Bioethanol Production from Coconut Fiber Wastes using *Saccharomyces Cerevisiae*

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Abstract - The principal energy source of the globe at present is non-renewable fossil fuels. With the arising of global dilemmas, the human population demands new energy sources. Coconut palm wastes have been identified as an important source of lignocellulosic biomass and it is underutilized in local industries, the accumulation of fiber has become a very problematic waste in Sri Lanka. Therefore, this study was aimed to determine the efficient coconut waste material for bioethanol production and to optimize the conditions for fermentation to enhance the yield. When the coconut husk fiber was inoculated with *Saccharomyces cerevisiae* (baker's yeast 2g/L) in the fermentation media (100ml, 8° Brix, Waste extract: distilled water = 1:3) composed of 10 g/L yeast extract, 10 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 2g/L peptone and 0.5 g/L $MgSO_4 \bullet 7H2O$ and allowed for fermentation for 24h at 30°C and 100rpm, the ethanol yield was 0.8% V/V. When different coconut palm wastes such as mature leaves (old leaves), young leaves (green leaves), young fruit fiber (kurumba), roots and husk fiber were used as the substrate with *Saccharomyces cerevisiae*, significantly higher quantities of bioethanol were produced with young leaves (green leaves) and husk fibers. However, coconut husk fiber was selected as a bioethanol source, for further studies due to its abundance and availability in the farms, slow natural degradation, and its role in providing a breeding ground for mosquitoes. The conditions were optimized sequentially by changing one factor at a time while keeping the other variables constant. When the fermentation time was optimized with coconut husk fiber the ethanol yield was significantly increased by 4 times at the 3rd day, than non-optimized conditions. When the amount of coconut husk fiber was increased by 2.5 times (12.5g/ 100 ml), bioethanol output was significantly increased by 6.4 times. Ethanol yield was significantly increased when 3.75g/ 100ml of yeast inoculum was used, compared to the non-optimized condition (1.25g/ 100ml). When the pH of the media was optimized as 4.8, significantly higher bioethanol yield was obtained, than the control (3.8). When the solution (V): air space (V) ratio was optimized to 1:1.3, bioethanol output was significantly increased by 6.6 times compared with the non-optimized condition (1:4). Large scale multi-centre fermentation study needs to be done in order to determine commercialization.

1. INTRODUCTION

Biofuel is a type of fuel that is produced through contemporary processes from biomass rather than by very slow geological processes. A higher percentage of the world's power requirement (electricity, transportation, and other energy sources) is basically met via fossil fuels. Coal, petroleum, natural gas is used for power generation through fossil fuels (Ayres *et al*., 2010). Usage of fossil fuel for energy generation provides not only affects human but also results in adverse effects by the gaseous emissions that are harmful to both the global living creatures and the natural environment. With the increasing human population, the usage of biofuels increasing in an exponential way (Menz *et al*., 2004).

Biofuels have the potential to reduce some undesirable aspects of fossil fuel production including exhaustible resource depletion, dependence on unstable foreign suppliers, and greenhouse gas emission. Therefore, they are considered carbon-neutral or greenhouse gas neutral (GHG neutral) fuels. Total CO_2 released

