

Integration of Ensiled Corncob to Diluted Molasses as Carbon and Microbial Sources for Ethanol Production

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Abstract

To improve ethanol production, carbon and microbial sources were derived from corncob and molasses. Before investigating simultaneous saccharification and fermentation (SSF), the boiled corncob was vacuum-ensiled and the molasses was diluted. The optimal ensiling time of 4 days increased not only the amount of fermentable sugar but also the population of beneficial microorganisms. The proper dilution of molasses at a ratio of 1:3 molasses to boiled water reduced both the contamination problem and the inhibition of ethanol fermentation. The combination of the ensiled corncob and the diluted molasses could provide a substrate that is suitable for SSF without the addition of enzymes, supplements, or yeast strains. The substrate contained the optimal concentration of initial sugar (174.631 ± 0.975 g/L) and viable microorganisms ($1.9 \times 10^6 \pm 0.1 \times 10^6$ CFU/mL of total bacteria and $1.0 \times 10^5 \pm 0.1 \times 10^5$ CFU/mL of yeast) that could continue to hydrolyze and ferment carbohydrates in the substrate for the SSF. Using a ratio of 1:3 of ensiled corncob to diluted molasses, a pH of 4.5, and a temperature of 27 °C for 72 h, the SSF produced the highest concentration of ethanol at 93.345 ± 0.062 g/L. This work provides a cost-effective process that requires no enzyme or yeast, is simply technology, and is friendly to the environment, while the remaining solid fraction can be utilized as a suitable feedstock for biogas production.

Keywords: Corncob, Molasses, Vacuum-ensiling, Hydrolytic microorganisms, SSF, Anaerobic digestion, Ethanol

Introduction

Thailand currently (in 2022) produces commercial ethanol from only two primary raw materials: sugarcane (cane juice and molasses) and cassava, which are sugary and starchy materials that are easily converted into ethanol. However, production costs are high due to sugarcane and cassava price fluctuations and seasonal availability. Both sugarcane and cassava are also used as raw materials in numerous industries, especially the food industry [1,2].

Utilizing inexpensive or alternative feedstocks reduces the cost of ethanol production while avoiding competition for resources. Typically, they are lignocellulosic materials known as agricultural wastes or residues. The lignocellulosic materials have to be pretreated at a high temperature with a low pH, followed by enzymatic hydrolysis, which is a common technique for scattering the carbohydrates in the materials into sugars prior to ethanol fermentation. Or, the pretreated materials undergo simultaneous saccharification and fermentation (SSF) to prevent product-induced enzyme inhibition [3]. These processes are intricate and expensive, requiring enzymes, chemicals, and yeast strains. In addition, lignocellulosic hydrolysate typically contains insufficient sugar for commercial ethanol production, necessitating an expensive additional evaporation process to increase sugar concentration. Researchers have improved the production method to reduce costs by co-fermenting the hydrolysate and molasses to provide appropriate sugar [4].

The sweet corncob, which is a byproduct of the frozen sweet corn industry, is an agricultural residue with composition and quantity potentials for biofuel production. It is possible to cultivate sweet corn year-round in nearly every region of Thailand, where it is a popular economic crop in many nations. Thailand is the world's largest exporter of frozen and canned kernels of sweet corn. The volume of exports tends to increase annually [5]. In the production of frozen corn kernels, the corn fruit without its husk and silk is boiled before the kernels are separated and frozen [6], resulting in a large byproduct of corn-core or corncob, comprising 40 - 50 %w of the whole corn fruit. The corncob is just one of many habitats that harbor hydrolytic microorganisms [7], and the pretreated corncob is suitable as a feedstock in SSF for ethanol

production due to its high carbohydrate content and low lignin and hemicellulose contents, allowing it to be easily hydrolyzed into sugar [8].

Pretreatment is a crucial step in the production of lignocellulosic ethanol, as it disentangles the network of polymers in lignocellulose and facilitates enzymatic hydrolysis [9]. Ensiling is a commonly employed technology that, unlike other pretreatments of lignocellulosic materials, is able to destroy the structure of lignocellulose while keeping the nutrient component as intact as possible [10]. It's a green pretreatment that improves the efficiency of hydrolysis and ethanol fermentation through a series of complex microbial interactions [11]. The ensiling process is analogous to the hydrolysis stage of anaerobic digestion, caused by microorganisms that can grow in the absence of oxygen, for biogas production. The process of anaerobic digestion includes 4 distinct metabolic steps. Hydrolysis breaks down biomass into soluble carbohydrates, which are then converted into short-chain carboxylic acids and alcohol via acidogenesis; hydrogen and carbon dioxide are produced via anaerobic respiration. Acetogenesis and methanogenesis are metabolic processes that convert acetic acid into biogas [12]. In order to maximize the efficiency of hydrolysis and methanogenesis, it is necessary to partition the process into 2 distinct stages: Hydrolysis-acidogenesis and acetogenesis-methanogenesis. While the slightly acidic pH of 5.0 - 6.0 is ideal for the hydrolytic microorganisms, the neutral pH and mesophilic conditions (30 - 45 °C), optimized at 37 °C, in which the methanogenic species thrive, are ideal for them [13]. Moreover, vacuum impregnation's main mechanism is to displace gas in porous material with aqueous mixture by pressure [14], and the vacuum condition, that is empty of gas (completely anaerobic), can enhance the activity of the hydrolytic microorganisms, leading to better biomass hydrolysis [15]. Therefore, improved biomass hydrolysis can be achieved through ensiling under a vacuum at an ambient temperature of 26 - 30 °C.

