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Optimization of Bioethanol Production Using an Enzymatic Hydrolysis Process with Green Algae (*Chaetomorpha*) as the Raw Material

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Abstract

Bioethanol is an alternative fuel derived from biological feedstock used to decrease the reliance on fossil fuels because of increasing energy consumption associated with population growth and increased use of oil fuels. Bioethanol production has been widely conducted using several types of algae, but the optimal conditions for the hydrolysis and fermentation processes are not explained in more detail. Therefore, this study focuses on determining the optimal conditions for hydrolysis and fermentation to maximize the bioethanol yield. This study uses optimization based on the hydrolysis time, temperature, and pH to increase the reducing sugar content using high-performance liquid chromatography in the enzymatic hydrolysis process. The process consists of liquefaction and saccharification steps, where 4% α-amylase enzyme and 2%, 3%, and 4% glucoamylase are used. Results showed that the optimal conditions for the hydrolysis time were 180 min at temperatures of 70 °C to 80 °C. The enzymatic hydrolysis process is conducted under optimal conditions, followed by the fermentation process. Finally, the distillation process was performed with a maximum bioethanol yield of 25.0%.

Keywords: bioethanol, distillation, enzymatic hydrolysis, liquefaction, optimization

1. Introduction

According to the Central Statistics Agency, Indonesia has a population of 278.69 million as of 2023. According to Muzayanah *et al*. [1], increasing economic growth and population have led to higher energy demand, particularly in the form of petroleum-based fuels. Holechek *et al*. [2] stated that approximately 80% of the world's energy consumption comes from petroleum-based fuels.

Bioethanol is one of the biofuels [3] considered a renewable energy source because it is derived from biological materials, such as green algae, in the form of carbohydrates, starch, and cellulose with the help of microorganisms. Bioethanol can be used as a clean and renewable energy source, helping mitigate environmental concerns associated with traditional fuels. Bioethanol does not cause the greenhouse effect compared with petroleum/natural gas [4]. Bioethanol, as a biofuel, can play a significant role in reducing the carbon footprint and supporting a more sustainable and circular economy, giving it the advantage of reducing carbon dioxide emissions by up to 18%. Bioethanol production contributes to energy security by diversifying energy sources and provides an alternative to traditional fossil fuels, reducing the reliance on finite and geopolitically sensitive resources.

According to Sui *et al*. [5], Indonesia is an archipelagic country with approximately two thirds of its territory covered by sea, amounting to 1.2 million ha of macroalgae ecosystem. Indonesia's status as the world's largest archipelagic nation plays a crucial role because it accounts for 6.42% of the world's seaweed resources, comprising 8,642 subspecies of seaweed from 555 species. The waters of Indonesia have approximately 6.42% of macroalgae germplasm resources. In 2017, a comprehensive survey documented 903 species of macroalgae from 268 genera, including 201 species of green algae (*Chlorophyta*), 138 species of brown algae (*Ochrophyta*), and 564 species of red algae (*Rhodophyta*) [6]. One noteworthy genus within green algae is *Chaetomorpha*, which is known for its starch content, ranging between 35% and 40%, surpassing many other algae types [7]. Green algae, such as *Chaetomorpha*, represent a potentially abundant and sustainable feedstock for bioethanol production. The use of green algae for bioethanol production can be environmentally sustainable.

Algae can grow in diverse environments and do not compete with food crops for land, addressing concerns related to food versus fuel competition. Certain types of green algae can be cultivated using wastewater or other nutrient-rich sources. Despite its potential, this particular green alga is sometimes regarded as a nuisance by fishermen [8].

As outlined by Aparicio *et al*. [9], bioethanol production from macroalgae encompasses diverse macroalgae types. In a study conducted by Yahmed *et al*. [10] employing *Chaetomorpha* algae, bioethanol production resulted in an impressive yield of 6.9% from the algae sample. This research underscores the adaptability of bioethanol production from macroalgae, with the choice of algae species and pretreatment methods significantly influencing the final output.