by the burning of biofuels can be completely absorbed by plants. Therefore, we can use biofuels as an alternative to fossil fuels. The main aim behind the bio fuel production is to replace traditional fuels with those made from renewable plant material or other feed stocks (Nikolopoulos *et al*., 2016). Replacing fossil fuels using biofuels has the potential to generate a number of benefits. Thus, the production and use of fossil fuels could be sustained indefinitely. Recently bioethanol has become one of the most prominent biofuels in the world as its production has been significantly increased all over the world, since the year 2000. Sugar and starch-based materials such as sugarcane and grains were used initially for the production of bioethanol (Dermirbas *et al.,*2009). Biofuels are categorized mainly by the type of feedstock that is used for biofuel production. First-generation biofuel production uses edible biomasses (sugar crops- sugar beet, sugarcane, starch crops- wheat, corn) as the carbon source (Amigun *et al.,* 2011) while the second generation biofuels use non-edible biomasses such as wood, straw, grass, waste as carbon source. There are some limitations in the second generation biofuel production which is related to the cost-effectiveness involved in scaling the production to a commercial level. For the production of third-generation biofuel, algal biomasses (macroalgae and microalgae) are used as feedstock. To achieve preferable hydrogen to carbon (HC) yield along with creating an artificial carbon sink to eliminate or minimize carbon emissions, genetically modified microorganisms are used as feedstock in the production of fourth-generation biofuels. These last two generations of biofuels are still in the early developmental stages (Alalwan *et al*., 2019). Bioethanol, biodiesel, biogas, bio methanol, and biohydrogen are some sub-categories of biofuels (Amigun *et al*., 2011, Amigun *et al*., 2008).

Motor vehicles are the major contributor to the level of environmental pollution where it emits approximately 70% of carbon monoxide and 19% of carbon dioxide globally (Balat*et al.,* 2009). Hence, transportation industries can utilize biofuels as a fossil fuel substitute. Therefore, biofuels can be utilized as a substitute for fossil fuels in transportation industries (Amigun *et al.,* 2008). Bioethanol is simply defined as ethyl alcohol or ethanol (CH_3–CH_2–OH), which are produced via biological processes that convert biomass (plant material or animal wastes) through biochemical processes, (Ex: hydrolysis and microbiological fermentation) (Balat *et al.,* 2008). Through the alcoholic fermentation of sucrose or simple sugars, which are produced from biomass, bio-ethanol can be derived (Edgard *et al*., 2005). It is a clear, colorless, flammable oxygenated hydrocarbon with a boiling point of 78.5ºC in the anhydrous state (Ding Teck et al., 2014). Bioethanol production of the world has been increased from 50 million m^3 in 2007 to over 100 million m^3 in 2012 (Kang *et al*., 2014, Saiful *et al*., 2020).

For the development of new technologies, biomass is an important strategic natural resource (Balat *et al.*, 2011). Among the natural biomass, lignocellulose residues are the most important which representing the most abundant sources of carbohydrates worldwide. The main importance of lignocellulose biomass is it does not compete with the food industry, and it has become an attractive substance for the production of bioethanol (Saha *et al.,* 2013). Coconut husk is very hard to degrade through natural degradative processes. (Carrijo *et al.,* 2002). Coconut husks disposed in open dumps, slopes, and landfills can be a major factor for the propagation of illnesses (e.g., Dengue) and unpleasant surroundings. In addition to that degradation of landscapes, creating stink are some examples. Methane gas is one of the most important greenhouse gas formed and coconut husk has become a huge environmental hazardous material when it is exposed to anaerobic conditions (Brito *et al*., 2004). Lignocellulosic substances are easily available from diverse natural environments as they are

wastes and therefore are really less expensive if purchased abundantly. The lignocellulosic biomass is made up of very complex sugar polymers and is not generally used as food source and they will never compete with food and feed sources (Croos *et al.,* 2019, Naik *et al.,* 2010; Vanniyasingam *et al.,* 2019). To avoid or reduce the impact of lignocellulose biomass on the environment and to add value to coconut husk, several alternatives have been proposed. Some of the alternatives include the use of coconut husk in agriculture (Carrijo *et al*., 2002), automobile industry (Costa *et al. ,*2001), civil construction (Pereira *et al.,* 2013), in the production of briquettes (Pimenta *et al*., 2015), enzymes and sound insulators (Senhoras *et al.,* 2004) and as a sorbent for heavy metals (Okafor *et al*., 2012).

There are several methods of pretreatment that can be divided into three categories, namely, chemical (alkaline, acid), physical, or a combination of both methods (thermal treatment and microwave-assisted-alkaline treatment) (Vivekanandaraja and Kapilan, 2021, Fernando and Kapilan, 2020, Mood *et al*., 2013, Rabelo *et al.,* 2009). For the enhancement of the enzymatic digestibility of lignocellulose materials, the pretreatment process is important (Chang, *et al.,* 2011).

