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RESEARCH ARTICLE

Maximising Bioethanol Production from Banana Stem Hydrolysate

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Abstract In recent years, various initiatives have been made to use low-cost renewable agricultural resources for biofuel production. Amongst these, banana waste has emerged as an alternative substrate for bioethanol production due to its abundance and rich carbohydrate content. This study explores the fermentable sugars in banana stem hydrolysate (BSH), which were utilised for bioethanol fermentation by *Saccharomyces cerevisiae*. The work was divided into preliminary investigations of BSH as a fermentation medium, optimisation of nitrogen sources used in bioethanol fermentation using BSH-based media, and scale-up of bioethanol fermentation from shake flasks to a 2 L bioreactor. Initially, BSH was prepared based on our novel 4-cycle enzymatic hydrolysis. Our results showed that the ethanol yield obtained using banana hydrolysate was 0.152, which was 1.43 folds higher than the ethanol yield produced using commercial glucose (0.106 g/g). Among the four nitrogen sources investigated (yeast extract, ammonium sulphate, urea, and peptone) 3 g/L yeast extract was found to result in the highest ethanol yield, which was 3.27 folds higher than that of the fermentation without the supplementation of nitrogen source. Scaling up the fermentation process from a shake flask to a 2 L bioreactor resulted in a higher specific growth rate, while maintaining a comparable bioethanol yield. In summary, this study proposes a novel approach to valorise bioethanol production from banana stems, implying the potential of banana stem hydrolysate as a feedstock for other bioproduction.

Keywords: Banana waste, banana stem hydrolysate, bioethanol fermentation, nitrogen source optimisation.

Introduction

Renewable energy is currently generating headlines worldwide due to concerns about diminishing fossil fuel supply. Governments worldwide have promoted the adoption of alternative energy sources due to the impending energy crisis. Oil prices have increased interest in biofuels, especially bioethanol, biodiesel, and biohydrogen. Among several biofuels produced, bioethanol is one of the most widely produced (1). In the beginning, first-generation ethanol is created by fermenting grains like corn, wheat, sugar cane, or sugar beets. However, these raw materials could also be utilised to make sustenance for humans or animals. Lignocellulosic biomass, which includes cellulosic plant biomass like stalks, stems, and wood, can serve as potential feedstock for second-generation biofuels. Numerous second-generation biofuels, including biohydrogen, bio-methanol, and mixed alcohols, are now being explored (2). Substantial quantities of lignocellulosic waste are produced by forestry and agricultural practices, and one of the major sources of lignocellulosic waste in Malaysia is the banana plantation industry.

With an annual planting of 530,000 metric tonnes, bananas are Malaysia's second most widely grown fruit (3). However, it also generates significant amounts of the pseudo-stem, leaves, and rachis residues, which are lignocellulose-rich, besides producing a high yield of edible carbohydrates with good nutrition (4). Approximately 3 tonnes of the pseudo-stem, 160 kg of stems, and 480 kg of leaves are produced for every tonne of bananas. Therefore, a well-established commercial usage for this lignocellulosic waste would aid in reducing the existing level of environmental pollution brought on by fossil fuels (5). Banana waste has valuable components such as lignocellulose that can be converted to value-added products such as fermentable sugars and renewable energy. The banana stem is revealed to have the highest

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amount of cellulose (37%) among other banana wastes (6). Previously, Amat-Jafar et. al established a novel approach, namely 4-cycle enzymatic hydrolysis, for recovering glucose from banana stems (6). Unlike most previous pre-treatment strategies that use harsh acids and bases, our approach is much greener and more cost-effective. The subsequent challenge is to undermine the utility of the sugar recovered from the proposed approach, particularly for fermentations.

Information on the optimal conditions for fermenting BSH, such as media composition, which can significantly affect ethanol yield and productivity, is still limitedly available. While some studies have investigated the viability of using banana stems for ethanol production, there is still a significant research gap in knowing the best medium formulation for maximising ethanol yield and productivity. Previously, in a study by Legodi *et al*., who reported the supplementation of banana stem hydrolysate with several types of nitrogen source, the ethanol yield was only 60% of the theoretical yield (7). In another study by Araguirang *et al*., who also explored the optimisation of banana stem hydrolysate based medium for bioethanol fermentation, the bioethanol production was relatively low (1.49 g/L) and the glucose consumption was only 65.26% (8). In both of the aforementioned works, the banana stem hydrolysate was recovered by alkaline pre-treatment followed by enzymatic hydrolysis.

The performance of fermentation can vary significantly with the medium formulation, thus necessitating ongoing research into medium optimisation for any fermentation. Furthermore, the consistency and adaptation of process parameters are essential components of the upscaling approach (9). In other words, the same or improved yields and rates should be attained using the same parameters when transitioning from small to large-scale fermentation (10). Establishing a complete approach for a product, process, or facility requires thorough characterisation and timely identification of significant aspects that impact yield, quality, and consistency. Maintaining consistency during the scaling-up process is crucial to achieve a cost-effective and economically viable bioprocess (11).

In general, there are still certain limitations in the strategies for producing bioethanol from banana stem hydrolysate. Therefore, this work aims to maximise the production of bioethanol from banana stem hydrolysate.

Materials and Methods

Microorganism and Media Preparation

Saccharomyces cerevisiae was used in this study. The stock culture was maintained at -20 °C in 5 mL vials containing 50% (v/v) glycerol. For inoculum preparation, cells were grown in a medium containing 20 g/L glucose and 5 g/L yeast extract. The culture was then incubated at 37 °C for 24 hours until the absorbance of the culture was between 0.6-0.7.

Enzymatic Hydrolysis of Banana Stem

The procedure was based on Amat Jafar *et al*. (6). The banana stems at 1% (w/v) were pretreated (boiling for 30 minutes) and hydrolysed using liquozyme at a concentration of 2 µL/g of the substrate was heated for 30 minutes at 90 °C. Then, spirizyme at a concentration of 1 µL per gram of substrate was added during saccharification, and the mixture was incubated at 60 °C for 24 hours. The aforementioned steps were repeated until the fourth cycle, whereby the resulting hydrolysate obtained after each cycle was topped up with fresh substrate. This procedure was performed to increase the amount of glucose in the substrate.

Bioethanol Fermentation

Shake Flask Culture

Banana stem hydrolysate obtained from 4-cycle enzymatic hydrolysis was used as a carbon source for bioethanol fermentation by *S. cerevisiae*. The glucose concentration in banana stem hydrolysate-containing medium was standardised at 30-40 g/L for all experimental runs. In the preliminary fermentation studies, no nitrogen source was added. Meanwhile, the optimisation studied the effect of different nitrogen sources (peptone, yeast extract, ammonium sulphate, and urea) and concentrations (1, 3, 5, 7, and 9 g/L) on bioethanol fermentation. The batch fermentation was carried out in 250 ml conical flasks with a working volume of 100 mL. The initial pH of the cultures was set at 6.5. The cultures were incubated at room temperature for 12 hours. During the fermentation, an aliquot of the culture broth was withdrawn for cell growth, glucose uptake, and bioethanol analysis.

