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Evaluation of The Biodiesel and Bioethanol Potential of *Hindakia tetrachotoma* Grown in Wastewater Including Various Nitrogen Sources

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Bioethanol,
Wastewater

Abstract: Microalgae can be used in many areas, including biofuel production. This process requires increasing the biomass yield of microalgae and producing high quantities of the product. This study investigated the biomass, biodiesel, and bioethanol potential of *Hindakia tetrachotoma* microalgae grown in wastewater containing various nitrogen sources. In addition, the antioxidant enzyme activities of superoxide dismutase and catalase were studied. Nitrate-containing samples yielded the highest biomass concentration (1487 ± 21 mg/L). The maximum lipid percentage was 38% at 1 mM NO_3^- concentration. In addition, the highest carbohydrate percentage was 28% at a concentration of 6 mM NO_3^-. The lowest carbohydrate percentage was 15% at a concentration of 1 mM NO_2^-. Furthermore, the highest biodiesel percentage was 118.8% at 1 mM NO_3^- concentration, whereas the highest bioethanol percentage was 112% at 6 mM NO_3^- concentration. Also, the maximum SOD activity was 132 ± 5 U/mg proteins at 1 mM NH_4^+. The lowest SOD activity was 39 ± 3 U/mg proteins at 6 mM urea concentration. The maximum CAT activity was measured to be 120 ± 7 U/mg proteins at a concentration of 6 mM NH_4^+. As a result, NO_3^- was the most effective nitrogen source for *Hindakia tetrachotoma* microalgae, making it appropriate for biodiesel and bioethanol production.

Çeşitli Azot Kaynaklarını İçeren Atıksularda Büyütülen *Hindakia tetrachotoma*'nın Biyodizel ve Biyoetanol Potansiyelinin Değerlendirilmesi

Anahtar Kelimeler
Hindakia tetrachotoma,
Azot,
Biyodizel,
Biyoetanol,
Atıksu

Öz: Mikroalgler biyoyakıt dahil birçok alanda kullanılabilirler. Bunun için mikroalglerin biyokütle veriminin arttırılması ve ürünün yüksek miktarlarda üretilmesi gerekir. Bu çalışmada, farklı azot kaynakları içeren atıksu içerisinde büyütülen *Hindakia tetrachotoma* mikroalginin biyokütle, biyodizel ve biyoetanol potansiyeli araştırıldı. Buna ek olarak antioksidan enzim aktiviteleri olan superoksit dismutaz ve katalaz aktiviteleri incelendi. En yüksek biyokütle konsantrasyonu 1487 ± 21 mg/L ile NO_3^- içeren örneklerde elde edildi. Dahası, maksimum lipid yüzdesi 1 mM NO_3^- konsantrasyonunda %38 olarak bulundu. En yüksek karbonhidrat yüzdesi 6 mM NO_3^- konsantrasyonunda %28 olarak bulundu. En düşük karbonhidrat yüzdesi ise 1 mM NO_2^- konsantrasyonunda %15 olarak bulundu. En yüksek biyodizel yüzdesi %118.8 ile 1mM NO_3^- konsantrasyonunda ve en yüksek biyoetanol yüzdeside %112 ile 6 mM NO_3^- konsantrasyonunda elde edildi. Buna ek olarak, maksimum SOD aktivitesi 1 mM NH_4^+'de 132 ± 5 U/mg protein olarak bulundu. En düşük SOD aktivitesi ise 6 mM üre konsantrasyonunda 39 ± 3 U/mg protein olarak bulundu. Maksimum CAT aktivitesinin 6 mM NH_4^+ konsantrasyonunda 120 ± 7 U/mg protein olduğu ölçüldü. Sonuç olarak NO_3^- *Hindakia tetrachotoma* için en kullanışlı azot kaynağı olarak biyodizel ve biyoetanol üretimi içinde uygun olduğu görülmüştür.

1. Introduction

The vast majority of microalgae are single-celled organisms capable of photosynthesis. Their photosynthesis means they can synthesize organic matter. However, some species can synthesize organic matter even in the absence of sunlight or when it is very scarce, and their ability to utilize any