Ramachandra and Hebbale [11] have also contributed to the literature on the role of macroalgae in bioethanol production, with a focus on algae varieties commonly found in Indian waters. However, their work provides limited elaboration on the pretreatment methods specific to *Chaetomorpha* media. In their research, they observed that *Chaetomorpha* media yielded 27.79 g/L of reducing sugar, with a bioethanol yield of 0.057 g/g, constituting a 10.15% yield in relative terms. Notably, the bioethanol content derived from *Chaetomorpha* could be further optimized, particularly within the hydrolysis phase.

However, the specific conditions that yield the highest sugar conversion for *Chaetomorpha* may not be well-documented. Optimization ensures that the process achieves the highest conversion of carbohydrates. Research on optimizing its production contributes to the development of sustainable alternatives to fossil fuels. By optimizing the bioethanol production process, this study addresses the global need to reduce the dependence on fossil fuels. By identifying the optimal conditions (i.e., enzyme hydrolysis time, temperature, and pH), the process can be designed to achieve bioethanol with minimal *Chaetomorpha* consumption. Understanding how to optimize the bioethanol content from green algae contributes to the diversification of feedstock sources.

The study of bioethanol optimization may contribute to the utilization of algae cultivation as a means of treating wastewater while producing valuable biofuel. Optimization studies aim to improve the efficiency of bioethanol production, which includes maximizing the yield of bioethanol from *Chaetomorpha* and minimizing the consumption of resources, making the production process more economically viable. This study has implications for the bioeconomy by contributing to the development of bio-based industries. Research on optimizing the enzymatic hydrolysis process for green algae bioethanol production may lead to technological advancements, which could include innovative process configurations. This study contributes to the scientific knowledge base by providing insights into the factors influencing bioethanol production from green algae. Thus, this study aims to enhance the bioethanol content using an enzymatic hydrolysis process while employing *Chaetomorpha* as the material.

2. Method

Materials. Green algae (*Chaetomorpha*) were from the coastal area of Lhok Bubon, Aceh Barat, and were identified in the Laboratory of Fisheries, Teuku Umar University, as *Chaetomorpha crassa*. The other materials used in this study included α-amylase enzymes (Boli, food grade), glucoamylase enzymes (Sunson, GA-01L), HCl (Merck, 37%), H_2SO_4 (Merck, 98%), baker's yeast (Fermipan), NPK Mutiara 16–16–16 fertilizers, and urea fertilizers (Nitrea, 46% N). The equipment used in this study included a high-performance liquid chromatograph, distillation apparatus, and autoclave.

Procedure. This research involved three stages, i.e., preparation of tools and materials, sterilization/ liquefaction, and saccharification. Bioethanol was obtained through fermentation and distillation. This study used five variables, i.e., concentration of the glucoamylase enzyme, saccharification time, temperature, and pH. Fermentation time (i.e., 7, 9, 11, and 13 days) was used to determine the conversion of reducing sugars into bioethanol. All variables were tested once because of the limited materials available.

Preparation of the materials. In the preparation stage, green algae (*Chaetomorpha*) were washed thoroughly to remove dirt. Clean green algae were dried using an oven at 105 °C for 24 h to obtain dried algae. Furthermore, the dried algae were crushed and mashed using a blender to obtain algae powder. The algae powder was first sieved using a 30-mesh sieve and then used in the enzymatic hydrolysis stage.

Sterilization/liquefaction. In the enzymatic hydrolysis stage, 300 g of green algae was placed in a 1,000-mL glass beaker, dissolved in distilled water, and stirred with the addition of one drop of 1 N HCl solution to adjust the pH to 5.5. Green algae liquefaction was performed at pH 5.5 and a temperature of 75 °C using the standard α-amylase enzyme from Jiangsu Boli Bioproducts Co., Ltd., with an activity of 40 U/mg for 90 min. Liquefaction of the hydrolyzed sample volume was performed using 4% α-amylase enzyme.