Bioreactor Culture

Batch fermentations were performed in a 2.0 L stirred glass reactor (BioFlo 120, Eppendorf, Hamburg, Germany). The reactor comprises two six-bladed Rushton impellers (D_i =52.4 mm, D_i/D_t =0.22) and four equally spaced baffles. The temperature was monitored by a thermocouple and controlled by the circulation of water in the external jacket of the reactor. The pH was monitored using an electrochemical gel-filled glass sensor (12 mm) (Mettler Toledo, Columbus, Ohio, the United States), while the DO was monitored using a polarographic oxygen electrode (12 mm) (Mettler Toledo, Columbus, Ohio, The United States). Prior to sterilisation, the pH probe was calibrated using standard buffers at pH 7 and 4. The culture was incubated for 12 hours and at 37 °C. The agitation speed was set at 300 rpm. During the fermentation, an aliquot of the culture broth was withdrawn for cell growth, glucose uptake, and bioethanol analysis.

Analyses

Cell growth Analysis

The dry cell weight (DCW) of the yeast cell was determined by centrifuging 10 mL of the fermentation broth at 3500 rpm for 10 minutes. The collected cells were then rinsed with 0.2 M HCl and centrifuged under the same conditions. This technique was carried out three times. The collected cells were dried in an 85 °C oven for 48 hours before being weighed.

Glucose and Ethanol Analysis

The hydrolysate obtained from the enzymatic hydrolysis process was centrifuged and filtered using a 0.45 µm Puradisc filter membrane to remove impurities. The supernatant was analysed for glucose concentration using High-Performance Liquid Chromatography (HPLC) (Shimadzu Chromatographic System, Shimadzu, Kyoto, Japan), which was equipped with a Shimadzu Refractive Index Detector-10A and BIORAD Fermentation Monitoring Column (Aminex HPX-87H column, 150 x 7.8 mm, BioRad Laboratories, Inc.). The column was maintained at 60 ºC at a flow rate of 0.8 mL/min, and 0.005 M sulphuric acid was used as the mobile phase.

Statistical Analysis

All data obtained from all sets of experiments were compared and analysed using Minitab (Version 17.1) software to perform the statistical analysis. A one-way analysis of variance (ANOVA) was used to identify significant differences in the mean of the ethanol yield obtained in each fermentation. The results were considered statistically significant if the p-value <0.05.

Results and Discussion

Preliminary Studies on Banana Stem Hydrolysate as a Fermentation Feedstock

Our previous finding Amat Jafar et al. (6), revealed that BSH contains a substantial glucose concentration upon recover via the 4-cycle enzymatic hydrolysis, our novel approach to recover glucose from banana stems Amat Jafar *et al*. (6). In this study, we investigated further the feasibility of BSH as a fermentation feedstock for bioethanol fermentation.

In the preliminary studies, we conducted batch bioethanol fermentations using BSH containing glucose in shake flasks. The initial concentration of glucose was standardised at approximately 30-40 g/L. The results were compared with those fermentations using commercial glucose. No nitrogen source was added. Table 1 shows the comparison of kinetic parameters obtained from bioethanol fermentations performed using BSH and commercial glucose (control) with no addition of nitrogen source

Table 1. Kinetic parameters during bioethanol fermentation using BSH and commercial glucose

	Media	
	Banana Stem Hydrolysate	Commercial Glucose
Maximum Cell Concentration (g/L)	1.65±0.01	1.60±0.02
Maximum Bioethanol Concentration (g/L)	3.59±0.06	0.57±0.05
Glucose Consumption (%)	65.03±0.08	11.98±0.14
Bioethanol Yield (g/g)	0.15±0.02	0.11±0.01
Fermentation Efficiency (%)	29.71±0.02	20.82±0.01
Bioethanol Productivity (g/L/h)	0.30±0.06	0.05±0.05
Specific Growth Rate (g/L/h)	0.08±0.01	0.05±0.08

It was observed that there was a notable and comparable growth of the yeast when cultured in both types of media. The maximum cell concentrations obtained in cultures using BSH and commercial glucose were 1.65 g/L and 1.60 g/L, respectively, attained after 12 hours of fermentation. The comparable results between the two cultures suggest the feasibility of the sugar from banana hydrolysate, mainly glucose, for growing *S. cerevisiae* cells. Nonetheless, since no nitrogen source was added to the medium, the cell concentration obtained was considered low. In terms of glucose consumption during the bioethanol fermentation, cultures employing BSH showed more rapid consumption than those using commercial glucose. After 12 hours of fermentation, *S. cerevisiae* in cultures employing BSH utilised 65.03 % of the initial glucose. In contrast, within the same time frame, only 11.98% of the initial glucose was used in cultures employing commercial glucose. The rapid consumption of glucose by the yeast cells in BSH cultures suggests there might be components in BSH that can boost the sugar consumption by *S. cerevisiae* (12).

A prior study by Legodi *et al*. shows that *S. cerevisiae* only consumed 51% of the available glucose in the BSH during bioethanol fermentation, even though the media was supplemented with few nitrogen sources (7). This result was caused by the alkaline pre-treatment applied before the enzymatic hydrolysis, which contributed to the remaining inhibitory substances in the BSH, resulting in incomplete bioethanol fermentation (13). Another finding by Araguirang *et al*. showed a result comparable to the current study, that the sugar consumption during the bioethanol fermentation of BSH was 65.26% (8). However, the BSH used in the Araguirang *et al*. study was added with 3 nitrogen sources (ammonium sulphate, potassium dihydrogen phosphate, and magnesium sulphate), unlike the preliminary study of this work that used BSH alone (8). Furthermore, our method of recovering glucose from BSH was based on an approach that did not use harsh acid and alkaline treatment. This is beneficial since it offers the advantage of bypassing the formation of fermentation inhibitors or other undesirable substances, which are common with acid or alkaline treatments (14). Such inhibitors and by-products are recognised as contributors to reduced bioethanol yield (13).

In terms of the corresponding bioethanol from each culture, the results showed that the maximum bioethanol concentration obtained in fermentations using BSH was 3.59 g/L, which was 6.26 higher than that attained in cultures employing commercial glucose. This clearly corresponded to the rapid glucose uptake in the fermentations using BSH. Compared to a study by Sukmaningtyas, the highest bioethanol concentration achieved was 4.32 g/L after 120 hours of bioethanol fermentation (15). Even though the bioethanol production was 16.9 % higher than the concentration achieved in the current study, the long fermentation time (120 h) is less cost-effective than the duration taken in the present study, which was 12 h. A shorter fermentation time is preferable as it reduces the fermentation cost (16). In another study by Thakur *et al*., the bioethanol production and yield from alkaline pretreated banana stem as the substrate were 3.8±0.05 and 0.35±0.02, respectively (17). Despite using various nitrogen sources such as ammonium sulphate, potassium dihydrogen phosphate, and yeast extract, these results are considered low.

