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Flocculation of *Oscillatoria* sp. using fungal mycelium isolated from tempeh

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Abstract
Oscillatoria sp. is known as one of the common algae that can cause harmful algal bloom and affecting the environment surrounding. Therefore, there are some methods that can be utilized to control the spreading such as aeration, the use of chemicals and biological additives, and ultrasonic technology. Despite the disadvantages that algae possess, there are some benefits if harvest and process them toproduce value added product. One of method in harvesting is flocculation, which are inexpensive and safe. This study is focused on flocculation of microalgal through biological methods which is fungal as flocculant. The screening of the potential flocculant was analyse based on its morphology using light microscope with 40X and 100X magnification. Further identification was carried out through analysing the Internal Transcribed Spacer (ITS) region. Phylogenetic tree and search BLAST using NCBI were carried out and identified as *Rhizopus microsporus.* Determination of growth performance of *Rhizopusmicrosporus* was carried out by calculating its dry cell weight. The optimized conditions during the cultivation conducted were modified media to pH 4, with 200 rpm rotation rate and at room temperature, 28°C. The maximum dry cell weight calculated was 1.43g/L. Next, the flocculation efficiency of *Oscillatoria* sp. using fungal flocculant was caried out using a series of floc jar test through one factor at a time (OFAT) method. Three parameters were studied to observe the effect on flocculation efficiency which are mycelia concentration, pH of the medium and time duration. The highest flocculation efficiency was recorded with 20% (v/v) mycelia concentration, medium with pH 8 and time duration of 4 hour. Therefore,fungal extracted from tempeh could be use as flocculant to flocculate the potential harmful algal cells for both controlling and harvesting.
Keywords: Bioflocculation; *Rhizopus microsporus; Oscillatoria* sp.; flocculation efficiency

Introduction

In recent years, some algal species that produces toxins are affecting marine fauna such as increasingtheir mortalities effecting the ocean ecosystem (Donald M.Anderson, 2009). However, there are severalmethods that we can use to control the spreading of algae such as aeration, using

chemicals and biological additives or ultrasonic technology. Even though some of the methods are effective and can be efficient, they also possess some cons including expensive, producing unnecessary pollution and non- target toxicity towards organism (Rui Sun et al., 2018). Because of this, more promising methods wereintroduced such as biological method. Furthermore, microalgae have been classified as an alternativesource for biofuel as various of species can produce a large quantity of lipid while up taking carbon dioxide (Trentacoste, E.M., Martinez, A.M. & Zenk, T., 2015).

This brings us to the methods in harvesting microalgae. Some common methods use for microbial mass harvesting includes centrifugation, filtration, flotation, and flocculation. Especially for flocculation, it is known as one of the inexpensive methods in harvesting microalgae due to the reasonthat it can increase the aggregation size of microalgae (Liu, C., Hao, Y., Jiang, J. et al., 2017). Li (2017) stated that, many researchers are focusing on flocculation through biological methods such as microbial flocculation due to the high efficiencyand safety compared to physical and chemical methods. However, for fungal assisted inmicroalgae harvesting, it was proven efficient for certain microalgae species, andit is difficult to separate the biomass produced from fungal and microalgae (Liu, C., Hao, Y., Jiang, J. etal.,2017).

In recent years, there were several discoveries about filamentous fungi that can be used in biological treatment under controlled conditions (More et al. 2010). In most of the studies, the fungi were cultivated separately. Not only that, according to Adam et al, 2016, there are several theories regardingthe interaction between fungi and microalgae to cause fungal pellet formation, but the details are still fuzzy. Another method in bio-flocculation is using bacteria as flocculant. However, the flocculation activity of other microorganisms is given little attention and according to Li et al. (2017), between both bacteria and fungi flocculation, fungi have greater advantages in aggregating microalgae biomass. Eventhough fungal flocculation has several advantages such as improving mass transfer and easier separation method, reports on applying fungi for direct microalgae harvesting are little (Luo et al. 2019).

