RESEARCH ARTICLE

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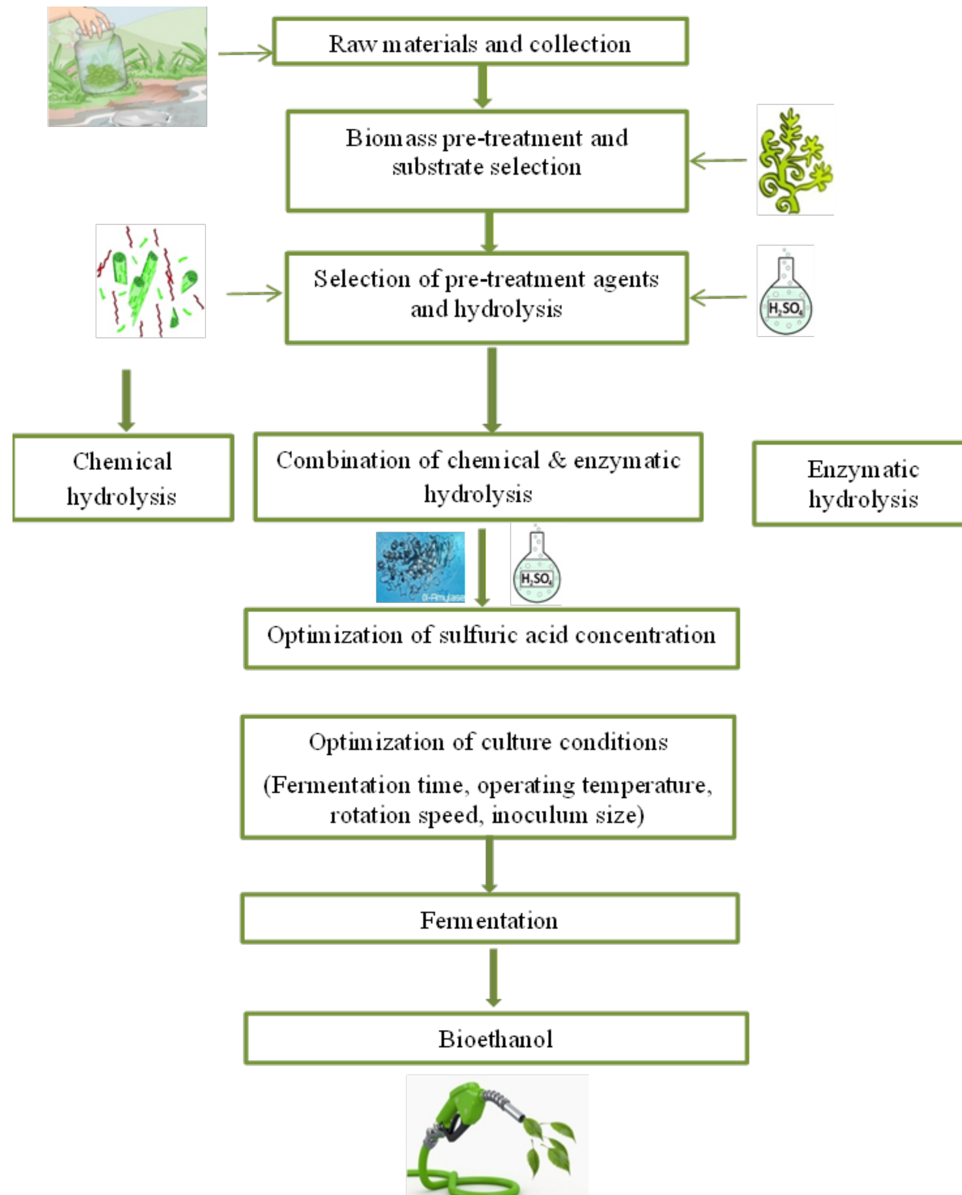


## Highlights

- Bioethanol is a renewable energy resource for fossil fuels and it can be produced from any low-cost raw material that contains sugar
- The objective of the study was to convert the low-value algae into high-value bioethanol using yeast
- Different pre-treatment techniques have been studied for the conversion of substrates to fermentable sugars.

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# Bioethanol production from *Chara globularis* using yeast and yield improvement by optimization of conditions

**E.J.S.B.A. Christy[1,2*], R. Kapilan[2], I. Wickramasinghe[1,3] and I. Wijesekara[1]**

[1]*Department of Food Science & Technology, Faculty of Graduate Studies, University of Sri Jayewardenepura, Nugegoda, Sri Lanka*
[2]*Department of Botany, Faculty of Science, University of Jaffna, Jaffna, Sri Lanka.*
[3]*Fakultät Physikalische Technik/ Informatik, University of Applied Sciences, Westsächsische Hochschule Zwickau, Germany.*

