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ISOLATION AND IDENTIFICATION OF HIGH OMEGA 3 FATTY ACID PRODUCING MICROALGAE THROUGH DAIRY WASTE EFFLUENT OF DHARASHIV, MAHARASHTRA

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ABSTRACT

The aim of the present study was to isolate and identify microalgae capable of overproducing omega-3 fatty acids using dairy waste effluent collected from Dharashiv, Maharashtra. Native microalgae were successfully cultivated in dairy effluent, which is rich in nutrients and organic matter. A total of ten green microalgal isolates were initially obtained using BG-11 medium. These isolates were then screened for omega-3 fatty acid productivity using autoclaved dairy effluent as the culture substrate. Among them, four isolates exhibited the highest concentrations of omega-3 fatty acids. These high-yielding strains were subsequently scaled up and subjected to molecular identification. DNA was extracted, and amplification targeting the 18S-rRNA gene was performed, followed by sequencing and phylogenetic analysis. The four isolates were identified as *Scenedesmus dimorphus*, *Chlorella vulgaris*, *Choricystis parasitica*, and *Chlorella conductrix*. All identifications were confirmed by high similarity in nBLAST results and clustering with reference sequences from the NCBI GenBank database. The findings demonstrated that dairy effluent serves as a promising source for isolating native microalgae with high potential for omega-3 fatty acid production. In addition to confirming the ability of local algal strains to produce bioactive compounds, the study highlighted the dual role of microalgae in both high-value biochemical synthesis and effluent bioremediation. These results support the application of microalgae in integrated wastewater valorization strategies and contribute to the growing interest in sustainable sources of omega-3 fatty acids.

KEYWORDS: Chlorella, Dairy waste, Dharashiv, Microalgae, Omega 3 fatty acid, Scenedesmus.

1. INTRODUCTION

Interest in sustainable microbiological sources has increased due to the growing need for omega-3 polyunsaturated fatty acids (PUFAs), such as EPA and DHA, on a worldwide scale. Environmental variability, contamination concerns, and overfishing are some of the sustainability issues facing traditional fish oil production (Qin *et al.*, 2023). Microorganisms such as plants, yeasts, bacteria, and fungi have all been investigated as potential sources of omega-3 fatty acids. However, microalgae stand out from the rest because they can grow autotrophically (using CO_2 and sunshine), quickly accumulate lipids, are the main producers of EPA and DHA in marine environments, and do not compete for freshwater or arable land resources (Jakhwal *et al.*, 2022).

Omega-3–producing microbial strains have been isolated from a variety of natural and industrial environments. Coastal and brackish waters have yielded Nannochloropsis, Tetraselmis, Chlorella, and Dunaliella, all known for high lipid and omega-3 content (Lim *et al.*, 2012). Marine environments rich in phytoplankton also provide *Phaeodactylum tricornutum*, Nitzschia, and haptophytes like *Tisochrysis lutea*, renowned for DHA production. In contrast, terrestrial waste streams such as dairy effluent remain relatively underexplored as sources of omega-3–rich strains (Ji *et al.*, 2015).

Targeting dairy waste effluent as a recovery site offers multiple advantages. Dairy effluents contain high organic loads and nutrients, which can support rapid microbial growth. Cultivating microalgae on these substrates provides a dual benefit: bioremediation of wastewater (nutrient removal, COD reduction) and valorization through biomass enriched with omega-3 fatty acids. Previous studies have shown that algae grown on dairy-derived substrates such as cheese whey or wastewater can yield ALA, EPA, and DHA while treating the effluent although direct isolation of such omega-3-rich strains from the effluent itself has not been routine (Lage *et al.*, 2019).

Microbial groups like fungi (e.g., Mortierella) and yeasts (e.g., Yarrowia lipolytica) have been engineered or naturally used to produce EPA, but their cultivation often requires organic carbon substrates, raising cost and sustainability issues (Tang *et al.*, 2018). Bacteria can produce small-chain PUFAs, but not typically EPA/DHA (Giner-Robles *et al.*, 2018). Plants, even when genetically modified, take longer growth cycles and often lack efficient pathways for long-chain omega-3 synthesis. In contrast, microalgae offer faster growth, direct sun-driven lipid synthesis, and high natural capacity for EPA/DHA biosynthesis in a single step, making them the most viable option.

