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Screening of Symbiotic Bacteria for Biodiesel Production in Algae Growth

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Abstract

A novel symbiotic bacterial strain was isolated from the algae culture and further analysed for sequence identification to determine the nature of the species. In this study, 3- to 4-month-old algae were cultured under nonaxenic conditions. Samples were taken to determine the presence of cross contamination, such as bacteria and other species, in the culture system. Microscopic analysis confirmed the presence of *C. pyrenoidosa* and *S. abundans* in the culture system. Additionally, bacterial identification revealed that stable colonies survived in the culture system. Several purifications of two isolated pure colonies were carried out, and growth curves were plotted for the isolated colonies (white and yellow colonies), which had maximum absorbance values of 0.467 and 0.154, respectively, at 660 nm. Moreover, the isolated bacterial colonies were cocultured with pure axenic cultures of *C. pyrenoidosa* and S. *abundans*. Studies have shown that yellow colonies support the growth of algae. Hence, isolated and purified yellow colonies were subjected to morphological, biochemical, 16S rRNA sequencing and FAME analyses via gas chromatography. This analysis confirmed that the isolated symbiotic bacterium was *Stenotrophomonas maltophilia*. The consensus sequence was deposited in the NCBI GenBank KX768757.

Keywords: *Stenotrophomonas maltophilia*; Algae, *Chlorella pyrenoidosa*, *Scenedesmus abundans,* Symbiotic, rRNA

Introduction

Global economic growth depends on the availability of energy to meet demand. This sudden and excessive level of energy consumption has resulted in an energy tragedy for the world, such as the depletion of the finite

supply of fossil fuel sources. Transportation fuels such as gasoline and diesel constitute 27% of all energy produced, in addition to the depletion of fossil fuel resources. Because of the serious environmental risks they pose, including acid rain, global warming, and ozone layer depletion, these fuels are widely acknowledged to be unsustainable (World Energy Outlook, 2007 and Ho et al. 2014). In particular, India imports between 75 and 80% of its petroleum fuel to meet its energy needs. According to statistical reviews, the rate at which fossil fuels are used to produce energy globally could completely disappear in 45 years (Rühl C 2008). Therefore, it is imperative to discover a fossil fuel substitute to lessen the excess shortages of gasoline and diesel and minimize the increase in the price of transportation fuel (Marousek J et al. 2023). Triglycerides (TAGs) are promising substitutes for fossil fuels in energy, according to a number of studies (Khan et al. 2009). The primary benefit of producing eco-friendly biodiesel from biomass is that it will boost the rural economy (Barnwal et al. 2005). Therefore, the creation of biodiesel from biomass was the main focus of this study.

According to Tica et al. (2010) and Zhang et al. (2022), biodiesel is currently used as a sustainable energy source to meet future energy needs because it releases more oxygen into the atmosphere and emits less sulfur, nitrogen, and traces of SOx, NOx, CO, benzene, and toluene. In accordance with Leung et al. (2010) and Vyas et al. (2010), biodiesel is primarily made from vegetable or animal fats and is composed of fatty acid alkyl esters that are produced during the transesterification process by converting triacylglycerols (TAGs) into diacylglycerols (DAGs), free fatty acids (FFAs), and phospholipids (PLs). Because biodiesel is made from edible and nonedible crop residues, first- and second-generation biofuel sources have reduced the production of transportation fuels and have their own limitations (Ulgiati 2001; Chisti 2007 and 2012; Borowitzka 1988; Borowitzka and Moheimani 2013; Pate 2013; Klein-Marcuschamer et al. 2013). Consequently, a third generation of algae-based fuel oil producers emerged through photosynthetic reactions (Chisti 2007, 2013; Hu et al. 2008; Brennan and Owende 2010; Huang et al. 2010). In comparison to other sources, algae offer a cost-effective way to

produce enough biodiesel to partially replace fossil fuels, although this approach is debatable (Malcata 2011; Pate et al. 2011; Klein-Marcuschamer et al. 2013; Pate 2013).