Although the use of sugar derived from banana waste as substrates for bioethanol fermentation has been reported in the literature, one of the underlying challenges is in terms of the optimisation of the fermentation. Furthermore, different parts of banana waste may need different strategies to maximise the derivation of sugars and, consequently, the corresponding fermentation. There has been limited research on utilising different parts of banana waste for bioethanol fermentation. Recently, a study by Suhag *et al*. shows that the enzymatic hydrolysate of the banana leaf could produce the highest ethanol concentration (15.43 g/L) after 12-hour fermentation (18). In another study by Shitophyta *et al*., 57.6% of the ethanol yield was produced after five days of fermentation utilising hydrolyzed banana peels as the substrate (19). Tan *et al*. reported the use of banana frond juice, which contained glucose (18.9 g/L), sucrose (13.29 g/L), and fructose (15.63 g/L) for bioethanol production where the yield obtained was 65% of the theoretical yield (20).

Numerous studies have reported enhancements in bioethanol production from various lignocellulosic materials. Based on the findings of Braide *et al*. findings, sugarcane bagasse, sugarcane bark, cornstalk, corncob, and cornhusk hydrolysate produced the highest ethanol yield (6.72%, 6.23%, 6.17%, 4.17%, and 3.45%, respectively) after 72 hours of fermentation (21). Similarly, Harinikumar *et al*. also reported that bioethanol fermentation using rice straw, millet, bagasse, and sorghum hydrolysate resulted in high bioethanol yields and with at least 85% sugar consumption (22). These findings indicate that bioethanol production, yield, and fermentation efficiency obtained from BSH as presented in this study are comparable to those achieved from other lignocellulosic material fermentations under similar conditions.

Optimisation of Nitrogen Sources in Bioethanol Fermentation Using BSH

Carbon source is not the only essential medium component for fermentation. It is widely acknowledged that nitrogen sources can boost the fermentation efficiency. Nitrogen is required for cell growth under anaerobic circumstances, and the rate of ethanol synthesis is higher in actively growing yeast (23). Liquefaction of lignocellulosic materials could also release complex nitrogenous compounds and nutrients into the medium. These nitrogenous compounds are broken down into free amino nitrogen, peptides, and amino acids before being used by fermenting microorganisms (24). Therefore, the subsequent investigation was conducted to determine the effect of four nitrogen source types, yeast extract, ammonium sulphate, urea, and peptone, and their concentrations on bioethanol fermentations using BSH-based medium. The initial glucose concentration of BSH was standardised to a range of 30-40 g/L in all fermentations employing all types of nitrogen sources. In the control fermentation, BSH was used as the sole fermentation medium.

Yeast Extract

The effect of yeast extract on bioethanol fermentation using BSH was studied by varying the concentrations from 1 to 9 g/L. The initial glucose concentration in BSH was standardised to a range of 30-40 g/L in all experimental runs. The control experiment was using BSH alone as a fermentation medium. Figure 1 shows the comparison of batch fermentation kinetics of *S. cerevisiae* when cultured using BSH and yeast extract at different concentrations.

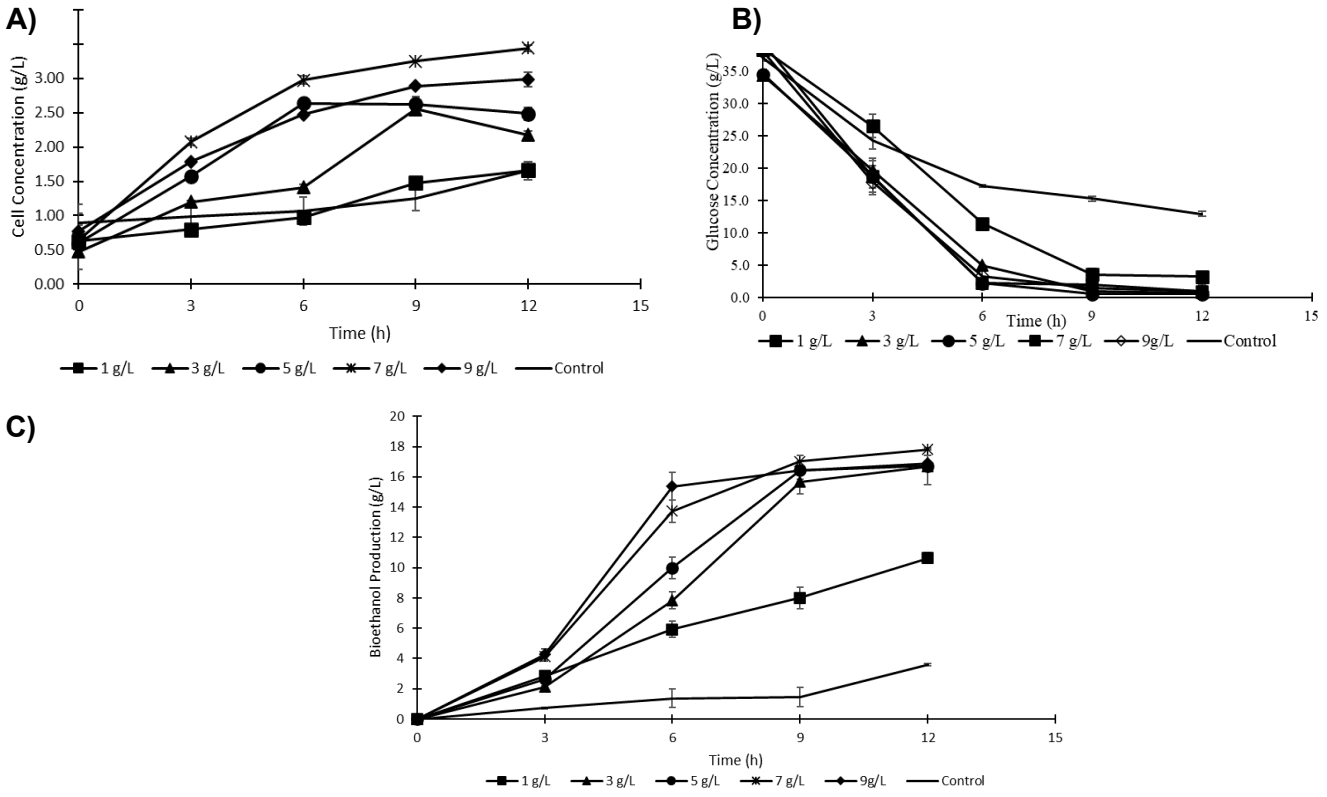

Figure 1. Comparison of batch fermentation kinetics of *S. cerevisiae* cultured in BSH and yeast extract at different concentrations (1-9 g/L): a) Cell concentration, b) Glucose consumption, and c) Bioethanol concentration

As shown in Figure 1 (a), in general, there was a significant increase in the concentration of yeast cells in all fermentations supplemented with yeast extract compared to the control fermentation that only employed banana stem hydrolysate. The notable log phase in fermentations employing yeast extract was attributed to the presence of growth factors in yeast extract (25). All cultures with yeast extract reached their exponential phase after 12 hours of fermentation, whereas the control fermentation remained in the lag phase within the same time frame. The peak cell concentration (3.44 g/L) was observed in fermentations using 7 g/L yeast extract. The value was 2.1-fold higher than the maximum value attained in the control fermentation.

There was a rapid decline in glucose levels in the culture employing yeast extract during the first six hours of fermentation. The control fermentation demonstrates the slowest rate of glucose depletion, reinforcing the role of yeast extract in accelerating glucose metabolism. Consistently, fermentations containing 5 g/L yeast extract showed the most efficient glucose uptake (98%), aligning with the trend of its cell growth profile. On the other hand, the glucose uptake in control fermentation was only 65.6%.