Several genera of microalgae are documented to produce high proportions of omega-3 PUFAs. *Nannochloropsis spp*. can accumulate up to 60–70% lipid content under nitrogen deprivation, with substantial omega-3 fraction. *Phaeodactylum tricornutum*, Nitzschia, and *Tisochrysis lutea* are known for DHA-rich lipid profiles. *Auxenochlorella protothecoides* and *Crypthecodinium cohnii* (though heterotrophic) are also industrially used for DHA production (Wen & Chen, 2003). These taxa serve as benchmarks for high-value omega-3 microalgal biotechnology.

In the current study, microalgae were isolated from the dairy waste effluent site from Dharashiv area of Maharashtra to produce omega 3 fatty acid and identify them through molecular identification.

2. MATERIAL AND METHODS

2.1. Collection and isolation of algae

The effluent samples (approx. 10-gram weight) were collected from dairy waste from Dharashiv, Maharashtra with latitude 18.544668 and Longitude 76.245724. the effluent samples were inoculated in 250 mL flasks containing 100 mL of BG-11 medium. The microalgae were cultured using BG11 medium, composed of the following (per liter): 1.5 g $NaNO_3$, 0.04 g K_2HPO_4, and a trace elements solution containing 0.075 g $MgSO_4 \cdot 7H_2O$, 0.036 g $CaCl_2 \cdot 2H_2O$, 0.02 g Na_2CO_3, 0.006 g citric acid, 0.006 g ferric ammonium citrate, and 0.001 g EDTA. Additionally, 1 mL of a microelement solution was added, comprising (in $g \cdot L^{-1}$): H_3BO_3 (2.86), $MnCl_2 \cdot H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.222), $CuSO_4 \cdot 5H_2O$ (0.079), $Na_2MoO_4 \cdot 2H_2O$ (0.390), and $Co(NO_3)_2 \cdot 6H_2O$ (0.049).

Continuous illumination at 120 $\mu E \cdot m^{-2} \cdot s^{-1}$ was used to keep the cultures at 25°C after a 16:8-hour light-dark photoperiod. The culture media was kept at pH 7.1 while the incubation was carried out at 90 rpm in an orbital shaker (Gomaa *et al.*, 2019). The medium was treated with 300 mg/L of the broad-spectrum antibiotic Cefuroxime in order to produce algal colonies free of microorganisms. 100 µL of the culture was transferred onto BG11 agar plates under the same incubation conditions when mixed algal growth was apparent. Microscopic analysis and repeated streaking on new BG11 agar were carried out until monospecific, axenic cultures of microalgae were isolated (Pandey *et al.*, 2019).

Pure isolates were then subjected to morphological characterization based on microscopic observations using standard taxonomic keys provided by Bellinger and Sigee (2015). A total of 10 green microalgal isolates were successfully obtained and are being maintained on BG11 agar slants at 4 °C for long-term storage and further analysis (Stanier *et al.*, 1971).

2.2. Screening of high omega 3 fatty acid producing microalgae isolates

Raw dairy effluent was collected from the effluent treatment facility's inlet at the dairy location in Dharashiv where the earlier effluent samples were taken. The effluent was autoclaved for 15 minutes at 121°C after the suspended solids were filtered out. The sterile dairy effluent was used right away as a microalgal growth culture medium when it had cooled to room temperature.

The microalgal isolates were first inoculated onto 6-well ELISA plates with 6 mL of sterile dairy effluent. They were then incubated for 12 days at 28 ± 2 °C with constant white, fluorescent light at 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Selected isolates were cultivated under the same circumstances after being expanded up to 500 mL Erlenmeyer flasks with 300 mL working volume based on observable growth and cell density.

Following the incubation time, biomass was collected by centrifugation, and the chloroform–methanol technique was used to extract total lipids. High-yielding strains were chosen for molecular identification after isolates were assessed according to their lipid content (Pandey *et al.*, 2019).