Several studies have reported that various algal species can secrete oils, confirming the capacity of algae to manufacture biodiesel (Banerjee et al. 2002; Chisti 2007; Griffiths and Harrison 2009; Griffiths et al. 2012) by limiting nutrient supplements and stressing cells to produce oil by starvation of some nutrients (Illman et al. 2000; Rodolfi et al. 2009; Griffiths et al. 2012). In addition to TAG, algae oil may also contain other components that are used to produce a high level of energy and carbon, such as non-triglycerides, polar lipids, carotenoids, chlorophyll, and terpenoid hydrocarbons (Banerjee et al. 2002). The energy content of algal crude oil is 35,800 kJ kg^{-1}, or 80% of the average energy found in petroleum fuels (Chisti 2012). Forty percent of the oxygen that has evolved in the atmosphere from 75% of microalgae is due to the growth of algae (Ponnuswamy et al. 2013). Therefore, algal crude oil is recognized as a possible replacement for transportation fuels. Thus, strains that produce a high yield of microalgae were selected for cultivation in this study. Numerous studies have been conducted to improve algae growth, either directly or indirectly, through the use of artificial sources. Furthermore, according to a literature review, triggered natural or artificial sources have several disadvantages, such as a lower yield or a longer time requirement for better lipid production. To address these problems, a novel symbiotic bacterial strain was isolated from the algal culture, and sequence identification was performed to determine the species's innate capabilities. To produce biodiesel, *Stenotrophomonas maltophilia* (*S. maltophilia*) has a symbiotic effect on the growth of algae. Most bacteria can terminate algae growth within a day.

Stenotrophomonas maltophilia for algae evolution

S. maltophilia is a member of the Xanthomonadaceae family. *S. maltophilia*, formerly known as *Pseudomonas maltophilia* or *Xanthomonas maltophilia*, has been designated the sole species within the recently established genus *Stenotrophomonas*. The non-fermentative gram-negative bacillus *S. maltophilia* grows

easily on a variety of bacteriological media (Adegoke et al. 2017). A number of characteristics give *S. maltophilia* its potential as a pathogen, particularly because of its ability to secrete a wide range of extracellular enzymes, such as lipases, fibrolyses, and proteases, which may be important in the colonization process. Molecular typing systems have advanced the understanding of the epidemiology of *S. maltophilia* infection (Gajdács et al. 2019; Brooke et al. 2021). Numerous techniques for typing have been developed, including ribotyping, DNA macro restriction analysis, random amplification of polymorphic DNA, enterobacterial repetitive intergenic consensus-PCR, and multilocus enzyme electrophoresis. Fungal lipases such as Lipozyme TL IM reportedly exhibit decreased activity in regard to the transesterification of various vegetable oils by bacterial lipases, such as *Pseudomonas* lipase. *S. maltophilia* isolated from algal culture itself sustains the algal life cycle. As a result, *S. maltophilia*, a type of symbiotic bacteria, was identified from the algal culture. To understand the symbiotic algae-bacterial growth behaviour for the production of biodiesel, a purified isolated symbiotic bacterial strain was sequentially coupled with various algal species. Many studies have been completed on algae growth enhancement, which may either directly or indirectly depend on artificial sources, but the yield efficiency has not reached a satisfactory level. To overcome this constraint, a novel symbiotic bacterial strain was isolated from the algae culture, and its growth profile was tested under different conditions for biodiesel production.

Methodology

Initially, this study involved collecting samples from algae cultivated for approximately three to four months in culture and grown in the laboratory under atmospheric conditions. This algae culture was grown under non-axenic conditions with good biomass production without additional nutrient supplementation or aeration. Therefore, to determine the culture status, the sample was further analysed for cross-contamination in the culture system. Microscopic analysis confirmed the presence of *C. pyrenoidosa and S. abundans* in the culture system. In addition, bacterial identification techniques such as the streak and pour plate

methods were used to identify stable colonies that survived in the culture system. Several purifications were performed, and two different pure colonies were found. Therefore, these colonies were taken up to construct growth curves. These curves were plotted for isolated colonies (white and yellow colonies), which showed the maximum absorbance value. Moreover, the isolated bacterial colonies were cocultured with a pure axenic culture of *C. pyrenoidosa* and *S. abundans*.