Figure 1 (c) illustrates the bioethanol production profiles, which show a positive correlation between the yeast extract concentration used in the media and the bioethanol yield over a 12-hour fermentation period. Notably, the fermentations using 7 g/L and 9 g/L yeast extract achieved the highest bioethanol production, significantly outperforming the control fermentation. The trend suggests an optimal nitrogen concentration range for maximising bioethanol yield, with diminishing returns observed beyond 7 g/L. This coincides with the cell growth and glucose uptake profiles implying a direct relationship between the variables. The highest bioethanol concentration attained in fermentations was 17.81 g/L, which was 4.96-fold higher than that achieved in the control fermentation.

Sugar or glucose are not the only essential substances in the medium for fermentation. It has been proposed that an appropriate amount of nitrogenous substances are crucial to achieve a complete fermentation process. A study by Appiah-Nkansah *et al*. reported that the liquefaction of lignocellulosic materials could result in the release of complex nitrogenous molecules (proteins) and nutrients into the medium (26). These nitrogenous compounds are broken down into free amino nitrogen, peptides, and amino acids before being used by microbes. This free amino nitrogen is a soluble protein broken down into small peptides and free amino acids, critical for yeast growth and metabolism (24). However, hydrolysate from lignocellulosic material only contains low nitrogenous compounds that might not be sufficient to promote yeast growth in fermentation (12). A lack of nitrogenous components in fermentation can cause delayed cell growth and product formation.

It is widely acknowledged that adding nutrients, particularly a sufficient nitrogen supply, can boost fermentation efficiency (shorter lag phase, rapid and complete fermentation). Nitrogen is required for cell growth under anaerobic circumstances, and the rate of ethanol synthesis is higher in actively growing yeast (23). Therefore, adding nitrogenous elements such as urea, yeast extract, peptone, wasted brewer's yeast, maise steep liquor, and ammonium salts may boost yeast cell growth and increase ethanol production (25). Numerous nitrogen sources can be used for bioethanol fermentation, and various sources can have varying impacts on yeast growth, metabolism, and the overall fermentation process. Numerous nitrogen-containing mediums are suitable for *S. cerevisiae* growth.

Table 2 shows a comparative analysis of the kinetic parameters during bioethanol fermentation using BSH alone (control) and BSH supplemented with different concentrations of yeast extract. The results clearly show a notable difference between all kinetic parameters achieved in the control fermentation and cultures with yeast extract. The optimal yeast concentration was found to be 3 g/L. Increasing the yeast extract to more than 3 g/L was found to result in a reduction of all parameters. In comparison to the control fermentation, the bioethanol yield achieved in BSH cultures supplemented with 3 g/L yeast extract was 3.3 times higher indicating a superior substrate-ethanol conversion efficiency. Furthermore, the fermentation efficiency, bioethanol productivity, and specific growth rate were improved by 3.3, 4.8, and 4.3-fold, respectively.

Table 2. Kinetic parameters of bioethanol fermentation using BSH at different concentrations of yeast extract

Kinetic Parameters	Control	Yeast Extract Concentration (g/L)				
		1	3	5	7	9
Bioethanol Yield (g/g)	0.15±0.02	0.30±0.01	0.50±0.02	0.49±0.01	0.47±0.02	0.46±0.02
Fermentation Efficiency (%)	29.71±0.02	59.24±0.21	97.24±0.93	96.01±0.77	91.09±0.29	89.38±1.11
Bioethanol Productivity (g/L/h)	0.30±0.06	0.89±0.02	1.39±0.02	1.39±0.12	1.42±0.05	1.45±0.03
Specific Growth Rate (g/L/h)	0.08±0.01	0.08±0.01	0.23±0.05	0.34±0.02	0.23±0.05	0.18±0.04

The literature has widely discussed the use of yeast extract as an additional medium component for bioethanol fermentation using feedstock from lignocellulosic biomass. The beneficial effect of yeast extract on *S.cerevisiae* growth was reported by Li *et al*. for bioethanol production from corn starch in which adding 6 g/L yeast extract to the fermentation media was highly advantageous to the growth of *S. cerevisiae* and could significantly increase the specific growth (25). Another study by Sheikh *et al*. also found a similar result: adding 2 g/L yeast extract to fermentation utilising potato peel waste resulted in the highest bioethanol yield (27). A comparable study by Joginder *et al*. also showed that the bioethanol yield improved when 2 g/L yeast extract was added to bioethanol fermentation utilising potato

(*Kufri Bahar*) hydrolysate (28). Another study by Kumar *et al*. also found that adding 2 g/L yeast extract in bioethanol fermentation sweet potato can increase bioethanol yield and fermentation efficiency (29). The presence of vitamins, amino acids, and minerals in yeast extract has made it suitable to promote cell growth and consequently the target product. The use of yeast extract in bioethanol fermentations has been reported widely (27, 29-31), where the findings showed significant improvement in bioethanol yield. The optimal range of yeast extract concentration for bioethanol production by *S. cerevisiae* is reported to be 2-6 g/L.

Based on the finding, the effects of each nitrogen sources tested was observed. High levels of ammonium sulphate and urea could negatively affect the fermentation by disrupting the pH balance. Ammonium sulphate releases sulphate ions that lower the pH of the medium, thereby hindering yeast growth and ethanol production (37, 38). Similarly, excess urea leads to ammonia buildup, raising intracellular pH and causing cell stress. Although low urea levels improve nitrogen availability, high concentrations can overwhelm detoxification systems and lead to cell lysis (39). Unlike urea and ammonium sulphate, which require precise dosing to avoid inhibitory effects, yeast extract performs optimally within a tested range. Peptone is less effective in bioethanol fermentation using BSH.

Although it cost higher, supplementing 3 g/L yeast extract in bioethanol fermentation with BSH is preferred over 1 g/L urea or ammonium sulphate, due to its broader nutritional profile enhances fermentation performance and efficiency (40). This improvement can offset costs by reducing overall production expenses and boosting profitability (41). Nonetheless, this study provides insights into the effects of each nitrogen source concentration for bioethanol fermentation using BSH as the main medium component. We propose 3 g/L yeast extract as the most optimal nitrogen source for bioethanol fermentation using BSH.

Ammonium Sulphate

The effect of ammonium sulphate on bioethanol fermentation utilising BSH was studied at concentrations ranging from 1 g/L to 9 g/L. In all experimental runs, the starting glucose concentration in the BSH was standardised to a range of 30-40 g/L. The control experiment used BSH as the fermentation medium. Figure 2 compares the batch fermentation kinetics of *S. cerevisiae* cultured with different concentrations of ammonium sulphate in BSH.