Molasses is an annual byproduct of sugar factories that is produced in vast quantities. Typically, 1 ton of raw sugar results in 0.38 tons of molasses. Thailand is the world's third-largest exporter of molasses and a significant sugar producer [16]. The primary components of molasses are carbohydrates (40 - 70 %w, such as starch and sucrose), water, and nutrients, required by all microorganisms, including amino acids, nitrogen, minerals, and vitamins. For the growth and metabolism of microorganisms including bacteria, yeast and mold, molasses is an essential carbon source. Compared to other carbon sources utilized in the production of bioproducts, it is a low-cost raw material [17]. The biological transformation of molasses generates a diverse array of bioproducts with added value, such as bioethanol, biogas, fructooligosaccharides (FOSs), docosahexaenoic acid (DHA), and enzymes [18-20]. However, raw molasses has a complex chemical composition. This brown, viscous liquid has the potential to inhibit yeast activity. Although molasses is known to be carbon- and nutrient-sufficient, it is tainted with contaminants and wild microorganisms that may disrupt the ethanol fermentation. Pretreatments, through sterilization, centrifugation, and filtration before fermentation, have solved the contamination problem [4,21], but they raise the production cost. The only usable component of the pretreated molasses is carbohydrates, the additions of supplements and yeast strains are thus required. The carbon source, nitrogen source, and inhibitor all have a significant impact on microbial growth and fermentation performance [22]. Some studies, however, found no statistically significant difference in ethanol production between sterilized and non-sterile molasses [23]. To retain the essential nutrients, it is recommended that raw molasses not be pretreated by heating, sterilization, or fine filtration prior to fermentation. The contamination effect can be reduced by dilution of molasses and control of fermentation conditions, which can improve the overall function of microorganisms and make them more efficient for ethanol production.

Consequently, research into the use of corncob and molasses as carbon and microbial sources to enhance ethanol production should be conducted. It is possible to design a cost-effective manufacturing process by eliminating steps and avoiding the addition of enzymes and yeast strains. The vacuum ensiling of boiled corncob was investigated in order to obtain an ensiled corncob that is more easily hydrolyzed into sugar and fermented into ethanol and contains live microorganisms that support further hydrolysis/saccharification and fermentation. The dilution of raw molasses was also studied to reduce the effects of contaminants and determine the appropriate sugar concentration to be combined with the ensiled corncob to produce an SSF-suitable substrate. The SSF of the substrate obtained by integrating the ensiled corncob and diluted molasses without the addition of enzymes, supplements, or yeast strains was referred to as simultaneous saccharification and fermentation with native microorganisms (SSFNM). A conventional SSF is operated at 30 - 40 °C with an initial pH of 4.8 - 5.5 [4,24], whereas the growth of beneficial microorganisms, such as yeast, is optimal at 24 - 34 °C with a pH of 3.5 - 4.8 [25]. Therefore, the SSFNM was then studied at low temperatures of 25 - 35 °C to retain nutrients for supporting microbial growth and at acidic pH levels of 4.5 - 5.5 to activate the functions of beneficial microorganisms for saccharification and ethanol fermentation for 84 h. SSFNM variables were investigated in the desire to produce satisfactory ethanol.

Materials and methods

Ensilage of corncob

The boiled corncob was obtained from a buttered corn kernel shop in Hat-Yai District, Songkhla Province, Thailand. The sweet corn, without its husk and silk, was first boiled at 95 °C for 12 min. Its kernels were removed and seasoned with sweet butter as the commodity, and its corn core (corncob) was the byproduct used as the material for this study. This process is fairly similar to that of the frozen corn kernel production, which remains the boiled corncob as the byproduct [6].

The boiled corncob, which contained 75.5 %moisture, was crushed into 2 - 3 mm pieces and mixed with boiled water at a weight ratio of 1:3 corncob to water before being placed in an aseptic box. At an ambient temperature of 28 ± 2 °C, the box was hermetically sealed under vacuum. Ensiling was performed in triplicate, and after 1 to 10 days, samples were taken for analysis.