Coconut palm is scientifically known as *Cocos nucifera L.* is a plant that can be found commonly in the tropics and growing more than 93 countries (Chan *et al*., 2006). "Tree of life" or "kapruka" are other terms which use to name coconut palm in Sri Lanka. Since the coconut palm is widely distributed throughout Sri Lanka, the wastes of coconut palm were chosen for the bioethanol production in this study. Initially, coconut husk fiber, young coconut (kurumba) fiber, coconut old leaves, coconut green leaves, and coconut root were selected as carbon sources for bioethanol production. Due to the excessive accumulation and difficulty in natural degradation, coconut husk fiber was selected for further optimization studies.

Therefore, the objective of the study was to select the part of the coconut palm waste that produces higher bioethanol yield and to optimize the culture conditions and media composition to enhance the ethanol yield.

2. MATERIALS AND METHODS

2.1 Chemicals and culture media
All the chemicals were obtained from standard sources. NaOH solution (5%) was used for the pre-treatment, α amylase enzyme (100g/L) and citrate buffer (50mM, pH 4.8) were used for the hydrolysis step. Basal medium containing 4 g/L yeast extract, 8 g/L KH_2PO_4, 4g/L $(NH_4)_2 SO_4$, 2g/L peptone and 4g/L $MgSO_4.7H_2O$ was prepared. After autoclaving, of conical flask containing 100ml media it was inoculated with 5g of *Saccharomyces cerevisiae.* (Commercial yeast, 50g/L).

2.2 Source of strain
Baker′s yeast (*Saccharomyces cerevisiae*) (mauripan- the commercial name) were purchased from Cargills food city, Jaffna and stored in the refrigerator.

2.3 Collection of samples
Coconut old leaves, coconut green leaves, coconut husk fiber, young coconut (kurumba) fiber, and old coconut roots were selected as substrates for ethanol production. They were collected from the coconut palms grown in Anuradhapura and Jaffna regions.

2.4 Substrate preparation
Old and young coconut leaves were collected from coconut palms and midribs (which are present at the middle of the leaf) were removed. Then they were cleaned with running tap water and allowed to air dry. Those cleaned leaves were cut into small pieces and were oven-dried at 50ºC, until a constant weight was obtained. Finally, they were ground and the fine powder was stored in plastic bottles separately. Coconut fiber from young fruits (Kurumba), old fruits, and old coconut roots were collected separately, and they were washed well using clean running

tap water and were air-dried. Then those substrates were cut into small pieces using a sharp knife and oven-dried at 50ºC until getting a constant weight. At the last step, they were ground well using a grinder and stored in plastic bottles separately (Gouveia *et al.,* 2009).

2.5 Production of biofuel and ethanol measurement

Physical digestion (size reduction): Cleaned, dried cut pieces of all five carbon sources were ground well-using grinder until a fine powder was obtained.

Alkaline pre-treatment: Five grams of each carbon substrate were added to 500ml conical flasks separately and 100ml of NaOH solution (5%) were added to aid the alkaline fermentation. Then autoclave them at 121ºC, 1atm pressure about 40min. Allow them to cool and filter the solid phase from the liquid phase. Wash the solid fraction with distilled water at room temperature until the pH becomes neutral (pH=7). Samples were stored in desiccators until their use (Gouveia *et al.,* 2009).

Enzymatic hydrolysis: Citrate buffer (350ml) was mixed with 3.5g of neutralized substrates (substrates which are obtained from alkaline pretreatment and oven-dried at 50ºC until gaining a constant dry weight). Citrate buffer solution (100ml) at separate conical flasks. Citrate buffer 350ml contains 60ml citrate buffer (50mM, pH 4.8), 38ml distilled water, and 2ml commercial enzyme (α- amylase). Then, those solutions were kept in an orbital shaker for 72 hours at 30ºC, 150 rpm for the enzyme activation. Then the liquid phase was collected by filtration (Shayanthavi and Kapilan, 2021).