2.3. Molecular identification of algal isolate

2.2.1. DNA Extraction of Algal Isolates

Total genomic DNA was extracted from axenic cultures of different algal isolates following the protocol described by Doyle and Doyle (1987). The samples were suspended in a pH 8.0-adjusted cetyltrimethylammonium bromide (CTAB) extraction buffer that contained 1% polyvinylpyrrolidone (PVP), 0.01 M EDTA, 1.4 M NaCl, 0.5% β-mercaptoethanol,

0.1 M Tris-HCl, and 3% CTAB. After being shaken intermittently every 15 minutes for an hour at 60°C, the suspension was allowed to cool to room temperature. An equal proportion of chloroform:isoamyl alcohol (24:1) was used to extract the DNA, and one volume of isopropanol was then used to precipitate it out of the aqueous phase. TE buffer was used to resuspend the resultant DNA pellet. Agarose gel electrophoresis was used to evaluate the quality and integrity of the DNA.

2.2.2. 18S-rRNA Gene Amplification and Sequencing

Using primers C-2 (5′-ATTGGAGGGCAAGTCTGGT-3′) and D-2 (5′-ACTAAGAACGGCCATGCAC-3′), the 18S ribosomal DNA section of the algal genomes was amplified using colony PCR, as stated by Moreno (2012). In a 25 µL total volume, PCR reactions were conducted using 30 ng of template DNA, 2 µL of each primer (10 pmol/µL), Master Mix (Takara, Japan), and water without nuclease. Prior to 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, the thermal cycling conditions comprised an initial denaturation at 95°C for 5 minutes. At 72°C, a last extension step was carried out for five minutes. After being resolved on a 0.9% agarose gel, amplified PCR products were purified using the Thermo Scientific GeneJET PCR Purification Kit.

2.2.3. Sequence Analysis and Phylogenetic Tree Construction

The acquired 18S-rRNA gene sequences were given accession numbers after being uploaded to a nucleotide database. BLAST analysis was used to compare the sequence's similarity to the NCBI GenBank database. Two bioinformatic processes were used to discover phylogenetic relationships. Initially, the online program Clustal Omega was used to align the retrieved sequences with their homologs. Second, the MEGA 7 program (http://www.megasoftware.net/) was used to import the aligned sequences and use the Maximum Likelihood approach to create a phylogenetic tree.

3. RESULTS

3.1. Isolation and screening of the potential microalgae from dairy waste effluent

In the current study, a total of 10 microalgae isolates were isolated from the enrichment procedure using BG-11 medium that is specifically known for the growth of algae. The cell density of all the isolates in the ELISA plate was more than 80% that shows that the. Further, the four best performing microalgae isolates that showed maximum omega 3 fatty acid production were chosen for molecular identification.

3.2. Molecular identification of the microalgae isolates

The four high omega 3 fatty acid producing microalgae isolates found from dairy waste effluent were *Chlorella vulgaris*, *Choricystis parasitica*, *Chlorella conductrix*, and *Scenedesmus dimorphus.*

Chlorella vulgaris

The 18S-rDNA sequence of the algal isolate showed 100% query coverage and identity with *Chlorella vulgaris* (Accession: FM205832.1) in the NCBI GenBank database. Other top hits also matched *C. vulgaris* strains with 99.88%–99.92% identity and 0.0 E-value, confirming the isolate's identity (Figure 1). Closely related chlorophyte species such as *Chloridium saccharophilum*, *Chlamydomonas chlamydogama*, and *Marvania coccoides* showed slightly lower identity scores.

Phylogenetic analysis using a neighbor-joining tree further confirmed the identification, as the isolate clustered tightly with reference *C. vulgaris* strains (e.g., CCAP 211/21A, 211/20, 211/11b) with high bootstrap support (≥99%) (Figure 2). These results highlighted minimal genetic divergence within the species complex and affirm the effectiveness of 18S-rDNA sequencing for accurate microalgal identification.

