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# The Comparison of Free Cell and Immobilization Cell Fermentation on Bioethanol Production from Sorghum Stem by SSF and SHF Method

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## ABSTRACT

Bioethanol is a new and renewable energy source that can be produced from plants or crops containing sugars, starch, and lignocellulose. Sorghum stem contain a significant amount of sugar and lignocellulose. This study utilized sorghum stem in bioethanol production using Separated Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) methods. These two processes are generally used in the prouction of bioethanol with raw materials containing lignocellulose. However, raw materials derived from sweet sorghum stems has not been widely used in the previous studies. This study aimed to determine the effect of fermentation using free cell and cell immobilization techniques on various pH, as well as to identify the most optimal fermentation method (SHF or SSF) for producing the highest ethanol content in sorghum stem fermentation. The fermentation was conducted at pH levels of 3, 4, and 5. Sorghum stem were processed into powder and followed by delingnification process by 3% of NaOH solution to degrade the lignin content. The hydrolysis process of sorghum stem used cellulase enzymes as the biocatalyst. Fermentation was carried out using Saacharomyce in term of dry yeast for 72 h. The results showed that the increasing within the range pH of 3-5 will increase the ethanol concentration. Freecell technique gave the better result over the immobilized. The best result reached out the ethanol concentration of 13.04 % by the SSF.

***Keywords*:** bioethanol, immobilized cell, SHF, sorghum stem, SSF.

## 1. INTRODUCTION

The increasing of energy necessity and awareness of the negative impacts of fossil fuels have driven the search for sustainable and environmentally friendly renewable energy alternatives. Bioethanol is one of renewable energy that can reduce the $CO_2$ emission up to 18% compared by fossil fuel emission [1]. It can be produced from the material containing sugar, starch, lignocellulose, etc.

Sorghum stems is an agricutural unused waste, which currently have no economic value [2]. According to the Ministry of Agriculture, Sorghum plants was cultivated in Indonesia in the 1970s. Nowadays, sorghum farming reached approximately 15,000 in Java, South Sulawesi, Southeast Sulawesi, West Nusa Tenggara (NTB), and East Nusa Tenggara (NTT). The utilization of sorghum is still limited to the grains as the raw material of some healthy food manufacture. However, sorghum stem have a potential biomass to be converted to bioethanol. The sorghum stem have the sugar content of 11-16% and lignocellulose consisting lignin, cellulose, and hemicellulose of 90.35%. Ethanol

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production from lignocellulose requires a hydrolysis process first to break down the lignin and convert the cellulose and hemicellulose into glucose for further fermentation into alcohol.

In the previous research, the ethanol production from sorghum stem juice, produced the ethanol concentration of 8.556 – 9.9996% [3]. To utilize the lignocellulose and sugar content in the sorghum stems, some methods can be used in the ethanol production. Separated Hydrolysis and Fermentation (SHF) is the process where the hydrolysis of cellulose become a monomer of sugar is carried out in separated process with fermentation to produce ethanol. On the other hand, the Simultaneous Saccharification and Fermentation (SSF) is the process that the hydrolysis of lignocellulose and fermentation was occured simultaneously in the same time. Some studies observed the ethanol production from some biomass waste by the SHF and SSF method. The production of ethanol using banana peels as raw material with SHF and SSF methods yielded ethanol concentrations of 51.1% and 33.7%, respectively. The ethanol production with pineapple peels as the raw material reached the ethanol concentrations of 7.99% the for SHF, and 13.01% for SSF. In this study, both of those method will be applicated to process the sorghum stems into bioethanol [4].

The acidity of the solution becomes one of the important conditions in the SSF and SHF processes. In the enzymatic hydrolysis process, the increase of acid concentration provided higher glucose levels [5]. However, conditions that are too acidic or low pH will cause the enzyme to not work well and the fermentation process by Saccharomyces cerevisiae to not take place properly. The optimal pH range for yeast growth (Saccharomycess cerevisiae) can vary from pH 4 to 6, depending on temperature, the presence of oxygen, and the strain of yeast [6]. The appropriate pH is needed for the acid hydrolysis process as well as for the growth of microorganisms during the fermentation process.