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available carbon source has contributed to their exceptional adaptability, which in turn has led to their industrial applications [1]. Environmental conditions are crucial for microalgae to grow and synthesize organic matter. Temperature and pH are paramount. Unless they are extreme, microalgae typically require room temperature. However, microalgae can be thermophilic and adapt to higher temperatures. The same applies to pH. Microalgae grow best at physiological pH values around pH 7. However, some microalgae thrive at lower or higher pH levels [2,3]. Light quantity and wavelength are also important for microalgae growth. While this is a species-dependent property, photoinhibition can occur at high light levels, slowing microalgae growth. The color of the lights used for growth can also be considered in this context. The optimal wavelength for some species may inhibit others [4,5]. The environment is crucial for microalgae growth. All microalgae can grow optimally in their natural environment. However, artificial media can be used to ensure reproducibility in scientific studies. If the microalgae are marine, a more saline medium is preferred, but if they are freshwater, a lower salt medium is preferred. Otherwise, microalgae growth will be limited [6]. Microalgae use the chemicals that make up the basic components in this media. These include carbon, nitrogen, phosphorus, hydrogen, and oxygen. In addition to key elements like calcium, potassium, chloride, and sulphur, they need trace elements [7]. Nitrogen is necessary for microalgae growth and can come from a variety of sources. Each microalgae species can make the most of a different nitrogen source by converting it into biomass, or they can produce tiny amounts of biomass while maintaining a high organic content. Microalgae can use nitrate, nitrite, ammonia, and urea as nitrogen sources. Using these compounds can lead to differences in biomass and metabolic content [8]. Wastewater can be used in addition to artificial media for growing microalgae. Because wastewater contains abundant nitrogen and phosphorus, it can create a suitable environment for microalgae growth. This environment both supports microalgae growth and contributes to wastewater treatment [9]. The types of wastewater can vary depending on their location. Wastewaters can be classified as agricultural, municipal, or industrial. Treatment of wastewater in an automotive industry area is likely to result in high levels of heavy metals. Such contaminants could limit the growth of microalgae. If the wastewater is from a sugar factory, the carbon content will be high. Or if it's from an olive oil factory, the wastewater may contain organic solvents and oil. These factors can affect wastewater treatment and microalgae growth. Municipal wastewater, on the other hand, can contain bathroom and kitchen waste, as well as other household materials [10,11]. Although it can be considered moderately dense wastewater, it can facilitate the growth of microalgae and the treatment of wastewater. When microalgae are grown in different media, changes in environmental factors such as temperature, pH, or medium concentration might cause variations in antioxidant enzyme activity [12]. The most significant enzymes are catalase (CAT) and superoxide dismutase (SOD). These enzymes detect any change in the environment and adjust their amounts accordingly. This is because these alterations typically produce reactive oxygen species (ROS), which these enzymes aim to deactivate or render less toxic [13,14]. In addition to the above, any changes in the nutritional content of microalgae can affect both the quantity and characteristics of the products produced in the industry. SOD and CAT activities can alter the quantity of microalgae in response to any perceived stress. When subjected to salt stress, *Chlamydomonas reinhardtii* produces more ROS, which boosts SOD activity [15]. In addition, CAT, like SOD, can suppress ROS production, increasing CAT abundance [16]. The most commonly produced products from microalgae are industrial ones such as energy. Given the ongoing need for cost-effective development in this field, it is crucial to increase the quantity of these products. Researching suitable microalgae strains or manipulating the environment can increase the quantity of these products [17,18]. Studies on energy production from microalgae have focused primarily on biofuels. The most important biofuels are biodiesel and bioethanol. Biodiesel is produced by extracting microalgae to release their oils, while bioethanol is produced by extracting their carbohydrates. While biodiesel is formed by the esterification of oils, bioethanol is formed by the fermentation of carbohydrates. Changes in nitrogen concentrations and nitrogen source types in microalgae can also alter their growth and metabolic processes. Microalgae generally utilize nitrite, nitrate, and urea as nitrogen sources. These changes, which are generally species-dependent, can contribute to product formation by fine-tuning nitrogen concentrations. For example, *Chlorella cf. ellipsoidea* can grow in different ammonium and nitrate concentrations [19]. Changes in nitrogen content and the carbon-to-nitrogen ratio can both alter lipid or metabolic content. *Chlorella sp.* can change lipid content in different nitrite, nitrate, urea, and nitrogen-free environments [20]. Since this situation is species-dependent, it manifested itself differently not only in Chlorella but also in Scenedesmus. Scenedesmus grown in sodium nitrate, glutamate, and acetate/nitrate at various C/N ratios and nitrogen concentrations gave different results in carbohydrate amounts [21]. Changing the amount or source of nitrogen can also cause changes in the final biodiesel product. This change may occur first by changing the type and amount of fatty acid methyl esters [22]. This change can also occur in another biofuel, biobutanol. Differences in the materials used can

alter the tendency toward different biofuels, leading to their formation [23].
Within the scope of this study, the fast-growing species *Hindakia tetrachotoma* (HT) was investigated. This species' biofuel potential has been investigated in the past, and its characteristics are currently undergoing further refinement.

This study aimed to determine the biodiesel and bioethanol potential of *Hindakia tetrachotoma* microalgae cultivated in wastewater containing various nitrogen sources such as nitrate, nitrite, ammonium, and urea. Furthermore, the study sought to explore changes in enzyme activity, such as catalase and superoxide dismutase, during the synthesis of these hydrocarbons.

2. Material and Method

2.1. Cultivation of *Hindakia tetrachotoma*

Hindakia tetrachotoma (HT) used in this study was obtained from Van-YYU Environmental Engineering Environmental Microbiology Laboratory. Medium was prepared by combining 50% BG-11 and 50% TAP mediums, and microalgae were cultured at a speed of 80 rpm, a temperature of 24+1°C, 16:8 (light:dark),and light intensity of 100 µmol m^{-2} s^{-1}. The test mixture was then obtained by mixing 80% of this medium with 20% municipal wastewater. This medium was used throughout the study, and it was carried out in a 1 L flat photobioreactor. The reactor has a volume of 1.3 L, an operational capacity of 1 L, and dimensions of 350-350-20 mm. The BG-11 and TAP mediums were prepared exactly according to Andersen 2005 [24]. The TOC value of the municipal wastewater was 181 mg/L, the TN value was 52 mg/L, and the TP value was 16 mg/L. The content and preparation of wastewater were given in detail in a previous study [25].

2.2. Preparation of nitrogen sources

Four different types of nitrogen were used in this study: nitrate, nitrite, ammonium, and urea. Sodium nitrate ($NaNO_3$) was the nitrate source, sodium nitrite ($NaNO_2$) was the nitrite source, ammonium chloride (NH_4Cl) was the ammonium source, and urea (H_2NCONH_2) was the urea source. These sources of nitrogen were prepared, and experiments were carried out at final concentrations of 1 mM, 3 mM and 6 mM.

2.3. Analitical experiments

Growth graphs of microalgae samples were monitored spectrophotometrically at OD_{680}. Stationary phase samples were centrifuged at 3600 g for 10 minutes, then dried, and dry weight measurements were made gravimetrically. For the purpose of determining the levels of catalase (CAT) and superoxide dismutase (SOD) activity, the nitroblue tetrazolium and thiobarbituric acid procedures were performed, respectively. Antioxidant enzyme activity measures were given in the previous study [25].

2.4. Lipid and carbohydrate content determination

Lipid determination in HT samples was performed using the Folch method [26]. Samples were obtained by mixing chloroform and methanol at a 2:1 ratio and then evaporating the chloroform and measuring the remaining oil gravimetrically. Carbohydrate content was determined using the acid hydrolysis method. Acid hydrolysis was conducted using 1M H_2SO_4. Carbohydrate determination was described in more detail in a previous study [27].