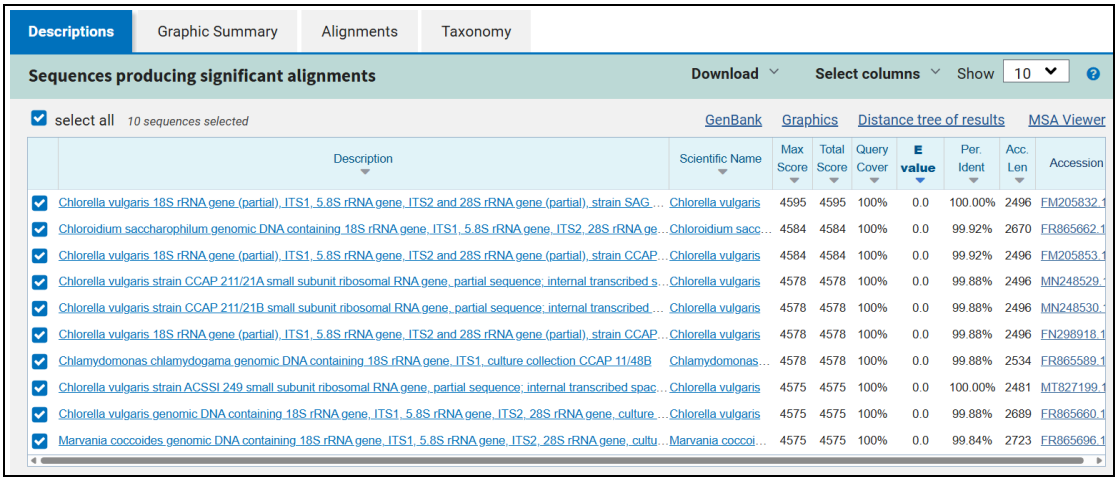


Figure 1: nBLAST analysis of *Chlorella vulgaris* 18S partial sequence.

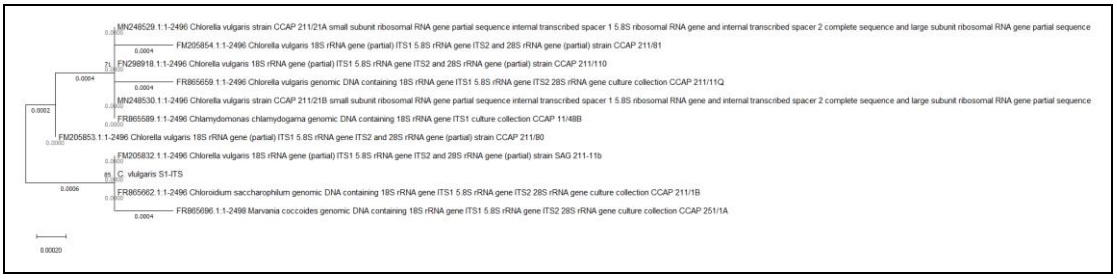
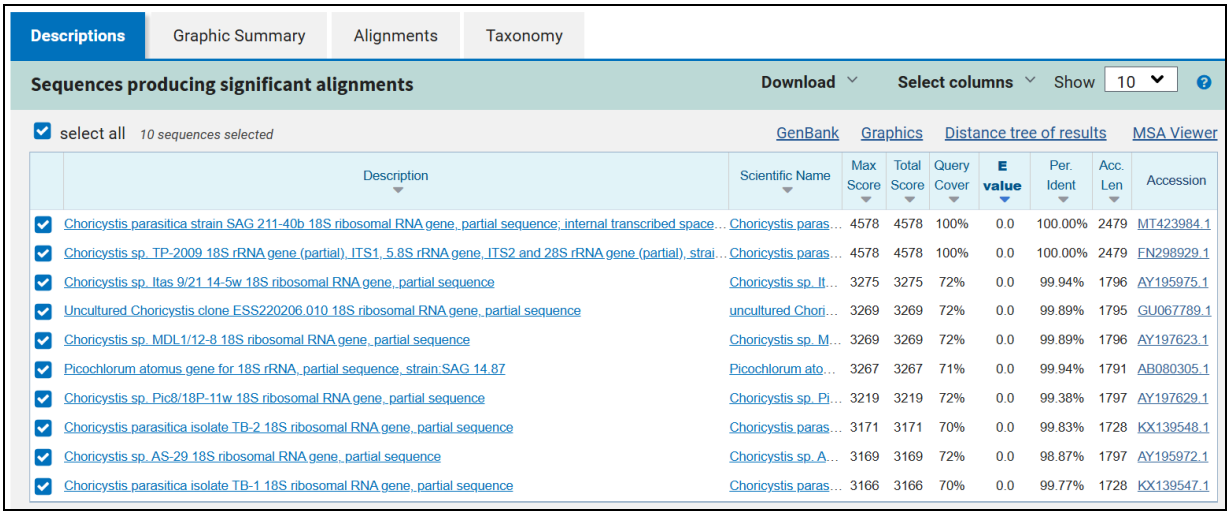


Figure 2: Phylogenetic analysis of *C. vulgaris* 18S sequence.

