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OPTIMISATION OF ANTARCTIC FILAMENTOUS ALGA GROWTH IN THE PRESENCE OF MOLYBDENUM

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Abstract

Elevated concentrations of heavy metals have been identified in Antarctica due to growing anthropogenic activities in recent years. Molybdenum (Mo) is a trace element that has not been extensively studied in terms of its toxicity towards the environment, especially in extremely cold weather. The algae communities in the Antarctic were less focused and explored, unlike indigenous bacteria consortia in their response to heavy metals. The study aims to optimise the physicochemical conditions for optimal growth of an Antarctic algal, *Klebsormidium* sp. in the presence of Mo via conventional one–factor–at–a–time (OFAT) and growth kinetics analysis. Algal cultures with aeration showed a higher growth rate (μ = 0.2352 d^{-1}) than those without aeration (μ = 0.1976 d^{-1}). Based on the optimised parameter, the overall biomass yields with and without aeration systems correspond to each other ($P > 0.05$). It was discovered that the *Klebsormidium* sp. showed maximal growth in terms of biomass at 20 g/L of sucrose, 2 g/L of ammonium nitrate, 4 g/L NaCl concentration and pH 7.5. The overall optimised conditions were further analysed using the Exponential growth model, which demonstrated no significant difference ($P > 0.05$) in the algae growth rate with aeration (0.020 ± 0.0018 h^{-1}) and without aeration (0.020 ± 0.0015 h^{-1}). The Antarctic filamentous algae exhibited the ability to grow in heavy metal, Mo at optimal growth conditions, but the aeration systems did not affect the algae growth significantly. Therefore, this study could help in understanding the capability of algae to grow in the presence of heavy metal through various manipulations of growth parameters and act as a preliminary study for bioremediation of Mo in Antarctic polluted sites.

INTRODUCTION

Antarctica is particularly vulnerable to pollution since the environment is generally pristine. The cold temperatures imply that the natural mechanisms, which assist in eradicating pollution, occur far more slowly and, therefore, have more chances to build up longer in the snowy and ice environment. Since the Antarctic is not connected to the rest of the continents, harmful pollutants such as pesticides, oil spills and inorganic heavy metals find their way to the land [1, 2], even as traces of man-made transported chemicals, weathering of the bedrock and sources of anthropogenic activities can be detected over the ice region [3, 4]. Eventually, these pollutants become concentrated in the bodies of local wildlife, such as marine or aquatic animals, seals and penguins [5].

Both acute and chronic toxicity of heavy metals or other trace elements such as molybdenum (Mo) from dumping sites, accidental oil spills, sewage outfalls, emissions of exhaust and mining found in living mammals and birds in cold regions of Antarctica is due to their non-degradable ability and persistency in the environment [6-8].

Minute traces of xenobiotic compounds in these pollutants may adversely deteriorate the health of animals and humans. A study found that penguin guano is an intermediate transport of heavy metals from marine to terrestrial ecosystems [7, 9].

The cost-effective and eco-friendly bioremediation technologies allow the removal of heavy metals from the environment to resolve the rising environmental pollution. Bioremediation of heavy metals has been investigated using bacteria [10, 11], algal species [12, 13], fungal species [14], and plants [15]. Mo is a poorly studied trace element in terms of its effects towards the environment. It is an essential micro-nutrient with lower toxicity for humans but causes fatality in some animals [16]. The study of Mo reduction into Mo-blue using microorganisms has been a keen interest among scientists over the past years [17].

Antarctica's cold condition is unfavourable for microbial growth and limits the efficiency of reducing heavy metals, causing long-term negative effects on the ecosystem. Mo-reducing bacteria have been successfully reported by optimising various nutrient sources through different growth adaptations to achieve maximum efficiency in remediating contaminants [17, 18]. Microalgae growth is influenced by carbon and nitrogen sources, pH, sodium chloride (NaCl) concentration, and incubation systems [19, 20]. The mathematical growth kinetics model was developed by Monod [21], Moser [22], Teissier [23], Haldane [24], Exponential [25], Luong [26], and Aiba-Edwards [27] that allows researchers to study the effects of several substrates and growth parameters on microbial growth rate.

Although many studies have reported different microalgae species' ability to bioaccumulate or reduce heavy metals, few have focused explicitly on Mo. Tropical microalgae species demonstrated Mo removal effectively, but utilising an Antarctic filamentous alga was relatively new. Although several studies have revealed the potential of various microalgae species to bioaccumulate or decrease heavy metals, few have mainly focused on Mo. Bacteria and tropical microalgae species exhibited efficient Mo removal. To the best of our knowledge, no reports have assessed Antarctica's filamentous algae growth responses in Mo. The present study provides a foundation for future novel Mo bioremediation investigations in polar environments. This study used the cold-adapted Antarctic freshwater filamentous algal *Klebsormidium* sp. to investigate its growth adaptations and kinetics towards Mo through various optimum conditions.