The followed techniques that used in this study was immobilised cell. Immobilised cell is a technique to trap the microorganism so it can be used repeatedly. Sorghum stem fermentation by immobilization cell technique have some advantages ease of product separation, improved process control, reduced contamination, and lower separation costs [7]. However, enzyme immobilization techniques have drawbacks, such as increased processing costs, longer processing times, and diffusion limitations [8]. The free cell fermentation is also done to become the control condition of this process.

The SHF and SSF need adjustment of the acidity level for the fermentation medium. The adjustment of pH level have to accomodate the condition both of the hydrolysis and fermentation process. The high and low pH level can inhibit the activity of enzymes and microorganisms. The controlling pH in the optimal condition during the fermentation process will improve the efficiency of ethanol production [9].

Previous studies have focused more on fermentation using the free cell method compared to cell immobilization. The gap in this research, compared to earlier studies, lies in the use of sorghum flour as the raw material, where fermentation is carried out using both free cell and immobilized cell methods. Additionally, this study involves variations in fermentation pH at levels 3, 4, and 5 to determine which pH is the most optimal.

## 2. MATERIAL AND METHOD

### 2.1. Material

The raw material used in this research was sorghum stemss obtained from Maja District, Majalengka City, West Java. The microorganism used a dry yeast (Saf-Instant). The chemicals used for the production of immobilized cells were Na alginate and $CaCl_2$, while for the activation

used medium glucose, $(NH_4)_2SO_2$, $KH_2PO_4$. All of them were pure chemical compounds.

## 2.2. Method

### 2.2.1. The Equipment Preparation

The main equipment used was 500 ml flask for the fermentation reactor while the supporting tools included a 500 ml beaker, a 250 ml Erlenmeyer flask, and other glassware which were sterilized at 121°C for 15 minutes.

### 2.2.2. Raw Material Pre-treatment

The sorghum stem were washed, cut, and dried until the moisture content reach 10%. The dried sorghum was then ground and sieved to approximately 60 mesh. The next step involved delignification to reduce and degrade the lignin in the sorghum stem flour using a 3% NaOH solution. The ratio of the sorghum stem flour to NaOH solution was 1:10 (w/v). The resulting slurry was then heated at 100°C for 80 minutes. Afterward, the slurry was filtered, and the solid phase was rinsed with distilled water until it reached a neutral pH (pH 7).

### 2.2.3. Inoculum Preparation and Production

The activation medium was prepared by dissolving 6 g of glucose, 0.2 g of $(NH_4)_2SO_4$, and 0.5 g of $KH_2PO_4$ into 100 mL of distilled water. It was then sterilized in an autoclave at 121°C and 1.5 atm pressure for 15 minutes [4], and cooled to room temperature. The medium was divided between two 100 mL Erlenmeyer flasks, with each flask containing 50 mL of the solution, followed by the addition of 0.1 g of *Saccharomyces cerevisiae* dry yeast [10]. This solution was referred to as the inoculum. The inoculum was incubated for 24 h in a shaker incubator at 37°C and 150 rpm [4]. The inoculation and activation medium were needed to grow the Saccharomyces cerevisiae and prepare it before the fermentation process is carried out. 24 h incubation time was prepared for the microorganism growth reached out the logharitmic phase.

### 2.2.4. Immobilized Cell Production

Sodium alginate was used as the immobilization supporting material. The 4 g of alginate was prepared by dissolving in 50 mL of distilled water. It was pasteurized at 80°C for 15 minutes and stirred thoroughly. The solution was then cooled to a temperature of 30°C - 40°C. The CaCl2 solution was prepared for 250 mL. Next, 50 mL of inoculum was mixed with 50 mL of the sodium alginate solution. This mixture was dropped into the $CaCl_2$ solution. They became beads with diameters of 3-4 mm. The beads then were rinsed by distilled water.

### 2.2.5. SHF Method

#### 2.2.5.1. Hydrolysis

10 g of sorghum stem flour was dissolved with 150 ml distilled water and placed into 250 ml erlenmeyer flask. 10 mL of cellulase enzyme (700 EGU/g) was added to the solution [11] which was then stirred at 30°C and 250 rpm for 2 h. A 3 mL sample of the solution was taken for sugar content analysis.

