

# Mangrove algae as sustainable microbial cell factory for cellulosic biomass degradation and lipid production

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## ABSTRACT

Human civilization faces a huge energy crisis due to climate alterations and reduction in fossil fuel reserves. Biofuels are sustainable platform that can combat this burning issue currently. Biodiesel is a potential fossil fuel alternative from cooking, soybean, or plant-derived oil resources. Existing biodiesel production processes are directly competing with food chain. Algae are impressive alternatives for biodiesel generation as algae grow without competition with food chain, have an inherently faster growth rate, and have higher lipid content under diverse nutrient-limiting environments. Lignocellulosic waste (LCW) accumulation has been increasing through anthropogenic activities. Accumulation of LCW causes ill effects on environment and human health. Thus, a faster paradigm shift needs to be required for establishing a single platform to perform lignocellulosic biomass degradation along with higher lipid production as biodiesel precursor. Few studies show algal endoglucanase or cellulase activities for degrading LCW to generate monomeric constituents which can further be channelized to produce higher biomass and lipid contents as biodiesel precursor. Thus, the present study focuses on a feasibility attempt to show that mangrove algal isolates could be a potential microbial cell factory for cellulose degradation as a major constituent of LCW and higher lipid production as potential precursor for biodiesel production in near future.

## 1. INTRODUCTION

Bioconversion of lignocellulosic waste (LCW) biomass represents a vast arena of violent exploration in research, development, and commercialization with distinct avant-garde activities at streaming ages [1]. Lignocellulosic biomass is one of the most generous substances in nature. It includes variant compositions of cellulose (40–50%), hemicellulose (25–30%), and lignin (15–20%). All components have been depicted as a higher amount of bioenergy resources towards sustainability. Therefore, the availability of renewable bioenergy assets has been drafted through these lignocellulosic biomass efficiencies [2-4]. LCWs are being comprehended with multiform categories of residues such as industrial waste, agricultural waste, domestic waste, agroindustrial waste, and municipal waste. Huge amounts of fermentable carbohydrates have been rescued from agricultural and food industrial LCW after the pre- and post-cultivation methods [5]. At the same time from these LCWs, immense amounts of value-added products have been generated. For example: Lipids, biodegradable plastic components, carbohydrates, protein, amino acid, resins, medicine, cosmetics, methane, ethanol, organic acids, biopesticides, surfactants, biofertilizers, feed additives, etc. [6].

In 2007, The Energy Independence and Security Act has declared that in 2008 from 9 billion gallons to 36 billion gallons in 2022, sustainable fuel volume has been raised by cellulosic biomass. That is why researchers are mainly focusing to attain a higher yield of bioenergy genesis throughout the cellulose conversion procedure [7] by the hydroxylation and ring cleavage mechanism the phenolic compounds through the usage of algal species [5]. Lignin and hemicellulose breaks down to release cellulose. However, it fails to release the cellulose from LCW following physical, chemical, biological, physicochemical, mechanical, irradiation, extrusion, and ozonolysis pre-treatment procedures time to time. With this enzyme-substrate interaction processes enable the hydrolysis possibilities for upward biodiesel formation [8]. After the transformation of lignocellulosic biomass to low fermentable sugars, also another microbial catalyst has been used, for example, *Zymomonas mobilis*, *Saccharomyces cerevisiae*, *Clostridium thermocellum*, *Clostridium phytofermentans*, *Trichoderma reesei*, etc. *Clostridium acetobutylicum* was used for butanol generation from hemicellulosic materials. *Basfia succiniproducens* and *Actinobacillus succinogenes* are used for fermentation during succinic acid generation [9,10]. Lignin contents in *Phanerochaete chrysosporium* produce high value-added products during the catabolic pathways metabolism [11].

Besides during the pretreatment methods for bioenergy consumption, high electric power supplementation, expensive equipment, and some high cost supplementary solvents reclamation are played as a barrier to LCW remediation [12]. Furthermore, there are many drawbacks

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for using these bacteria, fungi like microorganisms because they are costly, not economically feasible, as well as during the analysis period they should be highly contaminated and discharge some allergens materials toward impel the chances of health hazards. Dealing with these physical, chemical, physico-chemical issues, biologically active, and exclusive catalyst alga is being adopted for LCW conversion and bioenergy genesis. In addition, in this research work, a broad section has been briefly accelerated and examined the main reasons behind choosing algal regimes and how the algal isolates can break the lignocellulosic biomass toward mild green environmental energy generation. Some well-known algal species have demonstrated higher lipid origination yield with the lignocellulosic enzymatic assimilation [13,14]. *Chlamydomonas moewusii*, *Scenedesmus vacuolatus*, *Dunaliella salina*, *Dunaliella parva*, *Tetracystis aerea*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Hydrodictyon reticulum*, *Arthrospira platensis*, *Lyngbya* spp., *Cladophora* spp., *Tetracystis isobilateralis*, *Tetracystis aggregate*, *Tetracystis illinoisensis*, *Tetracystis dissociate*, *Chlorococcum echinozygotum*, *Chlorococcum elkhartense*, *Chlamydomonas noctigama*, *Tetracystis aplanospora*, *Desmodesmus communis*, *Scenedesmus ovaltemus*, *Acutodesmus obliquus*, *Neosporangiococcum gelatinosum*, *Chlorococcum oleofaciens*, *Lobochlamyssegnis* spp., *Chloromonas chlorococcoides*, *Spirulina platensis*, *Wolleea saccata*, *Anabaena oryzae*, *Nostoc* spp., *Oscillatoria* spp. are the most effective algal species for their enzymatic activities besides bioremediation. Some naive algal species can produce hemicellulase, cellulose, xylanase, glucosidase, mannose, lignin peroxidase, and laccase like various enzymes. Those are highly efficient for biofuel generation using LCW components [15]. The usages of strong biocatalyst like algal regimes for the generation of LCW to biodiesel are the most well accepted third-generation liquid biofuel considering its immense beneficial aspects likely renewability, biodegradability, non-toxicity, high flash point, and sustainability compared to conventional petroleum-based diesel [16]. It could be a most promising future biofuel in the transportation sector as it elucidates common features of petrochemical-derived diesel having lower greenhouse gas emissions during combustion and emission of the lower transferable carcinogenic end products [17,18].