Saccharification. Saccharification was performed after liquefaction. The sample was first cooled to temperatures of 50 °C to 60 °C and then stirred with the addition of 2 N H_2SO_4 solution to adjust the pH to 2.5. Subsequently, glucoamylase enzyme (with an activity of 150 U/mg), was added at 2%, 3%, and 4% *v*/*v* at 60 °C. The investigation

of multiple concentrations helped establish a dose–response relationship. The hydrolysis efficiency was observed to change as the enzyme dosage increased, providing insights into the optimal enzyme concentration for maximizing sugar conversion.

The saccharification process involved agitation at 130 shakes/min in a water bath for 60, 120, 180, and 240 min. The sugar produced was determined using the Nelson–Somogyi method and high-performance liquid chromatography (HPLC). Then, the identified optimal time was used as a reference for conducting further research with temperature variations of 70 °C, 80 °C, and 90 °C. The same procedure was performed at optimal times and various temperatures at pH values of 3.5, 4.5, and 5.5. The temperature and pH conditions that maximize the efficiency of the enzymatic hydrolysis process were identified. The optimal concentrations of the glucoamylase enzyme were used as a reference for the acquisition of total reducing sugar during the fermentation process.

Fermentation. The fermentation step involved cooling the mixture to approximately 37 °C. Then, urea (0.7%) and NPK fertilizer (0.7% of the total fermented sample) were added as additional nutrients for yeast growth. The bacterial starter *Saccharomyces cerevisiae* in the form of baker's yeast (0.5%) was mixed with each sample liquid (slurry) and placed in a closed container within the optimum temperature range of 27 °C to 32 °C. Subsequently, *Chaetomorpha* mixed with yeast, urea, and NPK was fermented for 7, 9, 11, and 13 days (anaerobic fermentation). The results of fermentation were liquid containing alcohol/ethanol (beer), which was centrifuged to separate the residue from the filtrate. The filtrate was obtained using filter paper. Then, the filtrate from the centrifuge was distilled through simple distillation at 76 °C to 82 °C for 4 h.

Distillation. The distillation process was conducted by boiling a mixture of ethanol and water. Ethanol had a lower boiling point (78 °C) than water (100 °C); thus, ethanol would evaporate faster than water. The percentage of bioethanol was determined using HPLC. Then, the ethanol content in each sample was analyzed using HPLC with an RID10A refractive index detector (Shimadzu) and a SIL20A autosampler. The column used was a YMC-Triart C8 measuring 150 mm × 4.6 mm, with a particle size of 5 µm. The mobile phases used were water and methanol (at a ratio of 70%/30% *v*/*v*), which were filtered using a cellulose nitrate membrane with a pore size of 0.45 µm and polypropylene with a pore size of 0.5 µm. The analysis time for each sample was 10 min with an oven column temperature of 40 °C, a flow rate of 1 mL/min, and an injection volume of 10 µL. Furthermore, the data analysis results were processed using Lab Software Solutions. The same procedure was performed for different variables. Parameter analysis was performed to test the total reducing sugar in the samples and the bioethanol obtained after distillation.

3. Results and Discussion

Effect of enzyme hydrolysis time on the total reducing sugar content. The enzymatic hydrolysis process consists of two stages, i.e., liquefaction and saccharification. According to Laga *et al*. [12], liquefaction is the stage wherein the α-amylase enzyme is administered to produce maltodextrin. Meanwhile, saccharification is the hydrolysis step of the maltodextrin compound into monosaccharides (glucose) with the help of the glucosidase enzyme under specified conditions.