MATERIALS AND METHODS

Algae Culture: Soil-based freshwater filamentous algal, namely *Klebsormidium* sp. was isolated from Greenwich Island of the South Shetland Islands, Antarctic (62.4692° S, 59.7963° W) provided by the International Medical University (IMU), Malaysia. The algal culture was cultivated in the freshwater media named Bold's Basal media (BBM) [28]. The final pH of the media was adjusted with 1 N KOH to 6.8 and subsequently sterilised at 121°C and 115 kPa for 15 min. The algal culture was maintained in a controlled-environment incubator at 10°C, illuminated with a cool white, fluorescent lamp at a photon flux of 42 µmol $m^{-2}s^{-1}$ on a 12:12h light-dark cycle [29]. The *Klebsormidium* sp. culture was subcultured every two weeks in 100 mL flasks containing 50% (v/v) algae to ensure sufficient fresh cultures [30, 31].

Standard Growth Studies: An inoculum size of 10%, standardised at an optical density at 620 nm (OD620) of 0.1 ± 0.1 from exponential phase culture, was used. The *Klebsormidium* sp. culture was grown in 250 mL Erlenmeyer flasks containing 100 mL BBM in triplicates fixed at two cultivation systems, without aeration and with aeration. The filtered atmospheric air was supplied continuously by a hi-blow diaphragm air pump (HAILEA HAP-100, output: 100 L/min) through a sterile 0.22 µm membrane filter attached to both inlet and outlet (4 mm). Growth was monitored daily for 14 days based on OD620, which was determined by spectrophotometry. 1 mL of the aliquot was homogenised using Potter homogeniser before being measured and BBM was used as a blank. The following equation was used to calculate the specific growth rate, µ (d^{-1}) of algae within the exponential growth phase: $\mu = \ln(N_2/N_1)/(t_2 - t_1)$, where µ is the specific growth rate, and N_1 and N_2 are the cell concentration at time 1 (t_1) and time 2 (t_2), respectively [32, 33].

Low Phosphate Media (LPM): To proceed with the optimisation of factors affecting the algae growth using a one-factor-at-a-time (OFAT) approach, the low phosphate media (LPM) was prepared. In brief, the chemicals required to prepare LPM in 1 L of dH_2O were as follows: 3 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract, 5 g of NaCl, 2.42 g of $Na_2MoO_4.2H_2O$, 0.73 g of Na_2HPO_4 and 10 g of glucose. The concentration of sodium molybdate was fixed at 10 mM and phosphate at 5 mM [34]. The pH of the media was adjusted to 7.5 and was autoclaved at 121°C and 115 kPa for 15 min prior to usage. The glucose was autoclaved separately before being mixed with the rest of the sterilised medium to prevent colour changes when exposed to very high temperatures.

Optimisation of Factors Affecting the Algae Growth using OFAT Approach: Optimisation of various growth parameters was conducted to maximise the growth of *Klebsormidium* sp. The following parameters were ascertained on the LPM; carbon sources (glucose, sucrose, fructose, mannitol and galactose) and concentration (10, 20, 30, 40 and 50 g/L), nitrogen sources (ammonium sulphate, sodium nitrate, ammonium nitrate, potassium nitrate and ammonium chloride) and concentration (1, 2, 3, 4 and 5 g/L), pH (6.0, 6.5, 7.0, 7.5 and 8.0) and NaCl concentration (1, 2, 3, 4 and 5 g/L). The effects of these parameters were

investigated by maintaining all factors constant except for one factor of interest. For all parameters, 5 mL of algae inoculum was obtained from exponential phase cultures (OD620 = 0.1 ± 0.1 nm) and transferred into 50 mL of LPM using 100 mL Erlenmeyer flasks fixed with aeration at incubation. The cells were harvested at the end of the experiment (day 7) by filtration for dry weight determination [11, 17, 18]. In brief, a known volume of the algal culture was filtered on a pre-weight blank filter paper. The filer was placed in the oven and dried at 50°C for 24 h. The algal biomass was determined using Equation 1 [35].

$$\text{Biomass (mg/mL)} = \frac{\text{[Weight of filter with algae (mg)] - [Weight of blank filter (mg)]}}{\text{Volume of algal culture (mL)}} \quad (1)$$