Likewise green and blue-green algae play a very pivotal role as third-generation biodiesel feedstock which shows few distinct benefits, that is, non-dependent on arable or agricultural fields for production purposes, higher photosynthetic efficacies, higher biomass yield having faster growth rate, higher lipid productivity, and higher cetane number. Algal biodiesel deals with around ten oxygen atoms in different fatty acids present and no aromatic constituents. Therefore, algal biodiesel combustion is comparatively higher environmentally benign [19,20]. Besides, algae-based lipids are predominantly neutral lipids or triacylglycerol which can easily be esterified to FAMES with the most preferable primary C16 and C18 chain lengths for high quality biodiesel production [21]. Algae can tolerate extensive environmental stresses and display flexibility toward mass cultivation. It can grow on marginal land in open or closed water reserves (i.e., waste waters), waste gases (i.e., flue gases), organic wastes (i.e., crude glycerol), etc. [22]. Even diverse metabolic routes of algae have shown greater adaptation, higher biomass yields, and lipid productivities. In general, algae can grow photoautotrophically using carbon dioxide as a carbon source and light as an energy source. Few algal regimes can grow heterotrophically utilizing organic ingredients whereas few can grow mixotrophically considering both organic compounds and carbon dioxide to carry out their growth cycle [23]. However, the first- and second-generation biodiesel production approaches require huge areas of arable agricultural lands. To this end, biomass-derived biodiesel generation is a highly beneficial alternative approach due to

extensive unique advantages including carbon neutrality, minimized greenhouse gas emission (i.e., carbon monoxide, carbon dioxide, and sulfur oxides), spontaneous cheaper biomass feedstock availability, and biosafety of biomass production through agriculture [24]. To this end, algae are impressive microbial candidates for biodiesel generation as algae grow without competing with the food chain, inherently faster growth rate, and have higher lipid content under diverse nutrient-limiting environments [25]. Based on this current scenario, the present study focuses on the characterization of biomass and Lipid production as well as emphasized the tremendous possibilities of lignocellulosic biomass degradation ability during the biological treatment procedure utilizing native algal isolates from the mangrove ecosystem for improving biodiesel production. Moreover, these mangrove algal isolates are promising as potential biofuel producers utilizing LCW biomass under variable open climatic circumstances. Hence, the present research work is a preamble attempt to demonstrate that mangrove algal isolates could be a potential microbial cell factory for cellulose degradation and higher lipid production toward potential sustainable biodiesel production using LCWs utilization in near future.

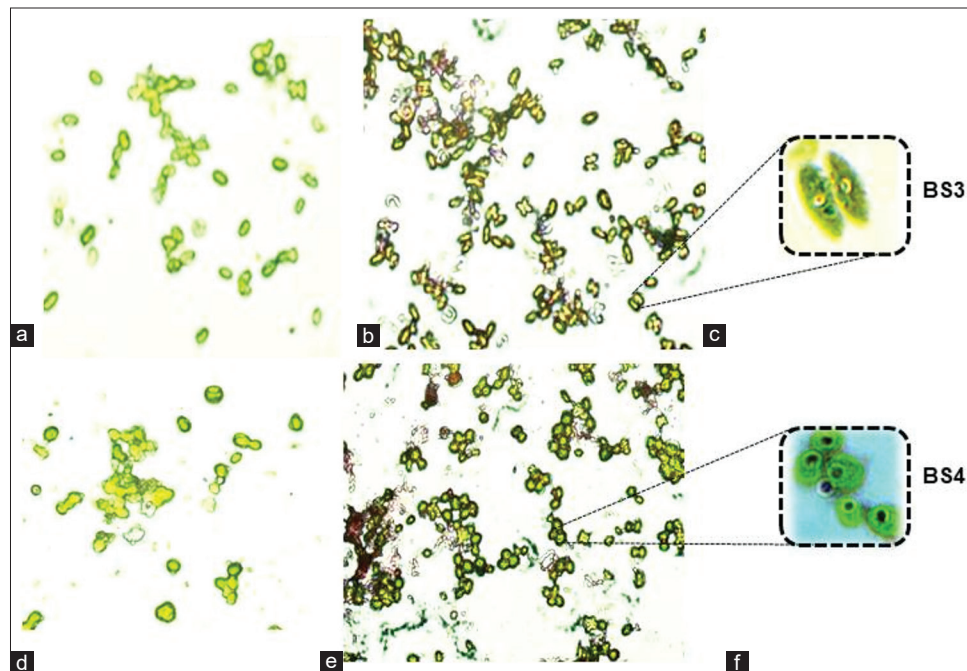
## 2. MATERIALS AND METHODS

### 2.1. Cultivation and Microscopic Observation On BS3 and BS4 Algal Isolates

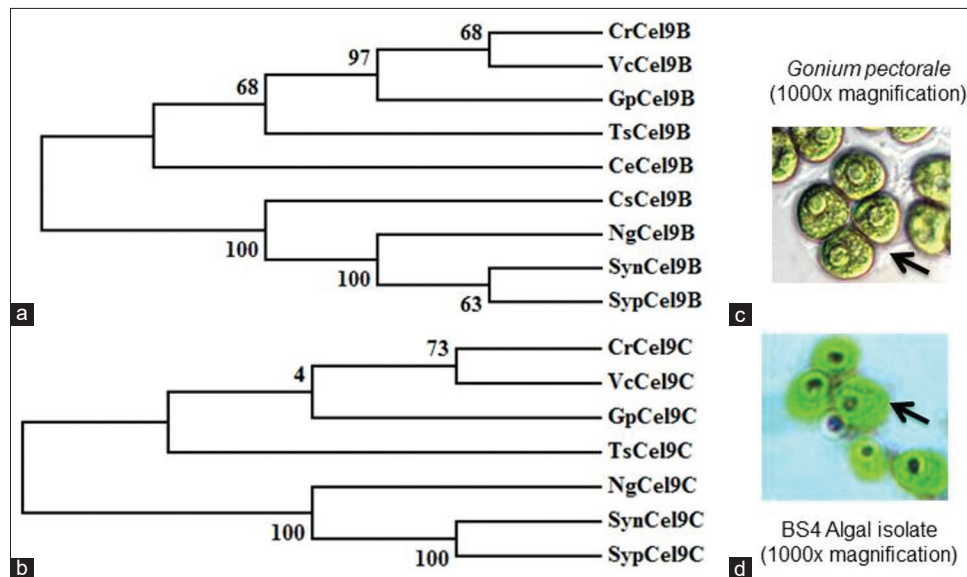
The experimental study had been initiated through water sample collection from the mangrove ecosystem in Sundarban islands. Two distinct types of water samples from the river stream have been collected and stored in a cold box for transportation to the research laboratory. First, these two river water samples were collected and had been filtered through Whatman filter paper (Grade 113, Sigma-Aldrich) to sieve out plant leaves and plant-associated debris. Afterward, 2 ml river water samples (filtered) were inoculated in 8 ml sterile BBM [26] algal growth media (pH 7.2) and incubated at 16 W/m<sup>2</sup> light intensity in shaking mode (50 rpm) for 7 days. After 7 days of incubation period, green color appears in the culture tubes. Then, these cultures from two different locations have been undergone for pure culture development on BBM agar plate (pH 7.2). 1 ml sample was transferred to a BBM agar media plate and spread evenly across the surface. Inoculated plates were placed in a temperature-controlled incubator (20–25°C, approximately) where the algae were allowed to grow for about 7–10 days. Grown algal cultures were streaked using a sterile technique onto additional sets of nutrient media plates and placed back in the incubator for isolation. This streaking method was repeated until isolation into an axenic algal culture was achieved. Subsequently, axenic algae isolates (BS3 and BS4) had been preserved in 15% glycerol stock at –20°C freezer for further usage. The pure isolates BS3 and BS4 had been regularly sub-cultured to maintain the optimal growth for light microscopic analysis ( $\times 400$ ,  $\times 1000$  magnification in Magnus MLXi Plus, Magcam DC3) and optical density-biomass analysis (using UV-Vis spectrophotometer from SHIMADZU, Model No: UV-1800).

