RESEARCH ARTICLE

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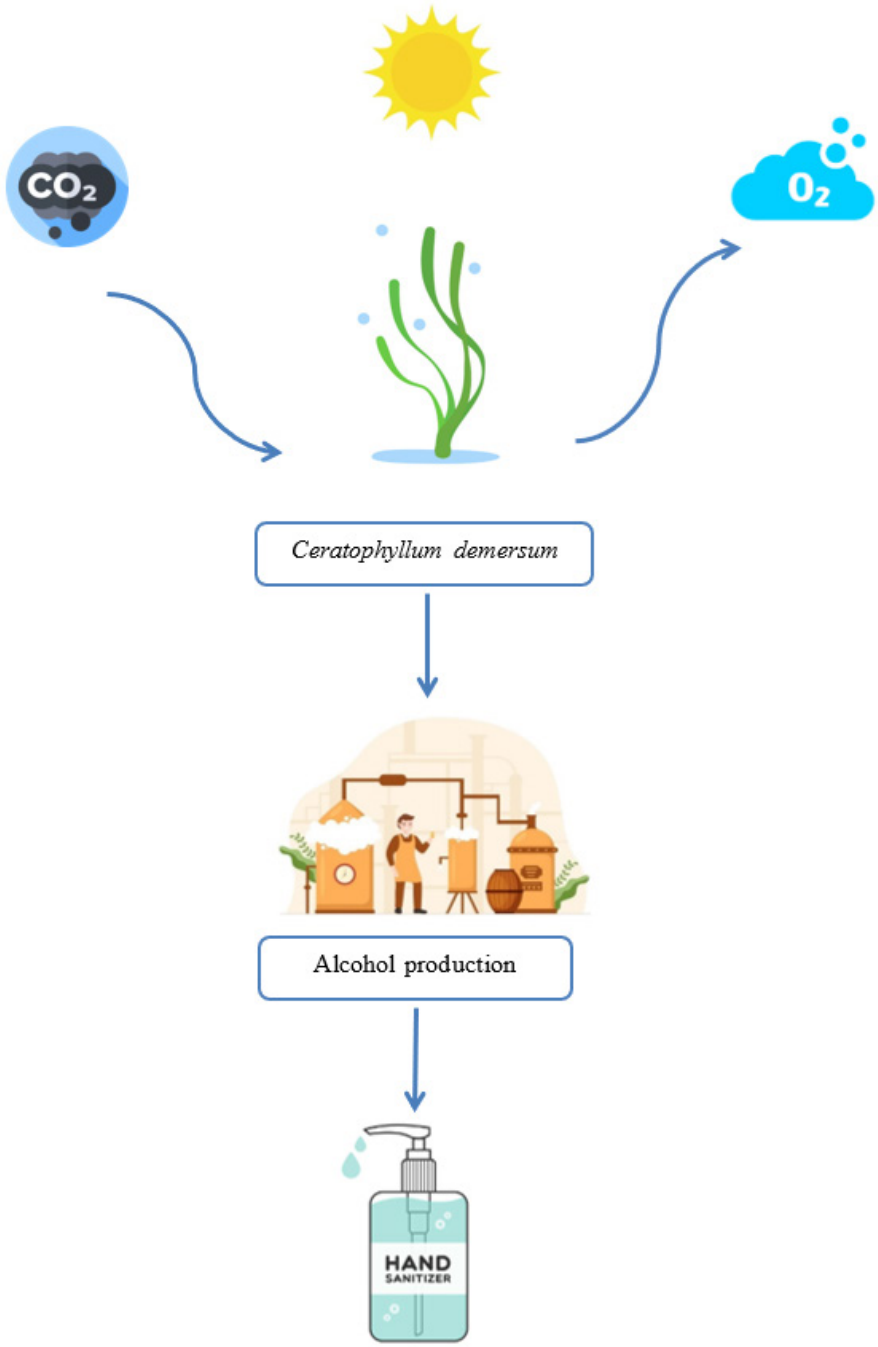

Highlights

- *Ceratophyllum demersum,* an aquatic weed, is a promising feedstock for bioethanol production in hand sanitizer manufacturing.
- Effective pre-treatment techniques were explored to enhance sugar extraction from *C. demersum* biomass.
- Fermentation parameters were optimized to maximize bioethanol yield.
- Bioethanol derived from *C. demersum* exhibiting a potential for producing hand sanitizers.
- Sustainable utilization of *C. demersum* for bioethanol production could facilitate weed management, while highlighting an eco-friendly practice.

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RESEARCH ARTICLE

Optimization of Bioethanol Production from *Ceratophyllum demersum* for hand sanitizers

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Abstract: Hands are the primary way to spread microorganisms, thus hand washing is the primary defence and an essential element of personal hygiene for infection control. Hand sanitizers that contain ethanol as their main constituent are used to kill a broad range of microbes. Bioethanol production has relied heavily on the use of first-generation feedstock. Therefore, the development and utilization of alternative feedstocks such as weed (*Ceratophyllum demersum*) and other non-food crops have gained more attention in recent times. This study explores the bioethanol production using *C. demersum,* a weedy species, for its potential use in hand sanitizer production. The substrate, *C. demersum* was subjected to mechanical pre-treatment and then pre-treated with varying concentrations of sulfuric acid, which was subsequently followed by enzymatic pre-treatment and allowed for fermentation using *Saccharomyces cerevisiae*. The results showed that a sulfuric acid concentration of 1 M resulted in a significantly higher amount of reducing sugar and alcohol yield compared to other concentrations, and this was selected for further studies. After optimization of fermentation parameters, a significantly higher alcohol yield of 2.6% was achieved using a *S. cerevisiae* inoculum concentration of 100 g/l and agitation at 150 rpm at 40 °C for 36 hours. Subsequent optimization of fermentation media components further increased the alcohol yield to 3.7%, with the use of 6 g/l yeast extract, 6 g/l $(NH_4)_2SO_4$, 5 g/l $MgSO_4$, and 8 g/l KH_2PO_4. The resulting alcohol mixture was analyzed and found to contain 84.9% bioethanol. An agar well diffusion assay was conducted against bacteria and fungi. The results showed that all the bacterial and fungal strains were sensitive to the bioethanol extract as evidenced by the presence of an inhibition zone.