2.5. Biodiesel and bioethanol production

Lipids of microalgae extracts were transesterified using methanol, including 0.1 N KOH for biodiesel. The mixture was incubated at 60°C for 4 h. The detailed procedure was given in the previous study [23]. In addition, S. cerevisiae was cultivated on agar medium, and fermentation was observed at 25 °C in a shaking incubator for bioethanol and bioethanol content was determined according to Rizza [28].

2.6. Statistical analyses

The experimental groups contained three parallel samples. All statistical analyses were performed by one-way analysis of variance (ANOVA) and Tukey's test. The confidence level was higher than 95%. In this report, the results are expressed as the mean ± standard deviation (SD). When $P < 0.05$, * shows significant and ** shows very significant.

3. Results

3.1. Biomass concentration of *Hindakia tetrachotoma*

Samples of *Hindakia tetrachotoma* (HT) were cultured in wastewater that included a variety of sources of nitrogen, and the biomass concentrations of these samples were then examined. Assessing the context, the highest value of biomass, which was 1487 ± 21 mg/L, was found at a concentration of 6 mM of NO_3^-.Conversely, the lowest biomass concentration recorded was 260 ± 10 mg/L when the concentration of NO_2^- was 6 mM. Upon general examination of the results, it was observed that the biomass concentration augmented with an increase in NO_3^- concentration. The biomass at a concentration of 1 mM NO_3 was 807 ± 15 mg/L. At a concentration of 3 mM NO_3^-, the biomass measured 1250 ± 20 mg/L. The biomass attained its peak at a 6 mM NO_3 concentration. Upon examining an

alternative nitrogen source, NO_2^-, the findings differed from those of NO_3^-. The peak biomass recorded was 447 ± 15 mg/L at a concentration of 1 mM NO_2^-. Subsequently, it was noted that the biomass ratio exhibited a decline as the quantity of NO_2^- grew. The biomass at a concentration of 3 mM NO_2^- was 393 ± 15 mg/L, whereas the biomass at a concentration of 6 mM NO_2^- was 260 ± 10 mg/L. The performance of NH_4^+ was better than NO_2^-. The highest biomass was 760 mg/L at 3 mM NH_4^+ concentration, 523 ± 15 mg/L at 1 mM NH_4^+ concentration, and 520 ± 10mg/L at 6 mM concentration. Urea, which is still another source of nitrogen, likewise produced a biomass concentration that was comparable to that of NO_3^-. The biomass reached its highest value, which was 1223 ± 15 mg/L, when the concentration of urea was 3 mM. When the concentration of urea was 6 mM, the biomass value that was obtained was close to the value that was acquired at 3 mM. At a urea concentration of 6 mM, the biomass concentration was measured at 1147 ± 6 mg/L. At a urea concentration of 1 mM, the lowest biomass was 743 ± 15 mg/L. Biomass concentration of HT grown in different nitrogen sources was given in Figure 1.

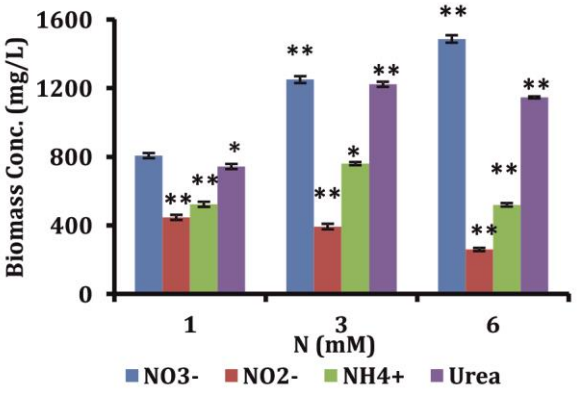

Figure 1. Biomass concentration of *Hindakia tetrachotoma* grown in different nitrogen sources

3.2. Lipid percentage of *Hindakia tetrachotoma*

Lipid percentages of HT, like biomass concentrations, showed a fluctuating trend as the nitrogen source changed. The maximum lipid percentage was 38% at 1 mM NO_3^- concentration. The lipid percentage decreased with increasing NO_3^- concentrations. The lipid percentage was 32% at 3 mM NO_3^- concentration and 28% at 6 mM NO_3^- concentration. When the nitrogen source was NO_2^-, the results were similar to those for NO_3^-. The highest lipid percentage was 31% at 1 mM NO_2^-. As the amount of NO_2^- increased, the lipid percentage decreased dramatically. At 3 mM NO, the lipid percentage was 23%, and at 6 mM, it was 22%. When NH_4^+ was used as the nitrogen source, the lipid percentage continued to decrease with increasing nitrogen source. The highest lipid percentage was found as 31% at 1 mM NH_4^+ concentration, while the lipid percentages at 3 mM and 6 mM NH_4^+ concentrations were 25% and 24%, respectively. Considering the lipid percentage, urea exhibited a similar pattern to NO_3^-. The highest lipid percentage was 35% at 1 mM urea concentration. On the other hand, as the urea concentration increased, the lipid percentage decreased. At 3 mM urea concentration, the lipid percentage was 30%, while at 6 mM urea concentration; the lipid percentage was 27%. Lipid percentages of HT grown in different nitrogen sources were given in Figure 2.

