis sp. PCC 6803 substrain GT-G	749	749	99%	0.0	85%
CP003265.1	Synechocystis sp. PCC 6803	749	749	99%	0.0	85%
AP012495.1	Bacillus subtilis BEST7613 DNA	749	749	98%	0.0	85%
AP012278.1	Synechocystis sp. PCC 6803 substr. PCC-P DNA	749	749	99%	0.0	85%
AP012277.1	Synechocystis sp. PCC 6803 substr.	749	749	99%	0.0	85%

	PCC-N DNA					
AP012276.1	Synechocystis sp. PCC 6803 substr. GT-I DNA	749	749	99%	0.0	85%

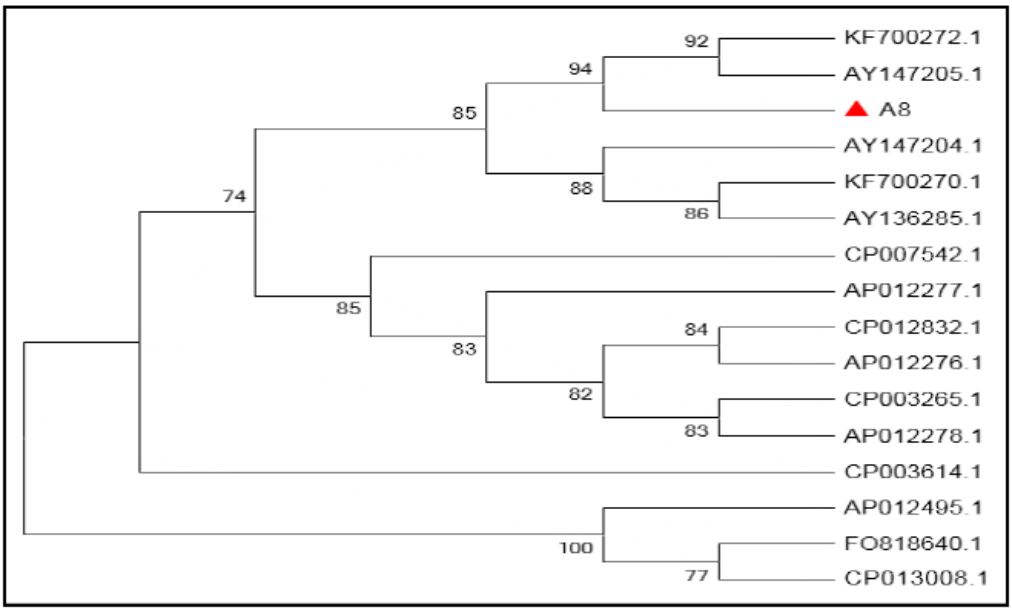

Fig5.*Evolutionary relationship tree for isolate A8*

Sequence Alignments and Phylogenetic Tree for D2

On the basis of Blast results (Table-5) and evolutionary relationship tree (Figure-6), isolate D2 shows highest similarity with ribulose-1, 5-bisphosphate gene of Dunaliella tertiolecta species.

Table5. *sequence alignment results on nucleotide Blast for isolate D2*

Accession	Description	Max score	Total score	Query coverage	E value	Max Ident.
KX530454.1	Dunaliella salina strain SQ chloroplast	1482	1482	100%	0.0	100%
JQ039069.1	Dunaliella tertiolecta ribulose-1,5-bisphosphate	1482	1482	100%	0.0	100%
AY882012.1	Dunaliella tertiolecta Ycf3 (ycf3) gene	1482	1482	100%	0.0	100%
GQ250046.1	Dunaliella salina strain CCAP 19/18 chloroplast	1454	1454	100%	0.0	99%
AB127992.1	Dunaliella primolecta chloroplast rbcL gene, strain:TS-3	1349	1349	91%	0.0	100%
AB127991.1	Dunaliella bioculata chloroplast rbcL gene , strain:TS-2	1310	1310	88%	0.0	100%
AB127990.1	Dunaliella salina chloroplast rbcL gene , strain: TS-1	1275	1275	87%	0.0	99%
KP202853.1	Dunaliella sp. DN1 ribulose-1,5-bisphosphate	1269	1269	98%	0.0	96%
KF975605.1	Dunaliella sp. AKS-21 ribulose-1,5-bisphosphate	1245	1245	87%	0.0	99%
AY531529.1	Dunaliella salina	1186	1186	95%	0.0	95%
AB127989.1	Chlamydomonas sp. LG-2 chloroplast rbcL gene, strain:LG-2	1155	1155	91%	0.0	95%
KC149893.1	Dunaliella sp. ABRIINW-M1/2	1142	1142	84%	0.0	97%
KT625418.1	Characiochloris acuminata culture-collection SAG:31.95	1133	1133	100%	0.0	92%
AB360752.1	Characiochloris acuminata chloroplast rbcL gene , strain: NIES-637	1103	1103	96%	0.0	92%
AB781586.1	Uncultured Dunaliella sp. cbbL gene, clone: 18AlexDunaliellacbbL	1083	1083	73%	0.0	99%