Materials and methods

Fungal sample was obtained from previous study in form of spore suspension to be cultivate and harvest. The spore suspension was pipette directly on potato dextrose agar (PDA) plates and cultivates for harvesting. The dominant fungal strains were screened based on its morphological characteristics such as colour, shape and size using light microscope. Further identification using Internal TranscribedSpacer (ITS) DNA barcoding method. The Potato Dextrose Broth (PDB) medium for the of fungal mycelium was prepared in Schott bottle based on the procedure preparing PDB media. For 1 litre of PDB,24 g of PDB powder was weight and mixed with distilled water. Potato Dextrose Agar was prepared based on the standard procedure for cultivation and spore harvesting. Both media were autoclaved at 121 degrees Celsius for 30 minutes.

For isolation and identification of fungal mycelium, the autoclaved PDA medium was poured into the plates and solidified. The ideal fungal sample that has been screened was grown on the PDA medium and incubated at 30 degrees Celsius for 5 days. After that, the grown fungal was lawn to collect the spores by using Tween-80. 1ml of the sample was inoculated in 100ml potato dextrose broth and incubated for 7 days at room temperature of 28 degrees Celsius with 160 rpm in an incubator shaker. The identification of the fungi species was determined using fungal Internal Transcribed Spacer (ITS) DNA barcoding method where its ITS gene was amplified using PCR. The amplified ITS product were sequenced at Apical Scientific Laboratory (Seri Kembangan, Selangor). A BLASTn search was performed using National Center for Biotechnology Information (NCBI) web to identify the obtained sequences.

Growth profile of the identified fungal species was carried out through dry cell weight measurement. Fungal samples were poured into a Falcon tube. Then, distilled water was added to fill the flacon tube in mixed solution. Pipette 1 ml of each of the Falcon tube into a microcentrifuge tube to measure the optical density at 680nm using spectrophotometer. After that, filter the

remaining mixture using vacuum pump and dried filter membrane. Weight and dried the filter membrane before starting filter procedure determine the initial weight of the filter membrane. After filtration, dry and weight the dried filter membrane to determine the final weight with the fungal residue. We can then calculate the dry cell weight using the formula dry cell weight (g/l) = filter membrane + dried residue (g) – initial weight of filter membrane (g)/ volume of sample (L) x 100%.

Flocculation efficiency were carried out using floc jar test. The floc jar test was optimized usingone factor at a time (OFAT) method. The chosen fungal mycelium was sub-cultured onto a potato dextrose agar (PDA) and potato dextrose broth(PDB) while microalgae was cultured in the BG-11 medium. The fungal mycelia were inoculated and mixed with microalgae for the flocking test. For the jar test, several parameters were measured including mycelia concentration, pH of the culture and time.The jar test for optimum dosage was determined by adding a certain quantity of centrifuged mycelium culture (v/v) into different microalgal biomass harvesting culture concentration. Centrifugation will be done at 3000rpm for 10 minutes. For the first parameter was to determine the flocculation efficiency with different mycelia concentration of 10%, 20% and 30% (v/v). After that, was based on pH of the culture which were modified to pH 6, pH 8 and pH 10. Lastly the flocculation efficiency was optimized at different duration of time (2h, 4h and 24h). Then, the clear supernatant was sampled for the determination of optical density at 685nm using UV-Visible spectrophotometer and proceed to calculate the flocculation efficiency (FE%) for the tests basedon the formula flocculation efficiency (%) = (A-B)/Ax 100. Where A is the initial reading and B are the final reading of absorbance of the microalgal cultureat 690nm.

Results and discussion

The fungal colonies that were screened appeared white on the Potato Dextrose Agar (PDA) plate at first, but they darkened to shades of brown, grey, and finally black as they spread across the plate andbecame overgrown. Based on the Figure 1, the rhizoids are predominantly simple, although they can also take the form of branches. Their lengths are either unequal or varied. The sporangiophores that emerge from the stolon and are located on the other side of the rhizoids. These sporangiophores havesimple, straight to slightly curved.