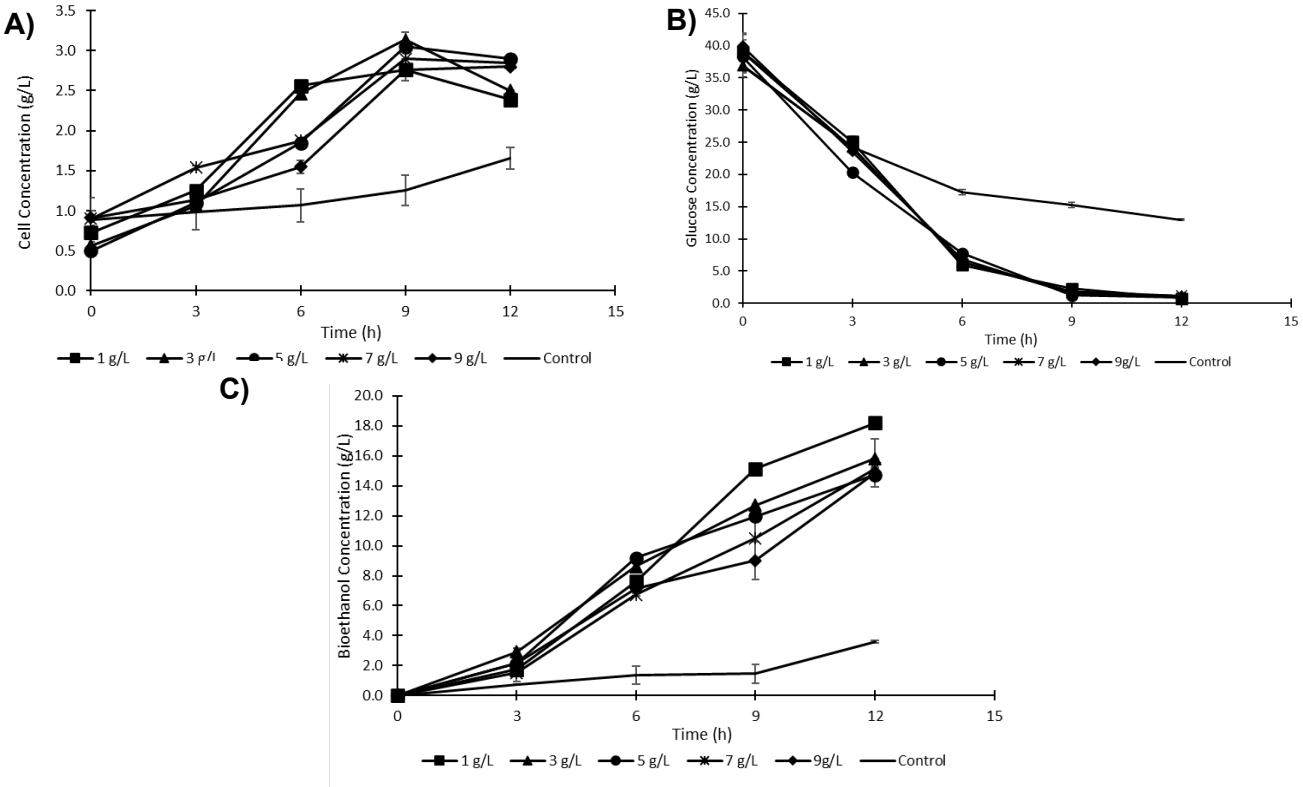

Figure 2. Comparison of batch fermentation kinetics of *S. cerevisiae* cultured in BSH and ammonium sulphate at different concentrations (1-9 g/L): a) cell concentration, b) glucose consumption, and c) bioethanol concentration

In general, the cell growth profiles in all fermentations supplemented with ammonium sulphate were higher than that using BSH alone. Figure 2 (a) shows that, the cell growth profiles in fermentations containing 1 to 3 g/L demonstrated a gradual increase over the fermentation period. Fermentations supplemented with ammonium sulphate of more than 3 g/L however, showed much lower cell growth profiles. The maximum cell concentration (3.14 g/L) was obtained in fermentation with 3 g/L ammonium sulphate, which was 1.9-fold higher than the maximum value achieved in the control fermentation.

The glucose uptake profiles of cultures supplemented with ammonium sulphate show a significant decrease during the early hours of fermentation. The control fermentation showed the slowest rate of glucose depletion, highlighting the importance of ammonium sulphate in increasing glucose metabolism. Fermentations containing 1 g/L ammonium sulphate demonstrated the most efficient glucose uptake (98%), which was similar to the trend observed in its cell growth profile. In contrast, the glucose consumption in the control fermentation was only 65.6%.

Figure 2 (c) indicates that fermentation with 1 g/L ammonium sulphate produced the highest bioethanol concentration, substantially surpassing the control fermentation. This corresponded to the cell growth and glucose uptake profiles described earlier. The maximum bioethanol concentration (18.21 g/L) was 5.1 times higher than that produced by the control fermentation.

Table 3 compares the kinetic characteristics of bioethanol fermentation using BSH alone (control), and BSH added with different concentrations of ammonium sulphate. All kinetic parameters obtained in the control fermentation and those in cultures containing ammonium sulphate were found to differ significantly. The bioethanol yield obtained in BSH cultures supplemented with 1 g/L ammonium sulphate was 3.2 times higher than that achieved in the control fermentation. Furthermore, the fermentation efficiency, bioethanol productivity, and specific growth rate were increased by 3.1, 5.1, and 3.1-fold, respectively.

Table 3. Kinetic parameters of bioethanol fermentation using BSH at different concentrations of ammonium sulphate

Kinetic Parameters	Control	Ammonium Sulphate (g/L)				
		1	3	5	7	9
Bioethanol Yield (g/g)	0.15±0.02	0.48±0.01	0.44±0.01	0.40±0.02	0.39±0.01	0.39±0.02
Fermentation Efficiency (%)	29.71±0.02	92.90±0.47	85.70±0.07	77.21±3.2	76.64±0.21	75.66±2.64
Ethanol Productivity (g/L/h)	0.30±0.06	1.52±0.02	1.32±0.00	1.23±0.08	1.26±0.01	1.24±0.02
Specific Growth Rate (g/L/h)	0.08±0.01	0.25±0.01	0.29±0.02	0.28±0.01	0.22±0.03	0.21±0.01

The use of ammonium sulphate as an additional medium component for bioethanol fermentation using feedstock from lignocellulosic biomass has been widely discussed in the literature. The beneficial effect of ammonium sulphate on *S.cerevisiae* growth was reported by Yalçin *et al*. for bioethanol production from glucose (30). According to Yalçin *et al*., adding ammonium sulphate to a fermentation medium could significantly affect yeast growth, and based on their findings, adding 0.8 g/L of ammonium sulphate into the fermentation of glucose media significantly improves the maximum specific growth rate of yeast (30). Previously, Joginder *et al*. also demonstrated that supplemented bioethanol fermentation utilising the potato (*Kufri Bahar*) hydrolysate with 3.0 g/L ammonium sulphate increased the bioethanol production to 6.98% (28). A similar study by Kumar *et al*. also showed that adding 3 g/L of ammonium sulphate to the fermentation media of sweet potato resulted in the highest ethanol production, and increasing the concentration of ammonium sulphate to more than 3 g/L reduced ethanol production (29). However, a study by Amutha obtained a higher ethanol yield by adding liquefied cassava starch with 1 g/L ammonium sulphate, and this finding is comparable to that of the current study (31). In summary, the optimal range of ammonium sulphate concentration for bioethanol fermentation by *S. cerevisiae* is reported to be 1-3 g/L. The presence of vitamins, amino acids, and minerals in ammonium sulphate has made it suitable to promote cell growth and consequently the target product.

