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*Research Article*

# Comparative Study on the Various Hydrolysis and Fermentation Methods of *Chlorella vulgaris* Biomass for the Production of Bioethanol

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**Abstract**. One of the microalgae that can be potentially used to produce bioethanol is *Chlorella vulgaris*, as it is rich in carbohydrates. However, the carbohydrates in *C. vulgaris* cannot be converted directly into ethanol. This study aimed to investigate the chemical and enzymatic hydrolysis of *C. vulgaris*, which is subsequently followed by fermentation. The catalysts used in the chemical hydrolysis were hydrochloric acid, sodium hydroxide, and potassium hydroxide, while the enzymes used were the mixture of alpha-amylase + glucoamylase, alpha-amylase + cellulase, and alpha-amylase + glucoamylase + cellulase. The hydrolysate obtained from chemical hydrolysis was fermented through Separate Hydrolysis Fermentation (SHF), while the one from enzymatic hydrolysis was fermented through Simultaneous Saccharification and Fermentation (SSF), in which both processes used *S. cerevisiae*. After undergoing five hours of enzymatic hydrolysis (using alpha-amylase + glucoamylase), the maximum glucose concentration obtained was 9.24 ± 0.240 g/L or yield of 81.39%. At the same time and conditions of the substrate on chemical hydrolysis, glucose concentration was obtained up to 9.23 ± 0.218 g/L with a yield of 73.39% using 1 M hydrochloric acid. These results indicate that chemical hydrolysis is less effective compared to enzymatic hydrolysis. Furthermore, after 48 hours of fermentation, the ethanol produced from SHF and SSF fermentation methods were 4.42 and 4.67 g/L, respectively, implying that producing bioethanol using the SSF is more effective than the SHF method.

***Keywords:*** Bioethanol, *C. vulgaris*, *S. cerevisiae*, Separate Hydrolysis Fermentation, Simultaneous Saccharification and Fermentation

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## 1. Introduction

The fossil fuel crisis is one of the indications that the world's energy reserves are continuously depleting. In addition, fossil fuels release large quantities of carbon dioxide when burned, which is the primary drive to global warming (Yanto *et al*., 2019). Therefore, the development of biofuels from renewable materials is necessitate. Bioethanol is a sustainable alternative energy that can substitute fossil fuels, and its production is divided into several generations characterized by biomass sources. According to Kumar *et al*. (2020), the first-generation bioethanol was derived from edible biomass, the second was from agricultural waste or lignocellulosic feedstocks, and the third was from micro and macroalgae. The use of microalgae as raw materials for bioethanol production has a considerable advantage due to their ability to grow five to ten times faster than terrestrial plants, easy to cultivate, and they do not require arable land (Zullaikah *et al*., 2019). Additionally, microalgae have the ability to utilize agricultural waste as a nutrient which can reduce adverse environmental impacts (Velazquez-Lucio *et al*., 2018). On top of that, microalgae are photosynthetic microorganisms whose photosynthetic efficiency is 10-15 times greater than terrestrial plants and can produce biomass as a food reserve that can be used for bioethanol production (Jayaseelan *et al*., 2021).

Theoretically, microalgae grow rapidly and contain a high carbohydrate composition consisting of cellulose and starch (Megawati *et al*., 2022). This feature makes the microalgae easier to be hydrolyzed into monosaccharides which are then fermented anaerobically by *Saccharomyces cerevisiae* (Selim *et al*., 2018). *C. vulgaris* is one of the microalgae that has a fairly large carbohydrate accumulation, i.e., 20.99% dry weight basis. The carbohydrates in microalgae cannot be fermented directly into ethanol because the yeast *S. cerevisiae* is only able to convert monosaccharides into ethanol. Instead, they have to be firstly hydrolyzed either by using a chemical or enzymatic catalyst into monosaccharide (Vasić *et al*., 2021). Hydrolysis provides a significant contribution to biomass microalgae conversion into ethanol (Seon *et al*., 2020). This process also has a great potential to enhance glucose conversion (Sabiha-Hanim & Halim, 2018). From

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the study by Offei *et al*. (2018), it is known that several studies on bioethanol production using microalgae with both acid and alkaline-catalyzed hydrolysis have been previously conducted. The hydrolysis using acid catalysts at high temperatures has been shown to be faster than alkaline catalysts (Kundu *et al*., 2021). As denounced in the available literature, sulfuric acid is capable of hydrolyzing carbohydrates in *Gracilaria birdiae* into 28.56 g/L of glucose (Albuquerque *et al*., 2021), and hydrolysis with a sulfuric acid catalyst in *Scenedesmus sp.* reached up to 1.113 g/L (Agustini *et al*., 2019). On the other hand, enzymatic hydrolysis using alpha-amylase, cellulase, and glucoamylase has been shown to be effective in breaking down carbohydrates into monosaccharides and can be conducted at a relatively low temperature, although it is fairly expensive in the production cost (Wang *et al*., 2020). Research carried out by Shokrkar *et al*. (2017) documented that enzymatic hydrolysis on microalgae could produce up to 57% of glucose.