2.6 Fermentation process
Three hundred milliliters of enzyme activated above solutions were added with 75ml fermentation medium in separate conical flasks and they were autoclaved at 121ºC, about 15min, 1atm pressure. Then those solutions were allowed to cool and 37.5 ml of pre-activated yeast inoculum was added and kept in

a shaker (at 100 rpm, 30ºC for 24 hours) for the fermentation. Oxygen limited (anaerobic) condition was provided by sealing the flask tightly by cotton plug and polythene, for the fermentation (Hahn- Hagerdal *et al.,* 1994).

2.7 Measuring the ethanol percentage
Fifty milliliters of each solution were taken to fulken tubes separately and they were centrifuged at full speed (3000 rpm). Then the supernatant was collected and the ethanol percentage was measured using an ebulliometer. This procedure for bioethanol production was carried out for five main carbon substrates selected from the coconut palm (Powell and Hill, 2013).

2.8 Analytical methods
Sugar concentration was measured by using the dinitrosalicylic acid method (Miller *et al.,* 1959). The supernatant obtained from the centrifugation was used to determine the bioethanol percentage (Wahab *et al*., 2005).

2.9 Optimization of conditions for bioethanol production

Selection of carbon substrate for optimizations: Old roots, green coconut leaves, coconut fiber, old coconut leaves, and young coconut fiber (kurumba) were subjected to alkaline pretreatment, enzymatic hydrolysis, and fermentation process and the amount of ethanol produced was determined. Optimization was done one after another in a sequence and once an optimized condition is fixed, then it was used as a constant for the rest of all optimization steps.

Optimization of time: After inoculation of activated yeast inoculum (50 g/L) into the final solution, (solution containing biomass which is already pretreated and hydrolyzed and mixed with fermentation medium) then it was incubated in an orbital shaker for five days at 100 rpm. Ethanol yield was determined at every 24 hours hour interval and the optimized time was determined.

Effect of amount of substrate: Different amount of coconut fiber (2.5g, 5.0g, 7.5g, 10.0g, 12.5g, 15.0g per 100ml) was taken in separate conical flasks. Then alkaline pretreatment, enzyme hydrolysis, and fermentation were carried out. Fermentation medium (75ml) and 37.5ml of activated yeast inoculum also were added. Then ethanol concentration was determined on the 3rd day (optimized time).

Effect yeast inoculum size: Coconut husk fiber (12.5grams) was pretreated with NaOH (5%) and then enzymatic hydrolysis was done.

(a)   (b)

(c)   (d)
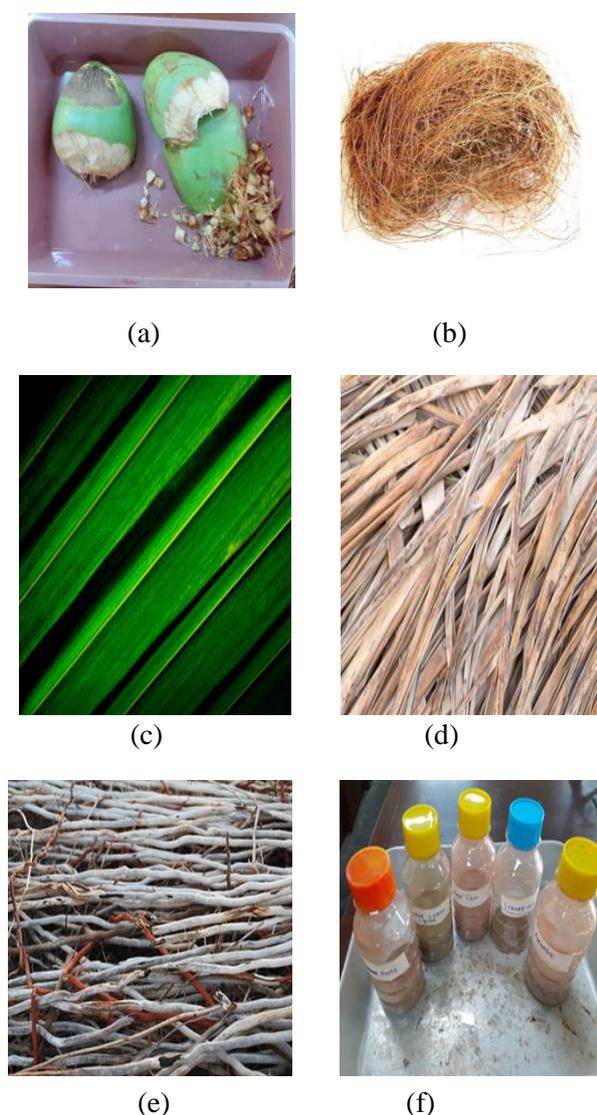
(e)   (f)

