Isolation and characterization of biological soil crust forming algae from Malaysia

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Abstract Soil erosion permanently reduces the quality of physicochemical and biological properties, soil fertility, and land productivity, all of which have a significant impact on cultivated areas. Communities that live on the top few centimetres of soil surfaces, are known as "biological soil crust" (BSC). This microorganism is essential for soil stabilisation, water retention, and soil fertility. A case study of a Japanese company that uses these organisms to combat soil erosion has inspired this study. Ground covers, slope drains, silt fences, blankets, and plastic covers are currently used to reduce soil erosion which are expensive and less environmentally friendly. An artificially induced BSC can be created on the specific targeted area. The first step in implementing this strategy in Malaysia is to collect, isolate,and identify the BSC-forming algae species. Our findings show that the isolated algae (C3 strain), based on its morphology, has been identified as the *Tribonema* species and has a yellow-green filament under a microscope. The C3 strain therefore, has been chosen as the potential native BSC forming algae. This research aims to provide information on isolated strains of Malaysian algae for application as BSC in tropical settings.

Keywords: Algae growth; Biological soil crust (BSC); Isolation; *Tribonema* sp.; Tropical environment

1. Introduction

Biological soil crust (BSC) is a community of cyanobacteria, lichens, mosses, and fungi, which are an essential part of dryland ecosystems. More than 300 cyanobacteria and 350 eukaryotic algae species have been discoveredin BSC formation. BSC are widespread in a range of settings, including deserts and polar regions, due to their remarkable tolerance to stress in extreme environmental conditions. Extreme environmental conditions such as wetland ecosystems are ideal for the growth of cyanobacteria and algae because wetland is rich in nutrients (organic and nutrient molecules) that feed other living species. Nitrogen (N) supply to the initial ecosystem is crucial for further development of BSC. One important input pathway is the biological N fixation that was shown to be the dominant process providing N to BSC [1]. Mineralization, nitrification, and denitrification are the three dominant microbial processes in the N cycle in the BSC. Polysaccharides are produced by algae and cyanobacteria with the ability to fix nitrogen(N) [1].

It is thought that the filamentous nature of *Microcoleus*, *Klebsormidium* and *Zygogonium* as well as their secretion of mucilage aids in the creation of BSC by entangling soil particles. The filamentous cyanobacteria *Microcoleus* are the most prevalent cyanobacteria. Soils are stabilised and aggregated due to the presence of the extracellular matrix in the filamentous cyanobacteria *Microcoleus*. When it comes to establishing themselves in unstable surroundings, these filament-forming organisms are the pioneers.

Recently, eukaryotic algae have been identified to assist in the creation of BSC. However, BSC created by eukaryotic algae is rarely studied. Their filamentous structure and mucilage secretion can

collect soil particles and aggregate them into BSC. *Klebsormidium* are eukaryotic [2] green algae that can tolerate dehydration by modifying its cell wall, cytoplasm, and mitochondria.

BSC which helps to maintain ecological balance has the capacity to repair soil conditions, water retention, and is utilised to preserve the fertility and health of degraded soils [3-4]. In deteriorated and eroded areas, the algae in BSC create sheaths and filaments to link the soil particles [5]. BSC are excellent at capturing eolian (wind-blown) dust. Dust is a critical component in enhancing soil fertilityand nutrient enrichment. BSC can also be used to cover seeds to keep them moist during germination [6]. A case study of a Japanese company that uses these organisms to combat soil erosion inspired this study. Therefore, a new approach has been embarked upon, which is through developing an artificially induced BSC. Thus, this paper reports on the work and progress of the isolation of BSC-forming algae in the native environment. This work covers the isolation, cultivation, morphology analysis of isolated algae, and the growth of the isolated algae.

2. Materials and Methods

2.1. Field collection

Algae samples with visible algae population were collected from soils that are located in the Department of Irrigation and Drainage (DID) Kuala Lumpur, around Kuala Lumpur campus of Universiti Teknologi Malaysia and Kolej Siswa Jaya (KSJ), and sites outside of Kuala Lumpur, which are Lojing, Cameron Highlands, Jalan Bangi Lama, and Cyberjaya. The criteria for sampling sites selection are the location must consist of any algae speciesthat are available on side hills or undeveloped areas that have previously experienced natural disasters such as landslides or soil erosion site.

2.2. Isolation and Cultivation

To isolate BSC-forming algae, soil samples were air-dried for one week on a petri dish following collection. It was considerably easier to separate the air-dried samples from the excess soil. The filamentous algae were the primary focus of this study. The soil sample was transferred in a container containing AF-6 medium. As a result, a single string of filamentous algae was picked up using a micropipette beneath the microscope (BS-2040 B/T, Best Scope, China) and transferred to a triple well microscope cavity slide containing distilled water for cleaning and washing purposes using a tweezer and needle.