### 2.2. Determination of Growth and Lipid Contents in BS3 and BS4 Algal Isolates

The growth profile of novel algae isolates (i.e., BS3 and BS4) had been determined following a spectrophotometric approach by preparing a standard curve between optical density (O.D 700 nm) and biomass (g/ml). Biomass quantification had been done using heating and drying in a hot air oven on filter paper [27]. Biomass content estimation was essential to determine cellular lipid content in regular time intervals during culture sample collection. *In vivo* qualitative estimation of lipid droplets or lipid accumulation had been determined by the classical



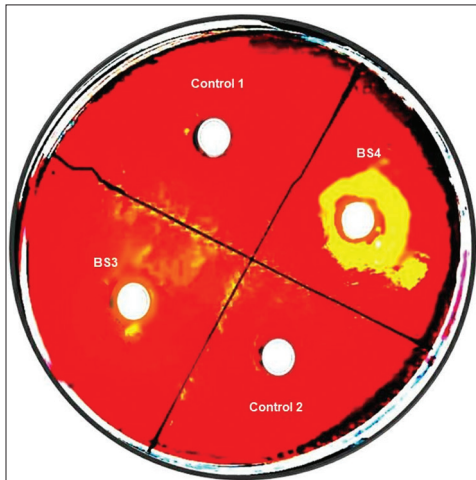
**Figure 1:** Microscopic observation of mangrove ecosystem derived algal isolates (a) BS3 isolate's microscopic view at  $\times 400$  magnification; (b) BS3 isolate's microscopic view after Sudan black staining under  $\times 400$  magnification; (c) BS3 isolate's microscopic view after SB stain in  $\times 1000$ ; (d) BS4 isolate's microscopic view under  $\times 400$ ; (e) BS4 isolate's microscopic view after staining; (f) BS4 isolate's microscopic view after SB stain in  $\times 1000$ .



**Figure 2:** Comparative overview on characterization of BS3 and BS4 algal isolates through microscopic observation and phylogenetic analysis (Phylogenetic tree has generated based on putative algal Cel9Bs and Cel9Cs protein sequences. The final phylogenetic tree presented here has been developed using MEGA5 with 500 times bootstrap replication and a substitution model p-distance (substitution type amino acids, complete deletion of gaps/missing data treatments). The evolutionary history was inferred using the Neighbor-Joining method [52]. The optimal tree with the sum of branch length is  $\sim 2.53$ . The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [53]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree; algae based Cel9Bs and Cel9Cs abbreviation are mentioned with all possible information in supplementary data file S1 microscopic observation depicts the morphological resemblance of BS4 algal isolate with *G. pectorale* strain under  $\times 1000$  magnification. Microscopic views are not in uniform scale. Here is the abbreviation of protein sequences and strains consider for phylogenetic tree development CrCel9B: *Chlamydomonas reinhardtii*; VcCel9B: *Volvox carteri*; GpCel9B: *G. pectorale*; TsCel9B: *Tetabaena socialis*; CeCel9B: *Chlamydomonas eustigma*; CsCel9B: *Chlorella sorokiniana*; NgCel9B: *Nannochloropsis gaditana*; SynCel9B: *Synechococcus sp.*; SypCel9B: *Synechocystis sp.*; CrCel9C: *Chlamydomonas reinhardtii*; VcCel9C: *Volvox carteri*; GpCel9C: *G. pectorale*; TsCel9C: *Tetabaena socialis*; NgCel9C: *Nannochloropsis gaditana*; SynCel9C: *Synechococcus sp.*; SypCel9C: *Synechocystis sp.* (strain PCC 6803/Kazusa). Moreover, microscopic views of *Gonium pectorale* (reference point) have been adopted from mentioned references in supplementary Figure S1 [50] as standard reference to make comparative overview in comparison to BS4 isolate in the present study.

Sudan black staining method [28]. It had been observed that cellular algal lipid droplets take blue-black or light blue color whereas non-lipid cellular materials look light pink under the light microscope. Lipid quantification had been carried out using standard lipid extraction and quantification approaches [29,30]. Cellular lipids constituents from algal isolates (BS3 and BS4) had been extracted using dried algae cells (0.5 g) using chloroform methanol solvent (15 ml) extraction method in 2:1 ratio (v/v) following sonication (30 Hz for 20 min on ice pack). Cellular lipid quantification had been carried out using the following molecular standard formula [31] where WL (g) is the weight of lipid and WB (g) the algal biomass dry weight.

$$C1 \text{ (g/g)} = \text{WL/WB} \quad (1)$$

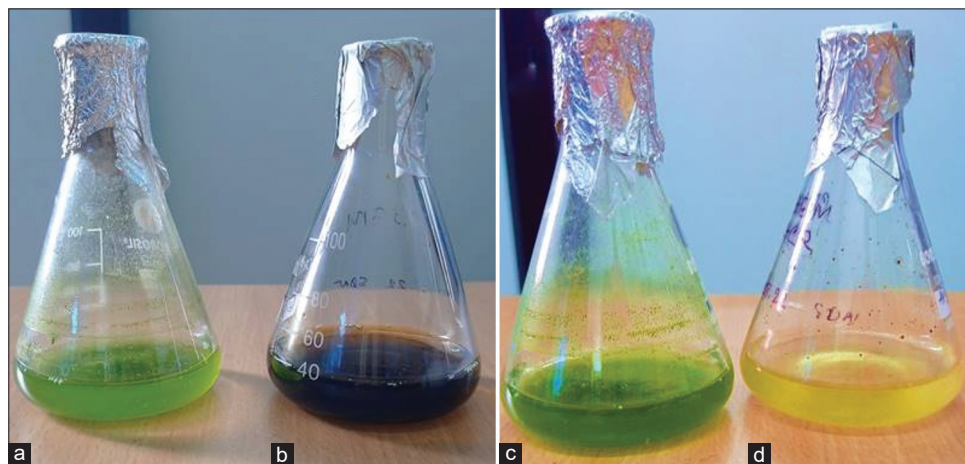


**Figure 3:** Profile on secretion of cellulolytic enzymes of BS4 and BS3 algae isolates using CMC supplementation and Congo red plate assay (Experimental results show that BS4 is the potential algal isolate which has extracellular cellulase and/or endoglucanase activity while BS3 does not bear this phenotypic trait through natural selection. Moreover, negative control 1 refers to sterilized distilled MiliQ water which reflects metal constituents do not show any impact on extracellular enzyme secretion and/or on its enzymatic activity; whereas negative control 2 refers to the sterile BBM medium which confirm not contaminants do exist in BBM medium, respectively).