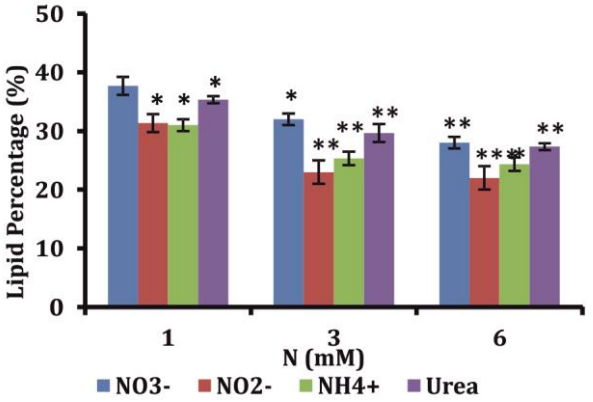

Figure 2. Lipid percentages of *Hindakia tetrachotoma* grown in different nitrogen sources

3.3. Carbohydrate percentage of *Hindakia tetrachotoma*

After studying the change in lipid percentages, the other metabolite, carbohydrate percentage, was investigated. Considering the overall experimental setup, the highest carbohydrate percentage was 28% at a concentration of 6 mM NO_3^-. The lowest carbohydrate percentage was 15% at a concentration of 1 mM NO_2^-. In addition, it was observed that the carbohydrate percentage increased as the NO_3^- concentration increased. At a concentration of 1 mM NO_3^-, the carbohydrate percentage was 22%, while at a concentration of 3 mM NO_3^-, the carbohydrate percentage was 25%. At a concentration of 3 mM NO_2^-, the maximum carbohydrate percentage was 19% when the nitrogen source was NO_2^-. On the other hand, when the concentration of NO_2^- was 1 mM, the carbohydrate percentage was 15%, and when the concentration of NO_2^- was 6 mM, the carbohydrate percentage was 16%. Using NH_4^+ as a nitrogen source resulted in similar carbohydrate percentages to NO_2^-. Carbohydrate percentages were highest at 3 mM NH_4^+ values (21%), followed by 18% at 1 mM and 6 mM NH_4^+ concentrations. When urea was the nitrogen source, the carbohydrate percentage increased as the urea concentration increased. The highest carbohydrate percentage was 25% at the 6 mM urea concentration, while it was 23% and 21% at the 3 mM and 1 mM urea concentrations, respectively. Carbohydrate percentages of HT

grown in different nitrogen sources were given in Figure 3.

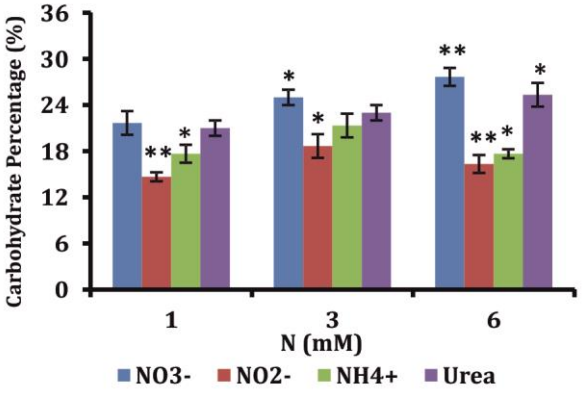

Figure 3. Carbohydrate percentages of *Hindakia tetrachotoma* grown in different nitrogen sources

3.4. SOD activity of *Hindakia tetrachotoma*

After analyzing the biomass, lipid, and carbohydrate contents of HT, enzyme activities were measured to determine whether these changes indicated differences in cell systems. The maximum SOD activity was 132 ± 5 U/mg proteins at 1 mM NH_4^+. The lowest SOD activity was 39 ± 3 U/mg proteins at 6 mM urea concentration. Although the concentration of NO_3^- increased, the activity of SOD dropped. However, when the concentration of NO_3^- reached 6 mM, the activity of SOD reached its lowest point (43 ± 4 U/mg proteins). When the quantity of NO_3^- was 1 mM, the maximum activity of SOD was observed to be 118 ± 7 U/mg proteins. For NO_2^-, the most SOD activity, which was 123 ± 7 U/mg proteins, occurred when the concentration of NO_2^- was 1 mM. SOD activity was measured at 96 ± 5 U/mg proteins when the concentration of NO_2^- was 6 mM, but the activity at 3 mM NO_2^- concentration was measured at 71 ± 7 U/mg proteins. When the concentration of NH_4^+ was 1 or 6 mM, the activity of SOD increased by a very notable amount; however, when the concentration was 3 mM, the activity of SOD was at moderate levels. At a concentration of 1 mM NH_4^+, it was 132 ± 5 U/mg proteins, while at a concentration of 6 mM NH_4^+, it was 106 ± 4 U/mg protein. The activity of SOD was measured to be 66 ± 3 U/mg proteins when the concentration of NH_4^+ was 3 mM. When it came to urea, a different source of nitrogen, the activity of SOD was inversely related to the concentration of urea. SOD activity went up as the amount of urea went down. As a result, the maximum SOD activity was seen at a urea concentration of 1 mM, with a value of 95 ± 7 U/mg proteins, and at a urea concentration of 3 mM, with a value of 58 ± 7 U/mg proteins. The lowest level of SOD activity was recorded at a concentration of 6 mM urea, which was 39 ± 3 U/mg proteins. SOD activities of HT grown in different nitrogen sources were given in Figure 4.

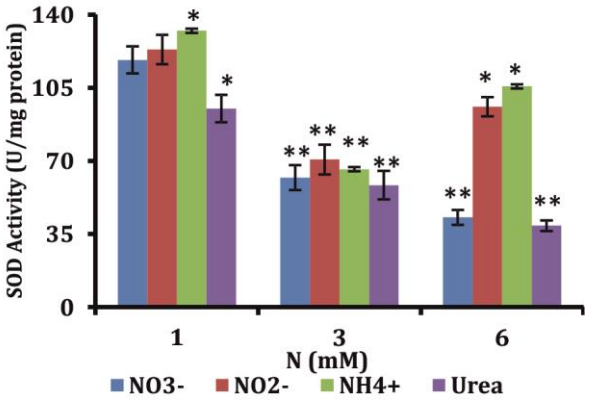

Figure 4. SOD activities of *Hindakia tetrachotoma* grown in different nitrogen sources