Following that, a single filamentous algae string was cultured in AF-6 media to provide nutrients and stimulate growth. To get other strains of BSC-forming algae, similar steps were repeated in a Mini Tray with Delta (12 well-plate, Thermo Fisher Scientific, USA) or 50 mL conical flasks were used to cultivate the isolated cells.

The requirements and conditions for optimum growth of algae indoors are the temperature is at room temperature, which is 25^{o}C, aeration of air supply with 2% of CO_2, placed under the light for 24 hours continuously. Fungicide (Polyoxin AL, Kaken Phamaceutical, Japan) were added to the medium at 0.5 ppm in final concentration if the algae were heavily infected with fungi.

The samples were maintained and observed under a microscope for isolation and morphological screening purposes. The microscope used is equipped with a light chamber and with the software provided by Motic Image Plus 2.0 and an image capturing camera.

2.3. Cultivation medium for isolated BSC forming algae (AF-6)

The following recipe was used to prepare the AF-6 culture medium: (1) Solution A-$NaNO_3$, 14g/L; NH_4NO_3, 2.2g/L; $MgSO_4.7H_2O$, 3g/L; $CoCl_2.2H_20$ 1g/L; Fe-Citrate 0.2g/L; Citric Acid, 0.2g/L (All stock in 200mL), (2) Solution B-KH_2PO_4, 1g/L; K_2HPO_4, 0.5g/L(Stock in 100mL), (3) PIV METAL $FeCl_3.6H_20$, 0.098g/L; $MnCl_3.4H_20$, 0.018g/L, $ZnSO_4.7H_20$, 0.011g/L, $CoCl_2.6H_20$, 0.002g/L; $Na_2MoO_4.2H_20$, 0.00125g/L; $Na_2EDTA.2H_20$, 0.5g/L (Stock in 100mL), (4) VIT MIX-Biotin, 0.0002g/L; Thiamine HCL,0.001g/L; Vit 6, 0.1g/L (stock in 100mL). Mix all stock solutions (Sol A-2mL, Sol B-1mL, PIV metal-1mL, and Vit mix-1mL), then adjust to pH 6.6. Autoclave sterilization (121^{o}C, 15 min).

2.4. Soil sample preparation & culture of algae outdoor (soil sample)

Soil samples (without algae added) were prepared and dry for two nights at room temperature (25°C). Mixture of C3 was prepared as: C3, water, fertilizers, fibre agents, and BSC-2. The mixture was then sprayed onto the soil surface as shown in **Figure 1**.

The surrounding temperature should be around 25-30 °C, and one layer of shade on the top of the soil is required to minimise direct sunlight for optimum growth of algae outdoors on the soil sample. This soil sample were then placed in the outdoor space of the Algae Biomass laboratory. The soil sample were then watered thrice a day for about 15 mL onto the surface of the soil sample. The samples were left for one week. The growth of C3 was evaluated through visual appearances (green colonies) after one week.

Figure 1. Soil Sample of C3 at 0 day.

2.5. Identification of isolated BSC forming algae through microscopic characteristics (morphology)

The samples were maintained and observed under a microscope for isolation and morphological screening purposes. The microscope used is equipped with a light chamber and with the software provided by Motic Image Plus 2.0 and an image capturing camera.

2.6. Monitoring algae growth indoor and outdoor

UV-VIS spectrophotometer (UV-1900, Shimadzu, Japan) is used to measure the optical density of the algae culture at 750 nm. The growth curve of the isolated BSC-algae was plotted. Outdoor culture growth after one week was monitor by visual appearance.