Glucose obtained from hydrolysis can be converted into ethanol through anaerobic fermentation using *S. cerevisiae*. Numerous studies on ethanol processing methods with polysaccharides substrates have been conducted, such as Separate Hydrolysis Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) methods. In the SHF, the chemically catalyzed hydrolysis of polysaccharides is conducted separately from the fermentation (Damayanti *et al*., 2021). Meanwhile, the SSF is an advanced bioethanol production where the enzymatic hydrolysis and fermentation are carried out at the same time and in one reactor (Angela *et al*., 2020; Aryanti *et al* 2013)). The ethanol concentration obtained from the SSF was reported to be the highest. A foregoing study carried out by Dahnum *et al*. (2015) reported that the SSF produced 6.05% of ethanol concentration with a fermentation time of 24 hours, while the SHF was only 4.74% for 72 hours. This phenomenon occurred because, in the SSF, sugar does not inhibit enzymes, so the sugar can be fermented directly into ethanol (Bader *et al*., 2020).

Studies emphasize the comparison between chemical and enzymatic hydrolysis, and SHF and SSF are still rare. Thus, this study aimed to investigate the chemical and enzymatic hydrolysis of *C. vulgaris*. Then, the hydrolysates obtained from the chemical hydrolysis were fermented using *S. cerevisiae* through the SHF, while the one obtained from the enzymatic hydrolysis was fermented through the SSF. The bioethanol yield and residual glucose from both SHF and SSF were compared. This study was expected to help to discover an effective way for bioethanol production using *C. vulgaris* as raw material and can be used as an initial stage for sustainable energy.

## 2. Materials and Methods

### *2.1 Microorganism*

Microalgae used in this study was green microalgae with the species Latin name of *C. vulgaris,* and it was obtained from Ugo Plankton Algae (Purworejo, Central Java, Indonesia). The dry powder of *C. vulgaris* was mashed and filtered to get granule size, and subsequently, it was kept in a sealed container box at 4°C. The instant dry yeast of *S. cerevisiae* was purchased from Lalvin 71B (France's National Agricultural Research Institute, France).

### *2.2 Chemical hydrolysis*

In the chemical hydrolysis (acid and alkaline), 20-100 g/L of *C. vulgaris* was hydrolyzed with some chemical catalysts (1, 2, 3 M of hydrochloric acid, 1, 2 M of sodium hydroxide, and 1, 2 M of potassium hydroxide) at the hydrolysis temperature of 90°C for 20, 40, and 60 minutes. All chemical catalysts were purchased from Merck (Darmstadt, Germany). Then, the samples were cooled in a water bath until they reached room temperature, and the suspensions were centrifuged (Thermo Fisher Scientific, Waltham, MA, the USA) at 3000 rpm for five minutes. Lastly, the glucose yields in the supernatants were analyzed, and the highest one was used in the SHF.

### *2.3 Enzymatic hydrolysis*

In the current study, the enzymes used were alpha-amylase (Liquozyme® Supra 2.2X, donated by Novozyme, Denmark) from *Bacillus licheniformis*, with enzyme activity of 300 KNU/g, cellulase (Viscozyme® Cassava CL donated by Novozyme, Denmark) from *Aspergillus aculeatus* with enzyme activity of 100 FBG/g, and glucoamylase (Dextrozyme® GA donated by Novozyme, Denmark) from *Aspergillus niger* with enzyme activity of 270 AGU/g.