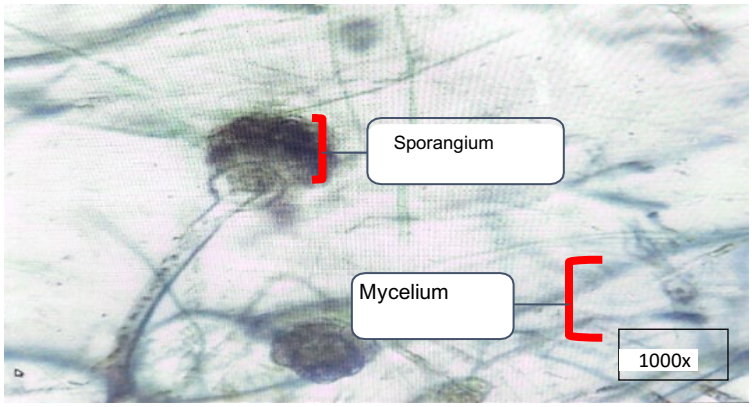

Figure 1 Morphology of the screened fungal under light microscope at magnification of 1000x

Prior to the fungal cultivation, the cultivation of *Oscillatoria* sp. was conducted to be used for screening based on the ability to flocculate. For the microalgae cultivation, pure culture of the *Oscillatoria* sp. was obtained from mixed microalgae culture. Next, 100 µl from the mixed culture was pipette and spread on BG-11 agar plate aseptically. Then, incubate under fixed light approximately ≈2000 Lux at room temperature for 2 weeks. From the plate, microalgae were inoculated into a 250ml conical flask for cultivation (Figure 2).

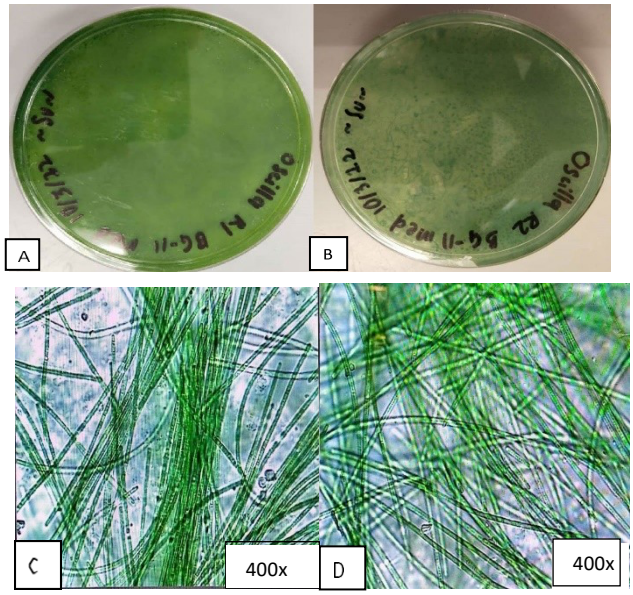

Figure 2 Isolation of *Oscillatoria* sp. vias spreading method for both plate (A and B) and the morphology observation of *Oscillatoria* sp. (C and D) under light microscope at 400x magnification.

The flocculation of Oscillatoria sp. using fungal flocculant was proceeded with the assumptionboth cultivated cultures were at exponential phase and met the optimum conditions. After 5 days, 25mlof the cultivated fungi were poured into the falcon tubes and centrifuge at 3000rpm for 10 minutes to obtain the fungal mycelium. The supernatants were discharged and 50ml of the microalgae culture werepoured into the falcon tubes containing fungal mycelium precipitate and mixed them. The flacon tubeswere left for about 2 hours to observe the flocculation activity.

To further identify and determine the fungal strain species, samples was aliquot and send to theApical Scientific Sdn. Bhd., a science service provider company. The identification was based on the nucleotide sequence analysis of the Internal Transcribed Spacer (ITS) region. The ITS region was amplified and undergo Polymerase Chain Reaction (PCR) to be sequenced. Then, a search from the GenBank with BLAST was conducted at the National Center for Biotechnology Information (NCBI) webto identify the PCR product obtained.