3. Result and Discussion

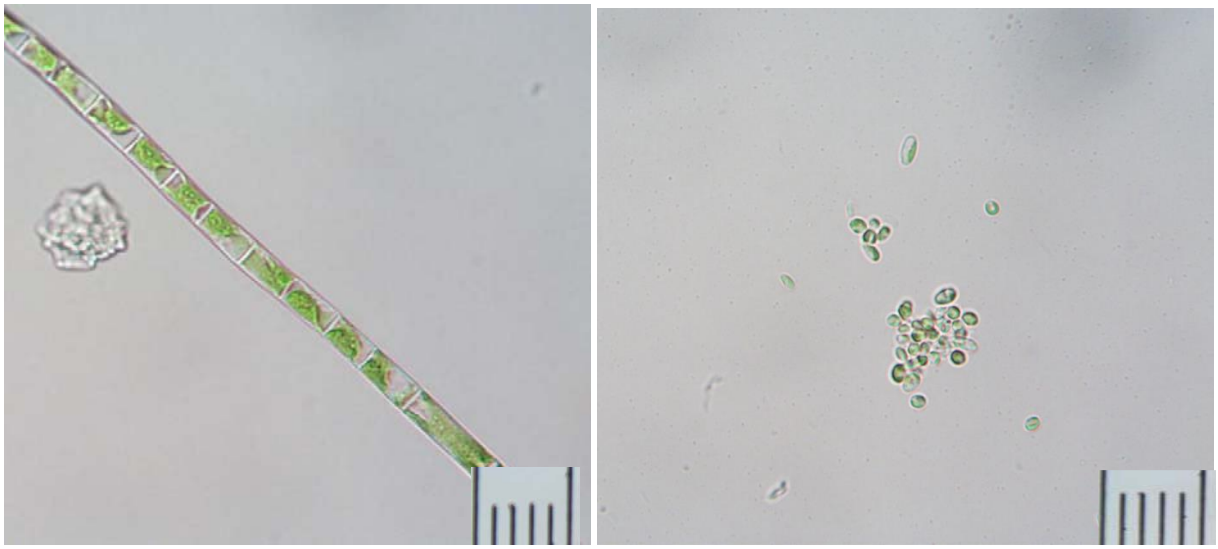
Soil samples from DID, Selangor, and Cameron Highlands that contained isolated algae were collected and kept in the agar plate as shown in **Figure 2**. The samples were then air-dried for one week on a petri dish.

(a) (b)

(c) (d)

Figure 2. Photo of soil samples from various sampling sites (a) DID, Kuala lumpur, (b) Cyberjaya, (c) Lojing Cameron, (d) Jalan Bangi Lama

Only two isolated strains from DID, Kuala Lumpur labelled as the D4 strain and Cyberjaya, Selangor labelled as the C3 strain were successfully isolated, cultured and evaluated as potential native BSC forming algae. Both strains were categorized based on the microscopic cellular appearance of isolated colonies. **Figure 3** shows the photos of the selected algae isolates from natural soil in Selangor and Kuala Lumpur under a microscope with a 40x lens: (a) C3 strain, (b) D4 strain.



(a) (b)

Figure 3. (a) C3 Strain under microscope with 40X lens and (b) D4 strain under microscope with 40X lens. The scale represents 10μm.

The isolated strains of algae are either spherical for D4 strains or filamentous for C3 strains, as determined by morphology analysis. The C3 strain's displayed yellow-green coloration, whereas the D4 strain displayed green colors. Later, since filamentous shaped algae are essentially our main focus, the D4 strain was kept first. We will use it for further studies. According to our hypothesis, the D4 strain comes from the *Chlamydomonas* sp., whereas the C3 strain comes from the *Tribonema* sp. In Gunong Jerai, Malaysia, there are 102 species of algae, including *Tribonema* sp. according to Ratnasabapathy [7].

Previous case studies by Shaharuddin [8] demonstrate the impact of nitrogen and phosphorus on the distribution of freshwater phytoplankton in stormwater, as well as the presence of *Tribonema* sp. at a sampling site. This research confirms our hypothesis that the C3 strain obtained from our soil samples may belong to the *Tribonema* genus.

The eukaryotic *Tribonema* sp. are yellow-green algae with unbranched filaments made up of a single row of elongated cylindrical cells. The dense cell wall is composed of open-ended double cylinders that overlap to contain the cell contents. With each cell division, a new section of the cell wall is produced. The sections of the walls resemble a 'H' word.

The entanglement of soil particles by the filamentous nature of algae and their release of mucilage is suggested to contribute to the development of BSC. The extracellular matrix found in filamentous cyanobacteria helps stabilise and agglomerate soils. These filament-forming organisms are pioneers when it comes to adapting to unsteady environments. Additionally, their contribution to C- and N-cycling enhances BSC formation and their ecological significance [2]. Eukaryotic algae are infrequently studied since they rarely contribute to BSC formation. However, eukaryotic algae also contributing to the development of BSC. Their mucilage secretion and filamentous structure allow them to gather soil particles and assemble them into BSC. The C3 strain has been selected as the possible native BSC-forming algae. However, molecular identification is required to validate our hypothesis.

The growth rate of C3 samples was then observed by measuring OD data every day for their growth analysis, as shown in **Figure 4**.