3.5. CAT activity of *Hindakia tetrachotoma*

HT was evaluated for both SOD and CAT activities. The maximum CAT activity was measured to be 120 ± 7 U/mg proteins at a concentration of 6 mM NH_4^+, which is comparable to the activity of SOD. At 1 mM NH_4^+ concentration, CAT activity was very close to 6 mM NH_4^+ concentration. CAT activity at a 1 mM NH_4^+ concentration was 116 ± 5 U/mg proteins. In contrast, at 3 mM NH_4^+ concentration, CAT activity was 56 ± 4 U/mg proteins. On the other hand, as the concentration of NO_3^- fell, the activity of CAT increased when the nitrogen source was NO_3^-. The maximum CAT activity measured was 99 ± 7 U/mg proteins. While CAT activity was 54 ± 2 U/mg proteins at 3 mM NO_3^- concentration, it was 45 ± 4 U/mg proteins at 6 mM NO_3^- concentration. The CAT activities of NH_4^+ were comparable to those of NO_2^-. The lowest CAT activity was 59 ± 4 U/mg proteins at 3 mM NO_2 concentration, whereas the maximum CAT activity was 118 ± 6 U/mg protein at 1 mM NO_2^- concentration. In addition, CAT activity at 6 mM NO_2^- concentration was 113 ± 9 U/mg proteins. Furthermore, the findings were comparable when urea was used as the source of nitrogen. At a urea concentration of 1 mM, the maximum CAT activity was measured at 115 ± 9 U/mg proteins, but CAT activities at urea concentrations of 3 mM and 6 mM were measured at 62 ± 3 U/mg proteins and 71 ± 8 U/mg proteins, respectively. CAT activities of HT grown in different nitrogen sources were given in Figure 5.

3.6. Changes in the biomass, biodiesel, and bioethanol of *Hindakia tetrachotoma*

This study also investigated the biomass, biodiesel, and bioethanol conversion percentages of HT in wastewater containing different nitrogen sources. A control concentration of 3 mM NO_3, which exhibited moderate growth, was selected for all studies and was evaluated as 100%. When the nitrogen source was NO_3^-, the highest biomass conversion rate was 119% in 6 mM NO_3^-. At a concentration of 1 mM NO_3^-, the biomass conversion was 64.6%. When the

nitrogen source was NO_2^-, the highest biomass change percentage was 35.8% at 1 mM NO_2^-. The lowest biomass change percentage was 20.8% at 6 mM NO_2^- concentration. When the nitrogen source was NH_4^+, the biomass change percentages were 49.8%, 60.8%, and 57.6% at 1 mM, 3 mM, and 6 mM NH_4^+ concentrations, respectively. The maximum biomass change was observed at a urea concentration of 3 mM, with a value of 97.8%. This occurred when urea was utilised. When the concentration of urea was 6 mM, the biomass change percentage was 91.8%, which is a value that is comparable to that of 3 mM urea. The lowest percentage of change in biomass was 59.4 at a urea concentration of 1 mM.

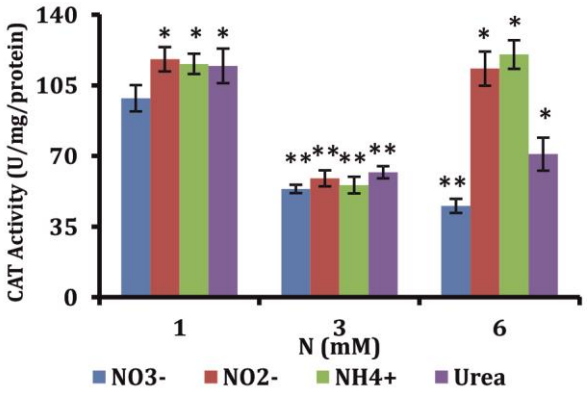

Figure 5. CAT activities of *Hindakia tetrachotoma* grown in different nitrogen sources

The percentages of biomass that were converted into biodiesel exhibited behavior that was significantly different from the percentages of biomass that were converted into other substances. The highest biodiesel conversion percentage was 118.8% at a concentration of 1 mM NO_3^-, whereas the lowest biodiesel conversion percentage was 68.8% at a concentration of 6 mM NO_2^-. In addition, the percentage change in biodiesel at 3 mM and 6 mM NO_3^- concentrations was 100% and 87.5%, respectively. When the nitrogen source was NO_2^-, the maximum percentage change in biodiesel was 96.9% at a concentration of 1 mM NO_2^-. The percentage change in biodiesel was 71.9% at an amount of 3 mM NO_2^- and 68.8% at a concentration of 6 mM NO_2^-. In addition, the biodiesel conversion percentages for NH_4^+ were similar to those for NO_2^-. The conversion percentages at 1 mM, 3 mM, and 6 mM NH_4^+ concentrations were 96.9%, 78.1%, and 75%, respectively. When urea was used as a nitrogen source, the maximum biodiesel change percentage was 109.4% at 1 mM, followed by 93.8% and 84.4% at 3 and 6 mM, respectively.

In this study, in addition to biomass and biodiesel conversion percentages, bioethanol conversion percentages were also examined. The highest bioethanol change percentage was 112% at 6 mM NO_3^- concentrations, while the lowest bioethanol change percentage was 72% at 1 mM and 6 mM NH_4^+ concentrations. In addition, the percentage changes in bioethanol at 1 mM and 3 mM NO_3^- concentrations were 88% and 100%, respectively. When the nitrogen source was NO_2^-, the bioethanol change percentages at 1 mM, 3 mM, and 6 mM NO_2^- concentrations were 60%, 76%, and 64%, respectively. The change in 3 mM NH_4^+ concentration was 84%. Finally, when urea was used, the maximum bioethanol change percentage was 100% at a concentration of 6 mM. The results were 84% and 92% at 1 mM and 3 mM urea concentrations, respectively. The percentage changes in biomass, biodiesel, and bioethanol of HT cultivated at various nitrogen sources were given in Table 1.