### 2.3. Qualitative Assessment on Cellulose Degradation Potentiality of BS3 and BS4 Algal Isolates

Cellulases randomly act concerning three different ways. The glycosyl bonds were hydrolyzed by exoglucanases, cellobiohydrolases (1, 4-β-D-glucanhydrolases) and cellobiohydrolases (1, 4-β-D-glucan cellobiohydrolase), and end of the cellulose polymer chain 1, 4-β-glucan-4-glucanhydrolases split the chain through the endoglucanases inwards. However, cellulose degradation was mainly convenient to the catalytic domains of the universal cellulase complex, which was a fourth and non-catalytic element. The β-glucoside glycohydrolases and 1, 4-β-D glucan cellobiohydrolases help to cleave the cellobiose glycosyl bond. Predominantly, in the cellulase complex, a carbohydrate binding module must be present. The attachment and position of the enzymes onto the cellulose and expedite of single molecules discharge helped enzymatic lysis [32-34]. Confirmation of cellulose degradability had been initiated through cellulose Congo red assay where cell-free supernatants had been used for the *in vitro* degradation assay including carboxy-methyl cellulose (CMC) as sole carbon substrate with a concentration of 1% (w/v). BS3 and BS4 algal isolates from an axenic glycerol mother stock had been picked and inoculated to BBM agar plate (to attain active subculture during experimentation) using an inoculating needle and inoculated onto basal broth medium (modified from Mandels medium) containing CMC sodium salt incubation period at 25°C (±2) for 48 h. Afterward, these exponential BS3 and BS4 algal axenic cultures had undergone for cell free supernatant generation under an aseptic environment. Then, cell-free supernatant of BS3 and BS4 algal isolates had been applied on CMC selective plates bearing grooves and incubated for 48 h at 25°C (±2). Finally, these incubated selective plates had been stained with 1% (w/v) Congo-red solution for 15 min and 1 M NaCl (15 min) have been used for discoloration [35-39]. The Congo-red assay had been used for the quantitative determination of the dye decolorization potentiality of the isolate BS4. The dye was degraded by cellulase enzyme produced from the aforementioned algal isolates in the selective BBM medium. Moreover, the Congo red breakdown rate was observed for 0, 3, 6, and 9 days at 417 nm wavelength as a standard outlet [40] including equation 2.

$$\text{Decolourization (\%)} = (\text{Initial absorbance} - \text{Final absorbance}) / \text{Initial absorbance} \times 100 \quad (2)$$



**Figure 4:** Dye decolorization analysis using algal isolate BS4 (0-9 days). These pictures signify the congo red decolorizing activity (96.04%) of BS4 in BBM algal growth medium within 9 days. Dye decolorization assay before the treatment (0 days) without Congo red (a), with Congo red (b) and dye decolorization assay after the treatment (9 days) Without Congo red (c), With Congo red (d).

Moreover, cellulose degradation potentiality had also been quantified using the di-nitro salicylic acid (DNS) assay to measure the release of reducing sugars after the breakdown of cellulose in the BBM media due to the potential activity of cellulase in BS4 algal isolates. In standard Bradford assay had been followed for estimation the protein concentration of BS4 isolates [41,42]. Afterward, we had measured the cellulase activity following standard protocol [43] including equation 3.

$$\text{Cellulose enzyme activity as U/ml} = W * 1000 / V \cdot t \cdot M \quad (3)$$

Where, W = Amount of release glucose, M = Molecular weight of the glucose, V = Volume of the sample, T = Reaction time.

#### 2.4. Studies on Phylogenetic Analysis of Algal Cellulase and Microscopic Observation of BS3 and BS4 Algal Isolates

The endo- $\beta$ -1, 4-glucanases of gut termites (i.e. UniProtKB\_Q64I76) and earthworms (UniProtKB\_O77045; UniProtKB\_B9A7E3) were the most potent and effective cellulase enzymes toward the degradation of lignocellulosic biomass [44]. These potential enzymes were belonging to the GH protein family 9 (GHF9). Protein sequence homology search among the GHF9 family proteins had helped to identify a few algae-based cellulase and/or endoglucanase group of enzymes, that is, Cel9Bs, Cel9Cs, respectively; however, exact function of these proteins was not yet clear or properly characterized. Based on this context and homology search, algal-based few cellulases and/or endoglucanase sequences, that is, *Cel9Bs* and *Cel9Cs* had been collected from online NCBI repositories and depicted in Supplementary Data file S1. *Cel9Bs* and *Cel9Cs* sequence alignment for molecular phylogenetic analysis had been generated using Clustal W and Clustal Omega [45] and optimized using MEGA5 [46]. Multiple sequence alignment dataset had been shown in the Supplementary Data file S2. Neighbor-joining (NJ), maximum parsimony, and maximum likelihood (ML) statistical models had been used to infer the phylogenetic relationship among strains of closely associated green algae based on *rbcL* gene sequences. A substitution model for phylogenetics of each nucleotide sequence data set had been selected using the jModel test [47] with model selection in MEGA5. Representative phylogenetic trees had been drawn using the NJ and ML methods. However, the main objective of doing this phylogenetic analysis was to classify the diverse ranges of algal cellulase and/or endoglucanase enzymes. Recently, *Cel9B*, *Cel9C*, and *Cel9D*-based cellulolytic activity in *Chlamydomonas reinhardtii* has been experimentally proven. However, the exact role of *Cel9D* on cellulolytic activity had still clearly needed to be understood [48]. Therefore, *Cel9B* and *Cel9C* sequences of *C. reinhardtii* were considered in this phylogenetic analysis as a positive set of control. Moreover, the phylogenetic analysis may also be conjugated with the comparative microscopic observation of BS3 and BS4 algal isolates using pre-morphologically characterized algal strains from literature due to non-availability of 16s rRNA sequencing dataset in this current wet research work. This conjugated approach may help to indirectly narrate that BS3 and BS4 belong to which class of algal regimes.

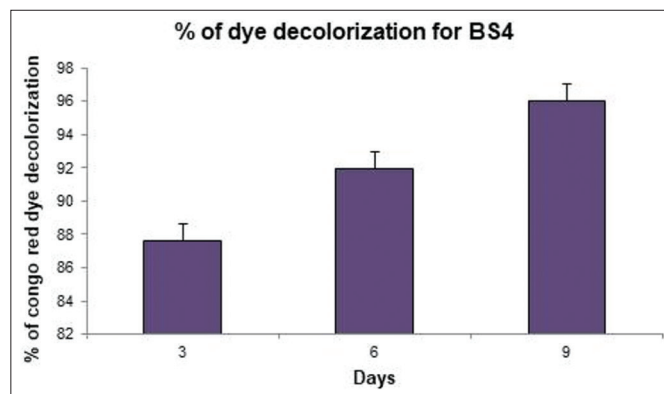
#### 2.5. Statistical Analysis

Statistical analysis was executed using IBM SPSS software (version 26.0). Correlation coefficient test (Pearson Correlation) had been accomplished for the confirmation of significance correlation at 0.05 significance levels (2-tailed) among the day, biomass concentration, and lipid concentration of pure axenic algal cultures inoculated in sterile BBM medium for small-scale experimentations [49].