Figure 1 shows the effect of different hydrolysis times (i.e., 60, 120, 180, and 240 min) with variations in the glucoamylase enzyme concentrations (i.e., 2%, 3%, and 4%) on the sample volume. In Figure 1, the hydrolysis time influences the extent of substrate conversion and, consequently, the amount of reducing sugar produced. Typically, as the hydrolysis time increases, more substrate is converted into reducing sugars. However, this relationship is nonlinear, and the optimal hydrolysis time beyond which the increase in the production of reducing sugar levels off or even decreases because of factors, such as enzyme denaturation, is identified. Some enzymes may become less stable over time, leading to decreased activity or denaturation at the hydrolysis time of 240 min, during which the total reducing sugar produced begins to decrease [13]. This decrease in the total reducing sugar can influence the effectiveness of hydrolysis over extended periods. The optimal hydrolysis time for this specific enzymatic reaction can be identified at which the maximum yield of reducing sugars is achieved. The highest total reducing sugar content of 1.2% to 2.2% for each enzyme was obtained during hydrolysis for 180 min. The data were obtained without any replication.

The effect of enzyme hydrolysis time on the total reducing sugar content is a key aspect of the optimization of the enzymatic hydrolysis process. Enzymatic hydrolysis is the step wherein complex carbohydrates, such as starch or cellulose, are broken down into simpler sugars (including reducing sugars) by enzymes. The hydrolysis time is a critical parameter because it influences the extent to which the substrate is converted into reducing sugars. A short hydrolysis time results in incomplete conversion, whereas a long hydrolysis time leads to diminishing returns.

Effect of hydrolysis temperature and pH on the reducing sugar content. Temperature optimization was performed to identify the temperature at which the enzyme exhibits the highest activity for a given substrate involving the hydrolysis reaction at different temperatures and the measurement of the resulting reducing sugar.

Based on the optimal time, the optimal temperature is determined from the highest increase in total reducing sugar.

Enzymes have an optimal temperature range for activity, and deviations from this range can affect their efficiency. Figure 2 shows that the temperature of the reaction can affect the enzyme activity. Figure 2 also shows the effect of different hydrolysis temperatures (i.e., 60 °C, 70 °C, 80 °C, and 90 °C) on the hydrolysis time of 180 min based on the first analysis. With the variation of the glucoamylase enzyme content (i.e., 2%, 3%, and 4%) in the sample volume, the highest total reducing sugar was obtained at a hydrolysis temperature of 80 °C. This enzyme has an optimal temperature range at which it exhibits maximum activity. This range is 70 °C to 80 °C, with 80 °C as the maximum temperature limit. The temperature that yields the maximum production of reducing sugar without causing enzyme denaturation is considered the optimal temperature for the enzymatic hydrolysis process. As the temperature increases within the optimal range, the catalytic activity of the enzyme and the rate of substrate conversion generally increase, leading to a higher production of reducing sugars. The optimal temperature is the range wherein the enzyme is most efficient in facilitating the hydrolysis reaction.

The peak value of the total reducing sugar is 1.3% to 3.0% Brix at 80 °C for each glucoamylase enzyme, which indicates that, for the glucoamylase enzyme used, hydrolysis or enzymatic activity is most efficient at this temperature. At this optimal temperature, the enzyme exhibits the highest catalytic activity, resulting in the maximum conversion of starch into reducing sugars. The range of 1.3% to 3% Brix represents the concentration of the total reducing sugars in the solution after the enzymatic hydrolysis process. Brix is a scale commonly used to measure the concentration of sugar in a solution. A higher Brix value indicates a higher concentration of the dissolved solid. In this context, the reported range indicates that, at the optimal temperature of 80 °C, the glucoamylase enzyme effectively converts a certain amount of substrate into reducing sugars, which corresponds to a total sugar concentration in the range of 1.3% to 3% Brix. Different enzymes have different optimal temperature ranges and substrate specificities. Glucoamylase hydrolyzes the α-1,4-glycosidic linkages at the nonreducing ends of starch, releasing glucose. Glucoamylase for glucose production is relevant in starch hydrolysis for bioethanol production.