Table 1. The percentage changes in biomass, biodiesel, and bioethanol of *Hindakia tetrachotoma* cultivated at various nitrogen sources

4. Discussion and Conclusion

Biomass (%)	**NO3-**	**NO2-**	**NH4+**	**Urea**
1 mM	64.6	35.8	49.8	59.4
3 mM	100	31.4	60.8	97.8
6 mM	119	20.8	57.6	91.8
Biodiesel (%)	**NO3-**	**NO2-**	**NH4+**	**Urea**
1 mM	118.8	96.9	96.9	109.4
3 mM	100	71.9	78.1	93.8
6 mM	87.5	68.8	75	84.4
Bioethanol (%)	**NO3-**	**NO2-**	**NH4+**	**Urea**
1 mM	88	60	72	84
3 mM	100	76	84	92
6 mM	112	64	72	100

HT samples grown in wastewater containing various nitrogen sources yielded different biomass concentrations. The biomass of samples at 1 mM NO_3^- concentration (807 ± 15 mg/L) was the lowest. The biomass increased as the amount of NO_3^- increased. The highest biomass was found at a 6 mM NO_3^- concentration (1487 ± 21 mg/L). This can be explained as an expected situation in microalgae. If the nitrogen source is the correct one, biomass increases with increasing nitrogen concentration in microalgae. However, when NO_2^- was used as the nitrogen source, the highest biomass (447 ± 15 mg/L) was observed at a concentration of 1 mM NO_2^-. Here, as the amount of NO_2^- increased, the amount of biomass decreased. The lowest biomass (260 ± 10 mg/L) was observed at nitrogen concentrations as high as 6 mM NO_2-. Here, NO_2^- had a toxic effect on the HT samples and limited the growth of microalgae. Biomass concentration decreased by 3- to 4-fold compared

to NO_3-. Moreover, this situation became more dramatic as NO_2^- concentrations increased. When the nitrogen source NH_4^+ is used, less biomass is obtained compared to NO_3^-, and more biomass is obtained compared to NO_2^-. While this situation shows the toxic effects of NH_4^+, it also explains that nitrogen cannot be usefully taken up by microalgae, as in NO_2^- concentrations. Using urea resulted in a biomass similar (1223 ± 15 mg/L) to that of NO_3^-, suggesting that urea could be beneficial for HT. The reduced biomass production from NO_3^- is most likely owing to the subsequent conversion of urea to NH_3. This conversion may contribute to a loss in biomass and increased toxicity as the concentration grows. Although the literature provides various results based on the species, there have been researches on Scenedesmus. In one investigation, *Scenedesmus sp.* samples were cultivated with various amounts of ammonium and nitrate. Samples containing 1.65 g/L calcium nitrate and no ammonium produced 1700 mg/L biomass. This value was the maximum biomass in the study. On the other hand, the lowest biomass value, 1300 g/L, was observed in samples containing approximately 2 g/L of ammonium and 0.05 g/L of calcium nitrate. In addition, in their study, the researchers used BG-11 as the medium, and the biomass of these samples was 1100 mg/L [29]. In another study, *Chlorella sp.* were cultivated in BG-11 medium with the use of $NaNO_3$, $(NH_2)_2CO$, KNO_3, and NH_4NO_3 as sources of nitrogen. The NH_4NO_3 samples, which had 670 mg/L, included the lowest biomass; on the other hand, the $(NH_2)_2CO$ samples, which had 930 mg/L, contained the highest amount of biomass [30]. As may be noticed here, ammonium inhibited biomass growth. When lipid amounts were examined in samples grown on various nitrogen sources, an almost inverse effect was observed with biomass concentration. The lipid amount of samples containing NO_3^- decreased as NO_3^- content increased. The highest lipid amount (38%) was observed in 1 mM NO_3^-. In samples containing NO_2^-, the amount of NO_2^- increased while the amount of lipids (23%) decreased down to a concentration of 3 mM NO_2^-. Then, the lipid concentration (22%) stabilized, with samples containing 6 mM NO_2^- exhibiting the same lipid concentration as those containing 3 mM. This was likely due to the fact that microalgae increase their lipid levels in stressful environments, but after a certain point, they stabilized as a result of the development of stress-coping mechanisms. Samples containing NH_4^+ showed almost the same behavior as those containing NO_2. As the NH_4^+ concentration increased, the lipid content (25%) decreased by 3 mM, while at 6 mM NH_4^+ concentrations, lipid concentrations (24%) were equal to those at 3 mM NH_4^+ concentrations. In this case, it can be explained as in other nitrogen source examples. With an increase in the nitrogen source, stress was eliminated, leading to a decrease in lipid levels. In the urea samples, a decrease in lipid levels (from 35% to 27%) was observed with an increase (from 1mM to 6mM) in nitrogen levels. Because the microalgae were able to utilize the nitrogen in the urea well, they didn't need to create lipids. However, when they began to extract urea from the environment, they quickly became stressed, increasing their lipid levels and attempting to adapt to the environment. There are some studies in the literature related with lipid extraction from microalgae. In another study, the analysis of the lipid content in samples of *Scenedesmus obliquus* BR003, which were cultivated in medium that contained ammonium and urea in varied quantities, showed that the highest lipid percentage was 20 to 25% when the samples were cultivated in a medium that contained only ammonium at a concentration of 0.08 g/L [31]. In other study, *Chlorella cf. ellipsoidea* was grown in Waris-H medium and then cultivated in varying concentrations of ammonium and nitrate. The samples that were grown in ammonium nitrate were found to have the maximum lipid concentration, which was 9.27 mg lipid/L day. In addition, this value was equivalent to a lipid percentage of 22.6% [19]. A distinct investigation was conducted for the purpose of determining the manner in which the metabolic contents of *Chlorella sp.* SE medium were changed via the application of various nitrogen sources and the manipulation of C/N ratios. The materials used included mediums that included NO_2, NO_3, urea, NH_4, and N-deficiency. The N-deficiency samples included the highest lipid percentage, with an estimated value of approximately 86% [20]. As shown in this study, when the amount of nitrogen decreased, the lipid percentage increased.