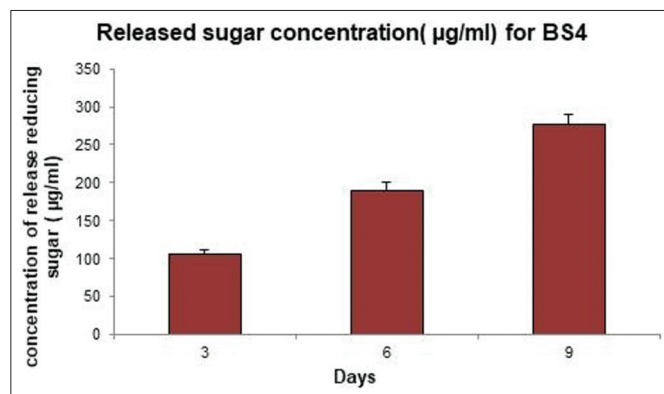
### 3. RESULTS AND DISCUSSION

#### 3.1. Studies on Morphological Characterization of BS3 and BS4 Algal Isolates through Microscopic Observation and Phylogenetic Analysis Using Putative Algal Cellulase Enzymes

Microscopic observation had played a very important role to identify the morphological characterization of algal communities through physical appearance with or without staining applications. Two mangrove algal isolates, that is, BS3 and BS4 had been successfully visualized under bright field microscopy without staining as algae contain photosynthetic pigments in its cellular biomass. Microscopic examination had demonstrated the morphological and physiological diversity of two different isolates at  $\times 400$  [Figure 1] and  $\times 1000$  magnification [Figure 1b and c] using algae culture of the 9<sup>th</sup> day. Microscopic observation had shown that BS4 has a very close morphological resemblance with the microscopic view [50] of the algal regime *Gonium pectorale* [Supplementary Figure S1, Figure 2c and d]. However, BS3 did not show any similarity considering the microscopic characteristics of chosen algal communities. Interesting phylogenetic analysis had also shown that *G. pectorale* algae [50] were closely associated clade to *C. reinhardtii* as phylogenetic analysis considering *Cel9B* and *Cel9C* cellulase and/or endoglucanase protein sequences in this present study [Figure 2a and 2b]. However, it would be impossible to place this alga taxonomically based on microscopic observation and



**Figure 5:** Determination of dye decolorization analysis using algal isolate BS4 (Decolorizing activity was highest (96.04%) of BS4 in BBM algal growth medium within 9 days;  $n = 3$  where  $n$  is the number of best replicates).



**Figure 6:** Measurement on reducing sugar release after cellulose degradation using algal isolate BS4 (Released reducing sugar has been estimated by DNS assay and BS4 released the highest amount of reducing sugar within 9 days, that is, 276.72  $\mu\text{g}\cdot\text{ml}^{-1}$ ;  $n = 3$  where  $n$  is the number of best replicates).

*in-silico* molecular phylogenetic analysis. Therefore, more work on the molecular and physiological levels would be required to firmly establish the taxonomy of this strain. For the purposes of this article, this strain will be referred to as BS4 algal isolate from the mangrove ecosystem.

### 3.2. Qualitative Analysis on Cellulolytic Activities of BS3 and BS4 Algal Isolates

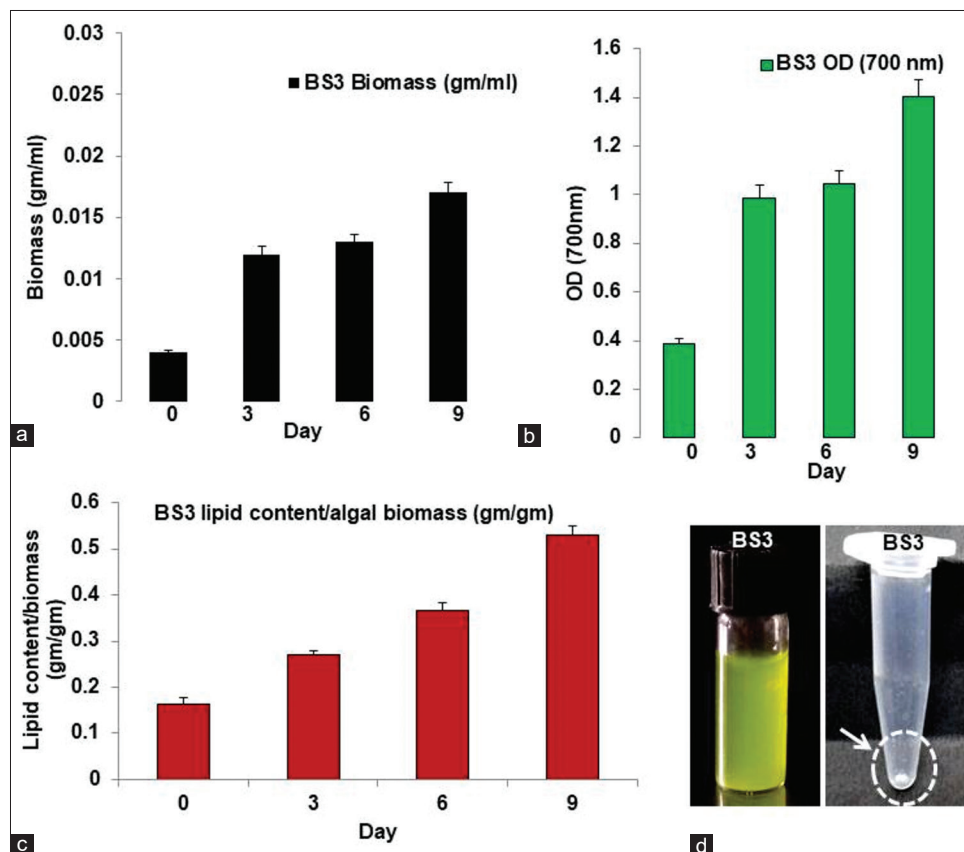
A qualitative analysis of cellulolytic enzymatic activities had been investigated for BS3 and BS4 algal isolates of the mangrove ecosystem considering Congo red plate assay approach. A clear hollow round zone had been observed on selective plates for BS4 algal isolate after 48 h of photo incubation at 25°C ( $\pm 2$ ) following Congo red plate assay technique. A clear visible yellow-color degradation zone had been shown around the groove where cell-free supernatant of BS4 algal isolate had been applied. Thus, it had clearly and qualitatively confirmed that BS4 algal isolate and its cell free supernatant did have

enormous potential to degrade or hydrolyze cellulose as potential cheaper feedstock. However, no zone had been observed for BS3 isolate on selective plates compared to the control sets (i.e., negative control 1 refers to sterilized distilled Milli Q water whereas negative control 2 refers to the sterile BBM medium). Whereas BS3 algal isolate did not show any cellulose-degrading ability while performing a similar experimental design. Extracellular secretion of active endoglucanases and/or cellulases degrading water soluble cellulose moieties had been indicated the presence of an extracellular secretory enzymatic platform toward extracellular cellulose decomposition [Figure 3, Supplementary Figures S2 and S3] in BS4 algal isolates. The dye decolorizing percentage (%) of BS4 algal isolate was attained as 96.04% on 9<sup>th</sup> day in this present study [Figures 4a-d and 5]. Simultaneously, it had also been shown as the continuation of this study where it had been shown that degradation products (i.e., precursor and/or intermediate hexose or pentose monomers) of these celluloses can be utilized toward value-added biomolecule generations likely biodiesel in this present