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**Abstract:** The rising population, depletion of petroleum-based fossil fuel and atmospheric contaminations by combustion of fossil fuel have opened avenues for alternative, eco-friendly and renewable energy sources. Bioethanol is an alternative and renewable source that has drawn attention due environmental concerns and energy security with non-renewable sources. This study was aimed at determining the potential bioethanol producing freshwater flora that are abundantly available in the Northern Province of Sri Lanka using *Saccharomyces cerevisiae* and to optimize the fermentation conditions to enhance the ethanol yield from *Chara globularis*. Freshwater flora such as *C. globularis, Cabomba caroliniana, Spirodela polyrhiza, Salvinia minima, Salvinia natans, Wolffia arrhiza* and *Wolffia globosa* were hydrolysed with 1M sulfuric acid solution to determine the reducing sugar and bioethanol yields. *C. globularis* produced a higher amount of reducing sugar and bioethanol than other species tested. When *C. globularis* was pre-treated with 1 M acid solutions (sulfuric acid, nitric acid, and hydrochloric acid) and alkaline solutions (sodium hydroxide and potassium hydroxide), a higher reducing sugar and bioethanol yields were obtained with sulfuric acid. When bioethanol was produced from *C. globularis* using *S. cerevisiae* following three different hydrolysis methods viz., acid hydrolysis (1 M sulfuric acid), enzymatic hydrolysis (1% alpha-amylase) and combination of chemical and enzymatic hydrolysis (1 M sulfuric acid and 1% alpha-amylase), the combination of chemical and enzymatic hydrolysis gave a higher yield, thus was selected. The conditions for fermentation of *C. globularis* substrate using *S. cerevisiae* were optimized sequentially by changing one factor at a time while keeping the other variables constant. After the optimization of fermentation time (24 hours), operating temperature (35 $^{o}$C), rotation speed (200 rpm) and sulfuric acid concentration for combined pre-treatment (0.75 M) with an inoculum size of 100 g $l^{-1}$, bioethanol yield was increased by 2 times compared with the non-optimized condition.

**Keywords**: Alpha-amylase; *Chara globularis*; Hydrolysis; Optimization; Sulfuric acid

## INTRODUCTION

The increasing population and extreme consumption of fossil fuels create the drive to generate alternative energy sources. Fossil fuel resources extensively contribute towards the generation of power from non-renewable resources that cause global warming and environmental pollution. Environmental issues involving climate change, increasing greenhouse gas levels, rising energy demands, and shortage of non-renewable fossil fuel have attracted the attention of the scientists globally to find a sustainable and alternative fuel resource to balance the demand-supply chain and improve the quality of life (Vasic *et al*., 2021). Moreover, a variety of biofuels such as biodiesel and bioethanol are alternative and renewable sources to current petroleum-based fossil fuels and are expected to minimize the dependence on petroleum-based fuels (Brennan and Owende, 2010). The bioethanol market has been expanding quickly in recent years, and bioethanol has become the world's leading biofuel. Developing nations have a competitive advantage in biofuel production due to favorable climatic conditions for agriculture production, land availability and lower production costs. Nevertheless, there could be some other socioeconomic and environmental effects influencing the potential for developing nations to profit from the rising energy demand for biofuel worldwide (Martins *et al*., 2017).

Bioethanol is generally produced from 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ generation of biomass (Scaife *et al*., 2015). The 1$^{st}$ generation of bioethanol is generated from edible biomass that contains higher levels of starch and sugar materials (Ho *et al*., 2014). Legumes and some cereals including wheat, sugar beet, corn, barley, sugar cane, and molasses are utilized as food sources for 1$^{st}$ generation of bioethanol production (Sarkar *et al*., 2012). The main advantages of the 1$^{st}$ generation feedstocks are high sugar production and less conversion cost (Sarkar *et al*., 2012). Usage of this 1$^{st}$ generation biomass for the production of bioethanol has led to various discussions about rising food prices and the occupation of agricultural land. The problems related to the use of 1$^{st}$ generation feedstocks are partially resolved with the use of the 2$^{nd}$ generation feedstocks (Nigam and Singh, 2011). The 2$^{nd}$ generation of bioethanol is based on non-edible food crops or products and it does not compete with the food supply (Thompson *et al*., 2013). Moreover, this is derived from lignocellulosic biomass such as municipal

**Corresponding Author's Email: arjunchristy17@gmail.com*
*https://orcid.org/0000-0002-2213-7302*

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waste or forest residues including grass, non-food crops, wood chips and straw (Nigam and Singh, 2011; Sims, 2003). The 2nd generation of biomass is inexpensive and readily available locally (Achinas *et al*., 2016). Second-generation feedstocks have a few advantages such as the lack of usage of food sources and low land requirements. Anyway, harvesting, purification and different hydrolysis methods made their production quite expensive (John *et al*., 2011).

Algae is recognised as an excellent alternative to 1st and 2nd generations feedstocks for bioethanol production. Algae are photosynthetic organisms; they can produce higher amounts of proteins, carbohydrates, and lipids in a short period of time (John *et al*., 2011; Yen *et al*., 2007). Algae can grow in saltwater, sewage water or fresh water and require less amount of water compared to other biomass for bioethanol production (Adey and Loveland, 2007). Algae are classified into macro and micro algae (Mata *et al*., 2010). *Chara globularis* though it appears to be a plant, is actually a multicellular macroalgae (Jang *et al*., 2012). Macroalgae are recognized as an ideal alternative for the production of bioethanol due to their higher content of carbohydrates and sugars than microalgae (Sirajunnisa and Surendhiran, 2016). Using non-edible macroalgae for bioethanol production has additional advantages, such as reducing conflict with agricultural products, high productivity per area, and non-dependence on fertilizer, freshwater and arable land (Adams, 2009).