Optical density method

The growth phases of the algal cultures were determined by measuring the absorbance at 660 nm with a UV-2600 Shimadzu, and the corresponding dry weight was calculated from the standard algal growth plot (Chellamboli et al. 2014 and 2016).

Genomic DNA Extraction

Agar plate colonies from a single streak were scraped, suspended in PBS, and centrifuged. After the pellet was evenly distributed, 600 µl of cell lysis buffer (SDS, Tris-EDTA, and *guanidium isothiocyanate*) was added. The mixture was gently stirred for five minutes while the vial was inverted, and the mixture was subsequently incubated for ten minutes until the suspension nearly became transparent. This solution was covered with 600 µl of isopropanol. The two layers were gradually combined until the solution was homogeneous, and white DNA strands were visible. Using a pipette tip, the DNA strands were spooled and placed into a new vial. The spooled DNA was added to 500 µl of 70% ethanol. For ten minutes, the spooled DNA was centrifuged at 10,000 rpm to precipitate the DNA. The supernatants were discarded. The pellet (not allowed to dry completely) was air-dried. After adding 50 µl of 1X TE, the pellet was suspended. The mixture was incubated at 55–60°C for 5 min to improve genomic DNA solubility. A 1% agarose gel was loaded with 5 µl of freshly extracted DNA and 3 µl of gel loading dye, after which electrophoresis was performed (Maha A. Rakaz et al. 2021).

Agarose Gel Electrophoresis

To make 1× TAE, distilled water was mixed with an appropriate amount of 50× TAE buffer. Fifty milliliters of 1X TAE and 0.5 g of 1% agarose were combined in a 250 ml conical flask and heated to a transparent liquid. The gel tank was

washed and cleaned completely. The combs were placed approximately 2 cm away from the center platform wall, facing the black electrode or cathode. The agarose gel was heated to approximately 60°C, at which point 0.5 µg/ml ethidium bromide was added. The mixture was then swirled twice before being carefully poured into the central platform of the gel tank. The agarose was allowed to solidify. The gel was filled with one X TAE buffer until the buffer level was 0.5–0.8 cm above the gel surface. The combs were gently removed. The samples were run at 50 V against voltage, and the power cords were connected. Following the run, the gel was examined using a UV transilluminator (Addgene 2018).

Column purification

After the column was set up in the collection tube, 400 µl of equilibration buffer was added, and the column was centrifuged for one minute at 10,000 rpm. The buffer that was collected was then removed. The DNA samples were mixed with 400 µl of equilibration buffer and then loaded into the column. This process was repeated until the DNA sample was finished. The flow that passed through the column was collected. After adding 500 µl of wash buffer 1, the mixture was centrifuged for one minute at 10,000 rpm, after which the buffer was extracted. After that, 500 µl of wash buffer 2 was added, and the buffer was collected after centrifuging for one minute at 10,000 rpm. An empty collection tube was used to centrifuge the column to fully remove the buffer for two minutes. After the column was placed in a new collection tube, 50 µl of the eluted buffer was added. After the column was placed in a new collection tube, 50 µl of the eluted buffer was added. The eluted sample was collected and examined on the gel after being incubated for two minutes at room temperature and centrifuged for one minute at 10,000 rpm. The amplification conditions are displayed in Table 1.

Table 1 Amplification properties for bacterium identification

Parameters	Quantity/Volume
Double distilled water	36 µl
10x Taq pol assay buffer	5µl
dNTP mix (100 µM each)	2 µl
Forward Primer(100ng/µl)	1 µl
Reverse Primer(100ng/µl)	1 µl
Taq pol (1u/ µl)	1 µl
Template	2 µl
Total Volume	**50 µl**

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Amplification of the 16S rRNA gene was performed using the primers. Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer: 5'- ACGGCTACCTTGTTACGACTT-3'. The conditions for PCR, which were carried out in 0.2 ml PCR tubes with a total volume of 50 µl, are listed in Table 2 (Maha A. Rakaz et al. 2021; Weisburget al. 1991).