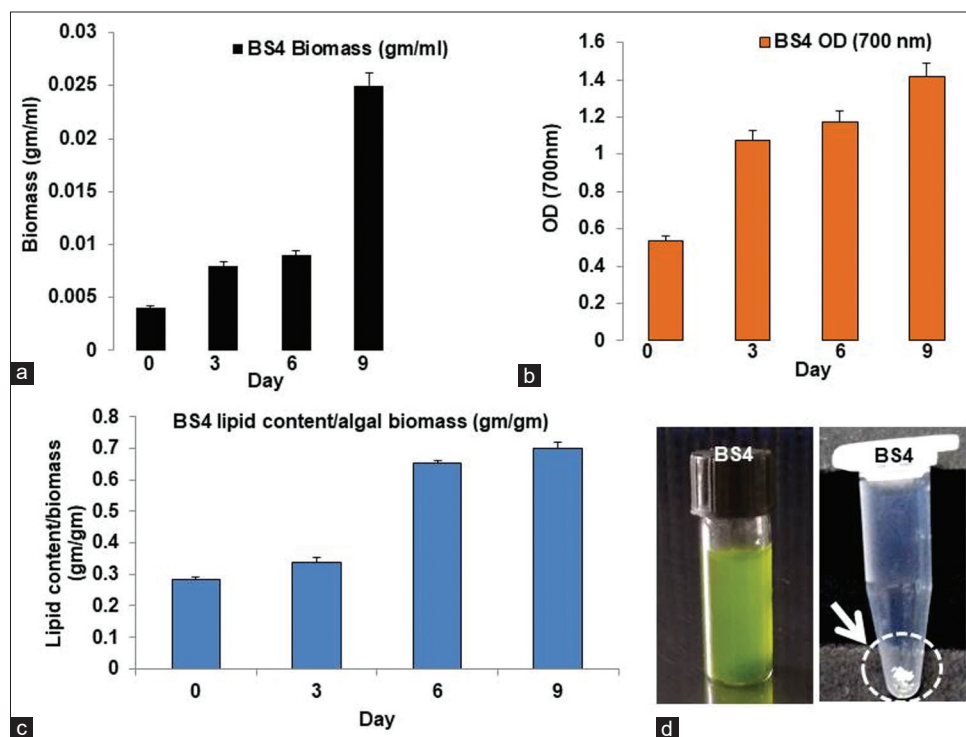
**Table 1:** Comparative overview on productions of biomass and lipid with cellulase enzyme activities in diverse algal assemblages.

Algal assemblage	Biomass production rate (g.L <sup>-1</sup> .day <sup>-1</sup> )*	Lipid production rate (g.L <sup>-1</sup> .day <sup>-1</sup> )*	Cellulase Enzymatic activity in U.mg <sup>-1</sup> * (ND refers undetermined)	References
<i>Chlorella sorokiniana</i>	0.23–1.47	0.044	$\geq 240,000$ (Cellulase)	[54,55]
<i>Chlamydomonas reinhardtii</i>	0.672	0.141	0.036 (crude enzyme)	[48,56,57]
<i>Nannochloropsis gaditana</i>	0.045	0.014	ND	[58,59]
Algal isolate BS4	2.77 ( $\pm 0.04$ )	1.93 ( $\pm 0.03$ )	0.284 ( $\pm 0.001$ ) (crude enzyme)	Current study

\*we have unified the values based on the dataset available in original references.



**Figure 7:** Biomass and lipid production profile using BS3 algal isolate from mangrove ecosystem (a) BS3 isolate's growth curve where day-wise biomass increases; (b) BS3 isolate's growth curve where day-wise OD increases; (c) BS3 growth curve while day-wise lipid content/algal biomass increases; (d) BS3 algal pure culture and the extracted lipid as dried white pellet).



**Figure 8:** Biomass and lipid production profile using BS4 algal isolate from mangrove ecosystem (a) BS4 isolate's growth curve where day-wise biomass increases; (b) BS4 isolate's growth curve while day-wise O.D. increases; (c) BS4 growth curve where day-wise lipid content/algal biomass increases; (d) BS4 pure culture and the extracted lipid as dried white pellet).

study. Released reducing sugar had been estimated by routine DNS assay and BS4 released the highest amount of reducing sugar on 9<sup>th</sup> day (i.e., 276.72  $\mu\text{g}\cdot\text{ml}^{-1}$ ) [Figure 6 and Supplementary Figure S4]. The protein estimation had been done by Bradford assay, to measure the total protein concentration of algal enzyme hydrolysate the concentration from BS4 90.77  $\mu\text{g}\cdot\text{ml}^{-1}$  [Supplementary Figure S5] [43]. However, the present result had specifically shown that higher cellulolytic activities were observed in the case of algal isolate BS4 0.284 ( $\pm 0.001$ )  $\text{U}\cdot\text{mg}^{-1}$  [Table 1]. In comparison, BS3 was not able to accelerate any enzymatic activities for the breakdown of the substrates for cellulose degradation. For BS3 isolate, the low and average molecular weight fragments formation process had been decreased by the split of glycosidic bonds because these fragments were soluble in water. Furthermore, during the photosynthesis growth condition, BS4 isolate helped to produce organic carbon acquisition toward degrading the cellulase. Hydrolysis of *in vitro* study had been focusing on CMC utilization for algal cellular higher growth rate and revealed that the secretion of cellulase and/or endoglucanase [51].