Molasses dilution

Molasses (with 72 %dry matter) was purchased from a molasses shop in Hat-Yai, Songkhla, Thailand. The molasses was diluted with boiled water before use.

Preliminary simultaneous saccharification and fermentation with native microorganisms (Preliminary SSFNM) at various molasses dilutions were conducted to consider an optimal dilution. The corncob ensiled with the optimal time, determined from subsection “Ensilage of corncob”, and a diluted molasses in weight ratios of 1:2 - 1:4 of molasses to boiled water, were mixed at 250 rpm, and a pH of each mixture was adjusted to 5.0. The preliminary SSFNM, which hydrolysis and fermentation happened by the metabolism or function of natural microorganisms, was investigated in a single fermentation flask (250 mL) without the addition of enzymes or yeast strains. Each flask was then filled with nitrogen gas to replace the air and sealed with an airtight lid, and carried out in triplicate by an incubator (WS300R, WIGGENS, Germany) under fixed predetermined conditions, e.g., at 30 °C with 150 rpm shaking for 48 h.

Simultaneous saccharification and fermentation with native microorganisms (SSFNM)

The ensiled corncob to diluted molasses as both carbon and microorganism sources, was referred to as SSFNM. The corncob ensiled with the optimal time, determined from subsection “Ensilage of corncob”, and the molasses diluted with the optimal dilution, determined from subsection “Molasses dilution”, were mixed, and delivered as a slurry. The SSFNM conditions were investigated at weight ratios of 1:2, 1:3 and 1:4 of the ensiled corncob to diluted molasses as slurries containing solid loadings of 16, 14 and 12 %. (%w dry basis), respectively, with pH levels of 4.5 - 5.5 and temperatures of 25 - 35 °C for 84 h. Each SSFNM proceeded similarly to the preliminary SSFNM described previously in the subsection “Molasses dilution”, with samples analyzed every 6 h. All experiments were carried out in triplicate, with averages and standard deviations reported.

Analytical methods

Before analysis, the sample was filtered through a 100-mesh cotton filter to obtain the liquid phase, and then through a 0.45 µm syringe filter to obtain the clear liquid.

A UV-Vis spectrophotometer (UV, HP8453 with Chem-Station software) was used to estimate total sugar concentration (sum of soluble sugars including glucose, fructose, and sucrose) using the modified phenol sulfuric method [26].

Gas chromatography (GC 6890 with auto injector, HP-FFAP polyethylene glycol TPA column, and flame ionization detector, Hewlett-Packard, USA) was used to determine the ethanol and acetic acid contents by feeding the injector at 240 °C and 9.9 psi, carrier gas helium flow rate 20 mL/min, and detector maintained at 240 °C. The flow rates for the combustion gases—hydrogen, nitrogen, and air—were 30, 30, and 300 mL/min, respectively. The oven temperature was set at 50 °C for 1 min, 180 °C for another minute, then held constant at 230 °C, and standard curves were prepared to estimate the concentrations of ethanol and acetic acid.

The yeast and mold, and total bacteria cell counts (in colony-forming units, CFU) were determined by the Department of Microbiology, Faculty of Science, Prince of Songkla University, in accordance with the FDA Bacteriological Analytical Manual (2001) and APHA (water) 2005. The total bacterial cells include lactic acid bacteria (hydrolytic bacteria), *Enterobacter*, which can hydrolyze carbohydrates to sugars before converting the sugars to lactic acid and ethanol, and acetic acid bacteria, *Acetobacter*, which can convert sugars to acetic acid in anaerobic conditions or ethanol to acetic acid in aerobic conditions.

Results and discussion

Ensilage of the corncob

The liquid fraction, of the mixture of 1:3 boiled corncob to boiled water, before ensiling had a total sugar concentration of 7.647 ± 0.935 g/L (at the beginning of ensiling). It indicated that the boiled corncob (solid part) contained some soluble sugars. This may be due to the partial starch in the corncob being degraded into soluble sugars during the boiling and crushing processes. Because hydrolysis of the starch, which uses water to break a bond in the molecule without requiring an enzyme, is easier than that of cellulose because starch dissolves in water but cellulose does not. In other words, because cellulose has a stronger structure than starch, it requires the use of an enzyme for dissolution [27]. During ensiling, hydrolytic microorganisms can produce cellulosic enzymes that break down partial cellulose into sugars such as glucose and cellobiose [13].