Keywords: Antimicrobial activity; Bioethanol; *Ceratophyllum demersum*; Hand sanitizer; Media composition

INTRODUCTION

Hygiene is important in maintaining the health of both humans and the various associated living organisms on earth, serving as a preventive measure against pathogenic diseases for centuries. Regularly emerging pathogens belonging to bacterial, viral, protozoans, and other classifications within the animal and plant kingdoms, have been accountable for illnesses such as cholera, chickenpox, measles, polio, hepatitis, and tuberculosis (Mahmood et al., 2020). Recently encountered pathogen "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) has caused the worldwide pandemic coronavirus disease 2019 (COVID-19) and many millions of people have contracted the disease globally. Hand hygiene practices include hand washing, antiseptic hand washing, and antiseptic hand sanitization hand hygiene is considered the basic principle of infection prevention and is vital to minimize the transmission and colonization of infection among healthcare workers and the general public (Hans et al., 2021).

Hand sanitizers are available in gel, liquid, and foam forms and are used to destroy a variety of micro-organisms (Jing et al., 2020). Hand sanitizers are classified as alcohol-free and alcohol-based sanitizers (Hans et al., 2021). Alcohol-based hand sanitizers have become increasingly prevalent in recent years, and their popularity has skyrocketed further due to their affordability, simplicity of preparation, and high efficacy in eliminating germs and bacteria. Alcohol-based sanitizers typically use ethanol, isopropyl alcohol, or n-propanol as their primary alcohol ingredient. For the effective elimination of microbes, the alcohol content in the sanitizer should be between 60-95% v/v. These alcohols disrupt by damaging the lipid membranes and denaturing the proteins of microbes. The World Health Organization recommends specific sanitizer formulations that have been found to be effective. These formulations contain either 80% v/v ethanol or 75% v/v isopropyl alcohol, in addition to 0.125% v/v hydrogen peroxide, 1.45% v/v glycerol, and water. Ethanol is generally considered to be less irritating to the skin and more effective at killing a wider range of microbes than isopropyl alcohol (Golin et al., 2020).

Bioethanol production is generated from edible biomass that contains higher levels of starch and sugar materials (Ho et al., 2014). The utilization of edible biomass, such as crops used for food, for the production of bioethanol has sparked concerns about potential increases in food prices and competition for agricultural land (Nigam & Singh, 2011). However, using non-food feedstocks such as *C. demersum* can offer advantages in terms of sustainability and avoiding negative impacts on the food industry (Kusolsongtawee et al., 2018).

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C. demersum is an invasive, submerged weed that has significant negative economic and environmental effects. *C. demersum*, which grows and completes its life cycle in water, harms the aquatic ecosystem and other connected eco-environments either directly or indirectly. Their rapid growth rate and difficulties in removal result in severe damage to aquatic ecosystems. They disrupt recreational activities, water irrigation, fish farming, electricity production, and drinking water supplies. *C. demersum* frequently decreases the effectiveness of water bodies to support fish growth. *C. demersum* may absorb a lot of nutrients from the water, which makes them less accessible for planktonic algae. They spread rapidly, covered the entire water surface, and resulted in a reduction of dissolved oxygen and sunlight penetration. Alternative strategies are required to minimize these issues (Lancar & Krake, 2002). There are various methods available to remove this weed from the water, including biological, chemical, and mechanical techniques that are widely employed (Hoque et al., 2017). As a consequence, these costs will become an investment if the aquatic weed can be used to produce bioethanol (Uddin et al., 2017). According to a study by Kusolsongtawee et al. (2018), the biomass of the *C. demersum* species contains a significant amount of total carbohydrates, with a weight percentage of approximately 39.37. This high carbohydrate content is promising for the production of bioethanol, as the sugars present can undergo fermentation to produce ethanol.

The effective production of bioethanol is heavily dependent on the pre-treatment of biomass to ensure optimal results. The pre-treatment process aims to maximize sugar yield either immediately or later through hydrolysis, minimizing sugar loss or degradation, reducing toxic substances that can prohibit ethanol production, minimizing the energy required for the process, and reducing production costs (Hill et al., 2006). There are three primary methods of pre-treating biomass: physical, chemical, and biological (Hill et al., 2006). The initial step in pre-treating biomass is physical pre-treatment, which involves mechanical grinding to reduce the size of the biomass and increase its surface area. This technique improves the accessibility of the biomass to subsequent chemical and enzymatic treatments (Rajendran et al., 2017). Chemical pre-treatment techniques are convenient to perform and may provide high conversion yields in a relatively short amount of time (Hill et al., 2006). Treatment with acids or bases can effectively break down the polysaccharides in the biomass and release simple sugars for subsequent fermentation processes (Tayeb et al., 2012).

Biological pre-treatment is an eco-friendly technique that requires less energy (Bhatia et al., 2017b;Sindhu et al., 2016). Biological pre-treatment using alpha-amylase and cellulase enzymes is a common approach in biomass conversion. The effectiveness of the biological pre-treatment is influenced by the polysaccharide structure 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, while cellulase enzymes break down cellulose into glucose (Zhang and Lynd, 2004; Yang et al., 2011).

Aquatic biomass such as *Azolla* (Chupaza et al., 2021; Christy et al., 2020), *Spirodela polyrrhiza* (Cui and Cheng, 2015), *Landoltia punctate* (Chen et al., 2012), and *Lemna minor* (Clark and Hillman, 1996) have been previously used in bioethanol production. Various micro-organisms have been utilized as biocatalysts for bioethanol production from biomass (Yu et al., 2009). *S. cerevisiae* has been identified as the most efficient micro-organism for enhancing bioethanol production (Taouda et al., 2017). In Sri Lanka, bioethanol production relies on sugar and starchy feedstocks, but there is an increasing interest in exploring alternative sources such as weeds (*C. demersum*) to support sustainable weed management practises and the production of bioethanol. The objectives of the research study were to produce bioethanol from *C. demersum* using *S. cerevisiae* and to determine the potential utilization of bioethanol generated from *C. demersum* for hand sanitizer production.

METHODOLOGY

Materials and Chemicals

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

Inoculum preparation

The *S. cerevisiae* (yeast strain) was sourced from a nearby market and subsequently incubated in 100 ml of sterile sucrose solution (50 g/l) for 18 hours at 100 rpm at room temperature (Inparuban et al., 2009).

Fermentation Medium preparation

The fermentation medium utilized for alcohol production consists of substrates (after liquefaction and saccharification) of 8% (w/v), *S. cerevisiae* extract (4 g/l), $MgSO_4.7H_2O$ (4 g/l), KH_2PO_4 (8 g/l) and $(NH_4)_2SO_4$ (4 g/l), respectively, in an Erlenmeyer flask sterilized using an autoclave at 0.15 MPa pressure.