In summary, our data showed that the optimal amount of ammonium sulphate in BSH-containing cultures might considerably improve bioethanol production, glucose uptake, and cell growth during fermentation. Our findings concluded 1 g/L ammonium sulphate as the best concentration for bioethanol fermentation using BSH-based medium with no indication of inhibition or adverse effects that greater concentrations may cause.

Urea

The effect of urea concentrations ranging from 1 to 9 g/L on bioethanol fermentation using BSH-based medium was studied. Figure 3 compares the batch fermentation kinetics of *S. cerevisiae* cultivated in BSH with different urea concentrations.

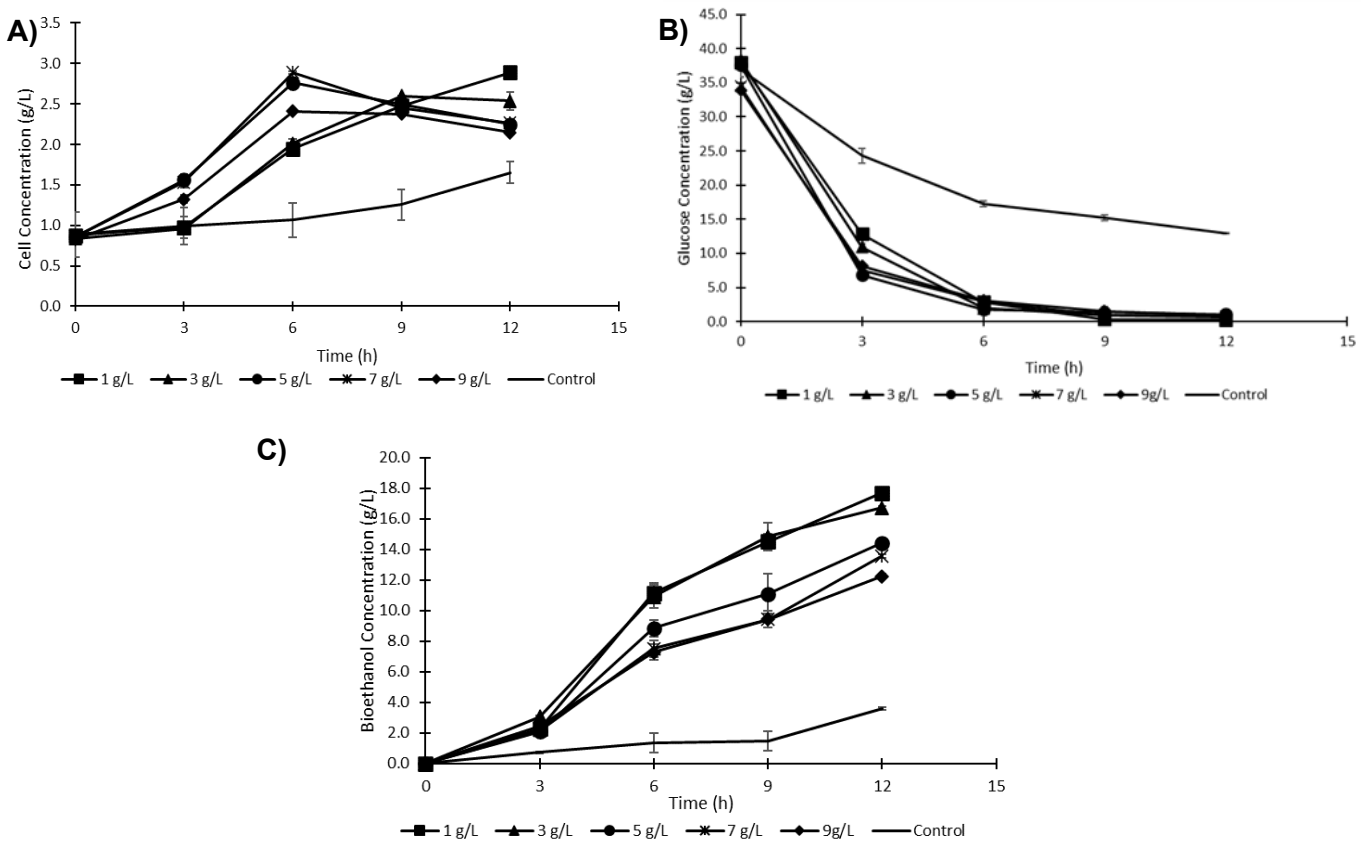

Figure 3. Comparison of batch fermentation kinetics of *S. cerevisiae* cultured in BSH and urea at different concentrations (1-9 g/L): a) Cell concentration, b) Glucose consumption, and c) Bioethanol concentration

Figure 3 (a) shows a steady increase in cell concentration during the initial hours of fermentation, with higher urea concentrations in the medium shortening the exponential phase of yeast growth. The *S. cerevisiae* cell concentration increased significantly in urea-supplemented fermentations compared to the control fermentation. All urea-based cultures reached the exponential phase at different times during fermentation, whereas the control fermentation remained in the lag phase after 12 hours. This result was due to the increase in urea concentration, which enhanced the formation of by-products such as glycerol, causing osmotic stress (34) and resulting in reduced yeast viability (35). The peak cell concentration (2.89 g/L) was observed during fermentation with 1 g/L urea, which was 1.8 times higher than the maximum value attained in the control fermentation.

The glucose uptake profiles of fermentations supplemented with urea demonstrate a significant decrease in during the first six hours of fermentation. The control fermentation, exhibited the slowest glucose depletion rate, demonstrating the relevance of urea in enhancing glucose metabolism. The highest glucose uptake (99%) was observed in fermentation with 1 g/L urea, which corresponded to its cell growth profile. The lowest glucose consumption rate was observed in control fermentation, which was only 65.6%. Fermentation with 1 g/L urea yielded the highest bioethanol concentration (17.71 g/L) outperforming that by the control fermentation by 4.9 times. Increasing the urea concentration to more than 1 g/L did not show any further increase in the bioethanol production.

Table 4 compares the kinetic parameters of bioethanol fermentation using BSH alone (control) and with different urea concentrations. The findings clearly show that all kinetic parameters attained in the urea-based cultures differ significantly from those in the control fermentation. The bioethanol yield obtained in BSH cultures supplemented with 1 g/L urea was 3.1 times higher than in the control fermentation, suggesting a higher substrate-ethanol conversion efficiency. Additionally, there was a 3.1-fold increase in fermentation efficiency, a 4.9-fold increase in bioethanol productivity, and a 2.6-fold increase in specific growth rate in comparison to those values obtained by the control fermentation.