In this process, 60 g/L dried samples of *C. vulgaris* were hydrolyzed with 0.9 mL alpha-amylase using a 250 mL volumetric flask with citrate buffer of pH 6.0 and in the enzyme active temperature (95$^{o}$C). After the alpha-amylase was added, the broth was cooled in a water bath until the optimum temperature of glucoamylase and cellulase was reached (80$^{o}$C). Subsequently, 2.7 mL of glucoamylase or 2.7 mL of cellulase was added, depending on the variation of the enzyme combination. The variations of the enzyme combination were alpha-amylase + glucoamylase (denoted as first combination); alpha-amylase + cellulase (denoted as second combination); and alpha-amylase + glucoamylase + cellulase (denoted as third combination). The hydrolysis was then performed at a speed of 400 rpm utilizing a magnetic stirrer (DLAB Scientific, MS-H280-Pro, China) for five hours. The samples were drawn at 30 minutes intervals to be analyzed. The enzyme combination with the highest glucose yields in this stage was used in the SSF.

### *2.4 Microorganism cultivation*

*S. cerevisiae* was activated through pre-inoculation. The inoculation media contained distilled water, 1% of yeast extract (Microgen, India), 2% of peptone (Oxoid, the USA), and 2% of glucose (Merck, Darmstadt, Germany). The media was sterilized using an autoclave at 121°C for 30 minutes which was then cooled in a water bath until room temperature was reached. The pre-inoculant was prepared in a 500 mL volumetric flask with 250 mL volume of fermentation medium with 0.5 g instant dry yeast of *S. cerevisiae*. The broth was incubated aerobically for 24 hours on the orbital shaker at 100 rpm and 30$^{o}$C. After the incubation, the activated *S. cerevisiae* was stored at 4°C for further ethanol fermentation.

*2.5. Fermentation*

*2.5.1. Separate Hydrolysis and Fermentation (SHF)*

In a 500 mL volumetric flask with 250 mL of distilled water, the *C. vulgaris* hydrolysate with the highest glucose yield from the chemical hydrolysis was added with nutrients (5 g/L of ammonium sulfate (Merck, Darmstadt, Germany); 6 g/L of magnesium sulfate (Merck, Darmstadt, Germany); 2.5 g/L of yeast extract (Microgen, India)). Acidity was controlled by using citric acid until reaching a pH value of 5.0. The medium was sterilized using an autoclave at 121°C for 30 minutes. The fermentation medium was cooled in a water bath until room temperature was reached. Then, 3% (v/v) of *S. cerevisiae* inoculum was added and incubated for 48 hours using an orbital shaker at 100 rpm and 30 °C. The anaerobic condition was employed for the fermentation. Samples were withdrawn every four hours for residual glucose and ethanol concentration analysis.

*2.5.2. Simultaneous Saccharification and Fermentation (SSF)*

In a 500 mL volume flask with 250 mL of distilled water, *C. vulgaris* (60 g/L) was added with nutrients (5 g/L of ammonium sulfate; 6 g/L of magnesium sulfate; and 2.5 g/L of yeast extract). The pH was controlled using citric acid until it reached pH 5.0. The media was sterilized using an autoclave at the temperature of 121°C for 30 minutes. Afterward, the media was heated to a temperature of 95°C. The enzymes combination with the highest glucose yield was added to the broth with the operating temperature conditions of each enzyme. The broth was then cooled in a water bath to the temperature of 30°C. Thereafter, 3% (v/v) of inoculated *S. cerevisiae* was added and incubated in anaerobic conditions for 48 hours. The sample was taken every 4 hours for glucose and ethanol concentration analysis.

*2.6. Analytical method*

*2.6.1. Glucose analysis*

The glucose content of *C. vulgaris* hydrolysates was determined using the Nelson-Somogyi method (Nelson, 1944). As much as 1 mL of diluted sample was put inside a test tube, and 1 mL of reagent Nelson was subsequently added. The test tubes were boiled for 20 minutes until red-colored sediment formed. The test tube was then cooled in a water bath until room temperature was reached. Afterward, 1 mL of arsenomolybdate reagent and 7 mL of distilled water were added. The solutions were analyzed using a spectrophotometer (GENESYS™ 20 Thermo Fisher Scientific, Karlsruhe, Germany) at 540 nm of wavelength.