#### 2.2.5.2. Fermentation

The fermentation process with free cells was conducted by mixing 50 mL of inoculum with the hydrolysis solution in separate Erlenmeyer flasks. The pH was adjusted to 3, 4, and 5, and maintained by adding NaOH solution. The flasks were tightly sealed and purged with nitrogen to create anaerobic conditions. The fermentation was carried out for 72 h [12], with samples taken daily over three days. After fermentation, the solution was centrifuged and analyzed for ethanol and glucose concentrations.

### 2.2.6. SSF Method

The Simultaneous Saccharification and Fermentation (SSF) process followed the same procedure as the Separated Hydrolysis and Fermentation (SHF) process, with the key difference being that in SSF, both hydrolysis and fermentation occurred simultaneously.

### 2.2.7. Analytical Method

#### 2.2.7.1. Lignocellulose Content Analysis

The method commonly used to measure lignocellulosic content, as proposed by Chesson (1978) in Datta (1981), involves gravimetric analysis of each component after hydrolysis or dissolution. The main steps of this method included removing extractives, followed by acid hydrolysis of hemicellulose without heating, and subsequent hydrolysis using dilute acid at high temperature. The insoluble residue at the end was lignin, whose content is corrected for ash content [13].

#### 2.2.7.2. Glucose Content Analysis

The glucose concentration in the hydrolysis and fermentation products was determined by using the DNS (Dinitrosalicylic Acid) method. The proposed formulation of the DNS reagent included dinitrosalicylic acid, Rochelle salt, sodium hydroxide, and sodium bisulfite. Prior research had indicated that impurities introduced by phenol in the DNS reagent led to an overestimation of total reducing sugars. It was important to note that sodium bisulfite was incorporated before the reagent was utilized to inhibit oxidation from the atmosphere. The standard addition method was a widely used analytical technique for quantifying the concentration of an unknown analyte. This method could be implemented by incrementally adding known quantities of the analyte to samples that contain equal volumes of the solution being analyzed. Subsequently, the samples were diluted with water to achieve uniform volumes. To ascertain the initial concentration of the analyte, a calibration curve can be constructed. In the presence of heat, the reducing sugar transfered a hydrogen atom to DNS, resulting in the loss of a hydrogen atom from the sugar itself. Consequently, the reducing sugar undergoes oxidation while DNS experiences reduction. This redox reaction was accompanied by a noticeable color change from yellow to orange/red. Subsequently, the absorbance can be quantified using a spectrophotometer to ascertain the initial concentration of glucose, which is the reducing sugar [14]. The instrumentation used was Shimadzu UV-Vis spectrophotometer set to a wavelength of 540 nm.

#### 2.2.7.3. Ethanol Level Analysis

To determine the ethanol content in the fermentation products, the concentration of ethanol was tested using refractometer for refractive index analysis and HPLC (High Performance Liquid Chromatography). The specification of HPLC used was Angilent 1120 HPLC with Zorbax Rx C 18 ODS column

## 3. RESULTS AND DISCUSSION

In this research, the fermentation was conducted under liquid fermentation conditions, anaerobically, at 37°C. Two fermentation techniques were employed: immobilized cell and free cell, using two methods: Separated Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). The process began with the raw material pretreatment and continued through the sample analysis. The fermentation results were observed and analyzed every 24h for 3 days. The analysis included measuring the lignin content, glucose content, and ethanol content.

### 3.1. Determination of Lignin Content

The delignification process using NaOH (%) on sorghum stems flour in this research is conducted after drying the sorghum stemss to a constant weight and reducing their size to 60 mesh. The delignification process involves the heating of the sorghum stems flour in a 3% NaOH solution at 100°C for 80 minutes. Before and after delignification, the sorghum stems flour was analyzed to determine the lignin content using the Chesson method as described in Datta (1981), and the results are presented in Figure 1.