The pre-treatment of biomass is the greatest difficulty in the production of bioethanol. Algal cell walls are the primary structures that must be depolymerized in order to obtain the polysaccharide contents (Vera *et al*., 2011). The hydrolysis of the polysaccharide will result in free monomer molecules that can easily be fermented to bioethanol during the conversion (Chandel *et al*., 2007). Chemicals such as acids and bases can greatly enhance the release of simple sugars from the polysaccharides component (Tayeb *et al*., 2012). This is a necessary step to alter the structure of biomass to make it more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars (Hsu *et al*., 1980). Alpha-amylase and cellulase are widely used enzymes in biological pre-treatment. Enzymatic pre-treatment of the substrate is affected by both the structural features of polysaccharides and the mode of enzyme action (Yang *et al*., 2011). Alpha-amylase specifically catalyses the hydrolysis of alpha-1, 4 glycosidic bonds of starch to produce maltose, dextrin, and a small amount of glucose (Zhang and Lynd, 2004). These molecules are converted into ethanol by *S. cereevisiae* (Mitiku and Hatsa, 2020). Many factors affect the cell growth of *S. cerevisiae* and the enzymatic chemical reactions within the living cells. The factors are sugar concentration, fermentation time, inoculum size, temperature, and agitation rate (Zabed *et al*., 2014). Khambhaty *et al*., (2012) reported that bioethanol production of 0.390 g/g was obtained from algal feedstocks (*Kappaphycus alvarezii*) after 48 h at 30 $^{o}$C at 150 rpm using 5% (v/v) *S. cerevisiae* concentration.

Micro and Macro algae have been previously used in the production of bioethanol employing *S. Cerevisiae* as the fermentation bio-catalyst using different pre-treatment methods (Azad *et al*., 2014). In a study by Meinita et al., (2012), 1.31% bioethanol production was observed with 0.2% $H_2SO_4$ pre-treatment at 130 °C for 15 min when macroalgae (*Kappaphycus alvarezii*) was used as substrate. Ge et al. (2011) reported that ethanol production of 11.3% was observed with *Laminaria japonica* substrate after 0.1 M $H_2SO_4$ acid pre-treatment at 121 °C for 1 h and enzymatic hydrolysis with cellulose and cellobiase. Kumar et al., (2013) reported that 43% bioethanol production was observed after enzymatic hydrolysis with cellulose and β–glucosidase when *Gracilaria verrucosa* was used as substrate.

The contribution of freshwater algae to the production of bioethanol is limited and there has been no literature mentioning bioethanol production from *C. globularis*, even though it produces a significant amount of bioethanol. Diverse under-utilized inland freshwater flora resources are abundantly available and widely distributed in Sri Lanka, and they could be utilized to produce bioethanol in the future by a continuous multiplication process. The objective of the study was to determine the best ethanol producer among the different freshwater flora in the Northern Province of Sri Lanka and to optimize its fermentation conditions for higher yield.

## METHODOLOGY

### *Chemicals*

The chemicals employed in this study were obtained from standard sources (Himedia).

### *Raw materials and collection*

Freshwater flora such as *C. globularis, Spirodela polyrhiza, Wolffia globosa, Salvinia minima, Salvinia natans, Cabomba caroliniana* and *Wolffia arrhiza* were collected from different freshwater bodies of Sri Lanka. The collected specimens were identified and confirmed through visual inspection.

### *Determination of reducing sugar*

The reducing sugar concentration was analysed using the DNS method (Miller, 1959).

### *Determination of bioethanol*

The bioethanol content in the fermented sample was determined by using Dujardin-Salleron ebulliometer and expressed in terms of percentage (v/v).

### *Inoculum Preparation*

The cells of *S. cerevisiae* were bought from the local market and incubated in 100 ml of sterile sucrose solution (50 g $l^{-1}$) for 18 hours at 100 rpm at room temperature (Inparuban *et al*., 2009).

### *Biomass pre-treatment and substrate selection*

All freshwater flora were washed, dried, milled and weighed and then substrate dissolved in distilled water was autoclaved for 15 minutes at high temperature. Then, 1 M sulfuric acid was added to the substrate solution for acid hydrolysis, before autoclaving for 15 minutes again, and

after cooling, the mixture was centrifuged and neutralized with sodium hydroxide. The samples were collected at regular time intervals and reducing sugar and bioethanol contents were determined. Flora that produced significantly higher amount of reducing sugar and bioethanol were chosen for further studies.

*Chemical hydrolysis*

The *C. globularis* was hydrolysed using alkaline and acid hydrolysis methods separately. Samples were added into the conical flask, 1 M sodium hydroxide and potassium hydroxide for alkaline hydrolysis; and 1 M sulfuric acid, hydrochloric acid, and nitric acid for acid hydrolysis. The flask was autoclaved for 15 minutes at a high temperature, and then the mixture was cooled down and neutralized. Then the supernatant was allowed to ferment with *S. cerevisiae* in the fermentation medium. From each solution, samples for analyses of reducing sugar content and bioethanol production were determined. This experiment was repeated with 1 M sulfuric acid.

*Enzymatic hydrolysis*

The *C. globularis* substrate was taken and 1% of the enzyme alpha-amylase, diluted with 0.1 M phosphate buffer was added to the mixture and kept at 60 °C for 2 hours and centrifuged. Then the supernatant was allowed to ferment with *S. cerevisiae* in the fermentation medium. The samples were collected at regular time intervals and reducing sugar and bioethanol contents were determined.

*Combination of sulfuric acid and enzymatic hydrolysis*

The *C. globularis* substrate was hydrolysed with 1 M sulfuric acid and neutralized to pH 7.0. This was followed by 1 % alpha- amylase hydrolysis. Then the supernatant was allowed to ferment with *S. cerevisiae* in the fermentation medium. The samples were collected at regular time intervals and reducing sugar and bioethanol contents were determined.

*Optimization of sulfuric acid concentration in the hydrolysis*

The *C. globularis* substrate was hydrolysed using the combined sulfuric acid and enzymatic (1% α-amylase) hydrolysis where different concentrations of sulfuric acid (0.50 - 1.75 M) for 15 minutes were used. Then the supernatant was allowed to ferment with *S. cerevisiae* in the fermentation medium. The samples were collected at regular time intervals and reducing sugar and bioethanol contents were determined.