This result is less than those reported in previous studies of acid hydrolysis, i.e., total reducing sugar of 50 mg/g from *Ulva intestinalis* [14] and 73 mg/g from *Amphiroa fragilissima* [15]. In addition, a high process temperature can reduce the quality and quantity of the resulting reducing sugar because of the production of by-products instead of sugars [16]. This phenomenon can be observed at process temperatures of 80 °C to 90 °C where the total reducing sugar produced begins to decrease. Beyond the optimal temperature range, enzyme activity starts to decline because of denaturation. Denaturation involves the disruption of the three-dimensional structure of the enzyme, rendering it inactive. The loss of enzyme activity at high temperatures can result in a decrease in the production of reducing sugar.

Effect of solution pH on the total reducing sugar content. Enzymes involved in carbohydrate hydrolysis typically have an optimal pH range within which they function most efficiently. Another variable that is reviewed optimally on total reducing sugar content is shown in Figure 3, wherein the optimal pH value is 3.5. This finding is in contrast to that reported by Abdulsattar *et al*. [17], who stated that the optimal pH for saccharification as the final step of enzymatic hydrolysis ranges from 5.8 to 6.0. This difference can be caused by several factors, including differences in the raw materials used. In this study, green algae were used, whereas, in the research conducted by Abdulsattar *et al*., wheat straw was used as the raw material. Their results showed that the total reducing sugar was 10.8 g/L for Cellic® CTec2 enzyme.

Figure 3 also shows that the total reducing sugar increases with the addition of glucoamylase enzyme concentrations of 2%, 3%, and 4% in the hydrolysis process. Under acidic conditions (pH 2.5), this enzyme exhibits reduced activity or denaturation. Acidic pH values can interfere with the ionization state of amino acids and residues in the active site of enzymes, affecting their capability to catalyze reactions. The total reducing sugars obtained were 2%, 3%, and 5% Brix. In this case, the total reducing sugar obtained can be increased again with the addition of a higher enzyme concentration. However, because of the limited materials available, the enzymes used are limited.

One limitation of enzymatic hydrolysis is the low concentration of sugars in the hydrolysate [18]. Thus, the effect of solution pH on the total reducing sugar content is a crucial aspect of the optimization of the enzymatic hydrolysis process. Enzymes responsible for hydrolyzing complex carbohydrates into reducing sugars have specific pH ranges at which they are most active and efficient. The pH of the solution can affect the ionization state of amino acid residues in the active site of the enzyme, affecting its catalytic activity. Enzymes involved in hydrolysis reactions exhibit different levels of activity at different pH values. Enzymes have an optimal pH range where their activity is maximized, and deviations from this range can lead to reduced activity.

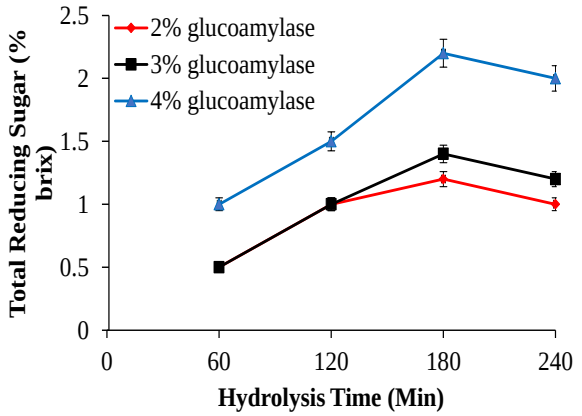

Figure 1. Effect of Enzyme Hydrolysis Time on the Total Reducing Sugar Content at 60 °C