Growth Kinetics and Statistical Analysis of Algae Growth: The simple exponential growth model is the most straightforward linear function [36] based on the Malthus or exponential model. This model assumes that the growth rate of the population increases at a rate proportional to its size under conditions of unlimited resource and space based on the equation (Equation 2) given:

$$N(t) = N(0)e^{\mu t} \quad (2)$$

where N(0) is the initial size of the population at time $t_0 = 0$, N(t) is the size of the population at t, μ represents the growth rate of the population at exponential or the rate of constant [37]. The population doubling time is converted to the parameter μ, known as specific growth rate and expressed in reciprocal time units [25, 38]. The growth was measured continuously for seven days via spectrophotometric analysis based on the optical density, OD620 nm. Growth kinetics were tested based on the factors that have been optimised as described above. Meanwhile, statistical analysis was performed using Microsoft Office Excel 2010 and Graph Pad Prism® 8 (2018). One-way ANOVA (Analysis of Variance) followed by Tukey post-hoc test was used to determine the significant difference between the growth of algae as compared to the respective control group. Data were presented as the mean ± standard error of the mean (SEM) and the data were significant when p value was ≤ 0.05.

RESULTS AND DISCUSSION

The freshwater filamentous Antarctic algae, *Klebsormidium* sp. isolated from Greenwich Island, was enriched with nutrients to make up for the deficiencies in the freshwater. A standard growth curve was developed by measuring the rate of cell population increase over time. A typical growth curve of algae should consist of five growth phases: lag, exponential, decline, stationery and death phase [39]. The aim of this experiment is to determine and compare the exponential growth phase for *Klebsormidium* sp. that was grown in different systems (with aeration and without aeration) over time. Aeration is the most common procedure to agitate algal cultures to ensure that the cultures are equally exposed to light and nutrients. Aeration is important to improve the gas exchange between culture medium and air, which contains the carbon source in the form of carbon dioxide that is required for photosynthesis [40, 41].

Generally, green microalgae absorb light energy for photosynthesis through a major pigment (chlorophyll) range of 450–475 nm and 630–675 nm [42, 43]. The optical density (OD620 nm) with an initial concentration of 0.10 ± 0.1 was measured using a UV-Vis spectrophotometer for 14 days without any optimisation factor affecting the growth. Apart from that, the increase of inoculum density can affect algal growth. The growth rate (μ) is inversely proportionate to the inoculum concentrations [44].

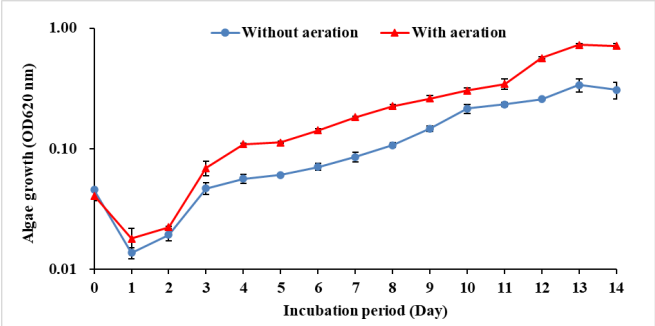

Figure 1. Semi-logarithmic growth curve based on OD620 of *Klebsormidium* sp in different systems. The culture was grown in Bold's Basal Media (BBM) for 14 days to determine the exponential phase. The error bars represent the mean ± standard error mean (n = 3).

Certain algae grow by increasing the cell number instead of size [40]. Based on **Figure 1**, in both aeration and without aeration systems, the algae underwent a lag phase from day 1 to 2 after a sudden decrease in its cell density, followed by a consistent exponential phase from day 3 to day 13 and a stationary phase by day 14. However, the death phase was not achieved by day 14. The *Klebsormidium* sp. culture in both systems achieved an exponential phase on the 3rd day after a lag phase. Algae culture grown with aeration (μ= 0.2352 d^{-1}) had an insignificant increase in specific growth rate compared to culture without aeration (μ= 0.1976 d^{-1}) during the exponential phase ($P > 0.05$). The cell growth gradually increased with the incubation period despite similar growth rate patterns in both aeration and without aeration systems. The exponential phase allowed rapid cell division, making the algae speedily reach the exponential phase and achieve the maximum cell concentration. The cell division declined during the lag phase as the cells adjusted to the new environmental conditions [46]. In contrast, cultures grown without aeration could not receive sufficient nutrients and light because without mixing, the algae cells will settle to the bottom [47].