Choricystis parasitica

The 18S-rDNA sequence of the second green algal isolate showed 100% query coverage and identity with *Choricystis parasitica* strain SAG 211-40b (MT243984.1) in the NCBI nBLAST database, confirming a precise species match (Figure 3). Additional high-scoring matches included various Choricystis species with 99.89%–99.94% identity and E-values of 0.0, further supporting the isolate's placement within the Choricystis genus. A single hit to *Picochlorum atomus* (99.89% identity) indicated phylogenetic proximity but less likely species-level match.

A neighbor-joining phylogenetic tree based on 18S-rDNA sequences placed the isolate firmly within the *Choricystis parasitica* clade, closely clustering with strains SAG 211-40b and TB-2 with high bootstrap support (Figure 4). Related Choricystis species such as TP-2009 and Itas 9/21-14-5w also formed part of this group, indicating intra-genus variation. More distantly related sequences like *Picochlorum atomus* and uncultured Choricystis clones formed separate clades, confirming the isolate's identity as *C. parasitica* and validating 18S-rDNA as a reliable marker for microalgal taxonomy.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 10

select all 10 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Choricystis parasitica strain SAG 211-40b 18S ribosomal RNA gene, partial sequence; internal transcribed space…	Choricystis paras…	4578	4578	100%	0.0	100.00%	2479	MT423984.1
Choricystis sp. TP-2009 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strai…	Choricystis paras…	4578	4578	100%	0.0	100.00%	2479	FN298929.1
Choricystis sp. Itas 9/21 14-5w 18S ribosomal RNA gene, partial sequence	Choricystis sp. It…	3275	3275	72%	0.0	99.94%	1796	AY195975.1
Uncultured Choricystis clone ESS220206.010 18S ribosomal RNA gene, partial sequence	uncultured Chori…	3269	3269	72%	0.0	99.89%	1795	GU067789.1
Choricystis sp. MDL1/12-8 18S ribosomal RNA gene, partial sequence	Choricystis sp. M…	3269	3269	72%	0.0	99.89%	1796	AY197623.1
Picochlorum atomus gene for 18S rRNA, partial sequence, strain:SAG 14.87	Picochlorum ato…	3267	3267	71%	0.0	99.94%	1791	AB080305.1
Choricystis sp. Pic8/18P-11w 18S ribosomal RNA gene, partial sequence	Choricystis sp. Pi…	3219	3219	72%	0.0	99.38%	1797	AY197629.1
Choricystis parasitica isolate TB-2 18S ribosomal RNA gene, partial sequence	Choricystis paras…	3171	3171	70%	0.0	99.83%	1728	KX139548.1
Choricystis sp. AS-29 18S ribosomal RNA gene, partial sequence	Choricystis sp. A…	3169	3169	72%	0.0	98.87%	1797	AY195972.1
Choricystis parasitica isolate TB-1 18S ribosomal RNA gene, partial sequence	Choricystis paras…	3166	3166	70%	0.0	99.77%	1728	KX139547.1

Figure 3: nBLAST analysis of *Chlorella parasitica* 18S partial sequence.