Figure 2.1: (a) Young coconut (Kurumba) fiber, (b) Coconut husk fiber, (c) Dried coconut leaves, (d) Green coconut leaves, (e) Old coconut roots, (f) ground carbon substrates in plastic containers.

Different amount of yeast inoculum (1.25g, 2.5g, 3.75g, 5g, and 6.25g / 100ml) was used for the preparation of the yeast activation medium and it was finally added with the enzyme hydrolyzed solution. Then ethanol concentration was determined on the 3rd day.

Optimization of pH: Alkaline pretreatment (5% NaOH) was done using 12.5g amount of coconut husk fiber. The pH values were changed as 2.8, 3.8, 4.8, 5.8, and 6.8 for citrate buffer solutions in each conical flask and enzymatic hydroxylation was carried out. Then fermentation was done with an optimized amount of (3.75g) of Baker's yeast and ethanol concentration was determined on the 3rd day.

Solution (v): free space (v) ratio optimization: Different fermentation solution: free space (v:v) ratios (1: 10, 1:5, 1:2.5, 1:1.6, 1:1.25, 1:1) were maintained at different conical flasks and those solutions were allowed to ferment at room temperature at 100 rpm. All the other conditions (carbon substrate- 12.5g, pH- 4.8, yeast inoculum size- 3.75g) were maintained at optimum, and ethanol quantity was determined on the 3rd day of fermentation.

Statistical analysis: All the experiments were done in triplicates. Statistical analyses were done using Minitab 18.0 version (Coffman *et al.,* 1972*).* The data were analyzed using one-way ANOVA. Tukey's multiple comparison test was used to determine the significant difference at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Effect of different substrates on bioethanol production

The amount of bioethanol produced was less than 0.2% after 24 hours of fermentation, when young coconut fiber (kurumba), coconut husk fiber, green and old coconut leaves were used individually as carbon sources for ethanol production (Under non-optimized conditions). From the old coconut roots 0.1 % ethanol yield
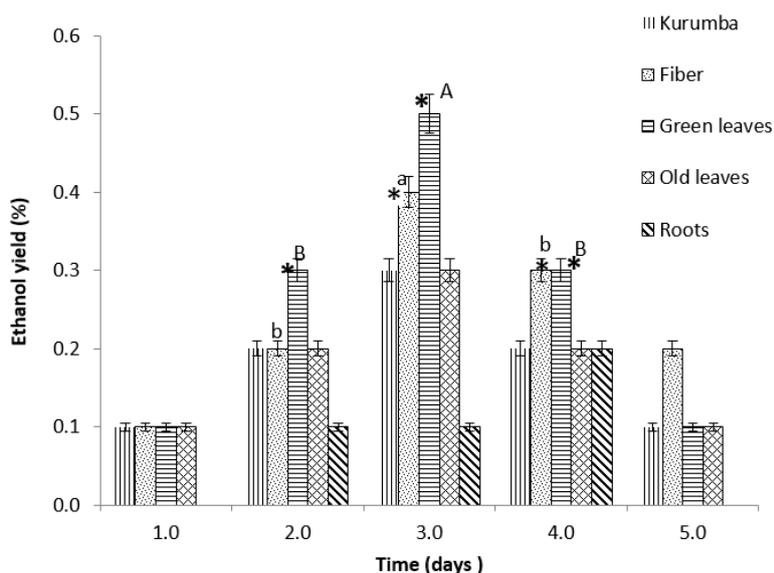
Figure 3.1: Effect of different coconut plant substrates on bioethanol production by *Saccharomyces cerevisiae*. Different letters (A, B and a, b) show significant differences between mean values of coconut green leaves and coconut husk fibers.