Carbon dioxide (CO_2)-enriched air increases cell biomass output and promotes gas exchange during photosynthesis. Furthermore, aeration culture provides filamentous microalgae with sufficient illumination, temperature, and nutrients. Aeration cultivation is strain- or species-dependent and the tolerance of vigorous or gentle mixing must be assessed individually [47]. For instance, a filamentous cyanobacteria (*Arthrospira platensis*) biomass was limited due to excessive hydrostatic pressure caused by increased aeration rates [48]. This finding can be confirmed by *Klebsormidium* UMACC 227, an Antarctic alga cultivated optimally with a minimal difference in specific growth, 0.17 ± 0.02 d^{-1} (6ºC) and 0.14 ± 0.01 d^{-1} (9ºC) while maintaining similar photon intensity and photoperiods [35]. Another Antarctic isolate *K. flaccidum* cultivated at 10ºC, exhibited a similar growth rate (≈ 0.20 μ/d^{-1}) recorded at 20ºC [49]. These findings highlighted the potential for establishing optimal conditions for *Klebsormidium* sp. cultivation applications.

The effect of different parameters on algae growth rate was evaluated by following the conventional OFAT experimental design. The following study aims to establish the optimal conditions for maximum cellular growth in the presence of Mo. The composition of low phosphate media (LPM) containing 10 mM Mo was manipulated with different types of carbon sources, carbon concentrations, nitrogen sources, nitrogen concentrations, NaCl concentration and pH, which were the most significant factors affecting the growth parameter of algae. The LPM appeared blue when microorganisms grew on it through Mo reduction. This is due to the formation of Mo blue, which occurred after microorganisms reduced Mo^{6+} in the medium and the Mo^{5+} that formed a phosphomolybdate complex [34]. This complex is unstable in the condition of ~pH 7 and elevated phosphate amount induced by the strong buffering capacity of phosphate buffer, inhibiting the molybdate reduction. Thus, the low phosphate concentration (2-5 mM) was optimal for any Mo reduction investigations [17, 18].

On the other hand, phosphate is essential for microalgal metabolic pathways, growth, cell division, lipid yield, and formation of cellular components [50]. Microalgae have greater efficiency for inorganic phosphate absorption collinear with algal growth and biomass production [51]. High lipid yield was observed by *Chlorella vulgaris* [52] under phosphate-sufficient conditions, unlike *Scenedesmus* sp. [53] and *Isochrysis galbana* [54] strive in phosphate-deficient conditions.

One of the most critical factors for alga growth is carbon sources. Microalgae can grow under heterotrophic conditions using various carbon sources such as carbon dioxide, glucose, acetate and other organic compounds [55]. The following set of experiments were executed to investigate the effects of various types of carbon source on the cell growth of *Klebsormidium* sp. based on biomass production. Increasing algal biomass over a period is known as the new production or growth rate.

Figure 2 shows the yield of algae biomass (mg/mL) from the cultivated cultures with different carbon sources: glucose, sucrose, fructose, mannitol and galactose. The carbon source was omitted in the control group. The highest yield of algae biomass was recorded for sucrose at 1.82 ± 0.01 mg/mL followed by mannitol and galactose with biomass yields of 1.78 ± 0.02 mg/mL and 1.79 ± 0.01 mg/mL, respectively, when the algae was subjected to low phosphate media. The statistical analysis showed no significant difference between sucrose and other carbon sources ($P > 0.05$). Glucose, followed by sucrose and fructose, is the most commonly used carbon source for heterotrophic or mixotrophic cultures of many microalgae [56]. The production of the highest amount of biomass was observed in green algae, *Chlorella* sp., *Chlorella vulgaris* CCAP211/11B, *Botryococcus braunii* FC124 and *Scenedesmus obliquus* R8 [57]. The maximal biomass concentration (2.71 g/L) of *Chlorella pyrenoidosa* was recorded for utilising sucrose as the sole carbon source produced by *Rhodotorula glutinis* in a co-culture system [58]. Thus, it agrees with the present study that optimising sucrose as a carbon source produces the highest algae biomass yield.

