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Bioethanol Production from Hydrolysate of Sago Starch Processing Effluent via Simultaneous Saccharification and Fermentation

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The extraction of sago starch generates substantial volumes of starch-rich wastewater, making the hydrolysate a potential substrate for bioethanol production. In this study, simultaneous saccharification and fermentation (SSF), using amylase and *Saccharomyces cerevisiae*, was conducted on sago effluent hydrolysate (SEH) to produce bioethanol. A commercial starch broth was used as the control to evaluate the efficacy of SEH as an alternative substrate. Both SEH and control exhibited comparable carbohydrate consumption profiles, with 91.70 % of total carbohydrates utilized from SEH, compared to 96.80 % in commercial starch. Ethanol production from SEH peaked at 8.39 g/l (78.02 % Theoretical Ethanol Yield, TEY) within 12 h, in comparison to 8.88 g/l, or 82.57 % TEY, for the commercial starch. However, statistical analyses confirmed that there was no significant difference between the ethanol yields of SEH and the commercial starch broth at the end of the fermentation period. These findings suggest that SEH is a viable and sustainable alternative feedstock for bioethanol production.

1. Introduction

The increasing global demand for energy, coupled with the volatility of fossil fuel prices, has raised concerns over energy security, leading to economic instability, supply disruptions, and environmental challenges such as global warming (Wang et al., 2024). In response to these issues, there has been growing scientific and public interest in developing renewable and sustainable energy sources such as wind, solar, biomass, and geothermal energy, as viable alternatives to conventional fossil fuels (Moreira et al., 2024).

Among the various alternative energy sources, biorenewable fuels have emerged as the fastest-growing category, primarily due to their potential to reduce harmful emissions (Wang et al., 2024) and lessen global dependence on fossil fuels (Lee et al., 2021). Biofuels are currently available in several forms, including bioethanol, biodiesel, and biogas, and are produced from a wide array of renewable feedstocks (Vincent et al., 2021). These include agricultural residues (e.g., corn stalk, sugarcane waste, rice straw), forestry waste (e.g., grass, hardwood, and softwood), cellulose-based materials (e.g., newsprint and waste paper), and municipal solid waste (Assan et al., 2023). Among these, bioethanol is widely recognized as the most promising and sustainable liquid biofuel, owing to its favorable characteristics such as clean combustion, biodegradability, environmental compatibility, high octane number of at least 108, extensive flammability limits (3.3 % to 19 %), and significant heat of vaporization (39.3 kJ/mol) (Sharma et al., 2025).

Bioethanol can be produced using either conventional or advanced biofuel technologies, depending on the degree of polymerization of the sugar feedstock (Sharma et al., 2025). A wide range of carbohydrate sources can be utilized for fermentation, ranging from simple sugars such as glucose and sucrose to more complex carbohydrates, including starch and lignocellulosic biomass (Hung et al., 2018). However, bioethanol derived

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from starch has faced criticism for relying on food-based resources and for being economically unfeasible without government subsidies. As a result, the use of non-food substrates, such as wastewater from starch processing plants, offers a promising alternative. Starchy effluents generated from starch processing plants are abundant, renewable, and represent a viable feedstock for bioethanol production (Mamat et al., 2021). This approach not only facilitates the recovery of residual carbon sources but also contributes to the treatment of organic-rich effluents, thereby providing both environmental and economic benefits (Barua et al. 2023).

Starch derived from the sago palm (*Metroxylon sagu* Rottb.), locally known as "rumbia" in Malaysia, has traditionally served as a staple food and dietary supplement (Awg-Adeni et al., 2012). The sago palm holds significant socio-economic importance for its high yield and cost-effectiveness in comparison to other starch-producing crops (Awg-Adeni et al., 2012). In the year 2024, Sarawak (Malaysia) was reported as the world's leading exporter of sago starch (Selikang, 2024). Despite the economic benefits of sago starch production, the extraction process produces bulk volumes of starchy effluent, commonly referred to as sago effluent. This effluent is characterized by a strong odor, dark coloration, and significant levels of chemical oxygen demand (COD) and biological oxygen demand (BOD) (Vincent et al., 2020). The routine discharge of untreated sago effluent into nearby aquatic environments contributes to significant environmental pollution, largely due to the presence of recalcitrant cellulosic fibrous materials (Vincent et al., 2020). Importantly, due to the inefficiencies in the extraction process, substantial amounts of residual starch remain in the effluent (Awg-Adeni et al., 2012). This residual starch represents a valuable resource that could serve as a promising substrate for bioethanol production (Mamat et al., 2021).