Table 2 PCR Condition for bacterium identification

Initial Step		**30 Cycles**	**Final Extension**		**Final Step**
Denat		**Anneal**		**Extend**	
HOLD		**CYCLE**	**HOLD**		**HOLD**
94 ˚C	94 ˚C	58 ˚C	72 ˚C	72 ˚C	4 ˚C
2 min	30 sec	30 sec	1 min 30 sec	5 min	∞

Results and Discussions

Screening of *S. maltophilia* as a symbiotic bacterium for algae growth

The screening of the symbiotic bacteria was carried out in the research laboratory. Normally, bacteria destroy algal species within a few days, as occurs for cross-contaminated strains (nonsymbiotic bacteria). However, few organisms may support algae growth via symbiotic pathway signalling. Therefore, bacterial colonies were isolated from several-month-old algae cultures. Initially, serial dilutions and strike plate methods were used to identify the most sustainable bacterial species. Furthermore, the bacterial colonies were purified three times by the repeated strike plate method to ensure that the pure colonies were not contaminated. Finally, the two colonies were isolated on the basis of the sustainability shown in Fig. 1. Subsequently, the bacteria were grown (Erin R. Sanders 2012; Pettipheret al. 2005).

The maximum OD values were approximately 0.467 and 0.154 for yellow and white colonies, respectively, within 12 hr. Bacterial depletion occurs due to a shortage of nutrients in the medium, and the death phase is recorded. Fig. 2 shows the growth curves of both pure colonies. Later, the yellow colony was selected for further analysis.

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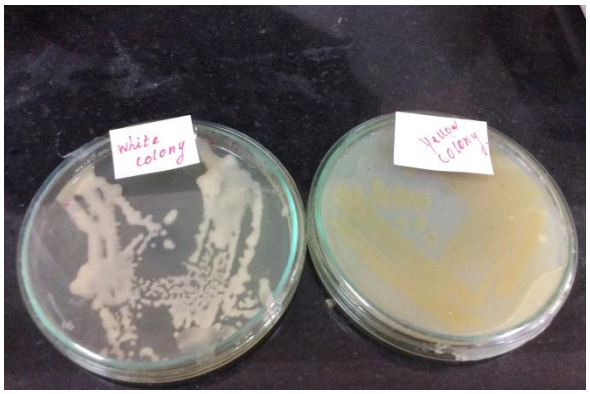

Fig.1 Isolated strains from algae culture a) White colony, b) Yellow colony

The initial growth and activation of algae take a minimum of approximately 12 hr, at which point the activity of symbiotic bacteria is needed. Furthermore, the two isolated colonies were cocultured with algal species. The experimental results showed that the algal species were supported by yellow colony bacteria. Hence, white colonies were excluded from the study, and the screened bacteria were subjected to gene sequence analysis. Studies have proven that yellow colonies support the growth of algae.

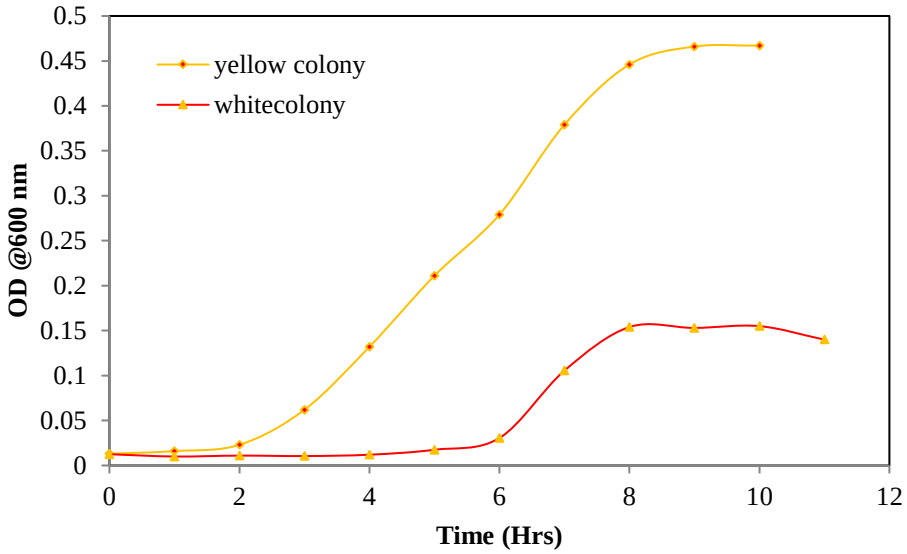

Fig. 2 Bacterial Growth curve for isolated species

Identification of microbial culture products using the 16S rRNA-based molecular technique