*2.6.2. Ethanol analysis*

The ethanol concentration was analyzed using a modified spectrophotometric method according to Sriariyanun *et al.* (2019), which involved dichromic acid oxidation. Potassium dichromate (Merck, Darmstadt, Germany) was weighed at 4.262 g and mixed using a magnetic stirrer with 100 mL of distilled water. Subsequently, 50 mL of sulfuric acid (Merck, Darmstadt, Germany) was conscientiously added into the flask and continued by adding distilled water until the volume became 1000 mL. Hereafter, 10 mL of dichromic acid was gently diluted with distilled water until the volume reached 100 mL. In the inner side of the Conway plate, 5 mL of dilute dichromic acid was poured. While on the outside side of the Conway plate, it was filled with 1 mL of 20% sodium carbonate (Merck, Darmstadt, Germany) and 5 mL of ethanol or sample solution. The ethanol concentration varied from 0.5 to 6 g/L. The Conway plate was covered and heated in an oven (Memmert 55, Schwabach, Germany) at 50°C for two hours. The solution in the inner side of Conway plate was measured using a UV-Vis (spectrophotometer GENESYS™ 20 Thermo Fisher Scientific, Karlsruhe, Germany) with a maximum wavelength of 446 nm. The absorbance values were taken from each solution with varying ethanol concentrations to obtain a linear correlation.

*2.7. Calculation of glucose yield and ethanol yield*

Eq. (1) expresses the formula to calculate the glucose yield. The equation describes that the yield is the glucose hydrolyzed per total carbohydrate of *C. vulgaris* (Shokrkar *et al.*, 2017).

$$\text{Glucose yield } \% = \frac{\text{Glucose concentration hydrolysed (g/L)}}{\text{Carbohydrate concentration in } C.vulgaris \text{ (g/L)}} \times 100\% \quad (1)$$

Eq. (2) was used while the ethanol yield refers to the equation proposed by Mithra *et al.* (2018), which states that the ethanol yield is the ethanol concentration obtained per glucose concentration.

$$\text{Ethanol yield } \% = \frac{\text{Ethanol concentration (g/L)}}{\text{Glucose concentration (g/L)}} \times 100\% \quad (2)$$

*2.8. Statistical analysis*

The analysis of variance (ANOVA) was performed using the Statistical Package for the Social Sciences (SPSS)® to determine the significance of the variables in this study.

## 3. Results and Discussion

*3.1 Effect of hydrolysis time*

In the present study, the first stage of the experiment was performed by diluting hydrochloric acid (1 M) with 60 g/L *C. vulgaris* at 90°C to obtain the optimum hydrolysis time. The hydrolysis was performed for 100 minutes, and the sample was taken in 10 minutes intervals. The time effect on the hydrolysis of *C. vulgaris* is presented in Fig. 1.

The glucose concentration increased markedly ($p < 0.05$) from 10 to 60 minutes. The highest glucose concentration was 9.24 ± 0.240 g/L at 60 minutes, with a glucose yield of 73.39%. However, the glucose concentration decreased gradually after 60 minutes onward with a final glucose concentration of 5.32 ± 0.00 g/L or 42.26% at 100 minutes. This phenomenon may be caused by the product decomposition into furfural. The furfural might be produced as a degradation product of pentose, such as xylose (Jeong & Lee, 2021). The furfural concentration increased during hydrolysis. The hydrolysis time is an essential factor in hydrolysis that needs further study to obtain an optimum glucose concentration. Shorter-time hydrolysis of *C. vulgaris* positively impacted

energy consumption and prevented the product from being further degraded. This finding concurs with a preceding research executed by Saha *et al*. (2005), who observed that the time increase in dilute acid hydrolysis of carbohydrate on wheat straw did not raise the glucose yield.

### *3.2. Effect of C. vulgaris concentration*

The effect of *C. vulgaris* concentration was studied at 20 to 100 g/L using 1 M of hydrochloric acid as a catalyst. The hydrolysis was performed at 60 minutes with sample analysis at 20 minutes intervals. The effect of *C. vulgaris* concentration on glucose concentration and glucose yield is depicted in Fig. 2. *C. vulgaris* concentration from 20 to 60 g/L increased the obtained glucose concentration. However, the glucose concentration decreased gradually with the *C. vulgaris* concentration from 60 to 100 g/L at all variations of hydrolysis time. The highest glucose concentration was obtained up to 9.23 ± 0.218 g/L with an experimental yield of 73.39% at 60 g/L of *C. vulgaris* and 60 minutes. It is likely that more concentration substrate added to the hydrolysis will have an impact on carbohydrate concentration that will be hydrolyzed by catalyst into glucose.