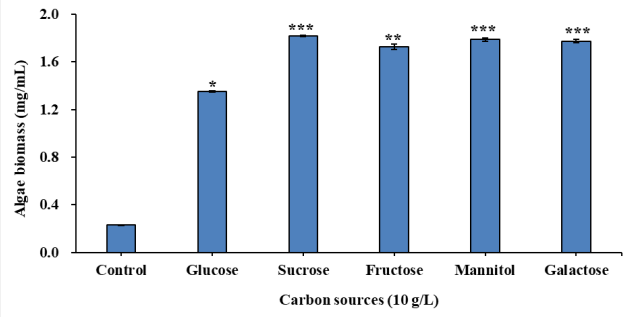

Figure 2. Effect of carbon sources on the biomass of *Klebsormidium* sp. The error bars represent the mean ± standard error mean (n = 3). The asterisk (*) represents the p-value of the statistical test compared to the control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Based on **Figure 2**, the algae culture subjected to sucrose as a carbon source produced the highest biomass yield compared to other carbon sources. Hence, the sucrose as a carbon source was studied using different concentrations at 10, 20, 30, 40, and 50 g/L. **Figure 3** shows that the optimal concentration of sucrose to produce the highest algae biomass was 20 g/L followed by 60 g/L. The lowest algae biomass was produced at 10 g/L of sucrose. Thus, the algae grow optimally at 20 g/L of sucrose concentration. No significance was identified among 30 g/L, 40 g/L and 50 g/L with 20 g/L concentration. Furthermore, there was a significant difference in algae biomass produced at the concentration of 20 g/L of sucrose compared to other sucrose concentrations ($P < 0.001$). It has been reported that a a higher amount of carbohydrates contributed to the inhibition

of algal growth and 20 g/L of sucrose was found as optimum for maximum growth rate [59, 60]. The addition of sucrose in *Chlamydomonas globosa, Chlorella minutissima* and *Scenedesmus bijuga*, were the most favourable carbon sources that supported the highest chlorophyll content [61]. *A. platensis* Gomont 1892, a known cyanobacterium, optimum biomass productivity (1.33 g/L/day) in a mixotrophic medium containing 2.5 mM sucrose, and the specific growth rate declined significantly with high sucrose concentration [62]. In the cultivation of *Chlorella vulgaris*, sucrose was supplied through waste materials from molasses and sugar cane as an alternative carbon source and to lower the cost [58, 63]. *C. vulgaris* utilise the fructose and glucose monosaccharides hydrolysed by sucrose to promote algae growth and metabolic process via hexose transport [64].

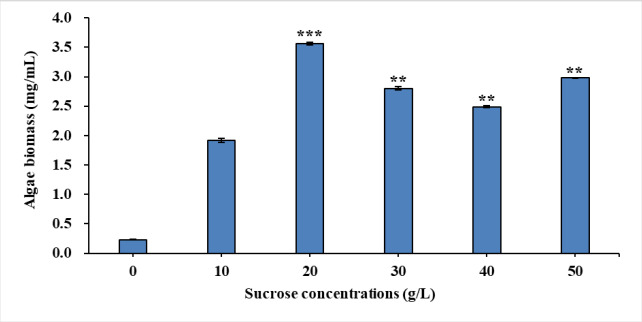

Figure 3. Effect of different concentrations of sucrose on the biomass of *Klebsormidium* sp. The error bars represent the mean ± standard error mean (n = 3). The asterisk (*) represents the p-value of the statistical test compared to the control (0 g/L), ** $P < 0.01$, *** $P < 0.001$.

Besides carbon sources, nitrogen is the second most required nutrient for algae biomass production. Nitrogen can be supplied in the forms of nitrate, ammonia, or urea for the utilisation of algae. Nitrogen is a key element in the nucleic acids, which are the most important of all biological molecules and crucial for all living things [65]. In this study, five inorganic nitrogen sources, namely ammonium sulphate ($(NH_4)_2SO_4$), ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3) and sodium nitrate ($NaNO_3$) were used in supplementing algae with low phosphate media (LPM). The control was exclusive of the nitrogen source. Ammonium nitrate (NH_4NO_3) gave the significantly highest yield of algae biomass at 1.92 ± 0.02 mg/mL as compared to other nitrogen sources ($P < 0.05$) (**Figure 4**). The result is supported by a study on cultivation of *Spirulina platensis*, in which ammonium nitrate and urea promoted maximum biomass yield instead of sodium nitrate [66]. Another study reported that the growth of a freshwater *Chlorella sorokiniana* was influenced by diverse sources of nitrogen and the cell growth was optimum in both $NaNO_3$ and NH_4NO_3; a higher growth rate was recorded with increasing concentration of nitrogen (6.0 M) [67].

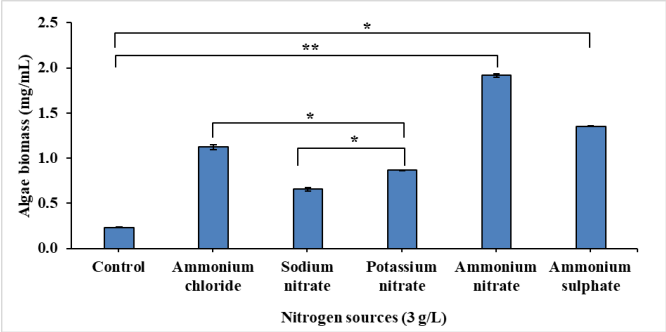

Figure 4. Effect of different nitrogen sources on the biomass of *Klebsormidium* sp. The error bars represent the mean ± standard error mean (n = 3). The asterisk (*) represents the p-value of the statistical test, * $P < 0.05$, ** $P < 0.01$.