Preparation of buffer solution

Initially, 13.2 ml of K_2HPO_4 was taken from an already prepared 1 M K_2HPO_4 solution and added to the 1 litre volumetric flask. Then 86.8 ml of KH_2PO_4 was taken from the already prepared 1 M KH_2PO_4 solution and added to the 1 litre volumetric flask which contained 13.2 ml of 1 M K_2HPO_4. Distilled water was added to a 1-liter volumetric flask containing a specific volume of K_2HPO_4 and KH_2PO_4, making it up to 1 liter in total. The pH was adjusted to 6.0 (Green, 1933). Then the solution was transferred to a 1 litre duran bottle and stored in the refrigerator.

Analytical method

Determination of Reducing Sugar and Alcohol Content

The reducing sugar content was determined by using 3, 5-Dinitrosalicylic acid (DNS) method (Miller, 1959) and the concentration of alcohol (v/v %) in the substrate was determined using an ebulliometer (Christy et al., 2021).

Analysis of Alcohol

GC/MS

Alcohol production from *C. demersum* substrate was injected into GC/MS for qualitative analysis using the Total Iron Conductivity model (TIC). The sample was filtered through an anhydrous sodium sulphate column and filtered effluent was directly injected into the GC/MS (Fini & Fattahi, 2021). The experimental setup involved employing the chromatographic conditions outlined in Table 1, while the temperature program utilized is detailed in Table 2.

Table 1: Chromatographic conditions for the GC/MS analysis.

GC Model	7890B GC System
MS	5975 inert MSD with triple axis Detector
Column	HP 5MS
Inlet	260 °C
AUX	320 °C

Table 2: Temperature program for GC/MS analysis.

	Rate	Temperature (°C)	Hold Time (min)	Total run time (min)
		50	1	1
Ramp 1	15	90	2	5.667
Ramp 1	10	300	2	28.667

GC-FID

The prepared samples were placed into the auto sampler carousel, transferred to the sample rack via a robotic arm, and injected into the column with the equipped auto injector. Ions were measured and displayed through the software as a chromatogram showing peak intensity per unit time. The carrier gas used in the mobile phase was ultra-high purity helium (Pourjabbar et al., 2020). Chromatographic conditions for the GC-FID analysis are described in Table 03.

Table 3: Chromatographic conditions for the GC-FID analysis.

GC Model	7890A GC System
Inlet	220 °C
Detector	250 °C
Inlet	260 °C
Column	DB 35MS
Temperature	40 °C (8 minutes)

Pre-treatment and Bioethanol Production

Physical pre-treatment and biomass preparation

The *C. demersum* substrate was collected from various freshwater bodies in different parts of the Northern Province of Sri Lanka. The collected substrates were cleaned, dried, chopped, and weighed (30 g). Then the substrate was dissolved in distilled water, and the container was autoclaved for 15 minutes at 121 °C.

Dilute Acid Pre-treatment

Six sets of conical flasks were prepared, each containing 100 ml of sulfuric acid with different concentrations ranging from 0.50 M to 1.75 M. Samples were then added to each of the conical flasks. Each flask was autoclaved and allowed to cool. Then the supernatant was centrifuged (8000 rpm for 15 minutes) and sodium hydroxide was used to neutralise the supernatant.

Biological pre-treatment and fermentation

The dilute acid pre-treated *C. demersum* supernatant 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. Afterward, *S. cerevisiae* was introduced into the supernatant, and fermented for a period of 5 days at room temperature in the fermentation broth, with agitation at 100 rpm. At regular intervals, samples were taken and the amounts of reducing sugar and alcohol content were analysed.

Optimization of Culture Conditions

The optimization process was conducted sequentially, with each variable optimized one after another. Once an optimized condition was determined, it was held constant for the subsequent optimization steps. The production of bioethanol from the *C. demersum* substrate was optimized using a combination of sulfuric acid and alpha-amylase enzyme hydrolysis followed by fermentation with *S. cerevisiae*. The optimization process involved varying the fermentation time (12-60 h), temperature (20-45 °C), rotation speed (50-250 rpm), and *S. cerevisiae* inoculum concentration (25-150 g/l). Samples were collected at regular intervals to analyze the alcohol contents.

Optimization of Media Composition

The screening process for the medium composition was done using the one variable at a time approach. A total of four media constituents, yeast extract (0-10 g/l), ammonium sulphate (0-10 g/l), magnesium sulphate (0-10 g/l), and potassium dihydrogen phosphate (0-20 g/l) were screened. The fermentation medium was added with the supernatant and allowed for fermentation using *S. cerevisiae*. The samples were collected and alcohol yield was determined.

Distillation

The distillation process was performed to collect alcohol and the sample was filtered. The fermented sample was distilled using a distillation unit and heated at 80 °C to get the alcohol. The filtrate was distilled using a distillation bath. Alcohol was distilled at 78 °C and water was distilled at 100 °C (Limayem & Ricke, 2012).

Study Antimicrobial Activity of bioethanol extract

Four different micro-organisms (bacteria and fungi), including *Pseudomonas aeroginosa* (ATC-27853C), *Escherichia coli* (ATCC-25922), *Staphylococcus aureus* (ATCC-25923), and *Candida albicans* (ATCC-1), were subjected to the agar well diffusion method to evaluate the antimicrobial properties of a bioethanol extract obtained

through distillation from *C. demersum* (Mandal et al., 2015). A sterile petri dish was filled with nutrient agar by pouring the appropriate amount and allowed to solidify. Fresh bacterial and fungal cultures (1.0 ml) were spread in the centre of each sterile petri dish, and 6.0 mm diameter wells were created using a sterile cork borer. Then, 100 µl of the bioethanol extract was added to each well, followed by incubating the Petri dishes for a duration of 24 hours at 37 °C. Following the incubation period, the clear zones' diameter around each well was measured to determine the extent of microbial growth inhibition. This measurement allowed for the evaluation of the extract's antimicrobial activity (Omar et al., 2013).