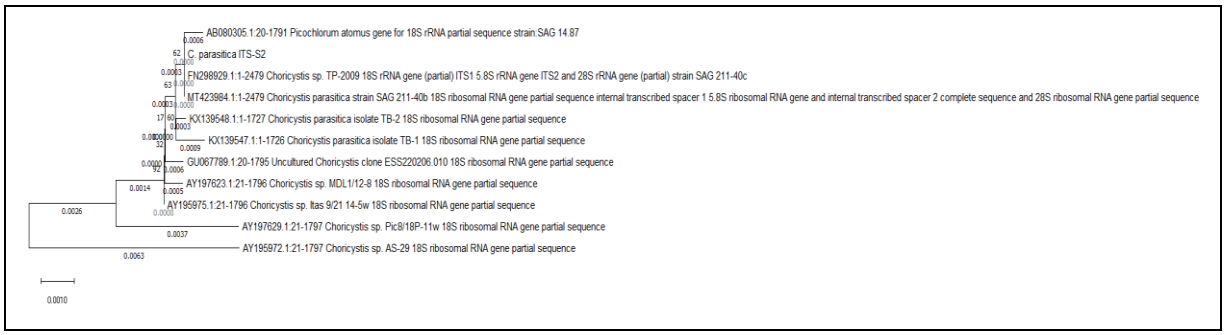


Figure 4: Phylogenetic analysis of *C. parasitica* 18S sequence.

Chlorella conductrix

The 18S-rDNA sequence of the third algal isolate showed 100% query coverage and 100% sequence identity with *Micractinium conductrix* strain CCAP 248/18 (Accession: MT253689.1), as revealed by nBLAST against the NCBI GenBank database, confirming a precise match (Figure 5). A second top hit, *M. conductrix* CCAP 248/20 (MT253690.1), exhibited 99.98% identity. Other closely related matches included *Micractinium reisseri* strains (e.g., AB437244.1, AB506070.1) with 99.86%–99.90% identity and 100% query coverage. Despite the high similarity, *M. conductrix* remained the most probable identity. All hits showed E-values of 0.0, confirming highly significant matches. According to the NCBI taxonomy browser, *Chlorella conductrix* is now recognized as a synonym for *Micractinium conductrix*.

The phylogenetic tree constructed using 18S-rDNA sequences placed the third isolate firmly within the *Micractinium conductrix* clade, clustering closely with reference strains CCAP 248/18 and CCAP 248/20, supported by high bootstrap values (Figure 6). Sequences of *Micractinium reisseri* formed an adjacent cluster, indicating close genetic relatedness within the genus but with clear species-level distinction. Additional nearby branches included uncultured Micractinium isolates from environmental samples, suggesting evolutionary proximity.

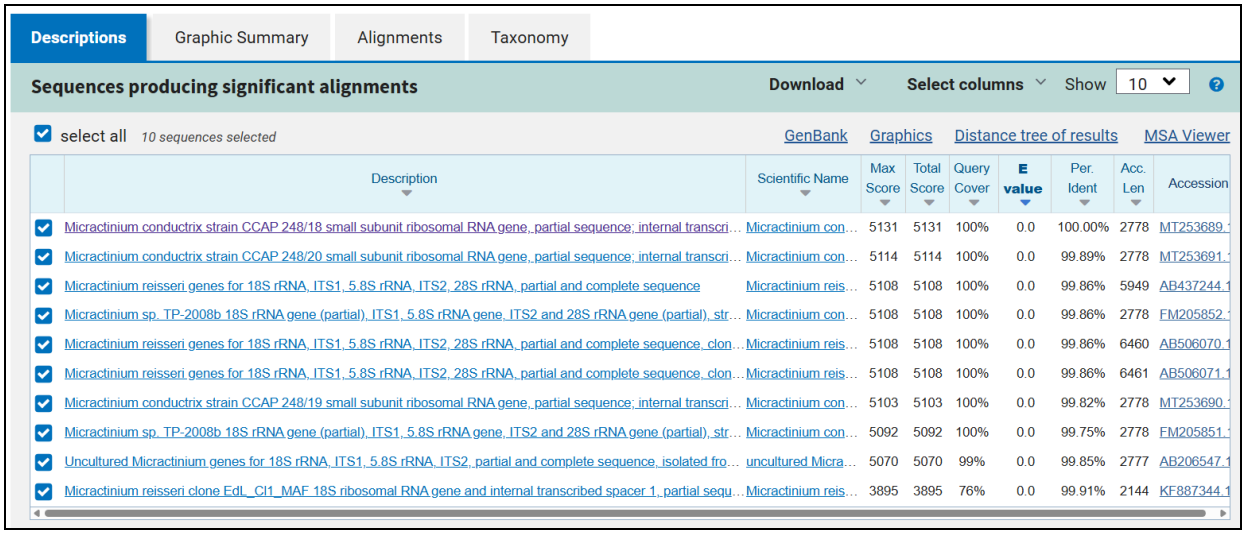


Figure 5: nBLAST analysis of *C. conductrix* 18S partial sequence.