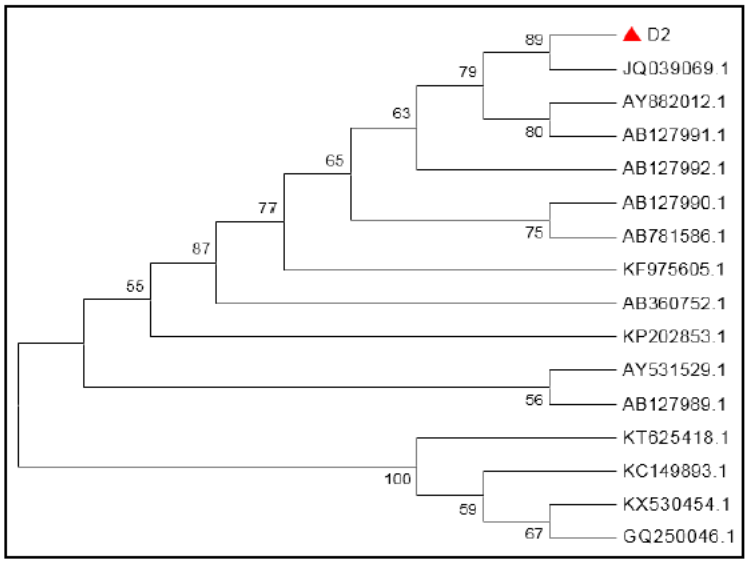

Fig6.*Evolutionary relationship tree for isolate D2*

DISCUSSION

Morphological approaches for identification of microbial isolates through microscopic observation are widely used by researchers at a higher success rate. However, morphological traits have major chances to mis-identification and molecular approach based confirmation is required to accurately identify the isolates at a species level [38,39]. The cultures harvested for extraction of DNA contained several species of microalgae along with symbiotic bacteria and ciliated protozoan. DNA extraction and PCR study based molecular identification approach was used to validate and confirm the morphological identification of isolates. DNA isolation is very common from most organisms including bacteria, fungi, parasites, insects, plants etc. [40]. However nucleic acid extraction from some microalgae species is quite difficult [41].

Amplification of 18S rDNA gene for identification of fungal and algal isolates is widely used and effective method [42]. However, amplification of conserved region of 18S gene by universal primers ITS 1F and ITS 4R for the isolates in this study resulted into variable size bands which indicated the amplification of 18S gene regions from DNA fragments of other microorganisms present in algae cultures. Hadi and his coworkers had also successfully sequenced ITS-1 to 5 region from more than 40 strain using same method. However, similar results of multiple PCR products were also reported by them for four samples amplified by ITS primers which were resulted into impaired direct sequencing [43]. On the other hand, using universal ITS region specific primers, Yicheng Wu and coworkers have successfully amplified 18S sequences of DNA extracted from axenic cultures (bacteria and parasite free) of microalgae isolates [44]. Above studies clearly indicate that 18S rDNA based molecular identification for microalgae isolates requires pure and axenic culture. However, as most of the microalgae species grow in close association with variable prokaryotic and eukaryotic microorganisms, development and maintenance of axenic culture of microalgae is time consuming and requires lots of efforts [13][45].