In recent years, considerable research has focused on mitigating the environmental impacts associated with sago starch processing and recovering the energy potential of sago wastewater through its use in fermentative ethanol production. For instance, Hung et al. (2018) demonstrated the feasibility of producing bioethanol from the solid fraction of sago effluent, known as sago hampas, using various processing approaches. In the present study, bioethanol production was carried out using simultaneous saccharification and fermentation (SSF) of sago effluent hydrolysate (SEH). This integrated bioconversion strategy represents a promising approach for transforming starchy wastewater into ethanol, offering both environmental and energy recovery benefits (Mamat et al., 2021).

2. Materials and Methods

2.1 Experimental setup

An overview of the experimental setup is shown in Figure 1. All experiments, including analytical procedures were performed in triplicate (n=3).

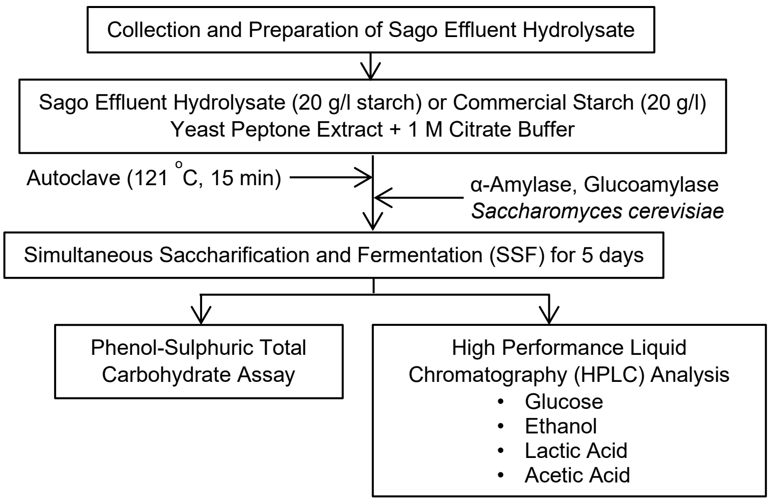

Figure 1: Flow chart outlining the steps for simultaneous saccharification and fermentation (SSF) of different feedstock loadings with amylolytic enzymes and the fermentative synthesis of ethanol of S. cerevisiae

2.2 Collection and preparation of sago effluent hydrolysate (SEH)

Sago effluent was collected from a local sago mill (Herdsen Sago Mill) in the district of Pusa, Sarawak. The effluent was filtered (using a fine stainless steel mesh) to remove large solid particles and sago hampas, yielding the sago effluent hydrolysate (SEH) utilized in this study.

2.3 *Saccharomyces cerevisiae* culture preparation

Saccharomyces cerevisiae ATCC 24859 was used as the fermenting microorganism in this study. The yeast culture was revived in Yeast Malt (YM) broth (HiMedia, India) by incubating at ambient temperature with agitation at 120 rpm using a multi-shaker (NB-101MT, N-Biotek, Korea) overnight. The inoculum for simultaneous saccharification and fermentation (SSF) was prepared by culturing an aliquot of the glycerol stock in 150 mL sterile YM broth in a 250 mL Erlenmeyer flask, with agitation at 120 rpm overnight at ambient temperature. The resulting culture was aliquoted into 50 mL centrifuge tubes and centrifuged at 3,240 × g for 6 min using a low-speed centrifuge (BK-1032J, Biobase, China) to harvest the yeast cells (Vincent et al., 2021). The harvested cell pellets were then aseptically added to the fermentation medium.

2.4 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) of sago effluent hydrolysate (SEH) was conducted in batch culture using *S. cerevisiae* and commercial amylases. The SSF was performed in 500 mL Schott bottles, each containing 200 mL of culture medium prepared in 0.05 M citrate buffer, supplemented with 3.0 g/L bacterial peptone (Bendosen Laboratory Chemicals, Malaysia) and 1.5 g/L yeast extract (Bacto, USA). The media were sterilized using an autoclave (Model 25X Electric Pressure Steam Sterilizer, All American, USA), which simultaneously ensured sterility and starch gelatinization. The starch concentration in the SEH was quantified using the phenol-sulphuric acid assay. A commercial starch solution (Bendosen Laboratory Chemicals, Malaysia) was used as the experimental control. Following autoclaving, the media were allowed to cool to 60 °C, after which commercial amylases (Sumson, China) were added. The mixture was then further cooled to 37 °C before aseptic inoculation with *S. cerevisiae*. Batch cultures were incubated under agitation at 130 rpm at ambient temperature for five days.