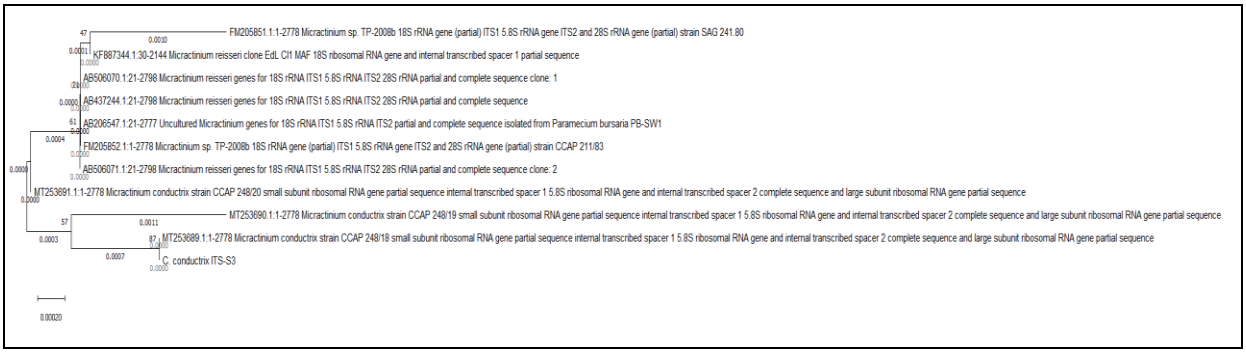


Figure 6: Phylogenetic analysis of *C. conductrix* 18S sequence.

Scenedesmus dimorphus

The 18S-rRNA sequence of the fourth algal isolate showed 100% query coverage and 100% identity with *Scenedesmus dimorphus* clone DM10 (KC790431.1), confirming a strong species-level match via nBLAST (Figure 7). Additional high-scoring hits included *Scenedesmus sp*. YSD-1, *Tetradesmus bajacalifornicus*, and *T. obliquus*, with 97% query coverage and up to 98.77% identity, indicating close phylogenetic relationships within the Scenedesmaceae family. While related species such as *Autodesmus obliquus* and *S. pectinatus* appeared in the results, the highest alignment score and identity uniquely supported *S. dimorphus* as the most likely identification. All hits showed E-values of 0.0, confirming statistical significance.

Phylogenetic analysis using 18S-rDNA sequences positioned the fourth isolate within the *Scenedesmus dimorphus* clade, clustering tightly with clone DM10 and supported by high bootstrap values (Figure 8). Closely related taxa, including *Scenedesmus sp*. YSD-1, S. pectinatus, Tetradesmus, and Autodesmus species, formed neighboring branches, reflecting intra-familial relationships. Despite their proximity, the isolate's distinct placement alongside *S. dimorphus* confirmed its species-level identity.