To identify the genera and phyla of bacteria in th e gut microbiota, 16S ribosomal RNA (rRNA) s equencing was used for a polymerase chain reaction to amplify a conserved part of the genome of a bacterium (Ranjan R et al., 2016). The 16S rRNA gene sequence was obtained via the base molecular technique used

for identification of enormous numbers of strains. The NCBI GenBank is one of the largest gene deposition databanks for rRNA sequences. The sequences of approximately twenty million genes have been deposited in the genebank, and the gene sequences of unknown microbial strains of the 16S rRNA gene have been compared (Jill E. Clarridge, 2004; Jacquelyn S. Meisel and Elizabeth A. Grice 2017).

Identification of symbiotic bacteria for algae growth

Hence, isolated and purified yellow colonies were subjected to morphological, biochemical, 16S rRNA sequencing and FAME analyses via gas chromatography. This analysis confirmed that the isolated symbiotic bacterium was *Stenotrophomonas maltophilia.* The consensus sequence was deposited in the NCBI GenBank as KX768757. Fig. 3 shows the identification of the bacterial genomic DNA in both isolated colonies. Lane 1 represents an isolated yellow colony, and lane 2 represents an isolated white colony.

Fig. 3 Genomic DNA

Identification of bacterial sequences via gel electrophoresis

The isolated DNA was further subjected to gel electrophoresis, and a single band of 1.5 kb (of 100 ng intensity) was observed. Gel electrophoresis was used to visualize the total RNA extracted from the symbiotic bacteria. Lane 1 shows the 500 bp DNA ladder of 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 kb. In addition, lanes 2-3 show the 16S rRNA amplification of two different colonies from the study experiments, i.e., the yellow and white colonies shown in Fig.4.

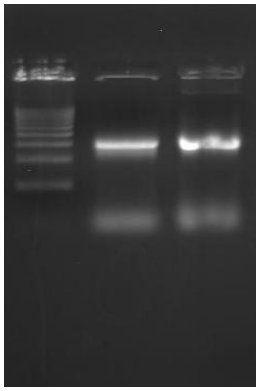

Fig. 4 Electrophoresis of 16s rRNA gene amplification

S. maltophilia STRAIN F-C06 16S ribosomal RNA gene; partial sequence in GenBank

The RNA sequence of *S. maltophilia* isolated from symbiotic bacteria has been deposited in the NCBI GenBank (KX768757). Fig. 5 shows electropherogram plots of DNA fragments from the symbiotic bacteria. The curve shows the relation of time(bps) to the fluorescence intensity of the isolated DNA from the bacteria.

Analysis of *S. maltophilia* via BLAST

BLAST of the gene bank website yielded the most significant result, which was identified by molecular testing. To identify the type of bacteria, GenBank BLAST was used for all bacteria. Several species genus conclusions were drawn from the sequences that produced meaningful alignments. The bacterial isolate was most likely *Stenotrophomonas maltophilia*, for which the maximum identification was above 99%, according to the results of the molecular test. The molecular sequence was 99% identical to the partial sequence of the *S. maltophilia* strain JB5-1 16S ribosomal RNA gene. Similarly, for the *S. maltophilia* AM3-1 16S ribosomal RNA gene, the partial sequence was 99% identical to that of the isolated bacteria. Similarly, the identified bacteria had 99% similarity with other partial sequences of bacteria deposited in GenBank for *Stenotrophomonas sp*., the CV8 16S ribosomal RNA gene, and the *Stenotrophomonas sp*. for the CV6 16S ribosomal RNA gene. *maltophilia* strain CV4 16S ribosomal RNA gene, *S. maltophilia* strain CV1 16S ribosomal RNA gene, *S. maltophilia* strain 257-B 16S ribosomal RNA gene, *Stenotrophomonas sp*. XBGRA2 16S ribosomal RNA gene, *S. maltophilia* strain L1 16S ribosomal RNA gene, *S. maltophilia* strain W8-1 16S ribosomal RNA gene, *S. maltophilia* strain Dh 16S ribosomal RNA gene, and *S. maltophilia* gene for 16S rRNA are shown in Table 3. The use of random primers for *S. maltophilia* was consistent with that of random PCR, which has been shown to be more successful at identifying *S. maltophilia* than gel electrophoresis (GE) and is a significant pathway of choice. PCR is also a much more potent way to demonstrate the degree of reliability of results between two distinct amplification systems (Walt Ream et al. 2013).