Statistical Analysis

The experiments conducted in this study were performed in triplicate to ensure the accuracy of the results, and the mean values were used to generate the graphs. Minitab 17.0 software was employed to perform the statistical analysis. A one-way ANOVA was employed to analyze the data, and significant differences were determined using Tukey's multiple comparison tests with a significance level of $p < 0.05$

RESULTS AND DISCUSSION

Optimization of Sulfuric acid Concentration and Bioethanol Production

The *C. demersum* substrate was pre-treated with different sulfuric acid concentrations and carried forward for the enzymatic hydrolysis process and the production of reducing sugar and alcohol yield was illustrated in Figure 1. When the sulfuric acid concentration was increased from 0.50 M to 1.00 M, the amount of reducing sugar was significantly increased and reached its peak at 1.00 M sulfuric acid concentration after which the amount of reducing sugar was significantly decreased until 1.75 M sulfuric acid concentration. Alcohol yield was significantly increased when sulfuric acid concentration was increased from 0.50 M to 1.00 M acid concentration. Afterward, the alcohol yield was drastically reduced from 1.00 M to 1.25 M acid concentration and then it was slightly decreased until 1.50 M. There was no alcohol yield was observed at 1.75 M sulfuric acid concentration. The optimum amount of reducing sugar and alcohol yield was observed at 1.00 M sulfuric acid concentration and which was chosen for further studies (Figure 1). Diluted acid pre-treatment using sulfuric acid is one of the most popular methods (Xue et al., 2009). Pre-treating the biomass with acid has the effect of releasing some of the fermentable sugars, making them more accessible to enzymes (alpha-amylase) during the subsequent hydrolysis process (Pandiyan et al., 2019). The macromolecules released after pre-treatment are subsequently hydrolyzed into simpler sugars by the enzyme alpha-amylase. These simple sugars can then be converted into ethanol through fermentation by *S. cerevisiae* (Zhang & Lynd, 2004). Increases in sulfuric acid concentration led to the charring or browning of the hydrolyzate at high sulfuric acid concentrations. Additionally, the formation of undesired by-products such as 5-dihydroxymethylfurfural and furfural, can be toxic to *S. cerevisiae* and inhibit fermentation (Mitiku and Hatsa, 2020). Sunaryanto et al. (2013) reported that bioethanol production of 7.98% (v/v) was produced from sago starch with a 2.5% H_2SO_4 concentration using α-amylase and dextrozyme DX enzyme.

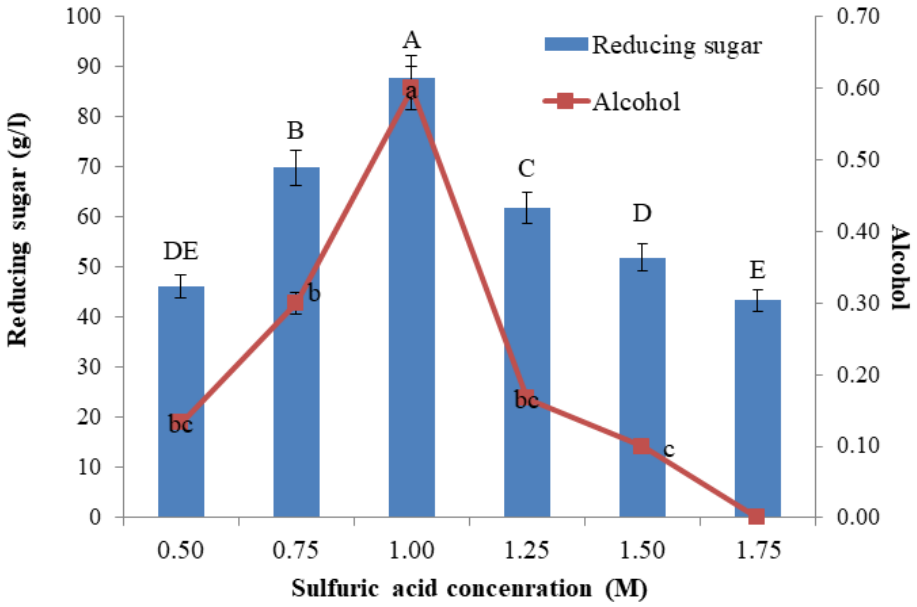

Figure 1: Optimizing alcohol production from *Ceratophyllum demersum* substrate using diluted acid and enzymatic pre-treatment: investigating the effect of different acid concentrations on yield. Different alphabets (A-E) (a-c) show significant differences between the mean values ($p < 0.05$).

Optimization of Culture Conditions

Optimization of the fermentation time

When the fermentation time was increased from 12 to 36 hours, alcohol yield was significantly increased and then alcohol yield was significantly decreased with *C. demersum* substrate using *S. cerevisiae*. Since, a significantly higher alcohol yield was observed at 36 hours of fermentation, this was chosen as the optimum fermentation time and used in further studies (Figure 2). Fermentation time directly has an impact on the growth of microorganisms. Shorter fermentation times result in inadequate *S. cerevisiae* cell growth, which ultimately results in a reduction in the amount of bioethanol produced. There will be minimal or no conversion of glucose to ethanol at the early stage of fermentation. The continuous reduction in ethanol concentration with increasing fermentation time may be due to ethanol having been lost through evaporation or its utilization by *S. cerevisiae* cells over time (Zabed et al., 2014). The co-culturing of amylolytic yeasts and *S. cerevisiae* in a starch medium resulted in a significant increase in bioethanol production, with a yield of 24.8 g/l observed after 48 hours of incubation (Verma et al., 2000).

Optimization of the temperature

When the temperature increased from 20 to 40 °C, alcohol yield was significantly increased and then alcohol yield started to drastically decline from 40 to 45 °C with *C. demersum* substrate. Since a significantly higher alcohol yield was obtained at 40 °C with the *C. demersum* substrate, this was chosen as the optimum temperature and used for further studies (Figure 3). Temperature is a crucial component that must be carefully regulated throughout the fermentation process, as it has a significant influence on the fermentation and enzymatic action. Higher temperatures not only create stress for micro-organisms to perform their metabolic activities but also form an unfavourable environment for their growth. Microbes produce heat-shock proteins in response to higher temperatures and inactivate their ribosomes (Phisalaphong et al., 2006). The behaviour was related to the enzymatic activity of *S. cerevisiae*, which was denatured by either too-low or too-high temperatures, which reduced the rate of fermentation and produced low bioethanol yields (Zabed et al., 2014). The higher bioethanol yield of 60 ml/l was achieved from sewage sludge broth at 10 days of incubation at a temperature of 30 °C by yeast (Manyuchi et al., 2018).

Optimization of the rotation speed

When the rotation speed was increased from 50 to 150 rpm, the alcohol yield was significantly increased and then it started to decrease with higher rotation speed. Since a significantly higher alcohol yield was obtained at 150 rpm with *C. demersum* substrate, this was selected for further studies (Figure 4). Effective agitation is an essential factor in facilitating the proper mixing of the various components within the fermentor and ensuring adequate mass transfer during the fermentation process (Rodmui et al., 2008), which is essential for the fermentation to proceed successfully. The agitation speed can improve micro-organism cell growth and efficiency. For yeast cell fermentation, between 150–200 rpm is the most regular agitation speed. A higher rate of agitation restricts the metabolic activities of the cells, leading to a decrease in ethanol production (Zabed et al., 2014). When the stem juice of sweet sorghum was used as a substrate, bioethanol production of 85.73% was observed at 200 rpm (Liu & Shen, 2008).