To selectively amplify micro algal DNA and to prevent the amplification of gene sequences from non- photosynthetic microorganism, rbcL gene region was targeted. The rbcL gene found in chlorophytes encodes the large subunit of the CO2 fixing enzyme RuBisCO only in green plants and autotrophic algae and absent it contaminating prokaryotic and eukaryotic microorganisms [46]. The Blast results and phylogenetic trees confirmed the morphological identification of microalgae isolates A8 and D2 as Arthrospira platensis and Dunaliella tertiolecta respectively.

Both isolates A8 and D2 are blue green and green algae respectively. These forms of microalgae are morphologically very diverse group of marine as well as freshwater algae. Although they are known since antiquity, they are still less understood taxonomically. Pigmented microalgae species are morphologically distinguished by pigment color, position of chloroplast, cell characteristics etc.

[47] however, identification is not easy since most species shares common morphology and lack obvious structures [48]. Different identification methods were evaluated with variable success rate. Gary and team also worked on evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers in green macroalgae and reported lower rate of success in case of ITS based barcoding in marine microalgae [49] however researches are still working to maximise the efficiency in this direction.

CONCLUSION

Sequencing and multiple alignment of amplified rbcL gene with Genbank database leads to species level identification of microalgae isolates with acceptable level of accuracy. The method adopted in this study for rbcL gene based primer designing and optimized PCR conditions can bypass the efforts and resources for developing and maintaining axenic culture and reduces the time for molecular identification. However this method is designed exclusively for unispecies green microalgae cultures since rbcL gene regions share some common codon sequence between closely related species. Moreover, species specific rbcL gene based marker design or barcoding can enhance the efficiency in microalgae diversity at specific environmental populations.

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REFERENCES

[1] Pathak C, Mandalia HC, Rupala YM. Biofuels : Indian Energy Scenario. Research Journal of Recent Sciences 2012;1(4):88–90.

[2] Gami B, Patel A, Patel B, Patel P. Micro Algae : Potential Weapon For CO 2 Sequestration And Biomass Generation At Bio-Power Plant In Gujarat. Proceedings of the India International Science Festival- Young Scientists' Meet, vol. 2010, 2015, p. 1–9.

[3] Patel A, Gami B, Patel P, Patel B. Microalgae: Antiquity to era of integrated technology. Renewable and Sustainable Energy Reviews 2017;71:535–47. doi:http://dx.doi.org/10.1016/j.rser.2016.12.081 .

[4] Chew KW, Yap JY, Show PL, Suan NH, Juan JC, Ling TC, et al. Microalgae biorefinery: High value products perspectives. Bioresource Technology 2017;229:53–62. doi:https://doi.org/10.1016/j.biortech.2017.01.006.

[5] Auinger BM, Pfandl K, Boenigk J. Improved Methodology for Identification of Protists and Microalgae from Plankton Samples Preserved in Lugol ' s Iodine Solution : Combining Microscopic Analysis with Single-Cell PCR. Applied and Environmental Microbiology 2008;74:2505–10. doi:10.1128/AEM.01803-07.

[6] Cronberg G, Laugaste R. New species of Uroglena and Ochromonas (Chromulinales, Chrysophyceae) from Estonia. In Nova Hedwigia: Zeitschrift für Kryptogamenkunde, vol. Suppl. 128, J. Cramer in der Gebrder Borntraeger Verlagsbuchhandlung; 2005, p. 43–63.

[7] Stern RF, Horak A, Andrew RL, Coffroth M-A, Andersen RA, Küpper FC, et al. Environmental Barcoding Reveals Massive Dinoflagellate Diversity in Marine Environments. PLOS ONE 2010;5:1–14. doi:10.1371/journal.pone.0013991.

[8] Zhao X, Pang S, Shan T, Liu F. Applications of three DNA barcodes in assorting intertidal red macroalgal flora in Qingdao, China. Journal of Ocean University of China 2013;12:139–45. doi:10.1007/s11802-013-2052-9.