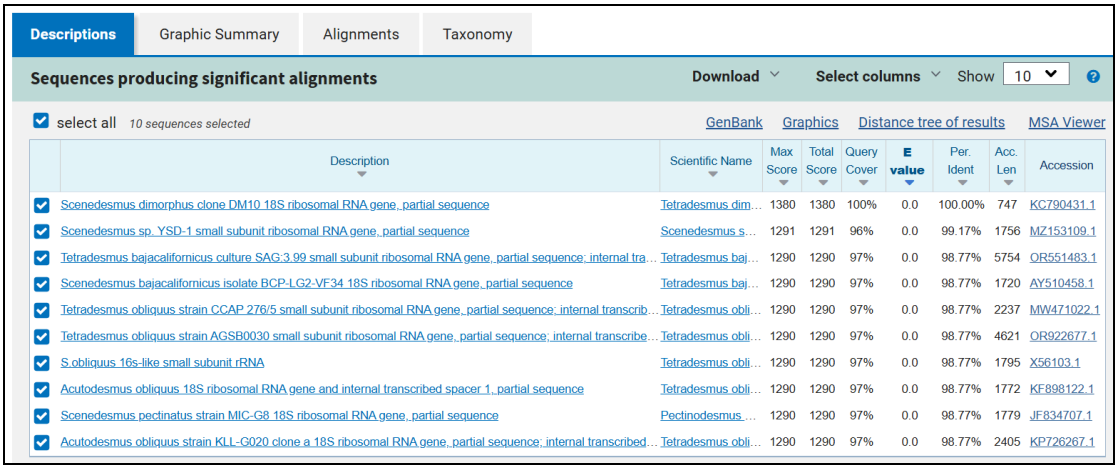


Figure 7: nBLAST analysis of *S. dimorphus* 18S partial sequence.

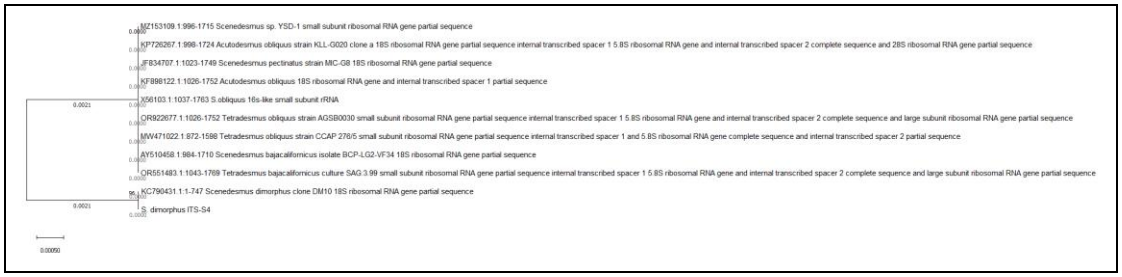


Figure 8: Phylogenetic analysis of *S. dimorphus* 18S sequence.

4. DISCUSSION

Isolation of native microalgal strains is a critical initial step in bioprocess development, as it enables the establishment of pure cultures and facilitates the selection of promising candidates for biodiesel production and wastewater treatment. According to Chen *et al.* (2015), microalgal species isolated directly from wastewater environments exhibited significantly higher nutrient removal efficiencies compared to those achieved with commercial strains. Several studies have further confirmed that the use of native isolates can enhance nutrient uptake, biomass yield, and lipid productivity simultaneously (Zhou *et al.*, 2011). Dairy waste has been an underrated site for the isolation of algae that has the potential to produce high omega 3 fatty acid. In literature, not many studies have been conducted in the past regarding isolation and exploring the fatty acid production from algae that has been recovered from dairy waste effluent.

Identifying microalgae based solely on morphological characteristics is extremely challenging due to their small size and structural simplicity. Molecular techniques, particularly those involving genetic markers, have enabled more accurate assessment of the validity of morphological species classifications in microalgae (Fawley *et al.*, 2004). However, several studies have reported that the 18S-rRNA gene is often too conserved to reliably distinguish between closely related genera and species (Luo *et al.*, 2010). The present study obtained microalgae of genera Chlorella, Scenedesmus, and Choricystis that produced high omega 3 fatty acid. Similar results have been obtained by Pandey *et al.* (2019) who reported isolation of high fatty acid producing microalgae genera Chlorella and Scenedesmus from dairy waste effluent.

5. CONCLUSION

In this study, microalgae were successfully isolated from dairy waste effluent collected from Dharashiv, Maharashtra, and screened for their omega-3 fatty acid production potential. Out of ten isolates, four strains *viz. Chlorella vulgaris*, *Choricystis parasitica*, *Chlorella conductrix*, and *Scenedesmus dimorphus* demonstrated the highest lipid productivity. Molecular identification based on 18S-rRNA sequencing confirmed their taxonomy with strong phylogenetic support. These findings demonstrated that dairy effluent can serve as a valuable resource not only for microalgal cultivation and effluent treatment but also for generating nutritionally important bioactive lipids. The study established a foundation for further exploration of local algal biodiversity for omega-3 production and supports the development of cost-effective and sustainable bioprocesses using native strains.

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