Optimization of the inoculum concentration

Increasing the concentration of *S. cerevisiae* inoculum from 25 to 100 g/l resulted in a significant increase in alcohol yield. Then alcohol yield started to decline with increasing *S. cerevisiae* inoculum concentration. Since a significantly higher alcohol yield was obtained at 100 g/l *S. cerevisiae*

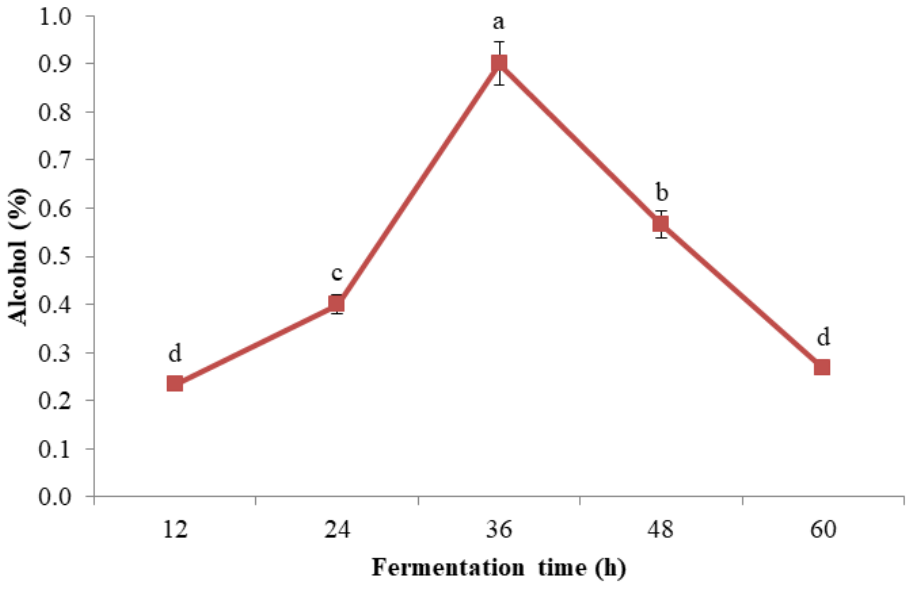

Figure 2: Changes in different fermentation times on the alcohol yield with *Ceratophyllum demersum* substrate using *Saccharomyces cerevisiae.* Different alphabets (a-d) show significant differences between the mean values ($p < 0.05$).

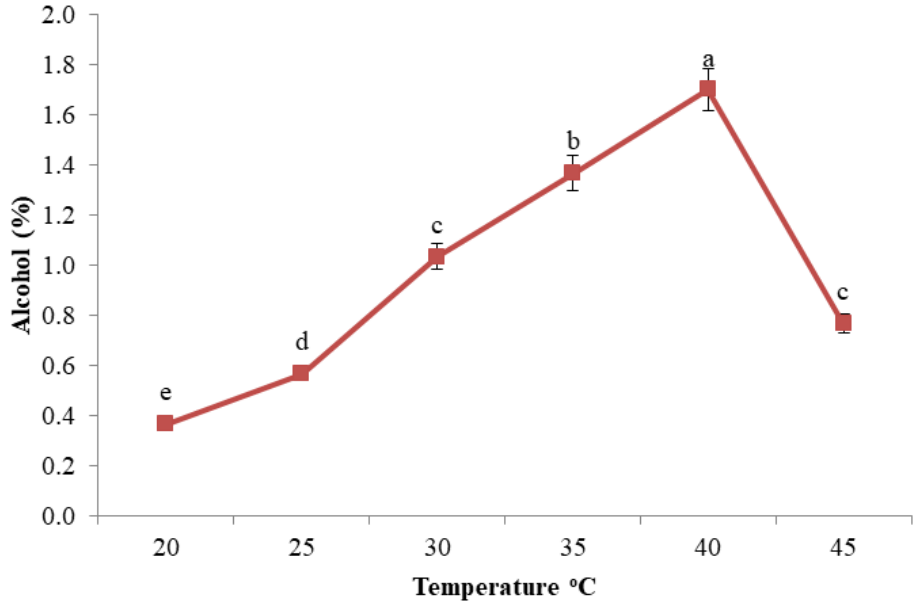

Figure 3: Changes in different temperatures on the alcohol yield with *Ceratophyllum demersum* substrate using *Saccharomyces cerevisiae*. Different alphabets (a-e) show significant differences between the mean values ($p < 0.05$).

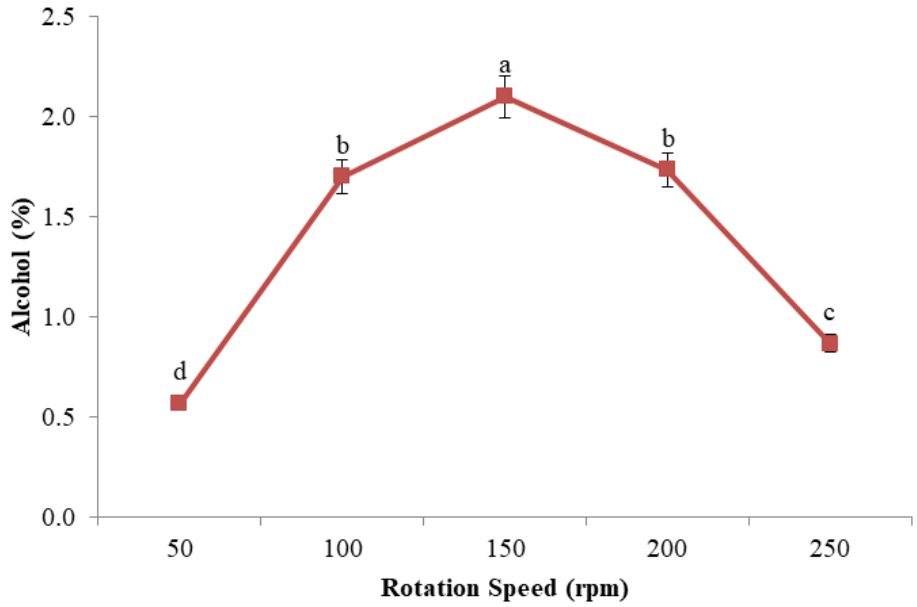

Figure 4: Changes in different rotation speeds on the alcohol yield with *Ceratophyllum demersum* substrate by *Saccharomyces cerevisiae*. Different alphabets (a-d) show significant differences between the mean values ($p < 0.05$).