Ensilage (anaerobic digestion) involves strictly anaerobic microorganisms, and it is generally believed that oxygen acts as an inhibitor. To ensure that the hydrolytic microorganisms can grow and function properly, the hydrolysis stage must occur in an anaerobic condition, which is a vacuum at ambient temperatures (26 - 30 °C) [13]. Since the space (above the mixture) in the vacuum-sealed box was empty at the beginning, the resulting gases formed during ensiling were undoubtedly products of the native-microbial hydrolysis. In addition, the products of ensiling (the hydrolysis stage) are soluble sugars and acids (short-chain carboxylic acids), therefore, the hydrolysis activity can be easily determined by measuring the pH of the liquid fraction of ensiled corncob.

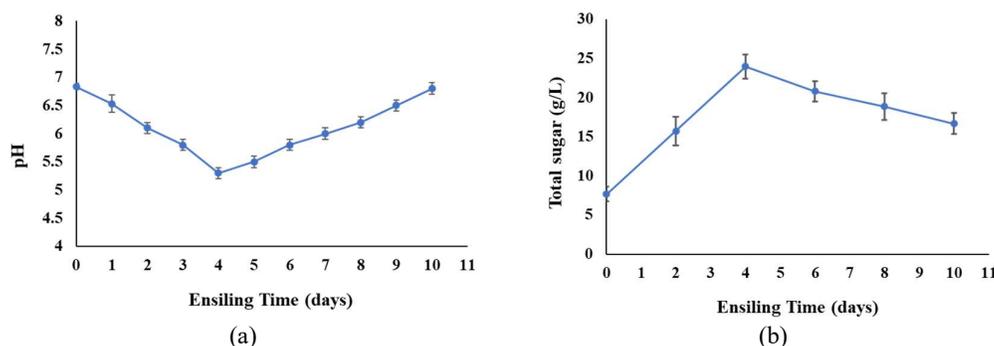


Figure 1 pH level (a) and total sugar concentration and (b) in the liquid fraction of ensiled corncob.

The pH level and total sugar (or soluble sugar) concentration, which is the most essential source of carbon for ethanol production, in the liquid fraction during the ensiling are depicted in **Figures 1(a)** and **1(b)**, respectively. From the experiments, gas formations, most likely hydrogen and carbon dioxide, started to be observed after 2 days of ensiling, and the pH started to be a slightly acidic after 3 days ($\text{pH} < 6.0$), indicating that the hydrolysis stage had already taken place. This could be confirmed by the increase in the concentration of total sugar in the liquid fraction of ensiled corncob.

As seen in **Figure 1(b)**, the total sugar concentration gradually increased during the first 4 days and then decreased. The higher levels of total sugar could be due to free sugars released during the decomposition of structural carbohydrates in the corncob, which could offset consumption by the hydrolytic microorganisms (mostly bacteria) [11]. The results indicated that 4 days of ensiling were suitable for the hydrolysis stage, yielding the greatest amount of total sugar (23.946 ± 1.529 g/L). After that, the pH increased slightly while the total sugar decreased significantly. This may be because methanogenic microorganisms, which consume the sugar and convert acids to biogas, begin to outcompete the hydrolytic microorganisms [28].

Before the ensiling, the boiled corncob (solid) contained $5.6 \times 10^3 \pm 0.1 \times 10^3$ CFU/g of total bacteria and $2.4 \times 10^3 \pm 0.1 \times 10^3$ CFU/g of yeast and mold, while the liquid fraction of the boiled corncob ensiled for 4 days contained $1.7 \times 10^5 \pm 0.1 \times 10^5$ CFU/mL of total bacteria and $2.8 \times 10^5 \pm 0.1 \times 10^5$ CFU/mL of yeast and mold. Thus, the ensiling not only increased the sugar concentration but also the number of microorganism cells. However, the sugar in the liquid fraction did not provide a sufficient initial sugar concentration for ethanol production. After 4 days of ensiling, if additional carbohydrates are added, the hydrolytic microorganisms may be able to continue their functions and increase the sugar concentration.

Consequently, the ensiled corncob should be combined with diluted molasses to serve as a satisfactory substrate for SSFNM.

Molasses dilution

Molasses must be diluted prior to use to reduce the contamination effect and be used to adjust the sugar concentration after mixing with the ensiled corncob in order to be a suitable substrate for the SSFNM.

Table 1 Components of molasses and their dilutions.

Component	Undiluted molasses	Molasses dilution with boiled water					
		Calculation			Analysis		
		1:2	1:3	1:4	1:2	1:3	1:4
Total sugar (g/L)	525.250± 2.350	175.083± 0.783	131.312± 0.588	105.050 ± 0.470	193.015± 3.520	157.481± 3.210	135.364± 2.980
Total bacteria (CFU/mL)	1.9×10 ³ ± 0.1×10 ³	NC	NC	NC	0.8×10 ⁴ ± 0.1×10 ⁴	0.5×10 ⁵ ± 0.1×10 ⁵	0.7×10 ⁵ ± 0.1×10 ⁵
Yeast and mold (CFU/mL)	< 10	NC	NC	NC	0.6×10 ³ ± 0.1×10 ³	0.7×10 ⁴ ± 0.1×10 ⁴	1.3×10 ⁴ ± 0.1×10 ⁴
Acetic acid (g/L)	0.130±0.009	0.043±0.003	0.032±0.003	0.026±0.002	0.062±0.003	0.057±0.003	0.059±0.006

Values are mean ± standard deviation for n = 3.