The changes in carbohydrate content, along with biomass and lipid content were examined. In samples containing the nitrogen source NO_3^-, carbohydrate content (from 22% to 28%) was observed to increase as NO_3^- content increased (from 1 mM to 6 mM). However, these increases were not as sharp or drastic as the lipid increase. A more proportional and moderate increase was observed. The highest carbohydrate content (%28) was observed in samples containing 6 mM NO_3^-. This suggests that the low lipid content affects carbohydrate synthesis systems. Samples with NO_2^- as a nitrogen source exhibit fluctuations. Carbohydrate content (20%) was highest at 3 mM NO_2^-, but decreased in samples with 1 mM and 6 mM NO_2^-. While these increases and declines were less substantial than increases and decreases in lipid content, carbohydrates were abundant in locations with the highest nitrogen levels. Carbohydrates reduced in places under stress or where a nitrogen source was lacking, but lipids increased. Samples containing NH_4^+ as the nitrogen source exhibited a similar behavior to the NO_2^- samples. The only difference was that carbohydrate amounts were higher in these samples. The maximum carbohydrate content (%21) was found

in the 3 mM NH_4^+ samples. This difference was likely due to the toxic effects of NH_4^+, causing the stressed samples to accumulate more toward lipid production. In samples containing urea as a nitrogen source, the amount of carbohydrates (from 21% to 25%) rose as the urea concentration increased (from 1 mM to 6 mM). Carbohydrates considerably replaced the decreased lipid content, with samples containing 6 mM urea having the highest carbohydrate concentration (%25), similar to NO_3^- samples. A variety of various nitrate sources were used to cultivate *Chlorella sp.* ABC-001, and it was discovered that the highest amount of carbohydrates was 41.5% at 15 mM NO_3^-. In their study, which was similar in our own investigation, the carbohydrate content increased but the lipid content fell as the NO_3 amount increased [32]. In another study, *Chlorella ellipsoidea* microalgae were grown in MBL medium at 0–10 mM sodium nitrate concentrations, and the maximum carbohydrate concentration was found to be approximately 223 mg/g at 1 mM [33]. *Scenedesmus vacuolatus* was cultivated in BG-11 medium with sodium nitrate, glutamate, and acetate/nitrate at various C/N ratios and nitrogen concentrations, and the effects on carbohydrate content of the samples were studied. The maximum carbohydrate concentration was 220 µg/mL at 12/10 (C/N) ratio. Similar to the current investigation, carbohydrate content increased with increased nitrogen level [21].

After examining the effects of HT on metabolic contents in response to various nitrogen sources, it was investigated whether this effect affected antioxidant enzyme activities. For this purpose, SOD and CAT enzyme activities were examined. SOD activities in samples containing the nitrogen source NO_3^- increased as NO_3^- concentration decreased. The highest SOD activity (118 ± 7 U/mg proteins) was observed at a concentration of 1 mM NO_3^-. This can most likely be explained by the fact that HT cells, stressed by a decrease in biomass, slow down their growth and increase their lipid levels, thereby increasing SOD activity. Oxygen radicals released after lipid peroxidation and cellular stress could be increased in amount to be converted to less harmful hydrogen peroxide by SOD. When NO_2^- was used as a nitrogen source, SOD activity was maximum (123 ± 7 U/mg proteins) at 1 mM NO_2^-, which was also a rare resource. This is most likely explained by the fact that nitrogen deprivation causes stress, which leads to lipid production and enhanced SOD activity. SOD activity (96 ± 5 U/mg proteins) was also high at 6 mM NO_2^- concentrations. This phenomenon can be explained as a result of the toxic effect of high NO_2^- concentration on HT. However, SOD activity (71 ± 7 U/mg proteins) was low at 3 mM NO_2^- concentrations, indicating that this concentration provided the most suitable growth conditions for HT including NO_2^-. The behavior of samples that used NH_4^+ as their source of nitrogen was comparable to that of those that used NO_2^- as their source of nitrogen. The highest SOD activity (132 ± 5 U/mg proteins) in the NH_4^+ samples was observed at a concentration of 1 mM NH_4^+. However, high SOD activity (106 ± U/mg proteins) was also present in samples that had a concentration of 6 mM NH_4^+.This result most likely suggests that a comparable pattern is responsible for the increase in SOD activity. This increase is induced by stress that arises from a lack of nitrogen and toxicity that is produced by an excess of NH_4^+. The highest levels of SOD activity (95 ± 7 U/mg proteins) were seen in samples where urea was used and the quantity of urea was 1 mM. Because of the fact that urea is an inadequate source of nitrogen, it is probable that the low amounts of urea that were present caused the stress levels within the cell to increase, which in turn resulted in an increase in the number of free radicals. This is the reason why the activity of SOD was shown to be elevated. When the amount of urea reached a sufficient level for HT cell growth, the cells began to grow rapidly, increasing biomass and decreasing SOD activity. Consequently, a decrease in lipid levels was observed. Thus, SOD activity was the lowest at 6 mM urea concentration, where growth was the highest. There are many articles in the literature on antioxidant enzyme activities. Those related to microalgal metabolism have either limited or indirect links. In one of these studies, *Chlamydomonas reinhardtii* was exposed to salt stress. At a sodium chloride concentration of 200 mM, increased ROS formation and an approximately three-fold change in SOD activity were observed [15]. In another study, *L.polyedrum* and *M.polymorphus* had grown in f/2 medium. SOD activities were shown to increase as cells entered the stationary phase. In this study, the increase was three times higher than in the initially stage [34]. In another study, *Borodinellopsis texensis* was cultivated at various copper concentrations and SOD activity was measured. SOD activity increased as Cu concentrations rose. SOD activity peaked at 8.87 µmol/mg at a Cu content of 0.025 g/L [27].