was obtained after 48 hours (2nd day) of fermentation (Figure 3.1). Next to the coconut green leaves, coconut husk fiber gave significantly higher bioethanol yield than the other substrates. The reason for the absence of ethanol at the end of 1st day may be due to a lack of saturated substances in old coconut roots. Higher bioethanol yield was obtained from green coconut leaves over the other substrates may be due to the capacity of green leaves to do photosynthesis and store the starch (Brito *et al*., 2004). However, coconut husk fiber was selected as the substrate for the further optimizations, because of the following reasons. 1. Easy to obtain from the environment (leaves, roots - hard) 2. Cheap. 3.Most problematic waste of coconut palm is coconut husk and it contains 80- 85 % of total fruit fiber. 4. Natural degradation is difficult and it takes nearly about 8 years, anaerobic digestion causes the emission of greenhouse gases such as methane. 5. Accumulation of fibers gives habitat for mosquito breeding (Dengue). 6. Harvesting leaves, roots and kurumba will affect the growth of the palm (Brito *et al*., 2004, Carrijo*et al*., 2002).

3.2 Effect of fermentation Time

When a hydrolyzed solution of coconut husk fiber was used to ferment at different time intervals (1-5 days), significantly higher ethanol yield was obtained on the 3rd day of fermentation (Figure 3.2). This optimization step increased the bioethanol yield by four times (from 0.1% to 0.4%). Since significantly higher bioethanol yield was obtained on the 3rd day of fermentation; the third day was selected as optimized time and used for future experiments. The fermentation rate was slower in the first two days as yeast needs some time to adapt to the media conditions. Later, significantly higher amount of ethanol yield was observed on the 3rd day than any other time. It may be due to the optimum conditions available for the growth of yeast on this day. Gradual reduction of bioethanol yield after 3 days may be due to the breakdown of glucose of the medium to ethanol (Olaniran*et al.,*2011).

The incubation period affects the growth of microorganisms. Short-term fermentation is inefficient because of the inadequate growth of microorganisms. Higher fermentation time

might lead to the accumulation of toxic substances in the microbe growing media especially in the batch mode where there are significantly higher concentrations of ethanol produced in the fermented broth (Vivekanandaraja and Kapilan, 2021; Nadir *et al*., 2009; Hossain *et al*., 2011).

3.3 Effect of amount of substratum

When different quantities of coconut husk fiber were used, significantly higher bioethanol yield (0.65% - six times) was obtained with 12.5g/100ml of husk fiber concentration, on the third day of fermentation (Figure 3.3). Therefore, 12.5 g of coconut fiber /100 ml was chosen as optimized substrate concentration and this was used in the further optimization experiments. At the end of this optimization step, the bioethanol production was increased by 6.5 times than the non-optimized conditions (from 0.1% to 0.65%). When the concentration of coconut husk fiber varies from 2.5g/100ml to 12.5g/100ml bioethanol production showed an exponential rise, taking more time for adaptation to the medium by yeast cells. At the same time, yeast cells will extensively multiply in the media under the existing conditions. Above the 12.5g/100ml, there was a small reduction in the ethanol yield. This drop of bioethanol may be due to the inhibition of ATP synthesis due to shortage of substrates, decrease in cell viability due to metabolically inactiveness as higher rate of ethanol was produced early and this led to the leakage of intracellular metabolites into the growth medium (Francois *et al.,* 2007).