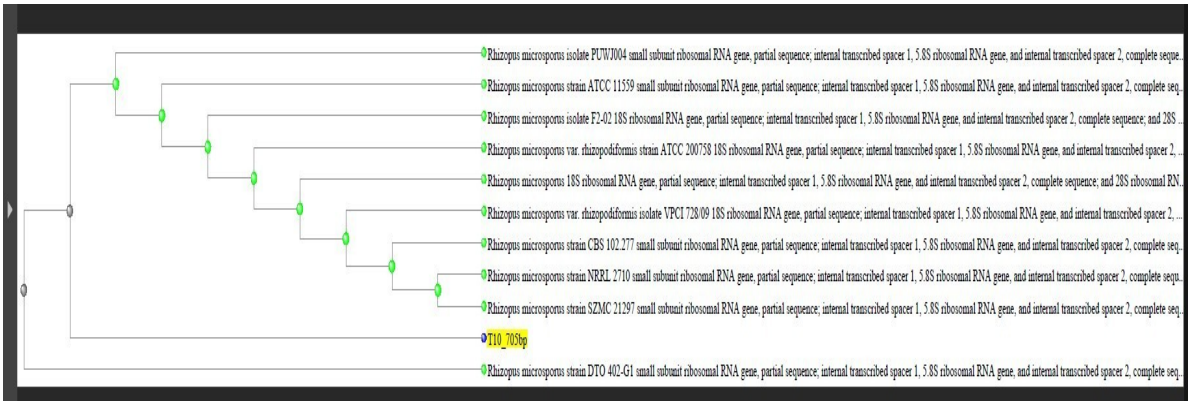

Figure 3 The phylogenetic tree constructed based on the sequence data of internal transcribed spacer (ITS) region for *Rhizopus* sp.

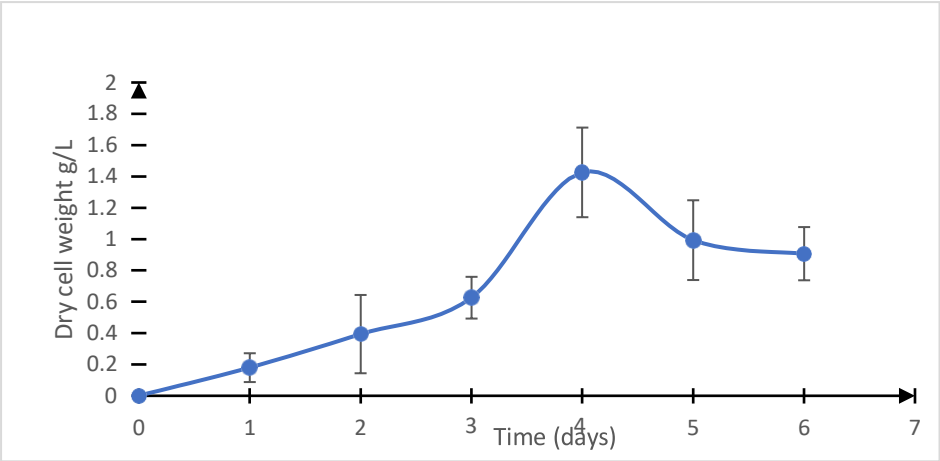

Figure 4 Growth profile of *Rhizopus microsporus* based on dry cell weight.

From the results obtained in Figure 3, the phylogenetic tree was constructedby NCBI Blast Tree method, and the isolated fungi strain was identified as *Rhizopus microsporus.* Thephylogenetic tree reveals that the sequence obtained from the ITS region was insufficient for classifying Rhizopus species down to the variety level. Within this subclade, the region that has been highlighted demonstrates thatno unique monophyletic clade was created.