2.5 Sample collection

Sample aliquots of 1.5 ml were taken aseptically at predetermined time intervals (0, 6, 12, 18, 24, 30, 36, 48, 72, 96 and 120 h). The samples were first centrifuged (WiseSpin® CF-10 High-Performance Microcentrifuge Set, Daihan Scientific, Korea) at 10,125 x g for 5 min, and the supernatants were filtered through a 0.45 µm nylon syringe filter (Whatman, NJ, USA). The prepared samples were then stored in 1.5 ml microcentrifuge tubes at -20 °C prior to analyses.

2.6 Phenol-sulphuric total carbohydrate assay

Phenol-sulphuric total carbohydrate assay was performed by recording the optical density of the filtered samples at 490 nm (OD490) using a Unicam dual-beam recording spectrophotometer (SP-880 Visible Spectrophotometer, Metertech, Taiwan). The total carbohydrate concentration (g/L) was determined based on a standard sugar concentration curve in accordance to Vincent et al. (2021).

2.7 High-performance liquid chromatography (HPLC) analyses

The filtered samples were analyzed using a High Performance Liquid Chromatography (LC-20A HPLC, Shimadzu, Japan) to quantify the concentration of residual sugars (maltose and glucose) and fermentation products (ethanol, acetic acid and lactic acid). The HPLC system was equipped with a column oven (CTO-20A, Shimadzu, Japan), refractive index detector (RID-10A, Shimadzu, Japan), communication bus module (CBM-20A, Shimadzu, Japan) and a computer controller. The separation of sample constituents (20 µl injection volume per sample) was performed on a 150.0 x 7.8 mm Aminex HPX-87H column (Bio-Rad Chemical Division, California, USA) with 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.8 ml/min, with the column temperature set at 65 °C (Vincent et al., 2021).

2.8 Statistical Analysis

Concentrations of total carbohydrates, maltose, glucose, lactic acid, acetic acid, and ethanol were quantified and plotted in their respective graphs. Error bars generated represent the standard deviation from the mean of triplicate (n=3) experiments. Statistical analysis was performed using one-way univariate analysis of variance (ANOVA, F-test) with IBM® SPSS® Statistics Software Version 22 to assess significant differences between the concentrations obtained from the two different substrates. A p-value > 0.05 was considered statistically insignificant.

3. Results and Discussion

Sago effluent, a by-product of starch production, represents an abundant and low-cost substrate for starch-based bioethanol production, offering both energetic and environmental benefits (Mamat et al., 2021). This carbohydrate-rich effluent is typically discharged into nearby water bodies without treatment, contributing to

significant environmental pollution (Hung et al., 2018). In this study, simultaneous saccharification and fermentation (SSF) was employed for the bioconversion of sago effluent hydrolysate (SEH) into bioethanol. SSF integrates enzymatic hydrolysis and fermentation into a single-step process, thereby minimizing end-product inhibition of enzymatic activity, reducing contamination risk, and lowering overall production costs. Previous studies have demonstrated that SSF achieves higher saccharification rates and ethanol yields compared to conventional multi-step processes (Vincent et al., 2021).
The initial concentration of total carbohydrates in sago effluent hydrolysate (SEH), as determined by the phenol-sulphuric acid assay, was 20.00 ± 0.22 g/L. This finding is consistent with a previous report by Bujang (2014). To evaluate the potential of SEH as a substrate for ethanol production via simultaneous saccharification and fermentation (SSF), a commercial starch broth was used as the control. All fermentation broths were sterilized by autoclaving, which also served to gelatinize the starch (Ritslaid et al., 2010). Gelatinization is a critical step, as it disrupts the granular structure of starch, rendering it more amenable to enzymatic hydrolysis into glucose monomers (Ritslaid et al., 2010).
Following gelatinization, α-amylase (12.5 U/mL) and glucoamylase (25.0 U/mL) were added to both SEH and control broths to catalyze the breakdown of glycosidic linkages between glucose units (Mamat et al.. 2021). During the SSF process, both SEH and commercial starch broths exhibited comparable trends in total carbohydrate consumption (Figure 2). A sharp decline in carbohydrate concentration was observed within the first 6 h of fermentation, followed by a plateau. At the 6-hour mark, the carbohydrate concentration in SEH was 1.98 g/L, slightly higher than the 0.83 g/L observed in the commercial starch broth. By the end of the fermentation, residual carbohydrate concentrations were 1.57 g/L in SEH and corresponded to a total carbohydrate utilization of 91.70 %. In comparison, the commercial starch broth achieved 96.80 % carbohydrate utilization.