Substrate concentration directly influences microbial cell growth, multiplication, and rate of fermentation. There is a direct relationship between initial sugar content with the fermentation rate. The increase of sugar concentration up to a certain level would lead to an increase in the rate of fermentation (Zabed *et al*., 2014). The use of excessive sugar concentration will lead to a steady-state of fermentation because of the elevated concentration of sugar usage is beyond the uptake capacity of the microbial cells (Laopaiboon *et al.*,2007).

3.4 Effect of different yeast inoculum concentration

When yeast inoculum was increased sequentially, the amount of bioethanol production was increased by six times from 0.1% to 0.6%. Significantly higher bioethanol yield was obtained when yeast inoculum was 3.75g/100ml than the non-optimized conditions (0.1%). Ethanol production showed an irregular trend with different concentrations of yeast inoculum (Figure 3.4) and this may be due to the mixture of different yeast varieties. Therefore, the yeast inoculum concentration was optimized as 3.75g /100 ml and it was used for further experiments.

Yeast activity rises up only to a point, where yeast cells reach their maximum efficiency. It is reported that the ethanol production was increased with the increase in initial yeast cell numbers from 1×10^4 to 1×10^7 cells /ml and no significant difference in ethanol production was observed between 10^7 and 10^8 cells/ml. Beyond this optimum concentration, yeast cells will start to die. Beyond a certain yeast cell concentration ability to show rapid growth will be inhibited due to the limitations of substances in the fermentation media that immediately consumes fed sugars producing ethanol (Torija*et al*., 2003).

3.5 pH optimization

When the pH value was 4.8, significantly higher bioethanol yield was obtained (Figure 3.5). Therefore, this pH (4.8) was selected as the optimized pH value for further studies. After optimizing the pH value, ethanol production increased by 6.7 times, compared to the non-optimized conditions. From pH 2.8 to 4.8 bioethanol production increased gradually and reached its maximum. Bioethanol yield started to decline beyond the pH value 5.8.
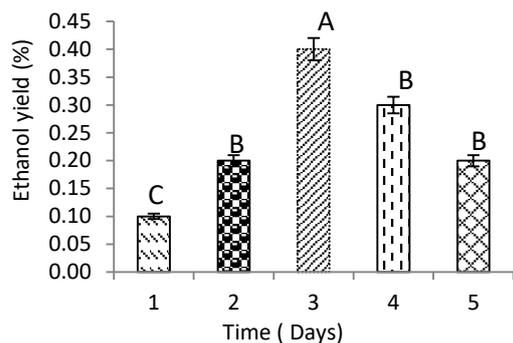
Figure 3.2: Effect of fermentation time on the bioethanol production from coconut fiber using *Saccharomyces cerevisiae*. Different letters (A, B and C) denote significant differences between the mean values.
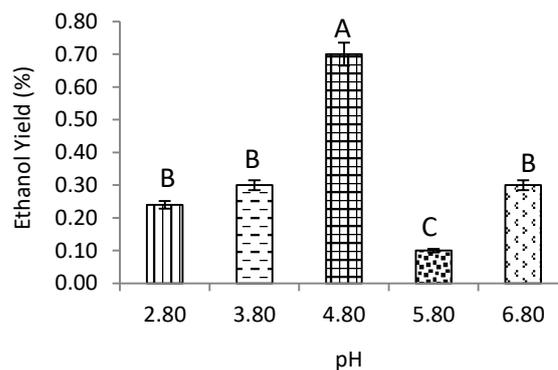

Figure 3.5: Effect of pH on the bioethanol production from coconut fiber using *Saccharomyces cerevisiae*. Different letters (A, B and C) denote significant differences between the mean values.
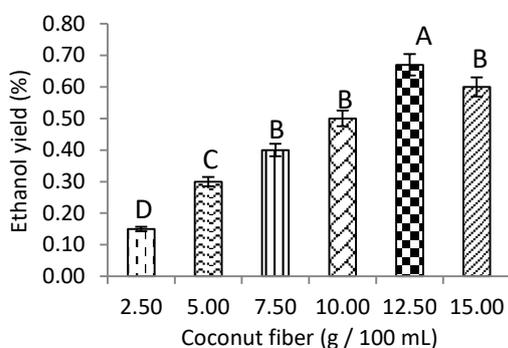