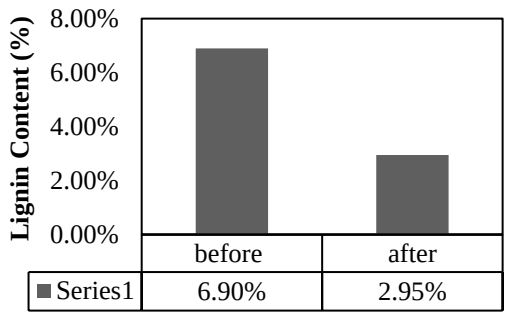



**Figure 1.** Lignin Content Before and After Pre-Treatment.

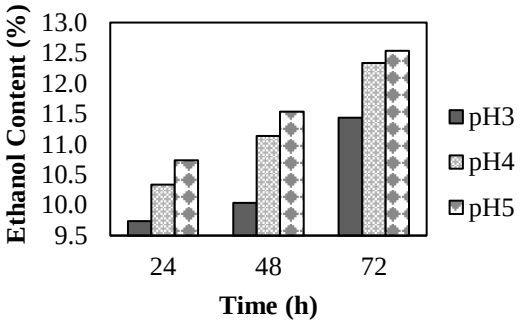



**Figure 2.** Ethanol Content in Free Cell in SHF Method.

Based on Figure 1, the lignin content in sorghum stems flour before and after heating in a 3% NaOH solution shows a decrease. This reduction in lignin content occurs because NaOH causes the breakdown of lignin compounds, leading to their solubilization in the solvent. NaOH also degrades lignin through hydrolysis and dissolves simple sugar groups still attached to the fibers [15]. Delignification with NaOH successfully reduces the lignin content by 57.25% (from 6.9% to 2.95%). These results demonstrate that treatment with NaOH can effectively reduce lignin content in lignocellulosic biomass. While the hemicellulose and cellulose content after the delignification process was 13.38% and 22.59%, repectively.

### 3.2. The effect of Freecell in SHF Method

One of the methods used in this research to produce ethanol is Separated Hydrolysis and Fermentation (SHF). The main objective of the SHF method is to optimize the conversion process of lignocellulosic biomass into bioethanol by separating the hydrolysis and fermentation stages. By maximizing the efficiency of each stage separately, SHF aims to increase bioethanol yield. The ethanol content gave the different result. The influence of free cells in the SHF method on the ethanol content produced can be observed in Figure 2.

Reducing sugar was sugar that is formed from the hydrolysis of cellulose and hemicellulose. Based on Figure 2, at pH 5 where the initial reducing sugar content of 1.03% decreases to 0.85%, this indicates a relatively small reduction. This finding is consistent with the observation that pH 3 is less than ideal for ethanol fermentation by Saccharomyces cerevisiae, as highly acidic pH inhibits yeast cell growth and activity, as well as the enzymatic activity necessary for fermentation [16]. The final ethanol concentration produced at pH 3, amounting to 11.4% (v/v), reflects low fermentation efficiency. At pH 4, where the initial reducing sugar content of 1.03% decreases to 0.52%, this indicates a more significant reduction compared to pH 3. This suggests that pH 4 is more supportive of ethanol fermentation, creating a more balanced environment for yeast cells to grow and function optimally, thereby enhancing fermentation efficiency [17]. The higher ethanol concentration produced compared to pH 5 indicates improved fermentation conditions. The most optimal condition is observed at pH 5, where the initial reducing sugar content of 1.05% decreases to 0.58%, reflecting a significant reduction, and the highest ethanol production reaches 12.3% (v/v) at 72 h. This indicates that pH 5 is the most optimal condition among the three pH levels tested for ethanol production using the SHF method with free cell technique. At pH 5, microorganisms can efficiently convert sugar into ethanol, achieving optimal ethanol production [18]. The yield obtained indicates the weight of ethanol produced

relative to the weight of the original biomass or substrate used in the fermentation process at pH 5, which is 6.2% (w/w).

In all pH conditions, the ethanol concentration increases over the fermentation period from 24 h to 72 h. This increase indicates that longer fermentation times allow microorganisms to convert more sugar into ethanol, thereby enhancing the final yield. The SHF method with free cell technique allows each stage (hydrolysis and fermentation) to be optimized separately. Cellulase enzymes used in the hydrolysis stage exhibit optimal activity at pH 5.0-6.5, with pH 5.0 showing the highest enzyme activity [19], enabling higher sugar production at pH 5. Microorganisms like yeast (*Saccharomyces cerevisiae*), commonly used in ethanol fermentation, generally have optimal activity around pH 4.5 to 5 [20]. Within this pH range, enzymatic activity and microbial growth are optimal, allowing for maximal ethanol production. pH levels lower than 4.5 can inhibit growth and enzymatic activity, while levels higher than 5.5 might reduce enzyme stability and disrupt the environmental balance needed for efficient fermentation. This aligns with the graph results showing the best performance at pH 5. Based on these findings, pH around 5 would be the optimal choice for the fermentation process using free cells and the SHF method.