Table 4. Kinetic parameters of bioethanol fermentation using BSH at different concentrations of urea

Kinetic Parameters	Control	Urea Concentration (g/L)				
		1	3	5	7	9
Bioethanol Yield (g/g)	0.15±0.02	0.47±0.01	0.45±0.02	0.41±0.01	0.39±0.01	0.37±0.03
Fermentation Efficiency (%)	29.71±0.02	92.04±1.03	87.36±0.25	79.36±0.80	77.10±1.50	72.63±1.84
Ethanol Productivity (g/L/h)	0.30±0.06	1.48±0.01	1.39±0.01	1.20±0.01	1.12±0.01	1.02±0.01
Specific Growth Rate (g/L/h)	0.08±0.01	0.21±0.04	0.27±0.01	0.32±0.01	0.34±0.03	0.26±0.02

The literature has widely discussed the use of urea as an additional medium component for bioethanol fermentation using feedstock from lignocellulosic biomass. The beneficial effect of urea on *S.cerevisiae* growth was reported by Li *et al*. for bioethanol production from corn starch hydrolysate (25). Based on the finding by Li *et al*., adding 9 g/L urea to the fermentation medium improved the ethanol yield and fermentation (25). Another study by Nofemele *et al*. concluded that 3 g/L urea was an ideal concentration for bioethanol production using sugarcane molasses as a substrate (34). A study by Tareen *et al*. found that supplementing fermentation media comprising oil palm trunk with 1 to 2 g/L urea improved the number of viable yeast cells and specific growth rate (35). Urea has vitamins, amino acids and minerals that are beneficial for increasing cell growth, glucose uptake and the target product. Our findings on the optimal concentration of urea for bioethanol fermentation by *S. cerevisiae* is in parallel with the range reported by the aforementioned reports. Our findings suggest that 1 g/L of urea is suitable for maximising bioethanol production using BSH-based medium.

Peptone

The effect of peptone on bioethanol fermentation using BSH as a medium was studied by varying the peptone concentration from 1 to 9 g/L. Figure 4 compares the batch fermentation kinetics of *S. cerevisiae* cultured with BSH and various concentrations of peptone.

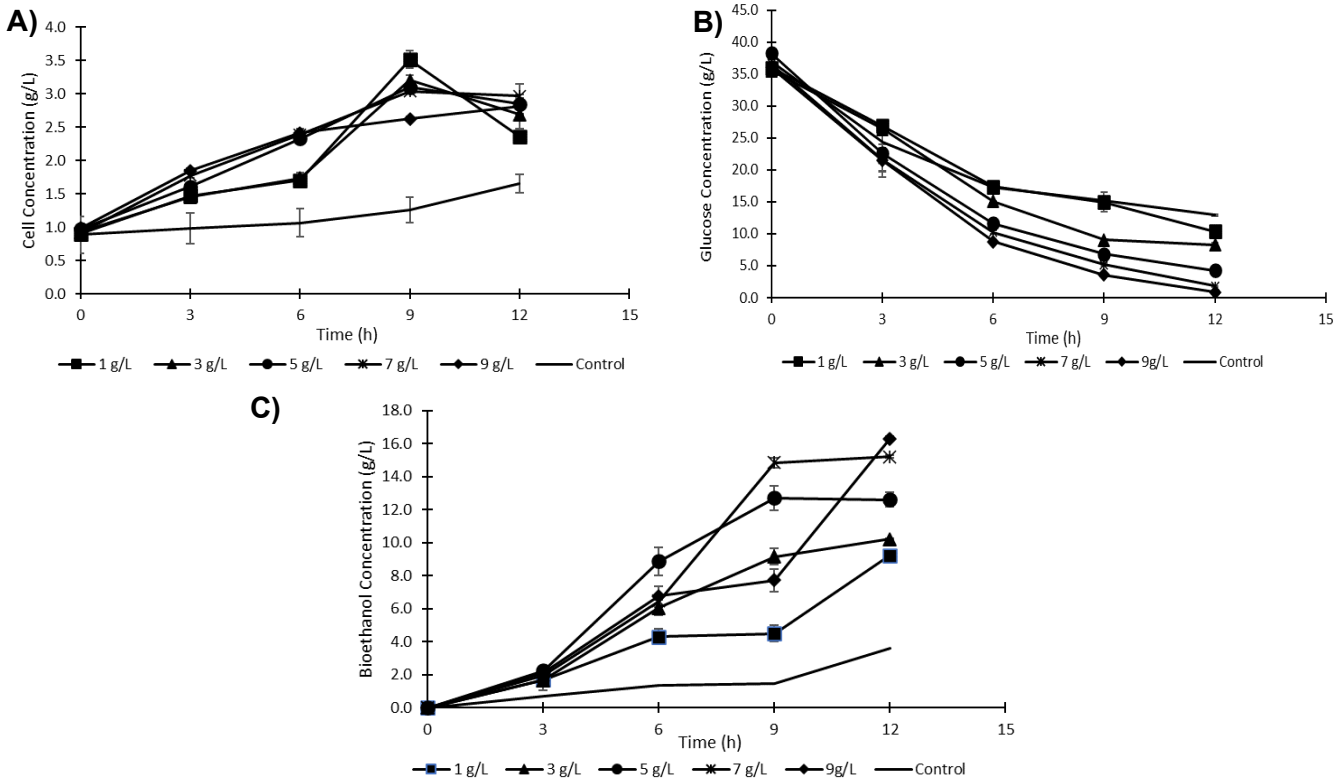

Figure 4. Comparison of batch fermentation kinetics of *S. cerevisiae* cultured in BSH and peptone at different concentrations: a) Cell concentration, b) Glucose consumption, and c) Bioethanol concentration

As shown in Figure 4 (a), the concentration of yeast cells increased significantly in peptone-supplemented fermentations compared to that in the control fermentation. All cultures using peptone reached the exponential phase after 9 hours of fermentation, whereas the control fermentation remained in the lag phase for the same duration. Fermentation with 1 g/L peptone yielded the highest cell concentration (3.51 g/L), which was 2.1 times higher than the maximum value achieved by the control fermentation.

The glucose uptake profiles in Figure 4 (b) show a significant decrease in glucose levels during the first six hours of fermentation. Fermentation with 9 g/L peptone had the highest glucose uptake rate (97%) whilst the slowest rate of glucose consumption was seen in the control fermentation. This clearly highlight the notable role of peptone in enhancing glucose metabolism. In terms of bioethanol production, fermentation with 9 g/L peptone yielded the highest concentration of bioethanol, outperforming the control fermentation by 4.5 times.

Table 5 compares the kinetic characteristics of bioethanol fermentation using BSH alone (control) and BSH added with different peptone concentrations. The highest bioethanol yield was achieved in BSH cultures supplemented with 9 g/L peptone, which was 3.1 times higher than that achieved in the control fermentation. Furthermore, the fermentation efficiency, bioethanol productivity, and specific growth rate were increased by 3.1, 4.6, and 2.6-fold, respectively. These data in general shows that the optimal amount of peptone in BSH-containing cultures may considerably improve the cell growth, glucose uptake and bioethanol production.