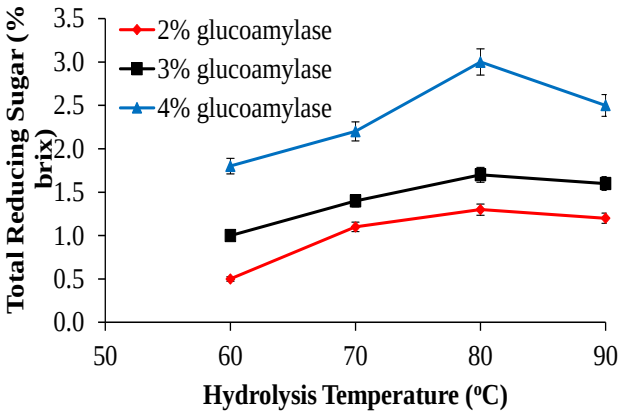

Figure 2. Effect of Enzyme Hydrolysis Temperature on the Total Reducing Sugar at 180 Min

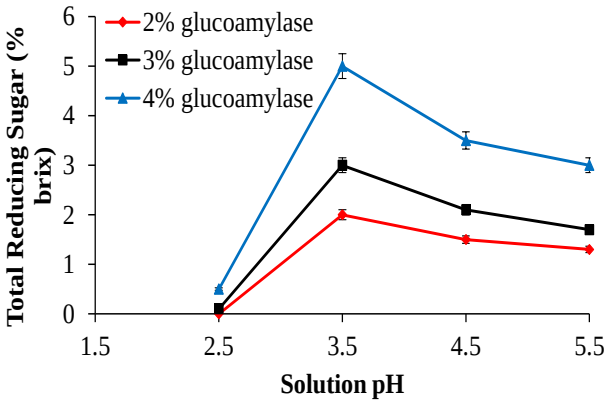

Figure 3. Effect of the pH of the Solution on the Total Reducing Sugar at 180 Min and 80 °C

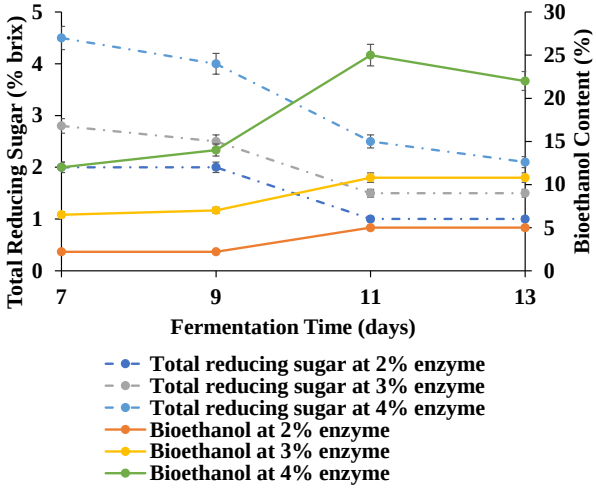

Figure 4. Effect of Fermentation Time on the Total Reducing Sugar and Bioethanol Contents

Effect of fermentation time on the total reducing sugar and bioethanol contents. After *Chaetomorpha* is hydrolyzed, the next step is fermentation. Fermentation is a process that converts larger organic compounds into simpler organic compounds in the form of total reducing sugars in the sample [19] and into bioethanol with the help of microorganisms in the form of yeast anaerobically. The fermentation process was performed anaerobically by incubating the samples in an incubator for 13 days. At the beginning of fermentation, a higher concentration of sugars is typically available for conversion. As fermentation progresses, microorganisms consume these sugars to produce bioethanol.

Microorganisms, e.g., yeast, have an active phase during fermentation, where they consume sugars and produce bioethanol. The rate of sugar consumption and ethanol production is usually highest during the logarithmic or exponential growth phase of the microorganisms. The yield of bioethanol per unit of sugar consumed changes during fermentation. A time–course study was conducted to monitor the progress of fermentation, measuring parameters, such as reducing sugar content and bioethanol production, at different times. Fermentation has its optimal time; after this time is reached, a longer fermentation process can form subsequent sugar and decrease the activity of yeast cells [20].