### 3.3. The Effect of Freecell in SSF Method

One of the methods used in the research to produce ethanol is SSF (Simultaneous Saccharification and Fermentation). The main objective of the SSF method is to combine the hydrolysis and fermentation processes into a single stage. This aims to enhance the efficiency of biomass conversion into ethanol by reducing process time. With SSF, cellulose is hydrolysed into glucose and immediately fermented into ethanol by microorganisms, resulting in a more efficient process.

The influence of free cells in the SSF method on the ethanol content produced can be observed in Figure 3.

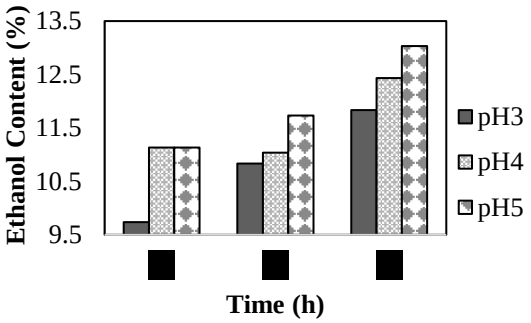



**Figure 3.** Ethanol Content in Free Cell SSF Method.

Figure 3. depicts the ethanol fermentation results using the Simultaneous Saccharification and Fermentation (SSF) method with free cell technique. At pH 3, there is an increase in ethanol concentration, albeit relatively slow, indicating that pH 3 is less ideal for maximizing ethanol production. The final ethanol concentration after 72 h is 11.8% (v/v).

At pH 4, the final ethanol concentration produced is 12.4%, demonstrating that pH 4 supports fermentation activity better compared to pH 3.

At pH 5, the final ethanol concentration reaches 13% (v/v) at 72 h. These results indicate that pH 5 is the most optimal condition for ethanol production. The yield obtained is 6.4% (w/w).

The increase in ethanol concentration is more significant at pH 5. This indicates that a higher pH supports enzyme and microbial activity better, while still remaining within the optimal pH range for fermentation. The SSF method combines saccharification and fermentation in a single process stage, allowing glucose to be converted directly into ethanol immediately after it is produced. This reduces the accumulation of glucose, which can inhibit enzymes, and enhances fermentation efficiency. From the graph, it is evident that longer fermentation times (up to 72 h) continuously increase the ethanol concentration, especially at pH 4 and pH 5. This indicates that extending the fermentation time in the SSF method can

enhance ethanol yield. pH 5 produces the highest ethanol concentration at each time point. This aligns with literature stating that pH around 4.5-5.5 is optimal for ethanol fermentation by Saccharomyces cerevisiae [16]. At this pH range, the activity of cellulase enzymes and fermentation by yeast are at their peak conditions.

Simultaneous saccharification and fermentation allows for more efficient and rapid conversion, resulting in higher ethanol concentrations in a shorter fermentation time. pH 5 is the optimal condition in this study, producing the highest ethanol concentrations at all time points.

### 3.4. The Effect of Cell Immobilization in SHF Method

The effect of cell immobilization on ethanol concentration produced using the SHF method can be seen in Figure 4.