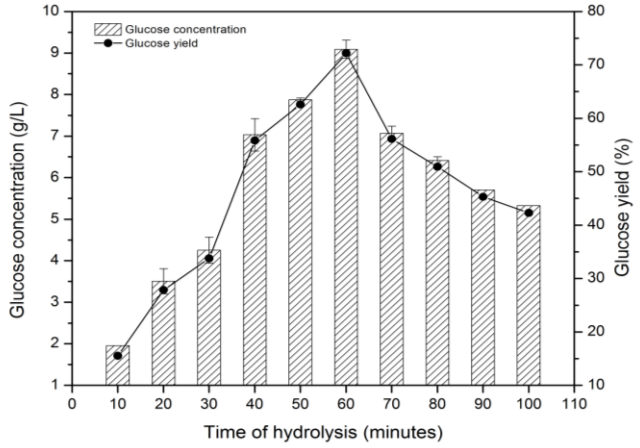


**Fig. 1** The effect of hydrolysis time on glucose concentration and glucose yield

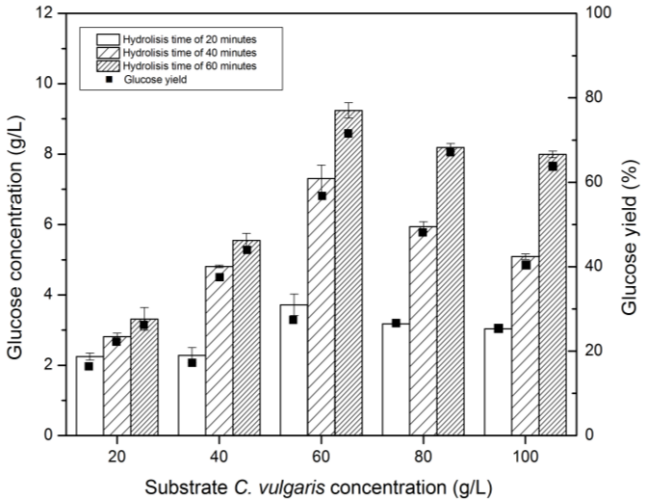


**Fig. 2** The effect of substrate *C. vulgaris* concentration on glucose concentration and glucose yield

Nevertheless, excessive disproportionate of catalysts and media addition will have an unfavorable impact on glucose yield. This phenomenon is caused by a higher substrate requiring a greater volume of catalyst and longer hydrolysis time (Mezule *et al*., 2019). A higher substrate concentration is also ineffective for acid hydrolysis of carbohydrates due to the increasing substrate viscosity, which can cause a rising number of undissolved materials (Alias *et al*., 2021). This can become the inhibitor of catalyst efficiency in hydrolysis. This finding is also in line with other studies in the hydrolysis of carbohydrates in mixed microalgae into glucose (Shokrkar *et al*., 2017).

### *3.3. Acid hydrolysis*

*C. vulgaris* comprised of several carbohydrate components, most of which are inside the cell wall *(Ru et al*., 2020). Therefore, a catalyst is needed to hydrolyze carbohydrates in the microalgae cell walls. In this stage, the effect of the acid catalyst type and its concentration on glucose concentration and yield were studied. Various concentrations of hydrochloric acid and sulfuric acid had interacted with *C. vulgaris*. The hydrolysis was conducted at 90°C and for 60 minutes as the optimum time, and the samples were drawn every 20 minutes. The effect of different acid catalysts and their concentrations is illustrated in Fig. 3.