Table 5. Kinetic parameters of bioethanol fermentation using BSH at different concentrations of peptone

Kinetic Parameters	Control	Peptone Concentration (g/L)				
		1	3	5	7	9
Bioethanol Yield (g/g)	0.15±0.02	0.37±0.01	0.38±0.01	0.39±0.01	0.44±0.01	0.46±0.01
Fermentation Efficiency (%)	29.71±0.02	71.97±0.51	73.44±0.29	77.05±0.92	85.70±0.81	90.74±0.53
Bioethanol Productivity (g/L/h)	0.30±0.06	0.77±0.01	0.85±0.01	1.05±0.04	1.27±0.01	1.36±0.02
Specific Growth Rate (g/L/h)	0.08±0.01	0.34±0.01	0.29±0.01	0.25±0.01	0.21±0.01	0.13±0.01

The use of peptone as an additional supplement in bioethanol production from lignocellulosic biomass has been highlighted in several works. Li *et al*. in their work on bioethanol fermentation using corn starch reported that supplementation of the fermentation medium with 3% peptone increased the cell growth, bioethanol yield, and fermentation efficiency (25). In another study by Laopaiboon *et al*., addition of 5 g/L peptone to media containing sweet sorghum juice produced high ethanol yield and fermentation efficiency (36). The optimal range of peptone concentration for bioethanol fermentation as reported by previous works is in line with the finding of this study. Our findings suggest that 9 g/L peptone as optimal to maximise the bioethanol production in BSH-based medium.

Based on the findings, the effects of each nitrogen source tested were observed. High levels of ammonium sulphate and urea could negatively affect the fermentation by disrupting the pH balance. Ammonium sulphate releases sulphate ions that lower the pH of the medium, thereby hindering yeast growth and ethanol production (37, 38). Similarly, excess urea leads to ammonia buildup, raising intracellular pH and causing cell stress. Although low urea levels improve nitrogen availability, high concentrations can overwhelm detoxification systems and lead to cell lysis (39). Unlike urea and ammonium sulphate, which require precise dosing to avoid inhibitory effects, yeast extract performs optimally within a tested range. Peptone is less effective in bioethanol fermentation using BSH.

Although it costs higher, supplementing 3 g/L yeast extract in bioethanol fermentation with BSH is preferred over 1 g/L urea or ammonium sulphate, due to its broader nutritional profile enhances fermentation performance and efficiency (40). This improvement can outweigh the costs by reducing overall production expenses (41). Nonetheless, this study provides insights into the effects of each nitrogen source concentration for bioethanol fermentation using BSH as the main medium component. We propose 3 g/L yeast extract as the most optimal nitrogen source for bioethanol fermentation using BSH.

Scalability of Optimal Bioethanol Fermentation Using BSH-Based Media: Transition from Shake Flasks to A 2-L Bioreactor

Following the identification of the best nitrogen source and its concentration, as discussed in the preceding subsections, the optimal bioethanol production using BSH and 3 g/L yeast extract was scaled up from shake flask cultures to 2 L stirred-tank bioreactor cultures. Figure 5 shows a comparison of the batch fermentation profiles using the aforementioned media in both platforms.

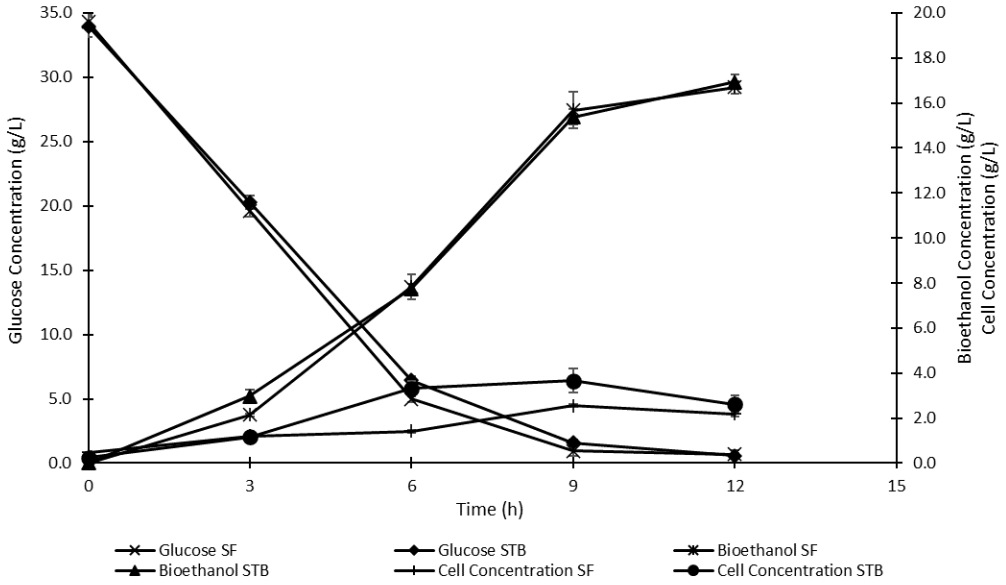

Figure 5. Comparison of batch fermentation kinetics (Cell concentration, Glucose consumption, and Bioethanol production) of *S. cerevisiae* cultured in BSH and 3 g/L yeast extract at different fermentation scales. (SF: Shake flask, STB: Bioreactor)

In terms of cell growth, the cell growth profile of bioreactor cultures is slightly higher than that in shake flask cultures. Although there is a slight difference in the maximum cell concentration produced in both cultures, the specific growth rate attained in bioreactor cultures was significantly higher, which was 1.7-fold higher than that achieved in shake flask cultures. The same trend of glucose consumption was observed on both platforms. After 12 hours of fermentation, *S. cerevisiae* in both shake flask and bioreactor cultures utilised 98% of the initial glucose. In terms of bioethanol production, there is a slight enhancement of bioethanol yield, fermentation efficiency, and bioethanol productivity rate in fermentations conducted in bioreactors (Table 6).

The scale of fermentation has the potential to influence biological, chemical, and physical factors throughout the fermentation process. Growth and production kinetics can be affected by physical and mechanical factors such as temperature, viscosity, and agitation rate during the scale-up process (10). The improved fermentation performance in bioreactor cultures is due to the controlled environment, such as pH and temperature. Moreover, bioreactors can support anaerobic conditions much better than in shake flasks. The good comparability of the fermentation performance conducted in both platforms suggests that the *S. cerevisiae* metabolism is consistent in both culture formats (shaken and agitated). These findings suggest the potential of scaling up bioethanol production using BSH as a substrate. Further scale-up studies involving different scales of bioreactors should adopt suitable strategies considering the type and design of bioreactors employed.

Table 6. Kinetic parameters of bioethanol fermentation using BSH at different fermentation scales

Kinetic Parameters	Shake Flask Culture	Bioreactor Culture
Bioethanol Yield (g/g)	0.50±0.02	0.51±0.01
Fermentation Efficiency (%)	97.24±0.93	99.30±0.77
Bioethanol Productivity Rate (g/L/h)	1.39±0.02	1.41±0.01
Specific Growth Rate (g/L/h)	0.23±0.05	0.38±0.04

Conclusions

In conclusion, this research has established effective strategies for maximising bioethanol production from banana stem hydrolysate. Our results showed that BSH-based fermentation yielded bioethanol 1.4 times more efficiently than fermentation using commercial glucose. Moreover, BSH cultures exhibited a 30% improvement in fermentation efficiency compared to glucose-based cultures. Notably, the study identified 3 g/L yeast extract as the optimal nitrogen source for bioethanol fermentation in a BSH-based medium. The successful scale-up of bioethanol production using BSH with 3 g/L yeast extract was evidenced by improved specific growth rates and maintained fermentation efficiency and yield. Future research should explore sustainable nitrogen sources like residual Baker's yeast from bioethanol processes and assess the potential of BSH as a carbon source for various bioproducts.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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