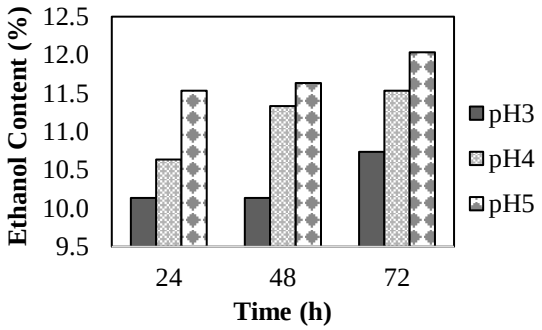



**Figure 4.** Ethanol Content in Cell Immobilization SHF Method.

The fermentation process using cell immobilization in the SHF method produces a relatively high ethanol concentration because the hydrolysis process is carried out separately, allowing the enzyme to maximize its efficiency in producing glucose. In the SHF method, the glucose conversion efficiency can be determined from the glucose concentration after hydrolysis and the glucose concentration after fermentation for 72 h. The glucose concentration after hydrolysis is measured to determine how effective the hydrolysis process is in breaking down cellulose into glucose. The glucose concentration after 72 h of fermentation is measured to see how much glucose has been consumed by the microorganisms to produce ethanol. The conversion rate ranges from 45% to 50%. The higher the glucose conversion, the more ethanol is produced.

Similarly, during the fermentation process, the beads are not disrupted by enzyme activity, which maximizes the activity of Saccharomyces cerevisiae in ethanol production. Based on Figure 4, at pH 3, the increase in ethanol concentration is relatively slow, resulting in a low ethanol yield with an ethanol concentration of 10.7% (v/v) after 72 h. At pH 4, the ethanol concentration is higher compared to pH 3, indicating that pH 4 is more supportive of fermentation activity, with an ethanol concentration of 11.5% (v/v) after 72 h. At pH 5, the ethanol concentration reaches 12% (v/v). It can be concluded that pH 5 is the optimal condition for the fermentation process in ethanol production with cell immobilization using the SHF method, yielding 5.92% (w/w).

### 3.5. The Effect of Cell Immobilization in The SSF Method

The effect of cell immobilisation on the SSF method can be seen in Figure 5.

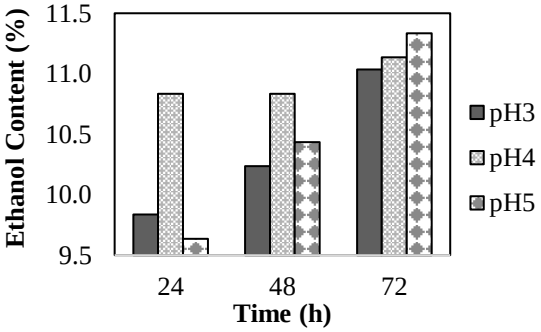



**Figure 5.** Ethanol Content in Cell Immobilization SSF Method.

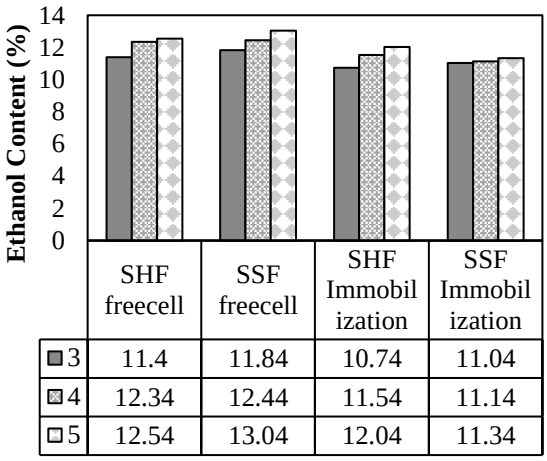
In the fermentation process using cell immobilization with the SSF method, hydrolysis and fermentation are carried out simultaneously. The hydrolysis process, which converts cellulose into glucose, occurs together with the fermentation process. The similar pH requirements for cellulase and *S. cerevisiae* facilitate the SSF process by avoiding the need for complex

pH adjustments, as found in literature by Olofsson et al. [21]. These studies have demonstrated that maintaining a pH around 5.0 optimizes the enzyme's hydrolytic activity and supports yeast viability and ethanol productivity. A study on cellulase enzymes also indicates that most retain high activity at pH 5.0, allowing efficient cellulose conversion in acidic conditions.