### 3.3. Growth and Lipid Profiles of BS3 and BS4 Algal Isolates with Statistical Constraints

Growth profile studies had shown that BS4 gives comparatively higher biomass production compared to BS3 on day 9<sup>th</sup> of cultivation [Figures 7a,b and 8a,b]. Extensive studies had also depicted that the biomass productivities for BS4 and BS3 isolates are 2.77 ( $\pm 0.04$ )  $\text{g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$  and BS3 1.88 ( $\pm 0.02$ )  $\text{g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ , respectively. BS4 was comparatively generated with a higher amount of lipid content compared to BS3 [Figures 7c and 8c, Figures 7d and 8d] on day 9<sup>th</sup> of cultivation. Moreover, BS4 and BS3 had shown lipid productivities, that is, 0.0775 ( $\pm 0.001$ )  $\text{g}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$  [i.e., 1.93 ( $\pm 0.03$ )  $\text{g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ ] and 0.0588 ( $\pm 0.002$ )  $\text{g}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$ , respectively. However, experimental

results had clearly shown that lipid production is highly proportional to biomass production in mangrove algal isolates BS3 and BS4. Preliminary screening had shown lipid droplet accumulation in the cellular fraction of two novel isolates, that is, BS3 and BS4. After Sudan black staining approach shows BS4 accumulates comparatively higher amounts of lipid droplet accumulation inside the algal cells than do the BS3. Algal isolates showed diverse intensity in stain (blue or blue-black) uptake considering the presence of different lipid content in BS3 [Figure 1b and c] and BS4 [Figure 1e and f], respectively. In addition, statistical analysis especially the correlation coefficient test had been done for the confirmation of significant correlation among the day, biomass concentration and lipid concentration by IBM SPSS 26 statistical software at 0.05 significance level and the result showed that there is significant relations present among all the three parameters in both BS3 and BS4 algal assemblages [Supplementary Tables 1 and 2] [45].

### 4. CONCLUSION

The present study has revealed several intriguing features of mangrove algal isolates, namely, BS4 and BS3. The BS4 algae isolate exhibits two distinct characteristics: Likely cellulose (the predominant constituent of LCW) degradation and higher lipid generation productivities as a single pot microbial cellular factory. Furthermore, at mesophilic and neutrophilic conditions, BS4 algal isolate has produced higher lipid contents, higher biomass productivities, and shown higher cellulose degradation. In contrast, BS3 does not show any cellulolytic efficacy though it produces a good amount of lipid and biomass during batch cultivation operations. Therefore, it seems BS4 algae isolate could be one of the most promising aspirants toward sustainable biodiesel production utilizing lignocellulosic biomass. However, microscopic observation along with *in silico* comparative phylogenetic analysis

tentatively and indirectly infers that BS4 is the closest clade of *G. pectorale*. However, it would be impossible to place this alga taxonomically based on microscopic observation alone, so molecular phylogenetic analysis needs to be carried out. However, BS4 exhibits the most similar phenotypic traits, such as cellulolytic efficacies harboring extracellular endoglucanase and/or cellulase activities (i.e., Cel9B and Cel9C), as seen in *Chlamydomonas reinhardtii*. Moreover, the function of another cellulose degrading module, that is, Cel9D, needs to be explored not only to understand the catalytic efficiency but also to comprehend the regulatory mechanism. Therefore, this study needs to be researched further to improve biomass and lipid productivities using BS4 and BS3 mangrove algal isolates.

#### 4.1. Supplementary Information

Supplementary Dataset S1; Supplementary Dataset S2; Supplementary Figure S1; Supplementary Figure S2; Supplementary Figure S3; Supplementary Figure S4; Supplementary Figure S5; Supplementary Table 1; Supplementary Table 2.

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#### 6. AUTHORS' CONTRIBUTIONS

Ms. Shrestha Debnath: All wet laboratory experimentation, data collection, data analysis, data set tabulation, data validation, investigation, wet laboratory experimental dataset visualization, original drafting, writing, and formatting. Dr. Dipankar Ghosh: Conceptualization, original drafting, writing, editing, reviewing, computation experimentation and computation dataset analysis, computational results visualization, and supervision.

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#### 8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

#### 9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

#### 10. DATA AVAILABILITY

All data supporting this study are available on request.

#### 11. PUBLISHER'S NOTE

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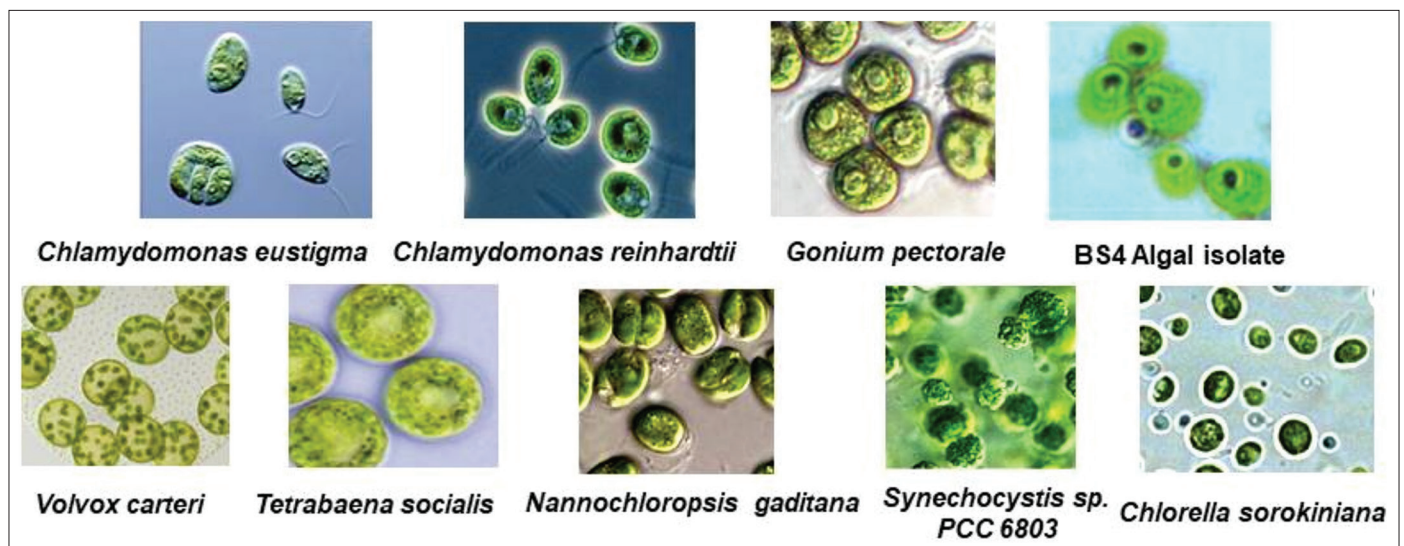
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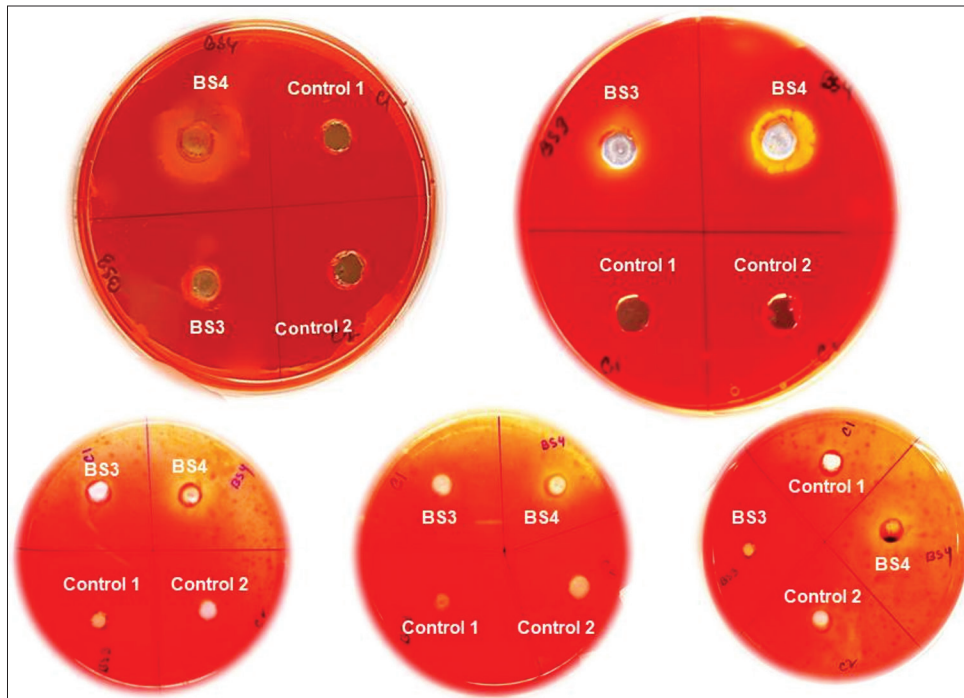
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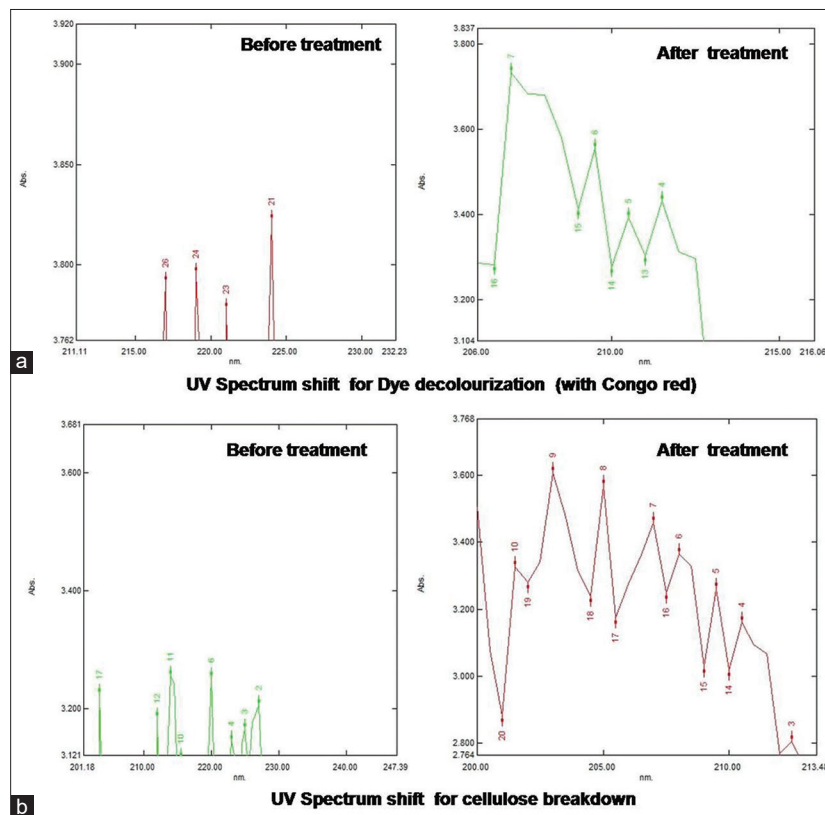
DOI: 10.7324/JABB.2023.76846