NC; can Not Calculation.

Table 1 shows the compositions of molasses and their dilutions, indicating that the compositions of diluted molasses cannot be calculated. This is because dilutions are water additions with some dissolved oxygen that are required for microorganisms. Raw molasses has a low water content (about 20 %w), which distinguishes it from juice or sap. This makes microbial growth difficult, and the high sugar concentration causes osmotic stress. While involved in a hydrolysis reaction that breaks bonds in carbohydrate molecules using water, sufficient water in diluted molasses with sufficient dissolved oxygen supports adequate microbial growth as well as microbial functions by providing hydrolytic enzymes. Thus, the higher total sugar concentration, which may be obtained from the hydrolysis of the partial carbohydrates in the more diluted molasses, demonstrates this. The acetic acid content, an important inhibitor, in the analyzed diluted molasses is also higher than the calculation. This meant that the dilution could support the activity of microorganisms, of which yeast can ferment sugar to ethanol and bacteria can oxidize ethanol to acetic acid. As a result, the analyzed sugar and acetic acid concentrations, as well as microorganism counts, are higher than those calculated for all dilutions.

Comparative SSFNM performances of different diluted molasses (The preliminary SSFNM) were evaluated using 14 %solid loading (%w dry basis), an initial pH of 5.0, and a temperature of 30 °C for investigating an optimal dilution (**Table 2**). The total sugar concentration in the various molasses dilutions reduced the ethanol concentration while increasing the sugar concentration to more than 174.631 ± 0.975 g/L. This could be because a high sugar content substrate inhibits yeast growth and fermentation to ethanol, and this inhibition is caused in part by osmotic pressure. When the sugar concentration exceeds 14 %w (about 160 g/L), plasma cells form and inhibit glycolysis enzyme activity [29]. In addition, acetic acid concentration increased as dilution increased due to increased dissolved oxygen content and bacteria cells, including *Acetobacter*.

As shown in **Table 2**, the highest ethanol was produced after 48 h by diluting 1:3 molasses with water. Hence, this dilution provided a suitable initial sugar concentration for the preliminary SSFNM, and the content of dissolved oxygen supporting microbial growth and causing the acetic acid had no negative effect, which could explain the increased yeast growth and ethanol productivity.

Table 2 Comparative SSFNM using the slurry having 14 %solid loading with a pH of 5.0 at 30 °C after 48 h for the preliminary SSFNM.

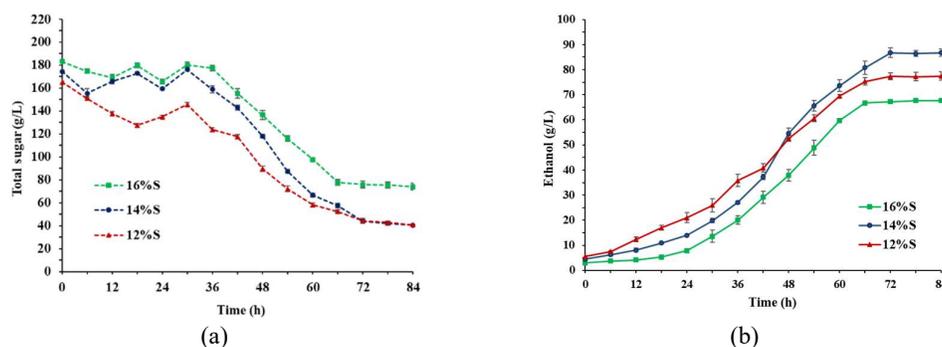
Parameter	Molasses dilution with boiled water		
	1:2	1:3	1:4
Initial total sugar (g/L)	212.396 ± 1.232	174.631 ± 0.975	154.493 ± 0.888
Ethanol (g/L)	40.983 ± 1.914	54.654 ± 1.982	54.468 ± 1.876
Productivity (g/L.h)	0.854 ± 0.040	1.139 ± 0.041	1.135 ± 0.039
Yeast (CFU/mL)	7.9×10 ⁴ ± 0.1×10 ⁴	1.0×10 ⁵ ± 0.1×10 ⁵	9.8×10 ⁴ ± 0.1×10 ⁴
Total bacteria (CFU/mL)	1.4×10 ⁶ ± 0.1×10 ⁶	1.9×10 ⁶ ± 0.1×10 ⁶	2.1×10 ⁶ ± 0.1×10 ⁶
Acetic acid (g/L)	0.912 ± 0.078	1.264 ± 0.082	1.481 ± 0.086

Initial total sugar is total sugar concentration in the liquid fraction of the slurry before the preliminary SSFNM.