inoculum concentration with *C. demersum* substrate, this was selected for further studies (Figure 5). *S. cerevisiae* was used as the inoculum for fermentation to produce bioethanol from *C. demersum* substrate. The bioethanol yield will be influenced by the inoculum concentration. The substrate's concentration was constant, an initial increase in *S. cerevisiae* inoculum concentration would result in an increase in ethanol concentration from an increase in the number of micro-organisms, which would cause ethanol production to reach a maximum. Thereafter, subsequent increases in *S. cerevisiae* inoculum concentration would result in a reduction in the bioethanol yield due to nutrient depletion (Hosny et al., 2016). Maximum ethanol yield was obtained with a concentration of 10% of inoculum size in sweet potato flour by coculture of *Trichoderma* sp. and *S. cerevisiae* (Swain et al., 2013).

Optimization of media composition

When the yeast extract concentration of the fermentation media was increased from 0 to 6 g/l, alcohol production was significantly increased with the *C. demersum* substrate. Further increasing the yeast extract concentration from 6 to 9 g/l resulted in a significant reduction in the alcohol yield. Since, no significant variation in the yeast extract

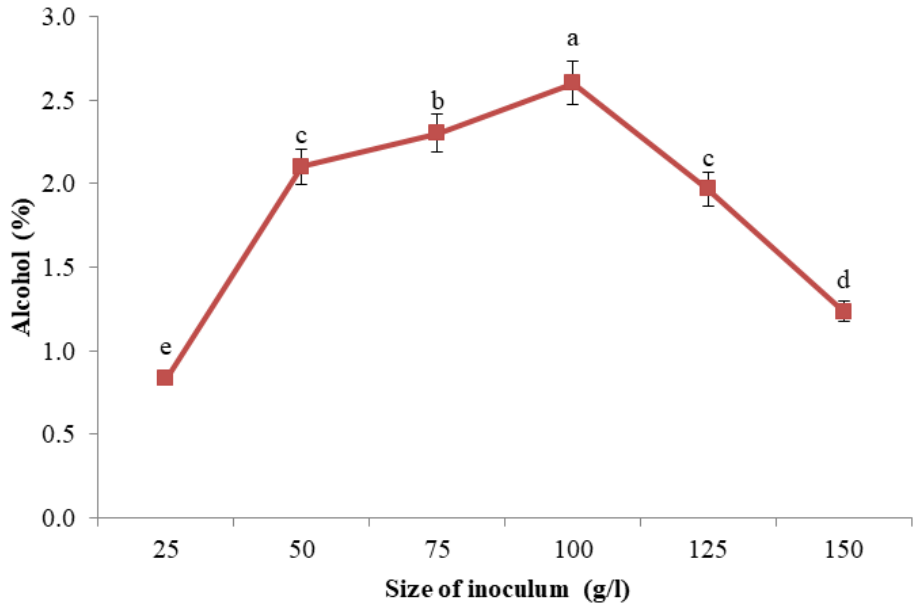

Figure 5: Effect of different concentrations of *Saccharomyces cerevisiae* yeast inoculum on alcohol yield enhancement from *Ceratophyllum demersum* substrate. Different alphabets (a-e) show significant differences between the mean values ($p < 0.05$).

concentrations was observed between the ranges of 9 to 10 g/l (Figure 6a). Since a significantly higher alcohol yield was obtained at 6 g/l yeast extract concentration with the *C. demersum* substrate, thus it was selected for subsequent studies.

When the ammonium sulphate concentration of the fermentation media was increased from 0 to 6 g/l, alcohol production was significantly increased with the *C. demersum* substrate. Further increasing the ammonium sulphate concentration from 6 to 10 g/l resulted in a significant reduction in the alcohol yield (Figure 6b). Since a significantly higher alcohol yield was obtained at 6 g/l ammonium sulphate concentration, thus it was selected for subsequent studies.

When the magnesium sulphate concentration of the fermentation media was increased from 0 to 5 g/l, alcohol production was significantly increased with the *C. demersum* substrate. Further increasing the magnesium sulphate concentration from 5 to 10 g/l resulted in a significant reduction in the alcohol yield (Figure 6c). Since a significantly higher alcohol yield was obtained at 5 g/l magnesium sulphate concentrations, thus it was selected for subsequent studies.

When the potassium dihydrogen phosphate concentration of the fermentation media was increased from 0 to 8 g/l, alcohol production was significantly increased with the *C. demersum* substrate. Further increasing the potassium dihydrogen phosphate concentration from 8 to 20 g/l, there was no significant difference was observed in the alcohol yield (Figure 6d). Since a significantly higher alcohol yield was obtained at 8 g/l potassium dihydrogen phosphate concentration, this was chosen as the optimum concentration.

Considering the results of the screening process, media elements such as yeast extract concentration, ammonium sulphate concentration, magnesium sulphate concentration, and potassium dihydrogen phosphate concentration showed a significant positive effect on the alcohol production with the *C. demersum* substrate. Essential cell nutrients are necessary for cell metabolism and growth. The intracellular activities will be impeded when one necessary nutrient is absent. Media components such as yeast extract, ammonium sulphate, magnesium sulphate and potassium dihydrogen phosphate are the nutrient sources that supply essential minerals and growth factors for yeast (Neto et al., 2005). The presence of yeast extract, which contains a lot of nitrogen, dramatically enhanced the amount of ethanol produced. When the nutrient concentration is too high, ethanol production decreases due to an excess of nitrogen sources. Nitrogen plays a vital role in industrial microbiology as it influences the enzymatic activities of both primary and secondary metabolism (Neto et al., 2005). Ammonium sulphate concentration, magnesium sulphate concentration, and potassium dihydrogen phosphate are nutritional components that might have an impact on the development and metabolism of yeast cells (Neto et al., 2005). Potassium dihydrogen phosphate contains phosphorus and plays a significant part in metabolic process which is started by substrate phosphorylation. This compound is also a constituent of ATP molecules, which are essential components of the cellular energy system. Ammonium sulphate can supply sulfur and it is necessary for the formation of proteins. Magnesium sulphate is an excellent source of sulfur and is highly recommended due to its additional supply of magnesium. Magnesium is necessary for maintaining the structural stability of specific enzymes and preventing the creation of vesicles on the outer membrane of cells. All the medium nutrients are essential to the metabolism of yeast (França & Rodrigues, 1985). According to research conducted by Martin et al. 2017, the

maximum concentration of ethanol (65 g/l) was achieved when *S. cerevisiae* was used to ferment sugarcane bagasse as a substrate. The optimal ethanol yield was achieved using a specific nutrient combination that included 12.5 g/l of yeast extract, 2.5 g/l of potassium dihydrogen phosphate, 1.25 g/l of ammonium sulphate, and 0.5 g/l of magnesium sulphate.