[9] Clarkston BECE, Saunders GWSW. A comparison of two DNA barcode markers for species discrimination in the red algal family Kallymeniaceae (Gigartinales, Florideophyceae), with a description of Euthora timburtonii sp. nov. Botany 2010;88:119–31. doi:10.1139/B09-101.

[10] Buchheim MA, Keller A, Koetschan C, Förster F, Merget B, Wolf M. Internal Transcribed Spacer 2 (nu ITS2 rRNA) Sequence-Structure Phylogenetics: Towards an Automated Reconstruction of the Green Algal Tree of Life. PLOS ONE 2011;6:1–10. doi:10.1371/journal.pone.0016931.

[11] Hall JD, Lewis LA, Karol KG. An assessment of proposed DNA barcodes in freshwater green algae. Cryptogamie, Algologie 2010;31:529–55.

[12] Huss VAR, Frank C, Hartmann EC, Hirmer M,

Kloboucek A, Seidel BM, et al. Biochemical Taxonomy and Molecular Phylogeny of the Genus Chlorella Sensu Lato (Chlorophyta). Journal of Phycology 1999;35:587–98. doi:10.1046/j.1529-8817.1999.3530587.x.

[13] Sensen CW, Heimann K, Melkonian M, Sensen CW, Heimann K, The MM. The production of clonal and axenic cultures of microalgae using fluorescence-activated cell sorting The production of clonal and axenic cultures of microalgae using fluorescence-activated cell sorting. European Journal of Phycology ISSN: 2017;28:93–7. doi:10.1080/09670269300650151.

[14] Wongsawad P, Peerapornpisal Y. Molecular identification and phylogenetic relationship of green algae , Spirogyra ellipsospora (Chlorophyta) using ISSR and rbcL markers. Saudi Journal of Biological Sciences 2014;21:505–10. doi:10.1016/j.sjbs.2014.01.003.

[15] Zarrouk C. Contribution to the study of cyanophyceae: influence of various physical and chemical factors on the growth and photosynthesis of Spirulina maxima (Setch and Gardner) Geitler. Ph.D. thesis, University of Paris, France., 1966.

[16] Patel B, Gami B, Bhimani H. Formulation of commercially feasible marine microalgae cultivation media for biofuel production. International Journal of Energy for a Clean Environment 2009;10:167–179. doi:10.1615/InterJEnerCleanEnv.2011002449.

[17] Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Analytical Biochemistry 1977;83:346–56. doi:https://doi.org/10.1016/0003-2697(77)90043-4.

[18] Bligh EG, Dyer WJ. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Canadian Journal of Biochemistry and Physiology 1959;37:911–7. doi:10.1139/o59-099.

[19] Lichtenthaler HK, Buschmann C. Extraction of Phtosynthetic Tissues:Chlorophylls and Carotenoids. Current Protocols in Food Analytical Chemistry, John Wiley & Sons, Inc.; 2001. doi:10.1002/0471142913.faf0402s01.

[20] Anthon GE, Barrett DM. Determination of Reducing Sugars with 3-Methyl-2-benzothiazolinonehydrazone. Analytical Biochemistry 2002;305:287–9. doi:https://doi.org/10.1006/abio.2002.5644.

[21] Desjardins P, Conklin D. NanoDrop Microvolume Quantitation of Nucleic Acids. Journal of Visualized Experiments : JoVE 2010:2565. doi:10.3791/2565.

[22] Gardes M, Bruns T. ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. Mol Ecol 1993;2:113–8. doi:10.1111/j.1365-294X.1993.tb00005.x.

[23] White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA Genes for ... PCR - Protocols and Applications - A Laboratory Manual, Academic Press; 1990, p. 315–22.

[24] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic Acids Research 2012;40:e115–e115. doi:10.1093/nar/gks596.

[25] Kibbe WA. OligoCalc : an online oligonucleotide properties calculator 2007;35:43–6. doi:10.1093/nar/gkm234.

[26] Bum Kim J, Blackshaw S. One-Step Enzymatic Purification of PCR Products for Direct Sequencing. Current Protocols in Human Genetics, John Wiley & Sons, Inc.; 2001. doi:10.1002/0471142905.hg1106s30.

[27] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America 1977;74:5463–7.