Values are mean ± standard deviation for n = 3

SSFNM

The viscosity of the slurry is critical for achieving high ethanol levels. A high solid loading generates the viscosity, which must be reduced prior to fermentation, and the mixing ratio becomes a significant challenge [30]. The slurry using 1:3 molasses dilution was subjected to SSFNM in order to increase ethanol concentration at high solid loadings. This was performed at solid loadings of 12 - 16 % to investigate ethanol production with an initial pH of 5.0 at 30 °C (**Figure 2**).

**Figure 2** Total sugar concentration (a) and ethanol production (b) during SSFNM with a pH of 5.0 and 30 °C, of different solid loadings (%S).

At these solid loadings, total sugar concentration appeared to be consumed as well as accumulated (**Figure 2(a)**). This means ensuring that the native microorganisms can grow and function in the slurry for SSFNM. The sugar accumulation was increased by increasing the solid loading. This was attributed to microbial growth adapting the environment for further metabolism during the initial period. Following that, the rate of sugar accumulation decreased, as increased the rate of ethanol accumulation. It was consistent with previous research by Ishola *et al.* [25], who suggested that a high solid loading could be used to control microbial functions, favoring ethanol yields.

As shown in **Figure 2(b)**, at the start of the SSFNM, the microbial cells begin to adapt to the slurry conditions by using the sugars and nutrients present and producing enzymes and other attributes required to adapt to the environment. For yeast, it should normally pass through the adaptation phase and begin primary fermentation within a few hours [25]. Once the yeast's cell walls are permeable, it can begin metabolizing sugars in the slurry as food, using anaerobic metabolism to convert the sugars to ethanol (fermentation). At high solid loadings of 14 and 16 % in a range of 0 - 12 h, the adaptation phase was similar, as was the trend in ethanol concentration.

The ethanol accumulation at 12 %solid loading during 42 h was higher than that of at other loadings, but it was lower after that. This could be because the sugar concentration in the liquid fraction was suitable for ethanol fermentation during the first 42 h. Following that, the accumulation of less sugar caused the

sugar concentration to fall below the appropriate range, lowering the fermentation rate. As a result, the carbon source at 12 %solid loading was insufficient to reach a desirable ethanol concentration.

However, the decrease in ethanol accumulation caused by increasing the solid loading was greater than 14 %, owing primarily to the difficulty of mixing as a result of poor mass transfer in such viscous slurries [31]. Additionally, the residual total sugar after 72 h for SSFNM with the solid loading of 16 % was the highest (75.835 ± 2.968 g/L), compared to 43.939 ± 1.557 g/L for 14 % and 44.396 ± 2.243 g/L for 12 %, indicating that the existing was difficult for mixing, which limited yeast viability and resulted in a reduction in ethanol accumulation. Moreover, the colony forming unit of living yeast cell dropped to $2.2 \times 10^2 \pm 0.1 \times 10^2$ CFU/mL at 16 % solid loading after 72 h, which was the lowest compared to $9.2 \times 10^3 \pm 0.1 \times 10^3$ CFU/mL at 14 % and $7.8 \times 10^3 \pm 0.1 \times 10^3$ CFU/mL at 12 %solid loading, whereas total bacteria expanded to $2.5 \times 10^6 \pm 0.1 \times 10^6$, $2.0 \times 10^6 \pm 0.1 \times 10^6$ and $8.3 \times 10^5 \pm 0.1 \times 10^5$ CFU/mL at solid loadings of 16, 14, and 12 %, respectively. These confirmed that the 14 %solid loading, which yielded the greatest concentration of ethanol for a time longer than 48 h (Figure 2), was suitable for the SSFNM. This loading may provide an acceptable viscosity, resulting in a workable osmotic stress that efficiently supports both bacteria for sugar accumulation and yeast for ethanol accumulation.

Furthermore, the genuine parental strains of natural hybrid microorganisms that lived in the slurry were unknown. To improve the functionality of the natural hybrids, SSFNM conditions were to be investigated by varying the pH and temperature, which are unquestionably important environmental factors in the survival, growth, and function of all microorganisms.