*Optimization of culture conditions for bioethanol production*

After a combined sulfuric acid and alpha-amylase enzyme hydrolysis, the fermentation was allowed to optimize at varying fermentation time (12, 24, 36, 48 and 60 h), temperature (20, 25. 30, 35, 40 and 45 $^{o}$C), rotation speed (50, 100, 150, 200 and 250 rpm) and *S. cerevisiae* inocula (25, 50, 75, 100, 125 and 150 g $l^{-1}$) with *C. globularis* substrate using one variable at a time approach. The samples were collected at regular time intervals and reducing sugar and bioethanol contents were determined.

*Distillation and gas chromatography*

Distillation was carried out to separate water and alcohol which was a product derived from the fermentation stage. The resulting distillate was tested by GC machine (Agilent 6890: Mass selective detector 5973N) to determine the characteristics of the ethanol compound.

**Statistical analysis**

The experiments were carried out with three observations, and the graphs were made using the mean values. Minitab 17.0 was used for the statistical analysis. One-way ANOVA was used to examine the data, and Tukey's multiple comparison tests were used to identify differences at 95% confidence intervals.

## RESULTS AND DISCUSSION

*Biomass pre-treatment and substrate selection*

The amount of reducing sugar produced by the freshwater flora *Cabomba caroliniana* (17.77 g $l^{-1}$), *Spirodela polyrhiza* (20.2 g $l^{-1}$), *Salvinia minima* (25.866 g $l^{-1}$), *C. globularis* (31.923 g $l^{-1}$), *Salvinia natans* (22.915 g $l^{-1}$), *Wolffia arrhiza* (14.589 g $l^{-1}$), and *Wolffia globosa* (8.289 g $l^{-1}$) fluctuated from 8.289 g $l^{-1}$ to 31.923 g $l^{-1}$ after the acid hydrolysis using 1 M sulfuric acid (Figure 1). Least amount of reducing sugar was observed at *Wolffia globosa*. *C. globularis* produced significantly higher amount of reducing sugar than the other species tested. When fermentation was done using *S. cerevisiae* among the chosen flora substrates, bioethanol was produced only from *C. globularis* substrate (Figure 1). Therefore, *C. globularis* was selected for further studies. The reason for bioethanol production by *C. globularis* substrate may be due to significantly higher amount of sugar production by *C. globularis* substrate in the acid hydrolysis. Since algae have a high content of carbohydrates in their composition, they are able to yield significantly higher bioethanol (Phwan *et al*., 2018) than the flora substrates chosen. Therefore, *C. globularis* substrate was selected for further studies.

When *C. globularis* substrate was hydrolysed with different acids (1 M sulfuric acid, 1 M nitric acid and 1 M hydrochloric acid) separately, and alkaline solutions (1 M sodium hydroxide and 1 M potassium hydroxide) separately, significantly higher amount of reducing sugar yield was obtained in acid pre-treatments than the alkaline pre-treatments. Among the three acids used for acidic pre-treatment, significantly higher amount of reducing sugar yield was obtained with 1 M sulfuric acid and nitric acid treatments. The least amount of reducing sugar was produced by hydrochloric acid in the acids used, and the least amount was produced by sodium hydroxide in the bases used (Figure 2 a). When acidic pre-treatment was done with sulfuric acid only, *C. globularis* substrate produced significantly higher amount of bioethanol after the 2$^{nd}$ day of fermentation by *S. cerevisiae* (Figures 2a and b). Therefore, acidic pre-treatment by sulfuric acid was chosen as the best pre-treatment agent for *C. globularis* substrate. The vital role in bioethanol production is the selection of the best pre-treatment method for the biomass. The objective of the pre-treatment was to break down

the structure of polysaccharides and facilitate the acids or alkalines to easily break down the polysaccharides into monomers. Pre-treatments that used nitric acid and sulfuric acid both yielded significantly higher amount of reducing sugar with *C. globularis* substrate (Figure 2 a). However, *C. globularis* substrate produced bioethanol after the fermentation using *S. cerevisiae* if they were pre-treated by sulfuric acid only. This may be due to the formation of toxic substances or inhibitors produced by the nitric acid hydrolysed samples (Christy *et al*., 2021). The enzymatic hydrolyses may not be affected by these inhibitors, but they restrict the growth of microorganisms and fermentation (Esteghlalian *et al*., 1997). Ren *et al*., (2010) observed, after using the hydrolysis procedure, that the maximum sugar production of 84 g $l^{-1}$ was attained when rice straw was treated with diluted sulfuric acid at 121 ºC for one hour.

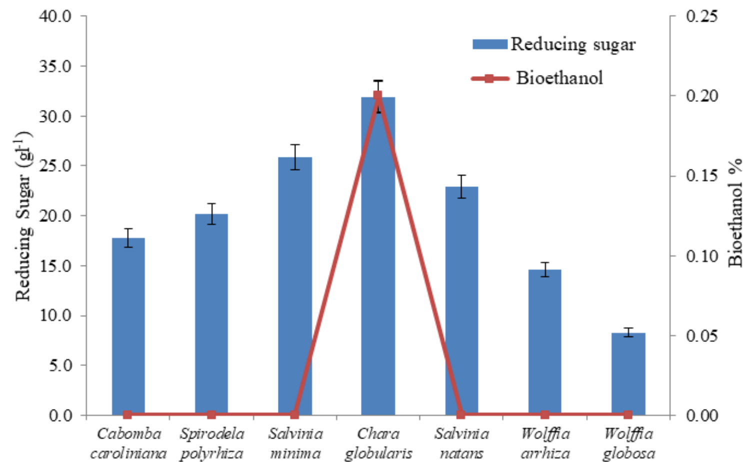


**Figure 1:** Changes in different amount of reducing sugar and bioethanol yield from diverse freshwater flora after the sulfuric acid hydrolysis.