[28] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of Molecular Biology 1990;215:403–10. doi:https://doi.org/10.1016/S0022-2836(05)80360-2.

[29] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 1994;22:4673–80.

[30] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5 : Molecular Evolutionary Genetics Analysis Using Maximum Likelihood , Evolutionary Distance , and Maximum Parsimony Methods Research resource. Mol Biol Evol 2011;28:2731–9. doi:10.1093/molbev/msr121.

[31] Saitou M, Nei N. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 1987;4:406–25.

[32] Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 1980;16:111–20. doi:doi: 10.1007/BF01731581.

[33] Ping Wang Z, Zhao Y. Morphological reversion of Spirulina (Arthrospira) platensis (Cyanophyta): from Linear to Helical. Journal of Phycology 2005;41:622–8.

[34] Teodoresco EC. Morphological and biological observations on the genus Dunaliella 1906:353–427.

[35] Butcher RW. An undescribed species of Dunaliella from the Cambridge collection of algae. Hydrobiologia 1959;12:249–50. doi:10.1007/BF00034911.

[36] Butcher RW. Part I: Introduction and Chlorophyceae. An introductory account of the smaller algae of British coastal waters., Fisheries Investigations, London; 1959, p. 1–74.

[37] Dieffenbach CW, Lowe TM, Dveksler GS. General concepts for PCR primer design. Genome Research 1993;3:S30–7.

[38] Borowitzka MA, Beardall J, Raven JA, editors. The Physiology of Microalgae. vol. 6. 6th ed. Switzerland: Springer International Publishing; 2016. doi:10.1007/978-3-319-24945-2.

[39] Manoylov K. Taxonomic identification of algae (morphological and molecular): species concepts, methodologies, and their implications for ecological bioassessment. Journal of Phycology 2014;50:409–24. doi:10.1111/jpy.12183.

[40] Dongyou Liu. Handbook of Nucleic Acid Purification. 1st ed. Boca Raton: CRC Press; 2009.

[41] Fiore MF, Moon DH, Tsai SM, Lee H, Trevors JT. Miniprep DNA isolation from unicellular and filamentous cyanobacteria. Journal of Microbiological Methods 2000;39:159–69. doi:https://doi.org/10.1016/S0167-7012(99)00110-4.

[42] Hadziavdic K, Lekang K, Lanzen A, Jonassen I, Thompson EM, Troedsson C. Characterization of the 18S rRNA Gene for Designing Universal Eukaryote Specific Primers. PLOS ONE 2014;9:e87624.

[43] Hadi SIIA, Santana H, Brunale PPM, Gomes TG, Oliveira MD, Matthiensen A, et al. DNA Barcoding Green Microalgae Isolated from Neotropical Inland Waters. PLOS ONE 2016;11:e0149284.

[44] Wu Y, Guan K, Wang Z, Xu B, Zhao F. Isolation, Identification and Characterization of an Electrogenic Microalgae Strain. PLoS ONE 2013;8:e73442. doi:10.1371/journal.pone.0073442.

[45] Lee H-G, Shin S-Y, Jin L, Yoo C, Srivastava A, La H-J, et al. Establishment and maintenance of an axenic culture of Ettlia sp. using a species-specific approach. Biotechnology and Bioprocess Engineering 2015;20:1056–63. doi:10.1007/s12257-015-0289-4.

[46] Ghosh S, Love N. Application of rbcL based molecular diversity analysis to algae in wastewater treatment plants. Bioresource Technology 2011;102:3619–22. doi:10.1016/j.biortech.2010.10.125.

[47] Watanabe S, Floyd GL. Considerations on the systematics of coccoid green algae and related organisms based on the ultrastructure of swarmers. Cytology, Genetics and Molecular Biology of Algae 1996:1–19.

[48] Zou S, Fei C, Wang C, Gao Z, Bao Y, He M, et al. How DNA barcoding can be more effective in microalgae identification: a case of cryptic diversity revelation in Scenedesmus (Chlorophyceae). Scientific Reports 2016;6:36822.

[49] W. Saunders G, Kucera H. An evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers from the marine green macroalgae. vol. 31. 2010.

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