Nitrogen is the major factor in growth medium and a limiting nutrient affecting various algae's biomass yield and lipid productivity [44, 53]. Any nitrogen source would be first reduced to ammonium and undergo assimilation to form amino acids via various metabolic pathways [68]. Based on the result in **Figure 4**, NH_4NO_3 was the preferred nitrogen source for *Klebsormidium* sp., which gave the significantly highest yield of biomass at 2.45 ± 0.02 mg/mL when subjected to 2 g/L of NH_4NO_3 (**Figure 5**) ($P < 0.05$). The *Klebsormidium* sp. growth in ammonium nitrate at a concentration of 2 g/L showed significant difference compared to the other concentrations of nitrogen ($P < 0.05$). Thus, this proved that algae could grow at limited nitrogen concentration, which triggers a high amount of carbohydrate and lipid production [69]. A common freshwater *Scenedesmus* sp. LX1 grew the fastest ($\mu = 0.82\ d^{-1}$) when ammonium was subjected to the growth medium [70]. Maximum cell concentration was observed in *Microcystis viridis* in the presence of both ammonium and nitrate compared to each treatment [71].

pH is a primary environmental factor in algae's physicochemical and biological processes. During photosynthesis, the algae consume carbon dioxide, which can elevate the pH level from 6 to 8 [72], with prominent bicarbonate formation [73]. The study on the effects of pH variations was conducted using sodium phosphate buffer with pH ranging from 6 to 8. Phosphate buffer is highly water soluble and has a high buffering capacity. Based on the algae biomass yield, the outcomes as in **Figure 6** show that the *Klebsormidium* sp. preferred a slightly alkaline condition with initial pH 7.5 of LPM. Significantly, the highest yield of algae biomass (7.53 ± 0.02 mg/mL) was produced in LPM with pH 7.5 as compared to other pH ($P < 0.05$). Most microalgae can favour alkaline and acidic pH for their growth and biomass production [74]. It was reported that *Chlorella vulgaris* and *Nannochloropsis salina* achieved optimal growth at pH between 7.5 and 9.0 [75, 76]; while the highest biomass of *Spirogyra* sp. (1.38 g/L) was observed at pH 7 [77]. Thus, a neutral pH demonstrated optimum

growth, as seen in *Mougeotia* spp., *Ulothrix* sp., and *Klebsormidium* sp. from the Río Agrio River [78].

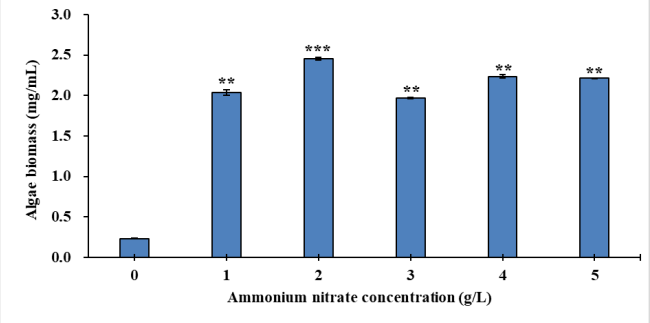

Figure 5. Effect of ammonium nitrate concentrations on the biomass of *Klebsormidium* sp. The error bars represent the mean ± standard error mean (n = 3). The asterisk (*) represents the p-value of the statistical test compared to the control (0 g/L), ** $P < 0.01$, *** $P < 0.001$.