Figure 3.3: Effect of substrate concentration on the bioethanol production from coconut fiber using *Saccharomyces cerevisiae*. Different letters (A, B, C and D) denote significant differences between mean values.
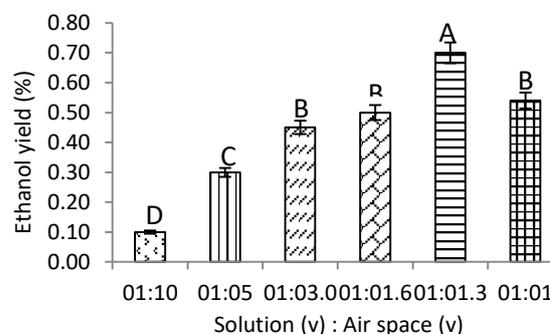

Figure 3.6: Effect of solution (v), free air space (v) ratio on the bioethanol production from coconut fiber using *Saccharomyces cerevisiae*. Different letters (A, B, C and D) denote significant differences between the mean values.
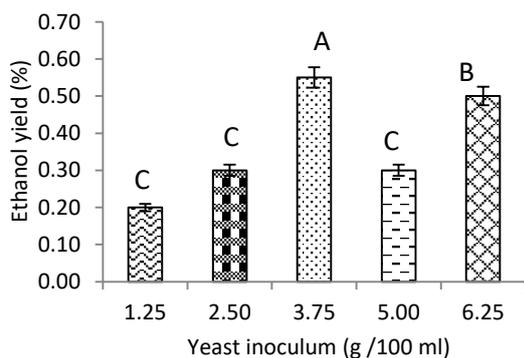

Figure 3.4: Effect of different yeast inoculum on the bioethanol production from coconut fiber using *Saccharomyces cerevisiae*. Different letters (A, B and C) denote significant differences between the mean values.

Bioethanol production is a process that is controlled by the pH of the fermentation medium as it has a direct influence on organisms as well as their cellular metabolism (Kasemets *et al.,* 2007). When the media of fermentation become more acidic, the rate of fermentation will increase. This may be due to the enzymes produced by yeast and their role in glucose fermentation leads to change the media into acidic. Yeast cells are more tolerant to acidic conditions than the basic conditions (Amanullah and Kapilan, 2021; Pirselova *et al.,* 1993).

3.6 Optimization of solution (v) and air space (v) ratio

When different fermentation solution (v) air space (v) ratio was maintained, significantly higher ethanol percentage was obtained with the solution volume was 400ml (solution (v): air space (v) = 1:1.3) (Figure 3.6). Therefore, this was considered as the optimized ratio. At this ratio, ethanol yield was increased by 6.6 times than the non-optimized conditions. This alcoholic fermentation converts sugars such as glucose, fructose, and sucrose into energy, ethanol, where carbon dioxide forms as by-products at anaerobic conditions (Waarde *et al*., 1993). During fermentation, an anaerobic environment is formed inside the conical flask due to the release of carbon dioxide as a byproduct.

4. CONCLUSION

Among the diverse coconut waste materials (coconut husk fiber, coconut green leaves, old leaves, coconut roots and young coconut (Kurumba) fiber), coconut husk fiber was the effective substrate for bioethanol production by fermentation by *Saccharomyces cerevisiae*. After the optimization of all the culture conditions and media composition, the bioethanol yield was significantly increased by 6.6 times with coconut husk fiber, compared to the non-optimized conditions. Large scale multi-center fermentation study needs to be done in order to decide whether this finding could be commercialized.

5. DECLARATION

The authors declare that they have no conflict of interest.

6. ACKNOWLEDGEMENTS

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