For the purpose to study the growth performance of the fungal flocculant, the experiment was conducted with standard parameters. The cultivation of fungal was carried out by preparing Potato Dextrose Broth (PDB) media and adjust to pH4 with several drops of 1M of hydrochloric acid (HCL) usingdropper. Then, 1ml of the fungal spores was introduced into 250ml conical flask and then cultivated for7 days on a rotary shaker at 200 rpm, 28°C room temperature. The parameters of the experiment werebased on the previous study due to limitation of time and material. The filtration of the fungal culture was carried out every day for 6 days using laboratory vacuum being that the data for 7th day of the experimentwas invalid due to the technical problems.

Generally, fungal growth and total biomass were influenced by several parameters suchas the chemicalcomposition of the media used and the experiment conditions (Jakovljevic et al., 2014). From the experiment conducted and data obtained, a simple growth curve was constructed. The figure 4 shows the growth profile of the *Rhizopus microsporus* with its dry cell weight against the cultivation days. Theresults show a consistent increase in the dry cell weight from inoculation until day 3, indicating that it isin exponential phase. The maximum total dry weight was recorded at day 4 which is 1.43g/L. After day4th, the dry cell weight was decreasing to approximately 0.99g/L, suggesting that the death phase of fungus. Overall, this growth profile needs to be revise again due to other factors that can affect the fungal growth such as temperature, pH and spore concentration.

This experiment was started by cultivating both fungus and microalgae culture based on the firstobjective. The dormant spores of the identified fungus were cultivated on Potato Dextrose Agar (PDA)plate to obtain a higher spore concentration to be used as inoculum for cultivation in shake flask containing Potato Dextrose Broth (PDB) for 5 days. Cultivation of *Oscillatoria* sp. was done in 1L shakeflask using BG-11 medium with modified pH of 7, 10% inoculum, aeration rate of 1.0 L/min, provided with light source of approximately 3000 lux for1 week. The jar test experiment was carried out in 50 mlfalcon tube with one factor at a time. As mentioned before, the cultivated fungal culture was poured intothe falcon tube and centrifuged to obtain the mycelium. After that, the supernatant was discharged andmixed with the already cultivated microalgal. Optical density for initial which is 0 hour and flocculation at given time which are 2 hour, 4hour and 24 hours were taken to calculate the flocculation efficiency. There are three parameters that were studied that can affect the flocculation efficiency which are mycelia concentration, time, and pH of the medium. All experiment were conducted in triplicates.

For preparation, different mycelia concentration was centrifuged at different fungal culture

volume. The microalgae were found to be spherically flocculated within the range of selected fungal dosage, and the dosage of fungi was found to influence the flocculation efficiency. The microalgae formed ordinary flocculation rather than spherical flocculation when there were no fungi present to aid in the process. The flocculation efficiency of the microalgae first increased with the increase in the number of spores added, and then it decreased as the number of spores continued to be added (Jiang et al., 2020b).

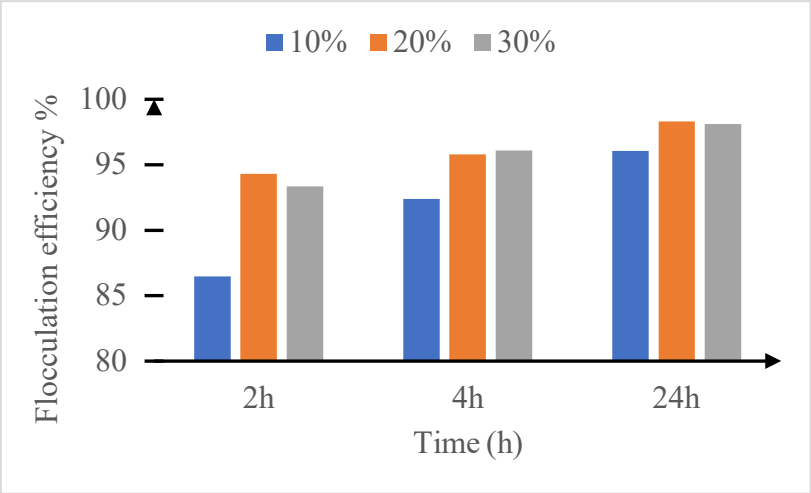

Figure 5 Flocculation efficiency of *Oscillatoria* sp. using fungal flocculant at different mycelia concentrations.