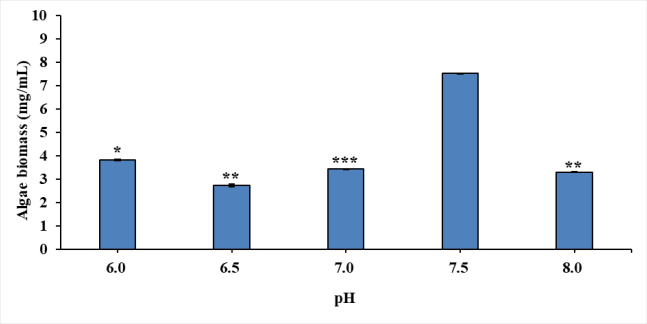

Figure 6. Effect of pH on the biomass of *Klebsormidium* sp. The error bars represent the mean ± standard error mean (n = 3). The asterisk (*) represents the p-value of the statistical test when compared to pH 7.5, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Sodium chloride concentration varied at 1 g/L (17 mM), 2 g/L (34 mM), 3 g/L (51 mM), 4 g/L (68 mM), 5 g/L (85 mM), 6 g/L (102 mM). The optimum activity of algae growth observed (**Figure 7**) in the concentration of NaCl 4 g/L (68 mM) at gave the highest yield of 2.12 ± 0.01 g/L with no significant difference compared to other NaCl concentrations ($P > 0.05$). The NaCl is another important factor that alters the biochemical composition of algae. *Klebsormidium* genus adapts and tolerates a diverse range of harsh environments and can thrive in hostile habitats [79]. The growth rate, lipid and carbohydrate contents of freshwater algae *Botryococcus braunii* increased at NaCl concentrations of 34 mM and 85 mM, whereas high NaCl concentrations are lethal (> 170 mM) [80, 81]. Another study reported that growth inhibition was exhibited in a typical freshwater microalgae *Chlorella vulgaris* at high concentration of 599 mM (35 g/L) of NaCl [82]; conversely, the highest production of biomass recorded in *S. obliquus* (0.63 g/L) was obtained at 50 mM NaCl [83]; *Chlamydomonas mexicana* (0.8 g/L) and *Scenedesmus obliquus* (0.65 g/L) with 25 mM NaCl [84]; *Chlorella vulgaris* YH703 at 30 mM NaCl [85]. Hence, the growth of freshwater algae mainly prefers a moderate level of NaCl concentration.

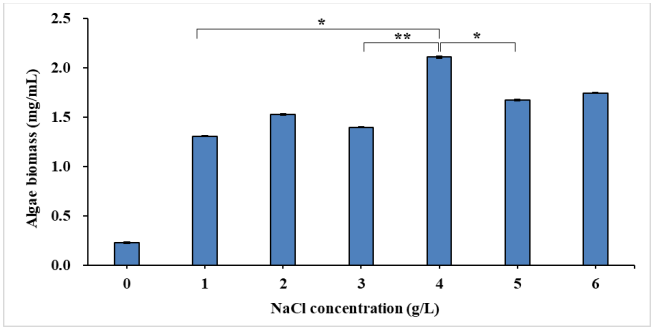

Figure 7. Effect of NaCl concentrations on the biomass of *Klebsormidium* sp. The error bars represent the mean ± standard error mean (n = 3). The asterisk (*) represents the p-value of the statistical test when compared to 4 g/L of NaCl, * $P < 0.05$, ** $P < 0.01$.

The overall yield of biomass was obtained after 7 days of algae cultivation in modified low phosphate media (20 g/L of sucrose, 2 g/L of ammonium nitrate, 4 g/L of sodium chloride and pH 7.5) containing 10 mM Mo, and the original LPM indicated as the control. Based on **Figure 8**, the biomass yield with aeration was significantly higher compared to without aeration ($P < 0.05$). The aeration promotes algae growth as algae culture efficiently uses all nutrients [39]. The impact of mixing on *Spirulina platensis* [86] and *Desmodesmus communis* [87] showed that using a bubble column and mixed culture, respectively, the growth and yield of the microalga were maximal. The exposure of shading and mixing appears to increase the biomass through mass cell distribution and nutrients [88] in *Chlorella* sp. [89] and *Scenedesmus obliquus* [90]. In this study, there was an insignificant difference between the growth rate, corresponding to García-Camacho et al. [91]; the cells may be damaged by direct air bubbles bursting at the surface of the microalgae culture.

Growth kinetic models provide an understanding of algae biomass production and nutrient consumption rate. The growth rate of microalgae can be altered by optimising the factors affecting their growth conditions through the modification of low phosphate media. Estimation of k (specific growth rate) from OD620 or cell concentration data was carried out using an exponential least square fit based on Equation (2) [92, 93]. In this study, *Klebsormidium* sp. was grown for 7 days of incubation to determine its growth kinetics using a simple exponential growth model in two different systems, with and without aeration. The aeration system is an important parameter for the cultivation of algae growth to ensure sufficient nutrients and light received by

the algae cells [47]. The isolate was tested in optimised LPM and different systems by keeping other conditions constant.

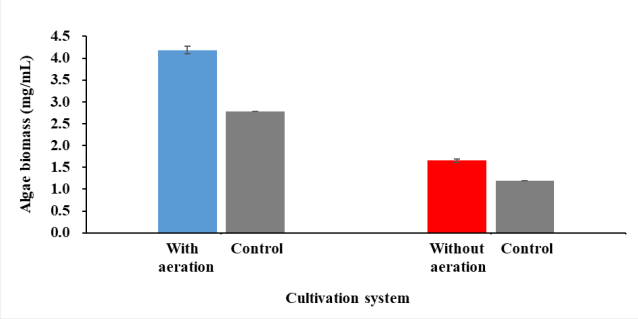

Figure 8. Biomass yield of *Klebsormidium* sp. under optimised factors with aeration and without aeration. The error bars represent the mean ± standard error mean (n = 3).