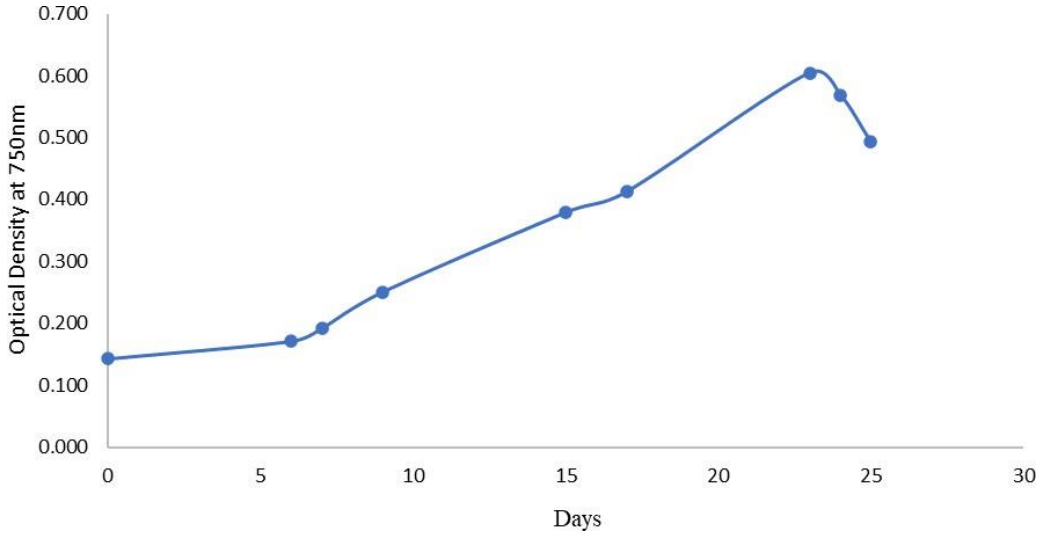
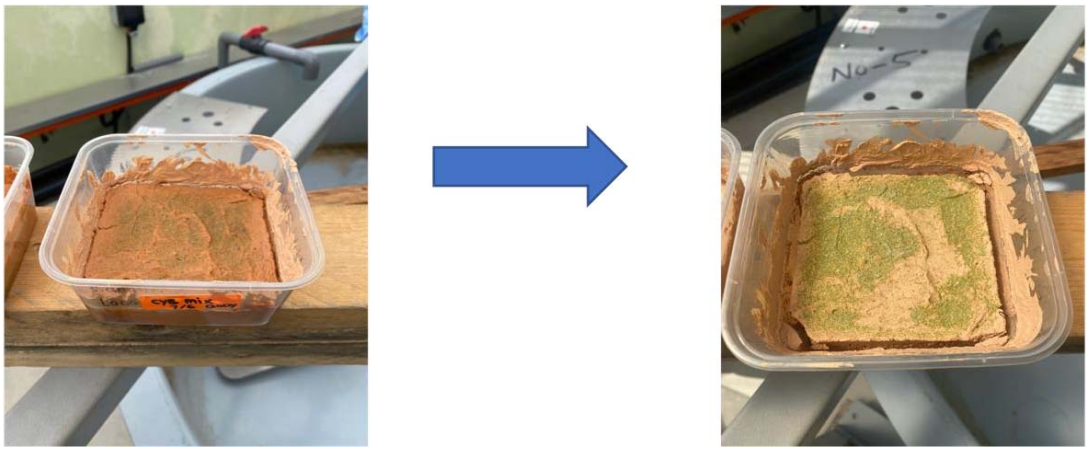


Figure 4. Growth Curve of C3 Strain

Figure 4 shows the growth of the C3 strain under normal CO_2 enriched and artificial lighting conditions. The culture is in AF-6 medium and placed under a light source for 24 hours continuously. The OD displayed a typical algae growth profile. From day 0 until day 6, there is a lag phase. The next stage of algae growth continuedfor roughly 14 days. The algae started to enter an exponential phase on Day 7 and continued until it achieved its maximum growth on Day 23. This might be due to the fact that algae are more productive and are dividing their cells more frequently at this time. After more than 23 days of culture, the algae go through a death phase. This indicated death rate is higher than reproduction rate.

Based on **Figure 5**, algae growth is indicated by the appearance of green colonies on the soil sample. The texture of soil during the experiment changed from loose particles into more compact structure. This may be due to secretion of the EPS that binds the soil particles together. After one week, a greener sign of C3 could be seen with the naked eye compared to day 0 on the surface of the soil. Therefore, the mixture of C3 according to the recipe is suitable for growth under tropical climate conditions.



(a) (b)

Figure 5. The visual appearance of the growth of C3 strain on soil sample at a) day 0 and b) day 7

4. Conclusion

The C3 strain has been isolated through the isolation process and the species of the algae obtained has been assumed as *Tribonema* sp. as the morphology of the C3 strain. Therefore, the C3 strain has been chosen as the potential native BSC-forming algae. Isolated filamentous algae can be used and explored more for its ability to grow outdoors, and soil stability tests can be performed to prove that this species is able to be used to prevent soil erosion.

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