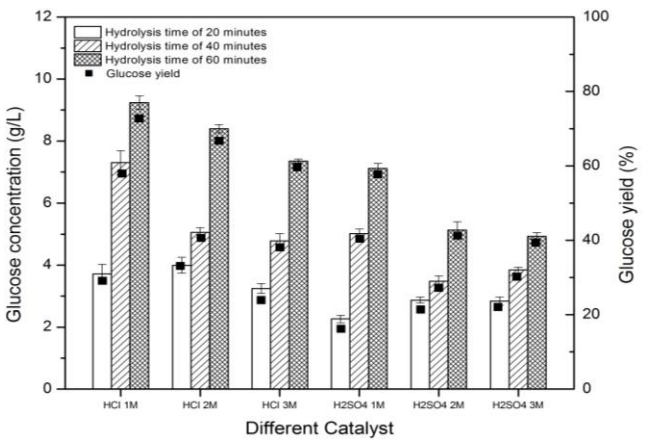


**Fig. 3** The effect of acid catalyst on glucose concentration and glucose yield

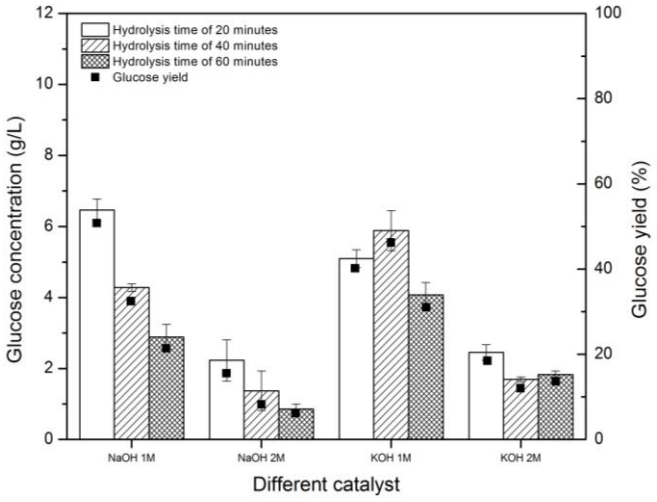


**Fig. 4** The effect of alkaline catalyst on glucose concentration and glucose yield

The highest yield of the hydrolysis obtained with 1 M of hydrochloric acid catalyst was 73.39%. The glucose yield decreased along with the increase of acid catalyst concentration. Hydrochloric acid catalyst with concentrations of 2 M and 3 M lowered the glucose yield from 66.65 to 58.40% at 60 minutes. A similar tendency was also identified at sulfuric acid catalyst with concentrations of 1, 2, and 3 M, which reduced the glucose yield from 56.54 to 39.09%. This might be caused by the glucose decomposition into furfural. However, the furfural concentration was not studied in this research as it is not the main component of fermentation. Thus, at the same concentration, the most effective catalyst for acid hydrolysis of *C. vulgaris* is hydrochloric acid. Although sulfuric acid is frequently used to hydrolyze polysaccharides, the glucose yields obtained from sulfuric acid were less than hydrochloric acid. In general, under acidic medium and high temperatures, a multiphase reaction will occur between the substrate and the medium, which leads to a reduction of the Maillard reaction. Therefore, it can increase the hydrolysis of carbohydrates into glucose (Buvé *et al*., 2021). Under acidic conditions, $H^+$ ions will encourage the interaction between amino acids and carbonyl groups which causes the polymers to decompose into monomers (Hafid *et al*., 2017). These results are also in line with the previous studies conducted by Lee *et al*. (2015), where the hydrochloric acid produced a higher yield in comparison with sulfuric acid in the hydrolysis of chlorella microalgae, and Miranda *et al*. (2012) on dilute acid hydrolysis of *Scenedesmus obliquus*.

### *3.4. Alkaline hydrolysis*

In this stage, hydrolysis using a dilute alkaline was also studied. Hydrolysis using dilute alkaline enables changes in the structure of carbohydrates of *C. vulgaris* into glucose which were then used in the fermentation. Sodium hydroxide (1 and 2 M) and potassium hydroxide (1 and 2 M) were used as alkaline catalysts in this study. The hydrolysis of 60 g/L *C. vulgaris* with an alkaline catalyst was performed for 60 minutes, and the sample was taken every 20 minutes. Hydrolysis temperatures were performed at 90°C. The effect of alkaline catalyst on glucose concentration and glucose yield is shown in Fig. 4.

Sodium hydroxide catalyst (1 M) yielded the highest glucose (51.30%) at 20 minutes. The yield kept decreasing along with the hydrolysis time to 22.91%. The increasing concentration of sodium hydroxide to 2 M also led to a reduction in glucose yield of 17.68, 10.89, and 6.81%, in which each sampling was taken every 20 minutes. This phenomenon was caused by the decomposition of glucose products. While potassium hydroxide catalyst produces lower glucose yield compared to sodium hydroxide catalyst, potassium hydroxide catalyst has a different profile on glucose yield. At 1 M and 2 M of potassium hydroxide, the highest yield of glucose reached 46.70% at 40 minutes and 19.46% at 20 minutes, respectively. However, prolonged hydrolysis time with the alkaline catalyst decreased the glucose concentration. Ellis & Wilson (2002) found that the addition of hot alkaline to glucose led to the degradation of glucose into lactic, formic, glycolic, and acetic acids. Generally, compared to the acid catalyst, an alkaline catalyst requires less time for hydrolysis and obtains less glucose yield. This phenomenon is due to the cell wall of *C. vulgaris,* which contains slight hemicellulose (Coelho *et al*., 2019). Dilute alkaline catalysts rupture the cell walls of *C. vulgaris* by cleaving the bond between hemicellulose molecules and other components (Nuhma *et al*., 2021). In addition, acquiring a high concentration of glucose concentration from alkaline hydrolysis of carbohydrates is reasonably difficult due to the $OH^-$ ion, which causes swelling and a decrease in crystallinity in the polysaccharide so that hydrolysis below 100°C become ineffective (Nawaz *et al*., 2020).