Figure 4 shows that the optimal time during 13 days of fermentation is on the 11th day with a remaining total reducing sugar content of 1.0%, 1.5%, and 2.5% Brix. The total reducing sugar is the sugar content after completing fermentation and not before starting fermentation because the total reducing sugar content on the 11th day of fermentation experienced the most significant decrease compared with the previous fermentation time. This finding was also reported by Olawale *et al.* [21], who determined that yeast achieved optimal activity in converting sugar into bioethanol. This microorganism may be more sensitive to high bioethanol concentrations, which can inhibit further fermentation.

As shown in Figure 4, the highest bioethanol contents of 5.0%, 10.8%, and 25% were obtained at the optimal

fermentation time of 11 days. However, after passing the optimal time of the fermentation process, the amount of bioethanol produced decreased. The reduction in reducing sugar content also occurred during the 13th day of fermentation in 4% glucoamylase enzyme, i.e., 0.4% Brix. Prolonged fermentation times can lead to the accumulation of inhibitory by-products, such as acetic acid or higher alcohols, which may negatively affect the fermentation process. The optimization of fermentation time involves finding a balance between maximizing bioethanol production and minimizing the negative effects of by-product accumulation or inhibition.

Even though the 4% enzyme concentration yielded the best results during the optimization processes, for bioethanol production, three enzyme concentrations were used because findings from experiments with different concentrations are more applicable to industrial-scale processes. This information is valuable for scaling up bioethanol production from laboratory-scale experiments to larger, practical applications.

In general, the relationship between fermentation time, total reducing sugar consumption, and bioethanol production is complex. The final concentration of bioethanol is influenced by both the initial substrate concentration and the fermentation time. Therefore, illustrating the effects of fermentation time, total reducing sugar, and bioethanol content in a single graph is appropriate and often beneficial. Combining multiple variables in a single graph enables a comprehensive visual representation of the relationships and trends within the data.

The highest value was detected in the fermentation time variation of 11 days with a pH value of 3.5 and a bioethanol content of 25%. The bioethanol content obtained in this study was higher than that obtained by Mellicha *et al*. [22], i.e., 21.64 ± 3.03 g/L ethanol. In the research, the fermentation process that occurs takes a shorter time (i.e., 5 days with a pH value of 4.7 ± 0.14). The effect of fermentation time on the total reducing sugar and bioethanol contents is a key aspect of the optimization of the fermentation process. Fermentation is the stage where microorganisms, typically yeast, convert sugars into bioethanol. The duration of fermentation influences the extent of sugar consumption and bioethanol production.

Bioethanol production is influenced by various factors, including the type of yeast used, the fermentation conditions, and the initial sugar content (Brix) of the fermentation medium. Although achieving a 25% bioethanol concentration with *S. cerevisiae* yeast is theoretically possible, the genetic modifications of yeast need to be considered. This research involves commercial yeasts that are genetically modified strains with enhanced ethanol tolerance. To achieve high ethanol concentrations, a distillation technique is typically required to concentrate the ethanol after fermentation.

4. Conclusion

Based on previous research, it can be concluded that the optimal conditions identified for the enzymatic hydrolysis of green algae were an enzyme concentration of 4% glucoamylase enzymes, a hydrolysis time of 180 min, a temperature of 80 °C, and a pH value of 3.5. The optimized conditions result in a total reducing sugar content of 5% Brix. The optimized enzymatic hydrolysis process increases the bioethanol content to 25% on the 11th day of fermentation with 4% enzyme. This increased bioethanol yield results from the efficient conversion of green algae biomass. This result is higher than that obtained by Mellicha *et al*. [22] with 5% enzyme and 27 h fermentation time, i.e., 8.16%. Optimization has economic implications, including potential cost savings, resource efficiency, and economic feasibility of large-scale bioethanol production.

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