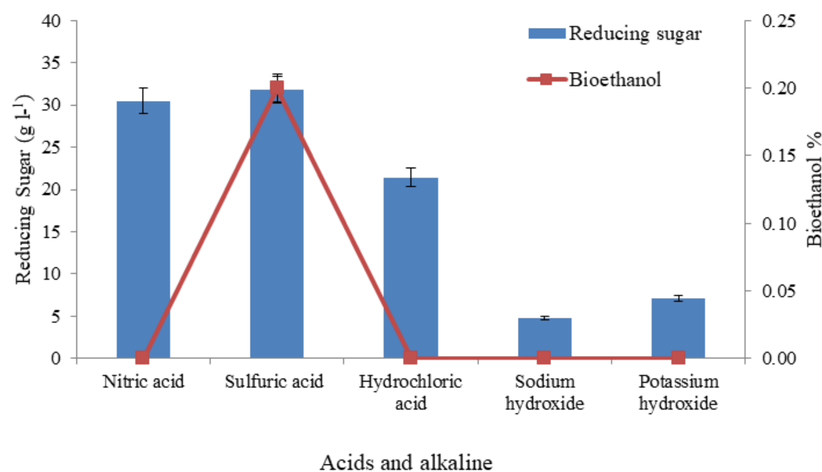


**Figure 2 (a):** Quantity of reducing sugar and bioethanol production from *Chara globularis* substrate after pre-treatment with acids and alkaline using *Saccharomyces cerevisiae.*

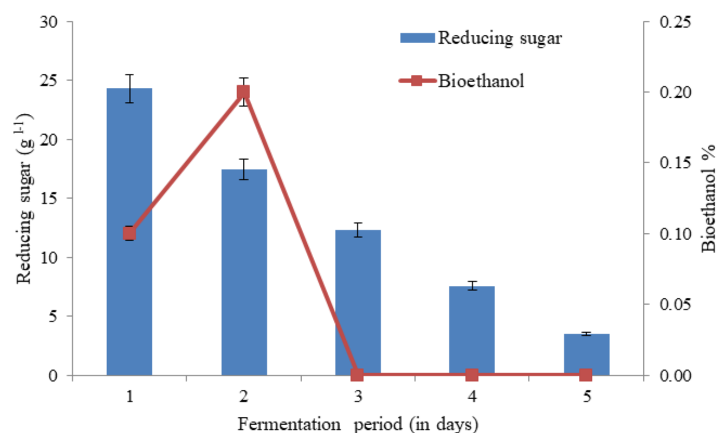


**Figure 2 (b):** Changes in bioethanol and reducing sugar yield during fermentation periods of *Chara globularis* substrate using *Saccharomyces cerevisiae* after the sulfuric acid pre-treatment.

*Enzymatic pre-treatment*

After the enzymatic pre-treatment by 1% alpha-amylase enzyme, significantly higher amount of bioethanol was produced after the 2nd day of fermentation with *C. globularis* substrate using *S. cerevisiae*. The amount of reducing sugar was significantly decreased from the 1st day towards the 5th day of fermentation for *C. globularis* substrate (Figure 3). The enzymatic process is a simple but well researched process. Enzyme α-amylase hydrolyzes the polymers to monomeric glucose units. Alpha-amylase specifically catalyzes the hydrolysis of α-1, 4 glycosidic bonds of polysaccharides to maltose, dextrin, and a small amount of glucose (Zhang and Lynd, 2004). These molecules are converted into ethanol by *S. cerevisiae* (Mitiku and Hatsa, 2020). Reduction of reducing sugar may be due to the rapid consumption of the reducing sugar by *S. cerevisiae* during the fermentation process (Agustini *et al*., 2019). The reduction in the quantity of bioethanol produced after 2nd day might be due to the evaporation of bioethanol produced at moderately high temperatures and the utilization of bioethanol by *S. cerevisiae* for its metabolic activities (Mitiku and Hatsa, 2020). Thontowi *et al*., (2018) reported that a higher ethanol yield (2.43 g $l^{-1}$) was obtained from sugarcane bagasse with a combination of cellulose and hemicellulose enzymes using *S. cerevisiae.*

*Combination of sulfuric acid and enzymatic hydrolysis*

After the combination of sulfuric acid and enzymatic hydrolysis with *C. globularis* substrate and fermentation by *S. cerevisiae*, significantly higher amount of bioethanol was produced after the 2nd day of fermentation. The amount of reducing sugar was significantly decreased from the 1st day towards the 5th day of fermentation for *C. globularis* substrate. Acid pre-treatment releases some of the fermentable sugars from the biomass and enhances the accessibility of enzymes (alpha-amylase) for subsequent hydrolysis process (Pandiyan *et al*., 2019). And then, enzyme alpha-amylase hydrolysis process hydrolyses the macro molecules into simple sugars that are converted into ethanol by *S. cerevisiae* (Zhang and Lynd, 2004). Sunaryanto *et al*., (2013) reported that bioethanol production of 7.98% (v/v) was produced from sago starch with 2.5% sulfuric acid concentration using alpha-amylase and dextroseDX.