Analytical Method

GC/MS is one of the most effective methods to qualitatively analyse and identify the compounds present in a sample. After the extraction of the *C. demersum* the liquid extract was subjected to GC-MS analysis. GC-MS spectrum revealed the presence of several chemical components with different retention times. Using the mass spectrometer, the eluted compounds were analyzed at different times to identify their type and structure (Tatipamula et al., 2019). The chromatogram of compounds extracted from the *C. demersum* obtained by the GC/MS analysis is shown in Plate 1. There are several compounds identified in this *C. demersum* extract such as acetaldehyde (52.8%), diethyl acetal (38.5%), ethyl acetate (2.7%) isobutanol (1.6%), pentane, 1-(1-ethoxyethoxy), octanoic acid, ethyl ester and decanoic acid using Total Iron Conductivity (TIC) model. The level of bioethanol produced is significantly influenced by the distillation process used for purification. High distillation temperatures can cause the vaporization of water present in the fermentation product, resulting in a decrease in the concentration of bioethanol (Juliarnita et al., 2018). The results revealed that there are two peaks. One was the internal standard (1-propanol) and the other showed the identification of ethanol. An ethanol percentage of 84.9 was identified through the GC-FID method (Plate 2).

Antimicrobial Activity of bioethanol extract

The graph shows the inhibitory activity of an alcohol produced by the *C. demersum* substrate against four different micro-organisms, namely *S. aureus*, *E. coli*, *P. aeroginosa*, and *C. albicans*. The inhibitory activity is represented by the size of the inhibition zone, which is measured in millimetres (mm). According to the graph, *E. coli* showed the highest inhibitory activity with an inhibition zone of 16.4 mm, followed by *C. albicans* with 15.2 mm, *P. aeroginosa* with 13.8 mm, and *S. aureus* with 13.4 mm (Figure 7). Therefore, the graph suggests that alcohol is more effective against selected bacteria and fungi.

Perturbation of membrane permeability is consequent to its expansion and elevated fluidity by causing the inhibition of enzymes in the embedded membrane. Moreover, this causes disruption of biological membranes, destruction of electron transportation, and perturbation of the cell wall. Ethanol kills microbes through denaturation. Alcohol molecules bind with the fat membrane of microbes' cells, making the cells vulnerable to leakage and eventual death (Kapilan & Anpalagan, 2015; Ingram, 1990; Kapilan & Thavaranjit, 2009). Amanullah & Kabilan, 2021, studied that bioethanol extracted from papaya peel waste exhibited antibacterial properties, as evidenced by the inhibition zone observed against several bacterial strains including *E. coli, S. aureus, Pseudomonas spp.,* and *Bacillus spp.*

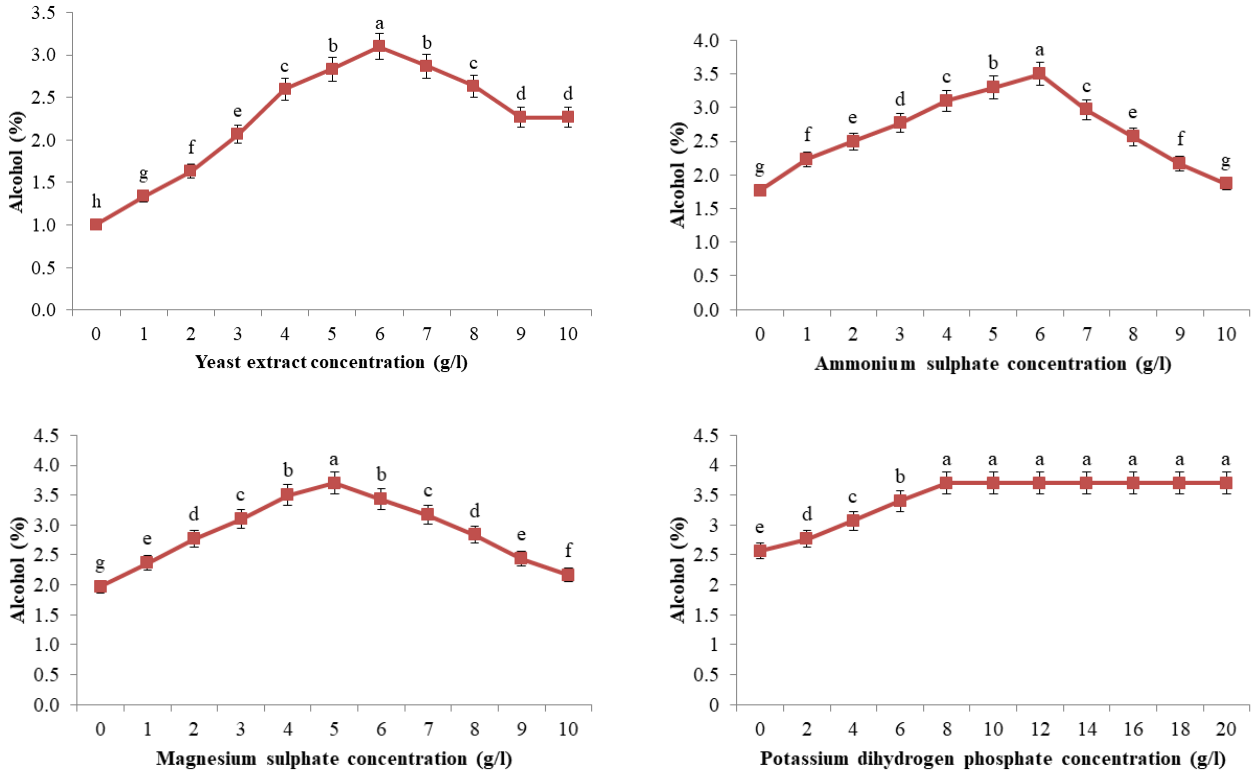

Figure 6: Effect of different quantities of yeast extract concentration (a), ammonium sulphate concentration (b), magnesium sulphate concentration (c) and potassium dihydrogen phosphate concentration on alcohol production from *Ceratophyllum demersum* substrate using *S. cerevisiae*. Different alphabets (a-h) show significant differences between the mean values ($p < 0.05$).