Another antioxidant enzyme, known as CAT, was investigated in addition to SOD activity. It was discovered that CAT activity (99 ± 7 U/mg proteins) was present in samples that contained NO_3^- at a concentration of 1 mM NO_3^-. This result was in accordance with the activities of SOD. There was also a high level of CAT activity, which was probably due to the fact that some of the hydrogen peroxide that was produced following SOD activity was transformed into a form that was less dangerous. When the quantities of NO_3^- were 3 mM and 6 mM, the formation of hydrogen peroxide decreased due to cell expansion and reduced stress, which subsequently led to a decrease in CAT activity (54 ± 2 and 45 ± 4 U/mg proteins). In samples containing NO_2^-, the highest CAT activity (118 ± 6 U/mg proteins) was found at 1 mM NO_2^-, while CAT

activity (113 ± 9 U/mg proteins) was also high in samples containing 6 mM NO_2^-. Hydrogen peroxides, which are produced by decreased cell division in areas with low nitrogen sources, are likely degraded by CAT, leading to increased enzyme activity. At a concentration of 3 mM NO_2^-, the relatively high amount of NO_2^- resulted in low CAT activity (59 ± 4 U/mg proteins) due to the high biomass of HT cells. In samples containing NH_4^+, the maximum CAT activity (120 ± 7 U/mg proteins) was at a concentration of 6 mM NH_4^+.This value is because the toxic effect of NH_4^+ likely predominated here, and high concentrations caused excess hydrogen peroxide production, resulting in high CAT activity. In the environment where the amount of NH_4^+ was 3 mM NH_4^+, CAT activity (56 ± 4 U/mg proteins) was low since cell divisions were in an ideal condition. The maximum CAT activity (115 ± 9 U/mg proteins) in urea-containing samples was found at 1 mM urea. At this concentration, cell development was slow and lipid levels were elevated. The significant CAT activity was most likely attributable to the generation of free radicals produced by lipid peroxidation and their subsequent conversion to hydrogen peroxide. CAT activities (62 ± 3 and 71 ± 8 U/mg proteins) were similar at 3 and 6 mM urea concentrations, although somewhat higher at 6 mM urea. The observed rise was most likely caused by the high concentration of hydrogen peroxide produced by enhanced cell growth and activity. CAT activity studies are frequently described in the literature in conjunction with SOD and can be used to measure stress variables. In other study, *Borodinellopsis texensis* microalgae were cultured in various amounts of wastewater and the change in CAT activity was explored. The highest CAT activity was reported to be 15 µmole/mg in 25% wastewater [25]. As noticed in studies similar to ours, ROS production and stress factors occur, and the microalgae system attempts to balance this with an increase in CAT activities.

The study also investigated changes in biomass, biodiesel, and bioethanol, in addition to the metabolic contents and enzyme activities of HT resulting from its growth in wastewater with various nitrogen sources. Samples with 3 mM NO_3^- concentration, with low enzyme activity and medium growth, were considered 100 and compared to other samples. Biomass increased as NO_3^- concentration increased in samples containing NO_3^-. The highest value (119%) was in samples containing 6 mM NO_3^-. The findings indicated that HT was using NO_3^- best as a nitrogen source, and 6 mM NO_3^- was the maximum value. In contrast, HT was least able to utilize NO_2^-, and toxicity increased as NO_2^- concentration increased. Consequently, biomass decreased (20.8%). The highest biodiesel yield (118.8%) was produced from samples containing 1 mM NO_3^- because the lipid content was high. The cells likely used lipid metabolism in response to stress. The lowest biodiesel (68.8%) was produced from samples containing 6 mM NO_2^- because the amount of biomass and lipids was minimal at this concentration. HT could not use NO_2^- efficiently, and the conversion of metabolic products was limited by its toxic effect. The highest bioethanol (112%) was produced from samples containing 6 mM NO_3^-. This was because HT used NO_3^- most efficiently at this concentration and had a high biomass as well as high carbohydrate content. It likely helped induce these by reducing lipids and keeping stress low. The lowest bioethanol (64%) was produced from samples with a 6 mM NO_2 concentration. NO_2^- could not be utilized by HT and had a toxic effect, resulting in low biomass and carbohydrate production, which ultimately led to the low bioethanol yield. There are various publications in the literature on how to produce biodiesel, bioethanol, and biobutanol by changing the ambient parameters of microalgae. Many of these research investigated extreme environmental situations or chemical alterations. In one of them, *Chlorella vulgaris* and *Scenedesmus dimorphus* were cultivated with varied nitrogen sources, resulting in varying biomasses. The maximum biomass was achieved with a 3.5 g/L mixture of nitrate, ammonium, and urea [35]. Another study found that *Chlorella vulgaris* FACHB-1068 microalgae increased lipid content despite 21.6 mg/L nitrogen limitation and starvation conditions, although biomass output dropped. Fatty acids containing 16, 18, and 20 carbons constituted approximately 80% of the total fatty acid composition. This form of algae could serve as a precursor for biodiesel manufacturing [22]. In another study, UV mutagenesis was used on *Micractinium sp*. microalgae. Samples with the highest lipid, carbohydrate, and biomass content were selected, and biodiesel and biobutanol conversion percentages were determined by exposing them to various concentrations of abscisic and salicylic acid. The highest biobutanol yield of 177% was found in 0.2 mg/L abscisic acid samples [23]. These studies indicate that changes in environmental conditions and microalgae species can alter the metabolic products of microalgae, thereby assisting the industry in obtaining the necessary products through manipulation.

This study investigated how different nitrogen sources and concentrations affect biomass, biodiesel, and bioethanol contents of *Hindakia tetrachotoma* samples grown in wastewater and determined their relationship with antioxidant enzyme activity. This study will be continued in larger reactor environments within the context of biorefinery.

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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