Among the three pre-treatment techniques, combination of sulfuric acid and enzymatic hydrolysis yielded significantly higher amount of reducing sugar and bioethanol after fermentation by *S. cerevisiae* than the other methods. Therefore, the combination of sulfuric acid and enzymatic hydrolysis was chosen for further studies. The results indicate that sulfuric acid pre-treatment improves the reducing sugar yield in the hydrolysis process. An acid solution degrades the hemicellulose component in lignocellulosic material by breaking the van der Waals forces of the covalent and hydrogen bonds that make up the component (Li *et al*., 2010). By having a simpler form of the hemicellulose, the enzymes are expected to have better access to perform the hydrolysis and yield more sugar (Aditiya *et al*., 2015).

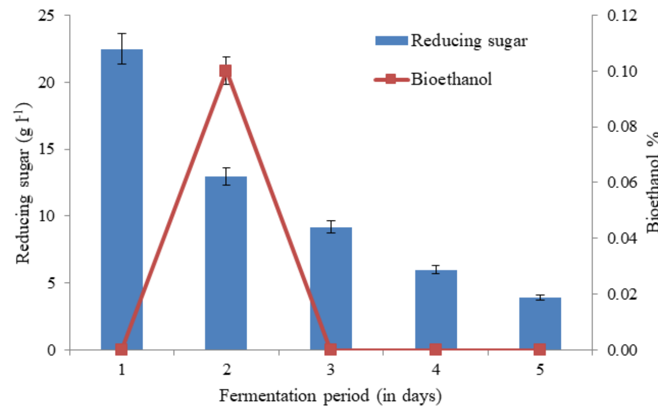


**Figure 3:** Quantity of reducing sugar and bioethanol production after the enzymatic pre-treatment using alpha- amylase from substrate of *Chara globularis* using *Saccharomyces cerevisiae*.

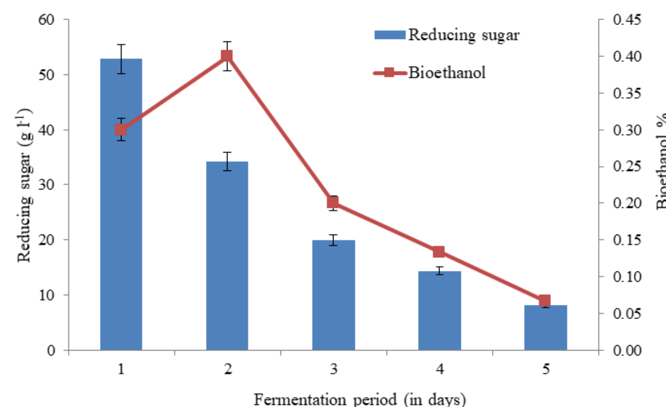


**Figure 4:** Quantity of reducing sugar and bioethanol production after the combination of sulfuric acid and enzymatic hydrolysis from *Chara globularis* substrate using *Saccharomyces cerevisiae*.

**Optimization of sulfuric acid concentration in the hydrolysis**

When 0.75 M sulfuric acid concentration was used as acid component in the combined hydrolysis with *C. globularis* substrate, significantly higher reducing sugar and bioethanol yields were obtained than the other acidic concentrations for the acidic component (Figure 5). Therefore, 0.75 M sulfuric acid concentration was chosen as the acid component in the acid alpha-amylase combined hydrolysis, for further optimization studies for *C. globularis* substrate. Sulfuric acid concentration plays a vital role in the hydrolysis technique of bioethanol production from *C. globularis* substrate. During this study, bioethanol production from *C. globularis* substrate increased significantly with the increasing sulfuric acid concentration and reached the maximum value, and then further increase in sulfuric acid concentration reduced the bioethanol production. This decrease in bioethanol concentration may account for the further sugar degradation that occurred under the severe status of acidity and would lead to an unfavourable effect on the sugar conversion (Kefale *et al*., 2012; Nutawan *et al*., 2010) which is toxic for *S. cerevisiae* (Mitiku and Hatsa, 2020). Novia *et al*., (2017) reportedthat when simultaneous saccharification and fermentation procedures using the commercial cellulase enzyme (Novozyme) were done, significantly higher ethanol concentration (13.68 g $l^{-1}$) was obtained from rice husk by *S. cerevisiae* with 3% sulfuric acid concentration.

**Optimization of culture conditions for bioethanol production**

*Optimization of the fermentation time*

When the fermentation time was increased from 12 to 24 hours, bioethanol yield was significantly increased and then bioethanol yield was significantly decreased with *C. globularis* with *S. cerevisiae.* Since, significantly higher bioethanol yield was observed at 24 hours of fermentation, this was chosen as the optimum fermentation time and used in further studies (Figure 6). The fermentation time affects the growth of yeast cells. Moreover, during the initial stage of fermentation, yeast cells spend energy to adapt themselves to the growth conditions. However, if the fermentation time is too long, the higher concentrations of bioethanol produced in the system might become toxic to the fermenting cells (Zabed *et al*., 2014). Dash *et al*., (2017) reported, when the sweet potatoes are used as a substrate, maximum ethanol production of 127.2 g/kg was observed in the 72 hours of fermentation time.

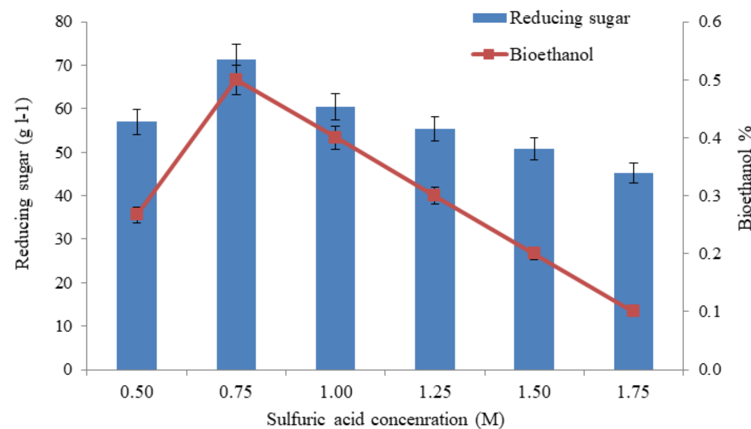


**Figure 5:** Production of reducing sugar and bioethanol yield after the combined hydrolysis using different concentrations of sulfuric acid from *Chara globularis* substrate using *Saccharomyces cerevisiae*.