Based on Figure 5. it is evident that the increase in ethanol concentration from the 24th h to the 72nd h experiences a relatively rapid rise because the hydrolysis and fermentation processes work simultaneously, pH significantly influences ethanol concentration in SSF processes. At pH 5, ethanol production reached the highest level of 11.3% (v/v), supporting the literature that pH 5 is ideal for maximizing ethanol yields. Studies like those by Zhang et al. [22] further confirm that lower pH levels (e.g., pH 3) reduce enzyme and yeast activity, leading to diminished ethanol yields. The rapid increase in ethanol concentration from 24 to 72 h observed in your findings is also typical for SSF methods, as both saccharification and fermentation proceed simultaneously, quickly converting glucose to ethanol as it is produced. Ethanol yield of 5.57% (w/w) in SSF at pH 5 is consistent with yields in similar research. For instance, research on SSF for bioethanol production from lignocellulosic biomass has shown yields ranging from 5–8% (w/w), depending on factors like substrate concentration and enzyme load [23]. The concurrent process efficiency in SSF often leads to higher yields than Separate Hydrolysis and Fermentation (SHF), as it reduces sugar accumulation and potential microbial inhibition.

### 3.6. Determining the Best Method for Producing Ethanol Content

Based on the research findings, it can be observed that the higher the fermentation pH, the higher the ethanol yield produced. Therefore, the comparison between free cell and immobilized cell fermentation to determine the best method is seen at pH 5, as shown in Figure 6.




| | SHF freecell | SSF freecell | SHF Immobilization | SSF Immobilization |
|---|---|---|---|---|
| 3 | 11.4 | 11.84 | 10.74 | 11.04 |
| 4 | 12.34 | 12.44 | 11.54 | 11.14 |
| 5 | 12.54 | 13.04 | 12.04 | 11.34 |

**Figure 6.** Ethanol Content in Free Cell and Immobilized Cell SHF and SSF Methods.

Based on Figure 6. comparing both fermentation methods, SHF and SSF, for free cell and immobilized cell configurations, SSF is the optimal method for free cell fermentation. This is because simultaneous saccharification and fermentation (SSF) allows for optimal hydrolysis and fermentation processes where hydrolytic enzymes and fermentation microorganisms work together. SSF enables the simultaneous breakdown of lignocellulosic biomass and fermentation of resulting sugars, which is efficient for free cell systems. This direct conversion of sugars minimizes product inhibition—a known challenge in bioethanol fermentation. Studies, such as those by Olofsson et al. [21] confirm that SSF reduces sugar accumulation by converting glucose to ethanol as it is released, which is particularly advantageous for free cells that can freely interact with enzymes and substrate without barriers. These studies further affirm that SSF leads to higher ethanol yields due to its streamlined process, with less lag between hydrolysis and fermentation steps.

SHF, on the other hand, allows for separate optimization of hydrolysis and fermentation steps, which is beneficial for immobilized cell setups. In SHF, hydrolysis can proceed without the interference of fermentation byproducts, potentially leading to more

effective enzyme activity. Literature supports that immobilized cells benefit from this approach, as they maintain optimal fermentation conditions within the beads, allowing ethanol production to continue at stable rates without enzyme inhibition concerns [24]. The stability of immobilized cells during fermentation can also lead to longer fermentation cycles and easier cell recovery, making SHF a suitable method for this setup.

The ethanol content results you obtained (13.04% for SSF with free cells vs. 12.04% for SHF with immobilized cells, using a refractometer) are consistent with these findings, where SSF tends to yield higher ethanol concentrations due to the efficient integration of hydrolysis and fermentation. However, High-Performance Liquid Chromatography (HPLC) analysis shows slightly lower ethanol values (11.52% for SSF and 9.57% for SHF). The approximately 2% discrepancy between refractometer and HPLC measurements is well-documented in studies, as refractometers often lack the specificity needed for precise ethanol measurement due to interference from other sample components [25]. HPLC, however, is more accurate as it separates ethanol from other compounds, offering a truer representation of ethanol content in complex fermentation samples

## 4. CONCLUSION

The Free Cell technique gave better result in producing ethanol compared to immobilization technique, both in SHF and SSF methods. This technique shows higher ethanol levels across all pH variations tested. The immobized cell gave lower ethanol yield because there was a diffusion barrier from the supporting matrix but the microorganism on the beads was reusable while it was not in the free cell method. The best method for this fermentation is SSF using the Free Cell technique, resulting in an ethanol concentration of 13% (v/v) at the optimal pH of 5.

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