The exponential model or Malthus law Equation (2) of population growth was proposed by Thomas Malthus [25], where the population can grow with sufficient resources at exponential rates. As shown in **Figure 9**, the *Klebsormidium* sp. growth curve was generated by using the exponential growth equation through non-linear regression analysis (GraphPad Prism® 8), where the cultivation of algae with aeration showed $N = 0.037e^{0.020t}$ ($r^2 = 0.9264$) meanwhile, for cultivation without aeration, $N = 0.028e^{0.020t}$ ($r^2 = 0.9390$). The coefficient of determination (r^2) value represents the goodness of fit value. The higher the values, the better the data fits the model; both cultivation systems have similar r^2. However, to determine whether the exponential growth model fits the data, a paired samples t-test was carried out to compare the growth between different aeration systems; both aeration systems are not significant ($P > 0.05$) with each other ($P = 0.0812$, n = 8, t = 2.036) [70, 94]. In a comparison modelling study for the growth of microalgae *Nannochloropsis* sp., the logistic model performed significantly better than the exponential model. The logistic model demonstrates that the population's exponential growth cannot proceed for an unspecified time, as the growth rate depends on the population's size to understand its productivity, contrary to the exponential model [36].

Based on the exponential growth equation, the specific growth rate (μ) of *Klebsormidium* sp. with the system was 0.020 ± 0.0018 h^{-1}, while without aeration was 0.020 ± 0.0015 h^{-1} after the 50th hour. The specific growth rates for algae cultivated in both culture systems were comparable with no significant difference ($P > 0.05$). The μ was determined from the slope of the growth curve of cell concentration against the time from the semi-logarithmic plot through a linear regression [95]. This growth model is the simplest to study the population of growth, as the growth rate is assumed to be constant in the exponential growth phase and does not depend on increasing cell densities [36]. Other literature has discussed growth modelling using the logistic and the Gompertz models, which estimated the growth parameter values from the absorbance data [96]. Thus, using the simple exponential growth model does not suffice to understand and investigate the parameters that affect algae growth. However, a comparison between different growth models may improve this study.

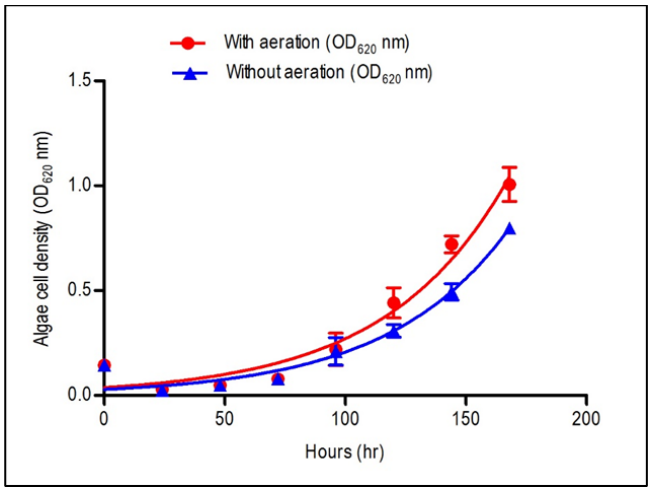

Figure 9. Exponential growth curve of *Klebsormidium* sp. in comparison with and without aeration. The error bars represent the mean ± standard error mean (n = 3).

In conclusion, *Klebsormidium* sp. grew in the low phosphate media containing Mo under varied optimised conditions. The culture of *Klebsormidium* sp. in both systems achieved an exponential phase on the 3rd day, with the aeration system exhibiting a higher growth rate (μ= 0.2352 d^{-1}) than those without aeration (μ= 0.1976 d^{-1}). The microalgal growth in Mo was based on the highest biomass yield for the following optimised conditions: 20 g/L of sucrose, 2 g/L of ammonium nitrate, 4 g/L NaCl concentration and pH 7.5. There was no significant difference in the microalgal growth between with aeration (0.020 ± 0.0018 h^{-1}) and without aeration systems (0.020 ± 0.0015 h^{-1}) in the optimised Mo media confirmed through the exponential growth curve model. Optimised *Klebsormidium* sp. growth adaptations provide fundamentals for a potential bioremediation process in mitigating Mo pollution.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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