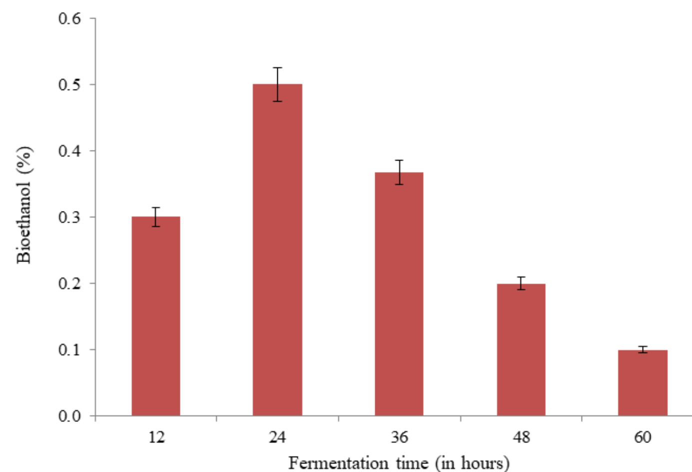


**Figure 6:** Effect of fermentation time on the maximum bioethanol production from *Chara globularis* substrate using *Saccharomyces cerevisiae*.

*Optimization of the temperature*

When the temperature was increased from 20 to 35 ºC, bioethanol yield was significantly increased and then bioethanol yield was significantly decreased with *C. globularis* substrate. Since significantly higher bioethanol yield was observed at 35 ºC with *C. globularis* substrate, this was chosen as the optimum temperature and used for further studies (Figure 7). Temperature has a direct impact on the growth rates of *S. cerevisiae* (Charoenchai *et al*., 1998). Higher temperatures can harm the enzymes that regulate fermentation and microbial activity. They become denatured and lose their functionality due to the inactivation of their tertiary structure (McMeekin *et al*., 2002). Use of temperatures that are too high or too low reduces ethanol production, inhibits the development of *S. cerevisiae*, and dramatically reduces the amount of fermentation (Manyuchi *et al*., 2018). They also reported that the higher bioethanol yield of 60 ml/l was achieved from sewage sludge broth at 30 °C during the 10 days of fermentation time by yeast.

*Optimization of the rotation speed*

When rotation speed was increased from 50 to 200 rpm, the bioethanol yield was significantly increased with *C. globularis* substrate and then it started to decrease with higher rotation speed. Since significantly higher bioethanol yield was obtained at 200 rpm rotation speed, this speed was selected as optimum for further studies with *C. globularis* substrate (Figure 8). Increased rotation speed led to an increase in dissolved oxygen concentration, which accelerated the growth rate of yeast and increased the creation of biomass. A low level of dissolved oxygen concentration is favourable for the production of bioethanol, whereas a high level of dissolved oxygen concentration is beneficial for yeast cell growth. The rotation speed produces shear force in the fermentation process, which will affect both cell growth and ethanol production (Rodmui *et al*., 2008). Zani *et al*., (2019) reported that, when oil palm frond juice was used as a substrate, higher ethanol production (0.5 g/g) was achieved at 100 rpm rotation speed with *S. cerevisiae*.

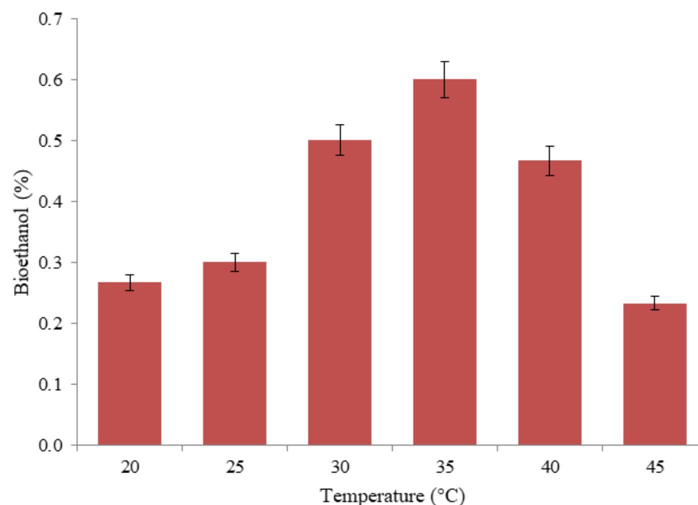


**Figure 7:** Effect of different fermentation temperatures on maximum bioethanol production from *Chara globularis* substrate using *Saccharomyces cerevisiae*.