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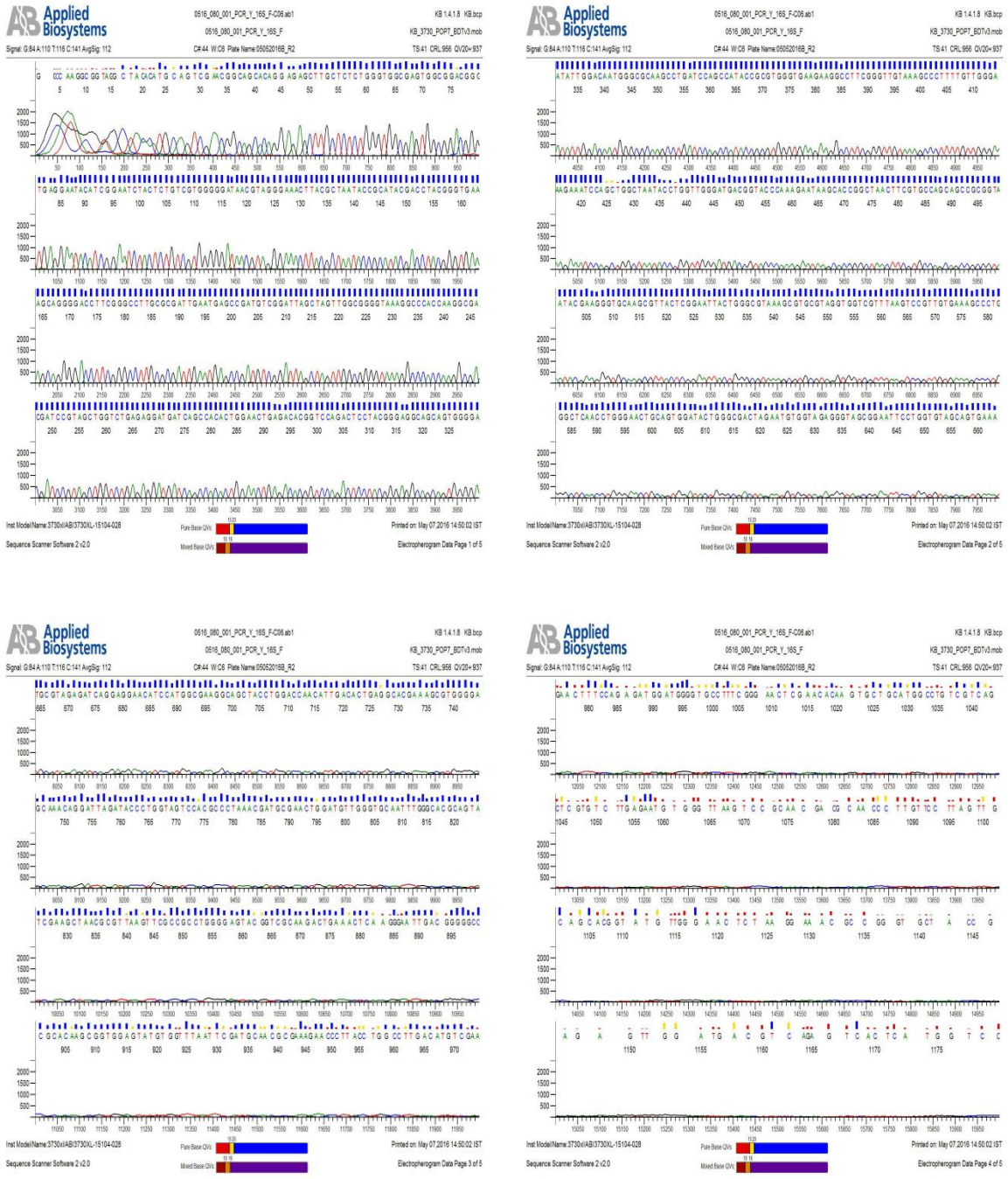