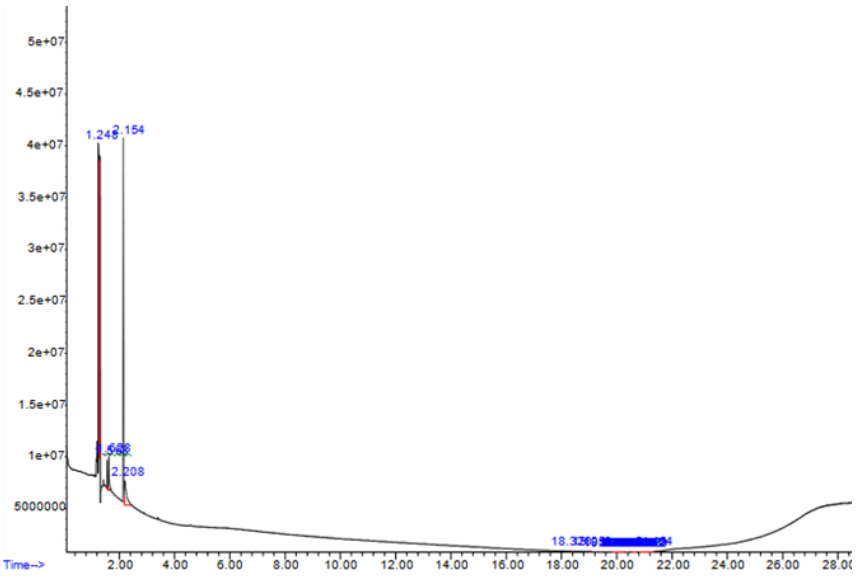

Plate 1: TIC model after the distillation of ethanol obtained from *Ceratophyllum demersum* extract sample.

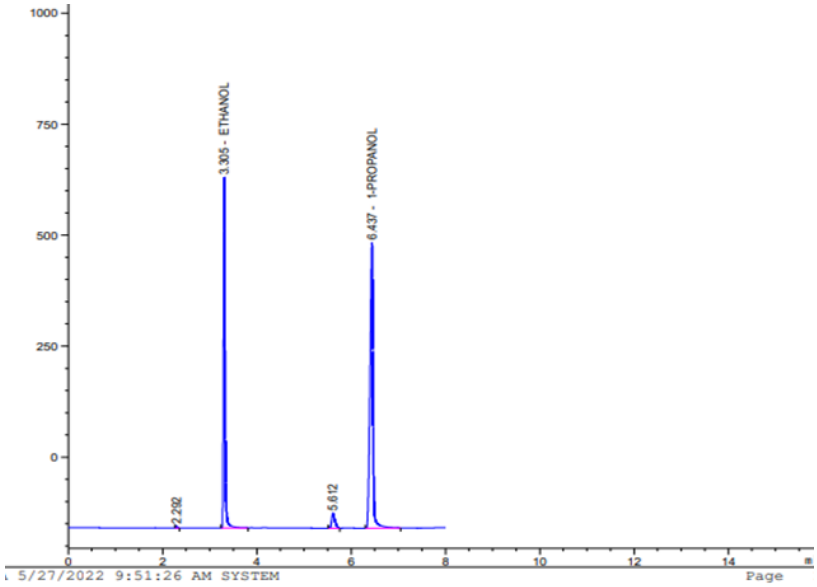

Plate 2: GC-FID chromatogram of ethanol extracted from *Ceratophyllum demersum* substrate

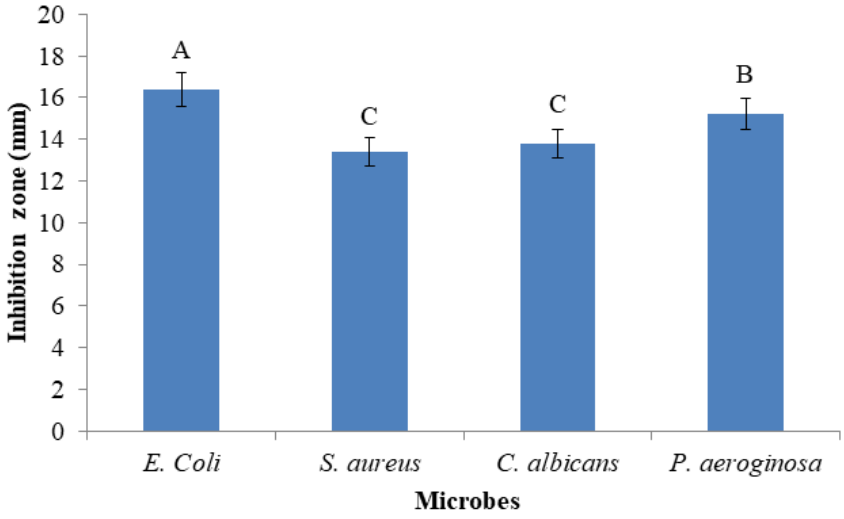

Figure 7: Antibacterial activity of ethanol against selected microbes (*Escherichia coli, Staphylococcus aureaus, Candida albicans and Pseudomonas aeruginosa*). Different alphabets (A-C) show significant differences between the mean values ($p < 0.05$).

CONCLUSION

The *C. demersum* substrate can be used as an efficient raw material for bioethanol production using *S. cerevisiae*. Bioethanol yield was significantly increased when the *C. demersum* substrate was pretreated with 1 M H_2SO_4, followed by a 1% alpha-amylase treatment and fermented by *S. cerevisiae*. After sequentially optimizing the culture conditions and media compositions in the following order: fermentation time (36 hours), temperature (40 °C), agitation rate (150 rpm), inoculum concentration (100 g/L), yeast extract concentration (6 g/L), ammonium sulphate concentration (6 g/L), magnesium sulphate concentration (5 g/L), and potassium dihydrogen phosphate concentration (8 g/L), a bioethanol yield of 3.7% was observed with the *C. demersum* substrate. The GC-FID method confirmed the presence of 84.9 % ethanol in the mixture, which is considered a satisfactory value for WHO standards for use as a hand sanitizer raw material. All the bacterial and fungal strains used in this study exhibited effective inhibition zones, indicating their sensitivity to the bioethanol extract. However, further studies are required to determine the antiviral properties of the bioethanol extract from the *C. demersum* substrate, and whether it can be used as a potential raw material for hand sanitizers.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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