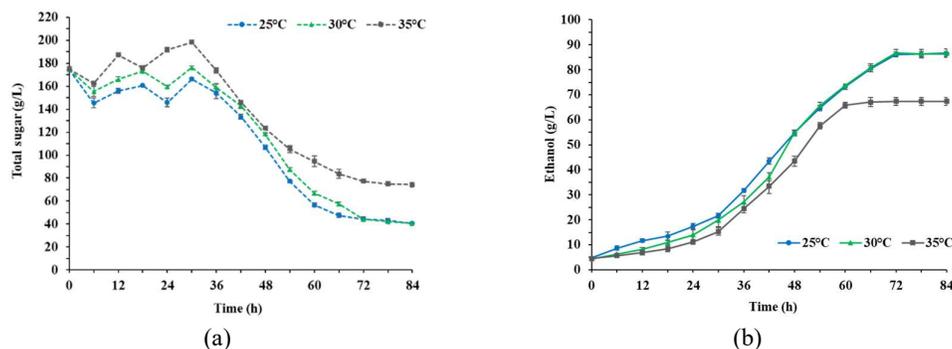


Figure 3 Total sugar concentration (a) and ethanol production (b) during SSFNM, at 14 %solid loading with a pH of 5.0 at different temperatures.

Temperature increases, as shown in Figure 3, increase sugar accumulation while decreasing ethanol accumulation. Higher temperatures cause carbohydrate hydrolysis into sugars with greater solubility, while yeast activity may decrease. After 12 h, there was an increase in sugar consumption and ethanol production at lower temperatures. Moreover, there was no improvement in ethanol accumulation at 35 °C after 66 h of SSFNM, whereas ethanol accumulations at 25 and 30 °C continued to increase until 72 h. These results suggested that the SSFNM should be conducted at a temperature lower than 35 °C, and 25 °C after 72 h could achieve the highest concentration of ethanol at 14 %solid loading and a pH of 5.0.

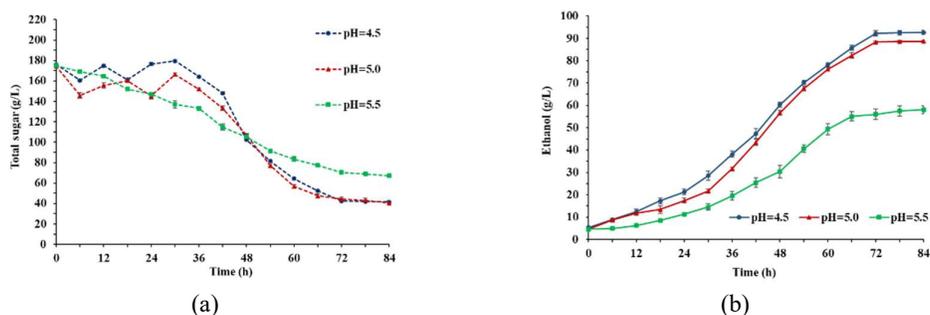


Figure 4 Total sugar concentration (a) and ethanol production (b) during SSFNM at 14 %solid loading and 25 °C with different initial pH levels.

As depicted in **Figure 4**, the pH has an effect on both sugar and ethanol accumulations. A more acidic slurry (pH is less than 5.0) leads to the dominance of yeast and *Enterobacter*, namely *Lactobacilli*, which can produce ethanol. The *Enterobacter* are effective at pH levels between 4.0 and 5.0, and the SSFNM with a pH of 4.5 could promote them to the saccharification and fermentation [13]. Additionally, the initial pH is crucial for batch fermentation without pH control. Organic acid production and carbon dioxide (fermentation byproducts) diluted in broth cause a decrease in pH, and an uncontrolled pH may reduce ethanol production [32]. However, ethanol production could potentially occur if the pH does not fall below 4.0 [33]. At the end of the experimental period (72 - 84 h), the results shown in **Figures 2 - 4** indicate that the yeast was inhibited by these conditions. There was no improvement in the accumulation of ethanol for times exceeding 72 h, during which the pH levels dropped to approximately 3.9 (for all initial pH levels).

Optimization based on ethanol concentration

As depicted in **Figures 2 - 4** it is evident that 14 %solid loading and 72 h are sufficient to achieve a satisfactory ethanol concentration. However, additional optimization was still necessary. Temperatures between 25 and 30 °C yielded roughly similar ethanol concentrations (**Figure 3**), and more acidic conditions (pH less than 4.5) may be preferable (**Figure 4**). Therefore, the temperature and pH level at which the highest ethanol concentration was achieved should be thoroughly investigated.

Using a central composite design (CCD) with 2 factors, 11 total experiments (8 distinct cases and 3 replicates of the central point) were conducted to investigate the effect of ethanol concentration. The pH levels were 3.8, 4.0, 4.5, 5.0 and 5.2, and the temperatures were 23, 24, 27, 30 and 31 °C. Based on the designed conditions, the ethanol concentration ranged between 61.894 ± 0.010 g/L and 93.345 ± 0.062 g/L in the 11 runs, which was comparable to the previously mentioned results, indicating that the designed range for SSFNM conditions was reasonable.