### *3.5. Enzymatic hydrolysis*

In this study, the enzyme mixtures used were alpha-amylase + glucoamylase (first combination); alpha-amylase + cellulase (second combination); and alpha-amylase + glucoamylase + cellulase (third combination). The hydrolysis temperature was adjusted to the active temperature of the enzyme. The results of enzymatic hydrolysis of carbohydrates on *C. vulgaris* biomass using three enzymes were presented in Fig. 5.

It can be seen that for all enzyme combinations, the glucose yields increase significantly during the hydrolysis. The first combination produced the greatest glucose yield of 81.39% after hydrolysis for five hours. Hydrolysis using the third combination yielded glucose of 80.19%. However, the addition of the second combination produced the lowest glucose yield, i.e., 54.24%. It can be stated that the combination of alpha-amylase and glucoamylase is the decent catalyst to hydrolyze carbohydrates from *C. vulgaris*. Compared to the other combinations, alpha-amylase and glucoamylase produced the highest glucose yield. This can be linked with the ability of alpha-amylase to degrade the surface of the granules in carbohydrates so that it forms many holes and releases glucose as a product at the optimum temperature. With the addition of glucoamylase, it can absorb carbohydrate granules to produce oligosaccharides (Xu *et al*., 2016). When compared to the chemical catalyst at the previous stage, enzyme catalyst took a longer time for hydrolysis. This phenomenon occurred due to the enzymatic hydrolysis that requires three stages of the process. The first stage is gelatinization which breaks the bonds between molecules by heat in the water. In this stage, the starch would swell because it absorbs the water by heating and starch grains form a viscous suspension.

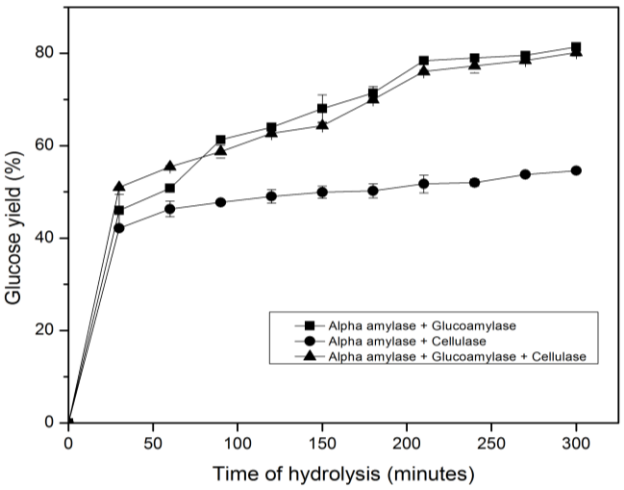


**Fig. 5 The** effect of enzymatic hydrolysis on glucose yield

The second stage is liquefaction, where the loss of viscosity and hydrolysis of starch into oligosaccharides occurs when alpha-amylase acts as a catalyst (Azmi *et al*., 2017). The third stage is saccharification using glucoamylase or cellulase to hydrolyze maltodextrin into glucose (Hossain *et al*., 2018). This phenomenon is in line with the research that hydrolyses *C. sorokiniana* biomass into glucose using enzyme catalyst (Souza *et al*., 2020).

### *3.6. Bioethanol Production*

In this stage, the SHF used the hydrolysates from the chemical hydrolysis of *C. vulgaris* using 1 M of hydrochloric acid, while the SSF used the hydrolysate from the enzymatic hydrolysis using alpha-amylase and glucoamylase. *S. cerevisiae* and nutrients work together to convert glucose in the *C. vulgaris* hydrolysate into ethanol. The fermentation was conducted for 48 hours with an incubation temperature of 30°C. The comparison of SHF and SSF processes in ethanol concentration, ethanol yield, and residual glucose is depicted in Fig. 6.