**SUPPLEMENTARY FIGURES AND TABLES**

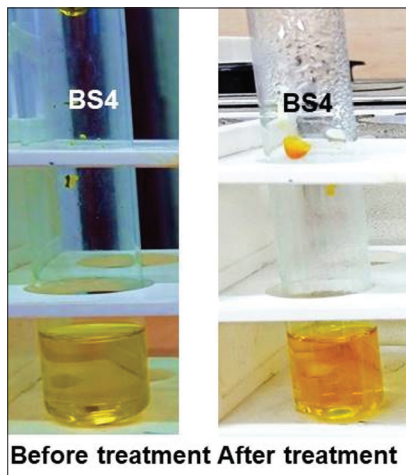
**Supplementary Figure S1:** Comparative microscopic overview on diverse algal regimes bearing Cel9B and Cel9C proteins as phenotypic trait to degrade cellulosic materials. Microscopic views are not in uniform scale but photographs have been taken at  $\times 1000$  magnification under oil immersion toward comparison with BS4 algal isolate used in this present study and reference microscopic views have been adopted from mentioned references as standard reference to make comparative overview in comparison to BS4 isolate in the present study [50,60-66].



**Supplementary Figure S2:** Cellulase plate assay (qualitative) for Congo red degradation. Breakdown the Congo red mixed BBM agar media enriched with carboxy methyl cellulose shows the orange hollow zone around the groove which signifies the cellulolytic activity of BS4.



**Supplementary Figure S3:** (a) These UV spectrum shifts for dye decolorization (with Congo red) signify the breakdown of Congo red within 9-day time span for BS4 isolate. Before the treatment, the highest UV absorbance spectra of Congo red were 224 nm. That was shifted to 207nm that clearly indicates the breakdown of Congo red. (b) UV Spectrum shift for cellulose breakdown signifies the breakdown of cellulose within 9-day time span BS4 isolate. Before the treatment, the highest UV absorbance spectra 214 nm. That was shifted to 203 nm that clearly indicates the breakdown of cellulose.



**Supplementary Figure S4:** DNS assay for sugar estimation. After the treatment, BS4 shows that the amount of released reducing sugar high (deep brown color).

**Supplementary Table 1:** Correlation among day, biomass concentration, and lipid concentration of BS3.

BS3	Correlation		
	Day	Biomass concentration	Lipid concentration
Day			
Pearson Correlation	1	0.930	0.398
Sig. (2-tailed)		0.002	0.003
Biomass Concentration			
Pearson Correlation	0.930	1	0.411
Sig. (2-tailed)	0.002		0.001
Lipid Concentration			
Pearson Correlation	0.398	0.411	1
Sig. (2-tailed)	0.003	0.001	

Correlation is significant at the 0.05 level (2-tailed). Statistical Analysis by IBM SPSS 26.



**Supplementary Figure S5:** Bradford assay for protein estimation. This picture shows that after reaction of bradford reagent with BS4 extract, it change its color to blue which signifies the presence of high amount of protein.

**Supplementary Table 2:** Correlation among day, biomass concentration, and Lipid concentration of BS4.

BS4	Correlation		
	Day	Biomass concentration	Lipid concentration
Day			
Pearson Correlation	1	1.00	0.889
Sig. (2-tailed)		0.003	0.002
Biomass concentration			
Pearson correlation	1.00	1	0.890
Sig. (2-tailed)	0.003		0.001
Lipid concentration			
Pearson correlation	0.889	0.890	1
Sig. (2-tailed)	0.002	0.001	

Correlation is significant at the 0.05 level (2-tailed). Statistical Analysis by IBM SPSS 26.