The flocculation of *Oscillatoria* sp. using *Rhizopus microsporus* was first conducted with different fungal mycelia concentration which are 10%, 20% and 30% (v/v) with fixed pH of 8 and time for 2 hour, 4 hour and 24 hour. Overall, the results show the highest flocculation efficiency is at 20% mycelia concentration (v/v) for 2 hour and 24 hours (Figure 5). Furthermore, the flocculation efficiency for 20% mycelia concentration is almost the same as 30%. This is most likely the result of insufficient nutrients or media to further assist the flocculation of fungal andalgal cells. In addition, neither performance wasimproved further by increasing the concentration of mycelia to 20 or 30 percent, which suggests that some algae cells were dissociated from fungal pellets and stabilized in suspension (Chu et al., 2021). It was not necessary for there to be an excessive amount of bioflocculants present for there to be effective harvesting, as complete coverage could give rise to steric repulsion and bring about the phenomenon of destabilization (Kumar et al., 2019). It was found that the presence of fungi could improve the flocculation efficiency byfacilitating the formation of spherical microalgal flocs. These flocswere more stable. We can see that there was an increase on the overall flocculation efficiency as the ratio of fungal and algal particles increased (Bhattacharya et al., 2017).

For the second parameter, the pH of the microalgal culture was modified using 1M of hydrochloric acid and 1M of sodium hydroxide. This experiment was conducted with fixed mycelia concentration which is 20% (v/v) and in triplicates. It is common knowledge that the flocculation of microalgal biomass is highly sensitive to the pH of the culture suspension. According to research that was conducted by McCausland and colleagues in 1999, elevating thesolution's pH encourages the precipitation of any additional flocculants that may have been added, which results in an improved flocculation efficiency. To add even more, a rise in pH may also influence the electrical charge of microalgal cells and alter the forms of metal cations that are already present in the culture medium (Danquah et al., 2009).

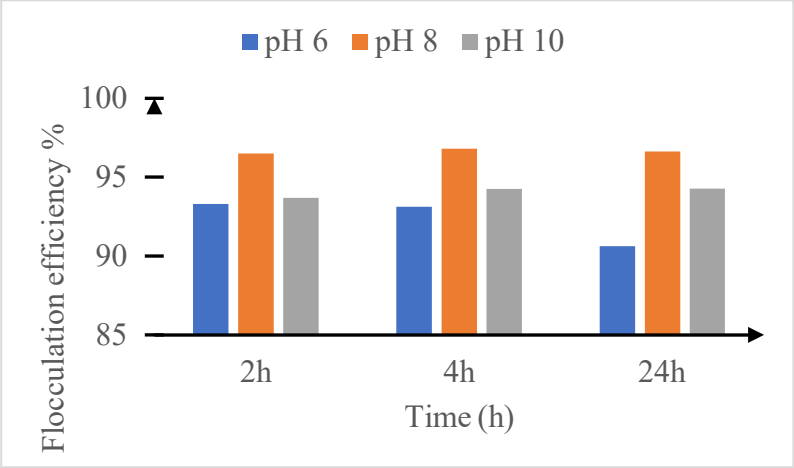

Figure 6 Flocculation efficiency of *Oscillatoria* sp. using fungal flocculant at different pH of the medium

According to Figure 6, it can be seen that pH 8 has the highest flocculation efficiency after two hours, four hours, and twenty-four hours have passed. It appears that the pH value of the algal suspension has a significant impact on the flocculation process, consequently, an increase in the pH value may result in the formation of instantaneous flocs. The flocculation phenomenon may be caused by the reduction of electrostatic repulsion, which leads to the neutralization of positively charged fungal mycelium and negatively charged microalgal cells. In general, the findings indicate that the flocculation of *Oscillatoria* sp. caused by fungal infection is most effective in environments with a pH that is slightly basic, which in this instance is 8. As can be seen in the graph, the flocculation efficiency for pH 6 is getting worse as the amount of time goes on. This is most likely because the fungal cells were unable to clump together with the microalgal cells in the slightly acidic environment. This causes an electrostatic repulsion between the fungal and microalgal cells because they both have the same charge. According to Jiang et al., (2020), the flocculation efficiency of microalgae increased initially with an increase in pH and then decreased, indicating that the pH requirements for flocculation were lower. This was shown by the fact that the flocculation efficiency increased first and then decreased.