Fig.5 Partial sequence of PCR amplification for *S. Maltophilia*

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Table 3: Sequences producing significant alignments

Description	Max score	Total score	Query cover	E value	Identification	Accession
Stenotrophomonas maltophilia strain JB5-1 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KT970985.1
Stenotrophomonas maltophilia strain AM3-1 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KT970982.1
Stenotrophomonas sp. CV8 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KP895794.1
Stenotrophomonas sp. CV6 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KP895792.1
Stenotrophomonas maltophilia strain CV4 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KP895790.1
Stenotrophomonas maltophilia strain CV1 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KP895787.1
Stenotrophomonas maltophilia strain 257-B 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KP993216.1
Stenotrophomonas sp. XBGRA2 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KJ184868.1
Stenotrophomonas maltophilia strain L1 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	KF358247.1
Stenotrophomonas maltophilia strain W8-1 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	JX393000.1
Stenotrophomonas maltophilia strain Dh 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	HQ200414.1
Stenotrophomonas maltophilia gene for 16S rRNA, partial sequence, strain: BL-9	2484	2484	100%	0.0	99%	AB194321.1

Phylogenetic analyses

To further identify the taxonomic position of *Chlorococcum* sp. GD, molecular phylogenetic analysis was carried out. The species used in each phylogenetic analysis were selected on the basis of the data available in the GenBank gene database (Chellamboli and Perumalsamy, 2017). A phylogenetic approach involves the study of relationships between and within groups of organisms, as well as their evolutionary history. Phylogenetic inference techniques, which concentrate on observed

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heritable traits such as DNA sequences, protein amino acid sequences, or morphology, are used to infer these relationships. The results of such an analysis constitute a phylogenetic tree, a diagram with a hypothesis of relationships that represents the evolutionary history of a group of organisms. The phylogenetic analysis tree is displayed in Fig.6. and the first five identities, the sample was determined to be *Stenotrophomonas maltophilia* (Jacquelyn S. Meisel and Elizabeth A. Grice 2017).

Symbiotic bacterial growth of *S. maltophilia* cultivated on different algae

The influence of the different treatments was positive at biomass concentrations of 0.154, 0.43, 0.8, and 0.637 g/L for *S. maltophilia*, *C. pyrenoidosa* cocultured with *S. maltophilia*, *S. abundans* cocultured with *S. maltophilia* and mixed algae cocultured with *S. maltophilia* obtained on the 15th day of culture, respectively. After the 15th day, the growth of algae decreased, as shown in Fig. 7. The biomass concentration of *C. pyrenoidosa* +*S. maltophilia*, *S. abundans* + *S. maltophilia* and mixed algae +*S. maltophilia* were 2.79-, 5.19-, and 4.13-fold greater,