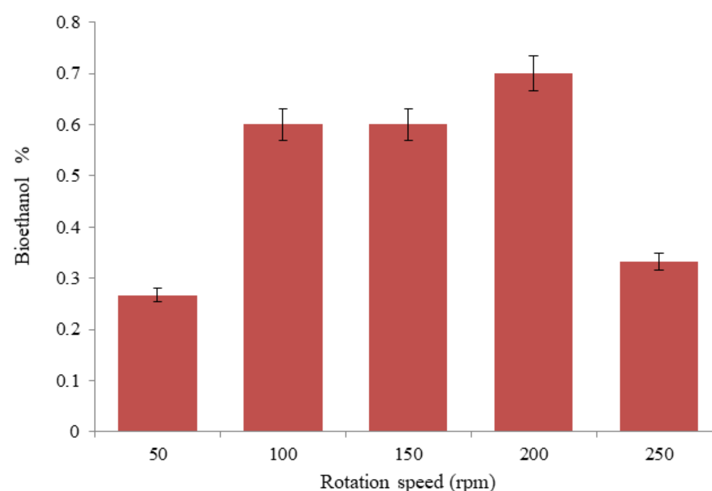


**Figure 8:** Effect of different rotation speed on maximum bioethanol production from *Chara globularis* substrate using *Saccharomyces cerevisiae*.

**Optimization of the *S. cerevisiae* inoculum concentration**

When *S. cerevisiae* inoculum concentration was increased from 25 to 100 g $l^{-1}$, the bioethanol yield was significantly increased with *C. globularis* substrate and then it started to decrease with higher *S. cerevisiae* inoculum concentration. Since significantly higher bioethanol yield was obtained at 100 g $l^{-1}$ *S. cerevisiae* inoculum concentration, this was selected as optimum for further studies with *C. globularis* substrate (Figure 9). The final ethanol concentration is not much affected by inoculum concentration, but it does have an impact on the utilization of sugar and the ethanol yield produced. The higher inoculum concentrations are not optimum for efficient bioethanol production, and the biocatalysts will saturate the system once they reach a particular concentration, which will result in a reduction in the amount of bioethanol produced (Laopaiboon *et al*., 2007). Swain *et al*., (2013) studied; maximum ethanol yield was obtained with a concentration of 10% of inoculum concentration in sweet potato flour by coculture of *Trichoderma* sp. and *S. cerevisiae*.

**Distillation and gas chromatography**

The GC results for *C. globularis* were presented in Figure 10. This indicated that ethanol was the dominant species (90.1%) found in the sample. The second dominant species was 3-methyl-1-butanol (7.3%). The least dominant species was 2-methyl-1-propanol (2.6%). However, the *C. globularis* included low undesirable chemical substances with a higher boiling point that may be favourable for engine performance (Fini and Fattahi, 2021).

## CONCLUSIONS

Among the seven freshwater species tested, *Chara globularis* obtained from the Northern Province of Sri Lanka could be used as an efficient raw material for bioethanol production using *Saccharomyces cerevisiae*. Among the chemical, enzymatic and combined pre-treatment conditions used for *C. globularis*, the combination of acidic (1 M $H_2SO_4$), and enzymatic (1% α-amylase) pre-treatment produced significantly a higher bioethanol yield (2 times) following the fermentation by *S. cerevisiae*. Higher bioethanol yield was obtained at an incubation period of 24 h with temperature of 35°C and 0.75 M sulfuric acid concentration with 100 g $l^{-1}$ inoculum concentration at 200 rpm. Distillation of the fermented sample of *C. globularis* using GC analysis confirmed the existence of 90.1% of ethanol in the fermented mixture.

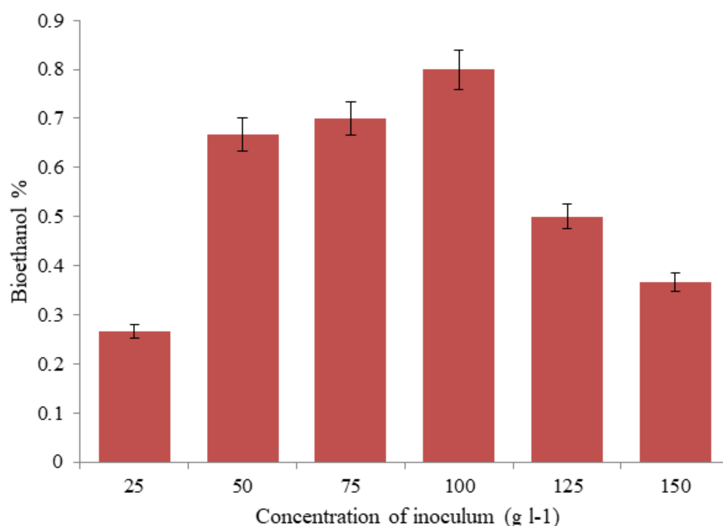


**Figure 9:** Effect of different concentration of *Saccharomyces cerevisiae* inoculum on bioethanol yield enhancement from *Chara globularis* substrate.

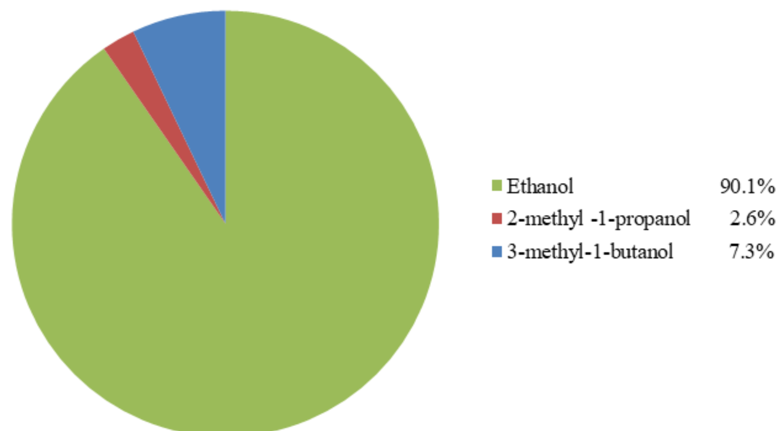


**Figure 10:** The results of the gas chromatography test for *Chara globularis* substrate.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no existence of a financial/non-financial competing interests.

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