The flocculation efficiency was calculated for the final parameter based on the time (2h,4h, and 24 h) with a fixed mycelia concentration of 20 percent (v/v) and a pH of 8. In terms of the general trend, the higher the flocculation efficiency that is calculated, the longer the time given for flocculation. According to the papers that have been published, various microalgae have varying time requirements in order to achieve the highest possible flocculation efficiency. According to Figure 7, the flocculation efficiency is at its peak after 24 hours; however, it is marginally higher at the mark of 4 hours. This suggests that a greater flocculation efficiency could be achieved in a shorter amount of time than previously thought. In addition to this, a simple gravitational sedimentation may also occur when the microalgal species are in an unfavourable condition (Matter et al., 2019), which results in a clearer culture and a small increase in the flocculation efficiency.

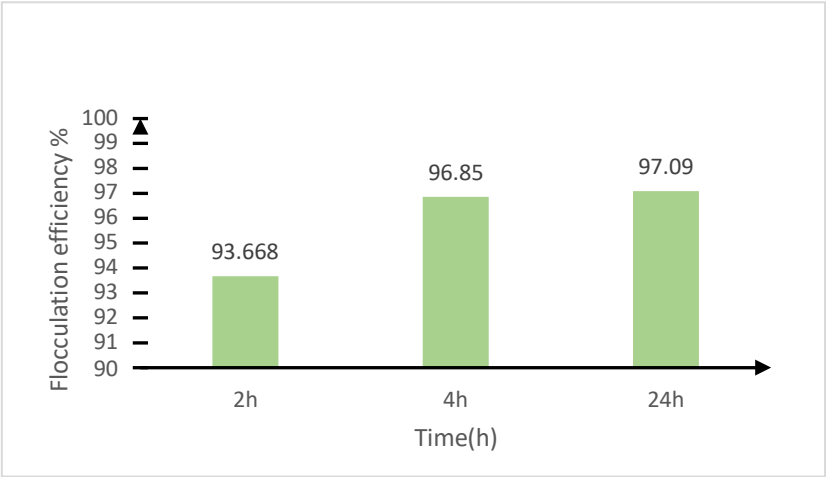

Figure 7 Flocculation efficiency of *Oscillatoria* sp. using fungal flocculant at different duration oftime with fixed pH and mycelia concentration.

Conclusion

The screening and identification of the possible fungal flocculant was accomplished by making use of the sample that was provided from the prior study in the form of suspension spore. The ability of the potential fungi to floc microalgae, which consisted of *Oscillatoria* sp. was used as a criterion in the screening. The morphology of the fungal growth was analysed, and the result were compared with previous published studies. Further identification of the screened fungi was carried out using DNA barcoding method of the Internal Transcribed Spacer (ITS) region and was identified as *Rhizopus microsporus.* Phylogenetic tree was constructed using NCBI Blast Tree method and BLAST search. The growth performance of the fungi was carried out successfully based on cell dry weight. The cultivation was carried out with the condition at room temperature, 28 degree Celsius, at 200 rpm for 1week. The data collected allowed for the construction of a straightforward growth profile of *Rhizopus microsporus*, which revealed that the maximum dry cell weight was 1.43 g/L.In order to calculate flocculation efficiency, we looked at threedifferent parameters: the concentration of mycelia, the pH of the medium, and the amount of time. Based on the results of the experiment that was carried out, the mycelia concentration thatwas used to achieve the highest level of flocculation efficiency was 20% (v/v), which resulted in a 98.3% efficiency. Additionally, a medium with pH of 8 and a time duration of 4 hours recorded the highest flocculation efficiency.

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