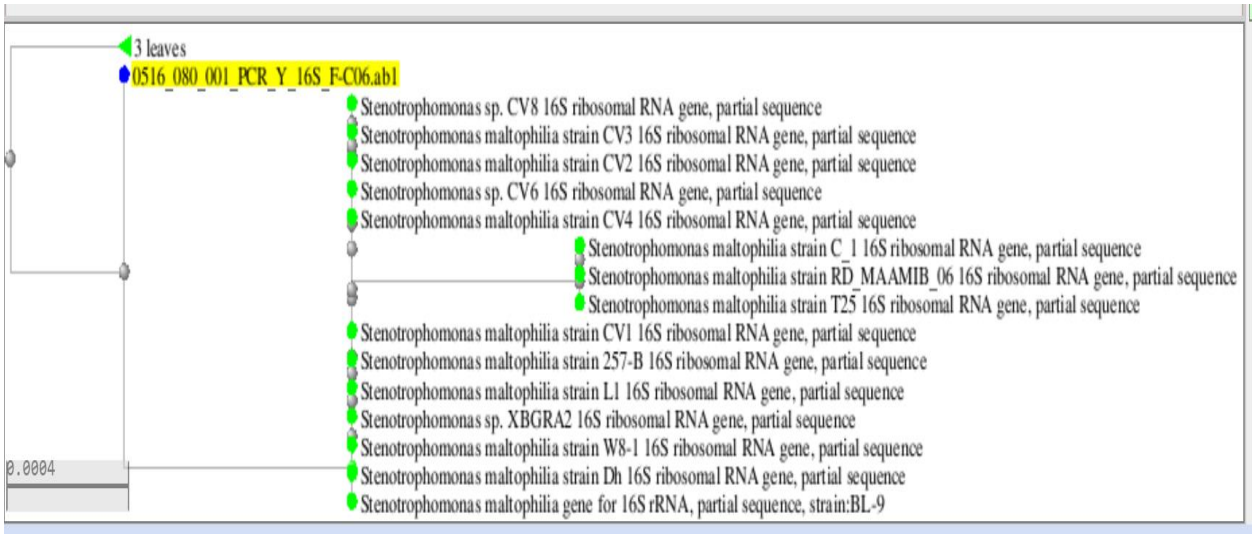

Fig.6 Phylogenic tree of *Stenotrophomonas maltophilia*

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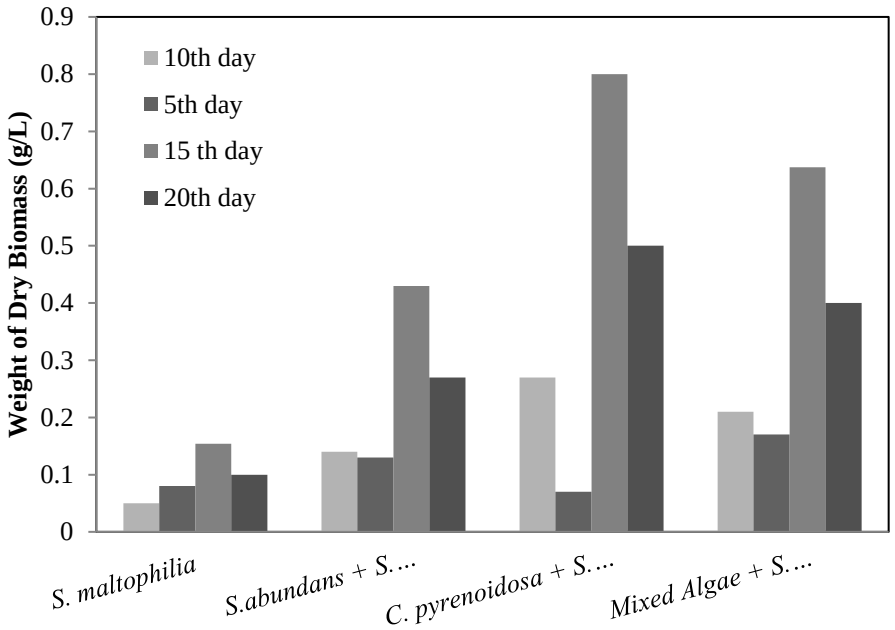

Fig.7. Influence of different treatment conditions such as *S. maltophilia, C. pyrenoidosa*+ *S. maltophilia, S. abundans*+ *S. maltophilia* and mixed algae + Symbiotic bacteria in biomass concentration

respectively. There was evidence that increased biomass production was obtained from *S. abundans* cocultured with *S. maltophilia, as* shown in Fig. 7.

Conclusion

The bacteria isolated from algae culture were subjected to microbial plating methods, and sustainable bacteria were taken for further study of algae cultivation. The standard yellow colony supports the growth of algae biomass production. Therefore, identification of bacterial cultures was performed using the 16S rRNA-based molecular technique. The results showed that 99% of the isolated and sustainable bacterial sequences matched *S. maltophilia.* Furthermore, the strain was deposited in the NCBI database. The increase in biomass production from algae was found for algae mixed with bacteria, and the corresponding lipid production was 4.13-fold. Hence, this study confirmed that *S. maltophilia*is an apt symbiotic bacterium for algae growth and lipid production.

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