The regression equation was derived by optimizing the SSFNM condition with response surface methodology (RSM) for Design Expert 12 software. The reduced quadratic model in terms of actual factors describing the ethanol concentration as a function of pH and temperature (°C) was listed in the Eq. (1).

$$\text{Ethanol (g/L)} = -1,336.075 + 477.281\text{pH} + 23.178\text{Temperature} - 51.152\text{pH}^2 - 0.423\text{Temperature}^2 \quad (1)$$

The model was deemed significant ($p < 0.002$) with $R^2 = 0.927$ based on the ANOVA results. At 14 %solid loading for 72 h, pH levels (3.8 - 5.2) had the most significant effect ($p < 0.05$), whereas temperatures (23 - 31 °C) had the least significant effect.

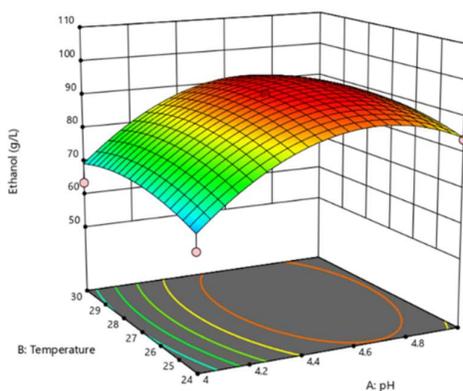


Figure 5 Response surface for ethanol concentration as a function of pH and temperature at 14 % solid loading for 72 h.

The RSM plot from the model Eq. (1) is shown in **Figure 5**. When the pH was less than 4.5 and the temperature was higher than 27 °C, there was no increase in ethanol concentration. It revealed that the clear optimum occurred at pH 4.5 and 27 °C. This was a reasonable requirement; because SSFNM was taken for 72 h, it should be conducted at low temperatures to preserve nutrients, and it is an optimal condition that supports both hydrolytic microorganisms and fermenting yeast but not methanogenic species (the neutral pH at 30 - 45 °C). Under the optimal condition, the highest concentration of ethanol was 93.345 ± 0.062 g/L, which was higher than the value discovered by Ishola *et al.* [25], for chemically pretreated hardwood lignocellulosic biomass via SO_2 impregnation at 18 bars and 215 °C for 5 min prior to delivery as a slurry.

After SSF processed the 10 %solid loading slurry with the addition of enzymes and yeast, this reached the highest concentration of ethanol (42.6 g/L). While this work produced less ethanol than that reported by Pandey *et al.* [4], relating to the integration of acid pretreated paddy straw hydrolysate into molasses as a diluent, it enhanced ethanol production. The hydrolysate was obtained through acid pretreatment followed by enzymatic saccharification. The molasses was heated at 80 °C for 2 h, centrifuged, diluted, and filtered before being mixed with the hydrolysate as the substrate for SSF at 40 °C for 72 h. This gave the highest ethanol of 104.2 g/L. However, the maximum ethanol of 93.345 g/L obtained from this work was considered high for a production with fewer steps, low operating conditions, friendly to the environment, and no addition of chemicals, enzymes, or yeast, which had never been published before. Furthermore, this concentration meets the requirement for commercial ethanol production (> 63.2 g/L) [34].

As a result, this work entails the production of bioenergy (bioethanol) utilizing genuine biological processes at all stages, including pretreatment and SSF. By controlling the conditions favorable for natural microbial activity, the corncob and molasses, which are both sources of nutrients and microorganisms, can be utilized economically. The ethanol production from the integration of the ensiled corncob and the diluted molasses as the substrate in SSF provides the following advantages: Inexpensive materials; no addition of enzymes, supplements, or yeast strains; no evaporation or sterilization step; low operating conditions; no requirement for high-pressure equipment; and straightforward technology. These are environmentally friendly and offer savings on investment, energy, and production costs. Furthermore, the remaining solid fraction after the liquid fraction has been extracted contains a high concentration of carbohydrates and useful microorganisms. Future work can utilize this as a suitable feedstock for biogas production, thereby simplifying the production process. Because it is a material that does not require pretreatment or the hydrolysis stage, biogas production may begin directly with the acetogenesis-methanogenesis.

Conclusions

The results indicated that the integration of corncob ensiled under vacuum conditions and diluted molasses could provide a substrate with sufficient sugar and useful natural microorganisms for SSF, which produces satisfactory ethanol without the addition of enzymes, supplements, or yeasts. This resulted in a high ethanol concentration that meets the specifications for industrial ethanol production.

Therefore, simple and cost-effective ethanol production from the integration could be competitive with ethanol production from conventional feedstocks, such as sugarcane juice and cassava starch. Noticeably, even if the SSFNM had no need for external supplements, external yeast should be added to increase the fermentation rate and efficiency in a commercial operation due to the sugar remaining after 84 h.

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