From 9.36 g/L of glucose, *S. cerevisiae* produced ethanol up to 4.42 ± 0.141 g/L at 48 hours of fermentation time using the SHF. At the end of the fermentation, the residual glucose concentration obtained was 0.52 ± 0.240 g/L. Meanwhile, the SSF produced 4.67 ± 0.098 g/L with a residual glucose concentration of 0.025 ± 0.007 g/L. The experimental yield obtained from the SHF and SSF reached 46 and 48.50%, respectively, at the end of the fermentation. The significant yield of ethanol in both processes showed the efficiency of glucose consumption by *S. cerevisiae* into ethanol. The ethanol concentration in the SHF and SSF increased significantly ($p<0.05$) in the first 24 hours of fermentation. Then, the ethanol production became slower after 24 hours. This is due to the glucose concentration depletion, or the yeast cell is already in the death phase (Kumoro *et al*., 2021).

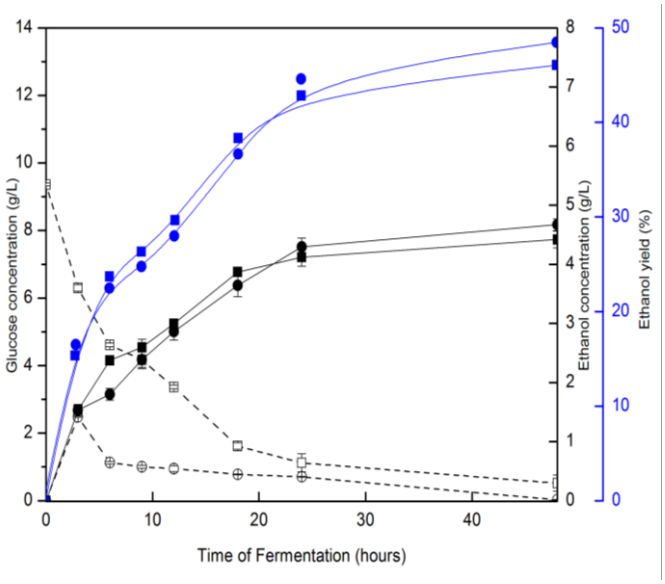


**Fig. 6** Comparison between SHF and SSF process on ethanol concentration, ethanol yield, and residual glucose concentration. (Symbols; ■: SHF ethanol concentration; ■: SHF ethanol yield; □: SHF residual glucose concentration ●: SSF ethanol concentration; ●: SSF ethanol; and ○: SSF residual glucose concentration)

In the SHF, the concentration of residual glucose lessened gradually during the fermentation, while the glucose concentration in the SSF increased in the first three hours, then continued to decrease until the end of the fermentation. Nevertheless, in the first 24 hours of the fermentation, the SHF produced more ethanol concentrations than SSF. This is due to the availability of glucose from the previous processes, which can be converted directly into ethanol (Maslova *et al*., 2019). In the first three hours of the SSF, the glucose concentration reached 2.48 ± 0.021 g/L, while the ethanol concentration obtained was 1.53 ± 0.028 g/L. This phenomenon occurred because hydrolysis and fermentation were carried out simultaneously at the same time. The glucose obtained from enzymatic hydrolysis will be immediately converted into ethanol (Liu *et al*., 2020). Compared to the whole process from the raw material of *C. vulgaris* into ethanol, the SSF was faster than the SHF. Besides, the SSF using the first combination was better due to the higher yield, lower utility, and less corrosion effect (Constantino *et al*., 2021). This phenomenon is in accordance with some previous studies performed by de Farias Silva *et al*. (2018) on hydrolysis and fermentation process using *C. vulgaris* biomass and El-Dalatony *et al*. (2016) on *C. Mexicana* biomass fermentation.

## 4. Conclusion

*C. vulgaris* has a considerable potential for bioethanol production. The present study demonstrates that the biomass concentration and hydrolysis time can remarkably affect the glucose yield. The most effective catalyst in chemical hydrolysis of *C. vulgaris* was 1 M hydrochloric acid, and the most effective in enzymatic hydrolysis is the combination of alpha-amylase and glucoamylase. The ethanol obtained from the SHF using the acid-catalyzed hydrolysis (1 M hydrochloric acid) and fermentation with *S. cerevisiae* was 92%, and the ethanol produced from the SSF using enzymatic hydrolysis (alpha-amylase + glucoamylase) and fermentation with *S. cerevisiae* was 97%. Overall, our findings suggest that the SSF is proven to be more effective and faster in bioethanol production. It is also possible to develop the integration of bioethanol production from *C. vulgaris* as alternative energy to substitute fossil energy and reduce emissions impact.

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**Conflicts of Interest**: The authors declare no conflict of interest.

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