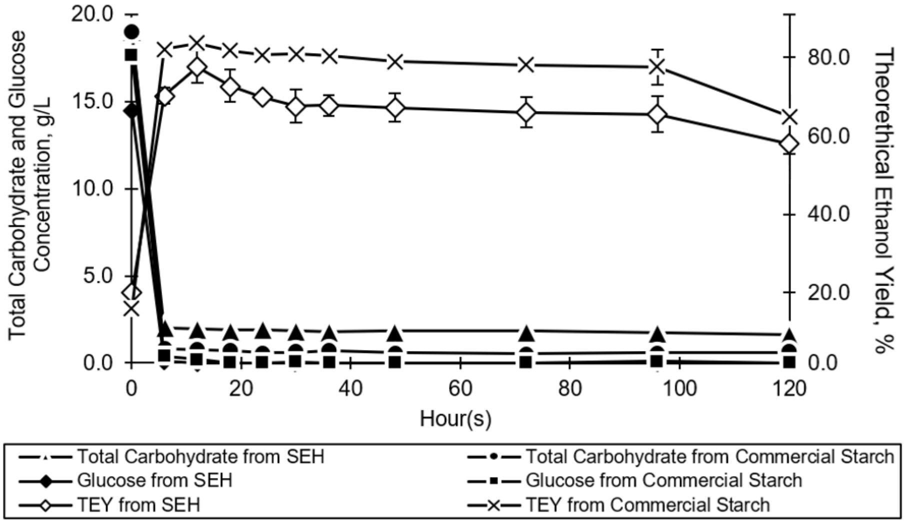

Figure 2: Time course of total carbohydrate, glucose, maltose and ethanol concentrations throughout the SSF period. Total carbohydrate was determined via the phenol-sulphuric acid assay, while glucose, maltose and ethanol concentrations were determined via HPLC. Note: The data points represent the average of three independent experiments (n=3)

To complement the residual carbohydrate analysis, glucose and maltose concentrations were quantified using high-performance liquid chromatography (HPLC). These sugars are the primary products of starch polymer hydrolysis. Glucose was detected immediately at the start of SSF (1 hour after initial saccharification), with concentrations of 14.50 g/L and 17.66 g/L in SEH and commercial starch, respectively (Figure 2). These concentrations decreased sharply within the first 6 h, followed by complete glucose depletion by 18 h, indicating rapid glucose consumption by *S. cerevisiae*. Awg-Adeni et al. (2012) attributed this pattern to the preferential hydrolysis of the amorphous regions of starch during the early saccharification stage. Furthermore, their study reported that the amylolytic process in SSF yields higher conversion efficiency when elevated glucose concentrations are present at the initial fermentation stage.
As shown in Figure 2, ethanol production exhibited an inverse correlation with the concentration of fermentable sugars in the fermentation medium. Although both substrates initially contained comparable total carbohydrate concentrations at 0 h, a significant difference was observed in residual carbohydrate levels at the end of the

fermentation period (120 h). Residual carbohydrates were lower in the commercial starch broth (0.61 g/L) compared to SEH (1.57 g/L). Initial fermentable sugar concentrations were 17.66 g/L for commercial starch and 14.50 g/L for SEH. Zabed et al. (2014) reported that initial sugar concentration is a critical factor influencing ethanol production and yield, consistent with the findings of this study.

During the first 6 h of SSF, ethanol concentration in SEH increased sharply to 7.75 g/L, corresponding to 72.07 % of the theoretical ethanol yield (TEY) based on the initial starch content. Ethanol production continued to rise, reaching a maximum of 8.39 g/L (78.02 % TEY) at 12 h. This yield was comparable to that of commercial starch, which peaked at 8.88 g/L (82.57 % TEY) at the same time point. Both substrates demonstrated a gradual decline in ethanol concentration over the remainder of the fermentation period.

Fermentation co-products, namely lactic acid and acetic acid, were monitored throughout the SSF period and were generally higher in the commercial starch fermentation compared to SEH (Figure 3). In commercial starch, lactic acid concentrations increased rapidly during the first 6 h, reaching 2.16 g/L, and peaked at 12 h at 2.19 g/L, before declining to 1.93 g/L by the end of the fermentation. In contrast, SEH exhibited a more gradual lactic acid production, peaking at 1.58 g/L at 24 h and decreasing to 1.22 g/L at 120 h. According to Vincent et al. (2021), *S. cerevisiae* experiences inhibitory effects on ethanol production only at lactic acid concentrations exceeding 30.00 g/L. Therefore, the concentrations observed in this study were not detrimental to yeast metabolism or ethanol yield in either fermentation broth.

Acetic acid, another common by-product in lignocellulosic ethanol production, has been reported to exert a synergistic effect on ethanol yield to glucose consumption (Liu et al., 2017). In both SEH and commercial starch fermentations, acetic acid levels exhibited an initial rise within the first 18 h, after which concentrations remained relatively stable throughout the remainder of the SSF process. Final acetic acid concentrations were 0.85 g/L in SEH and 0.91 g/L in commercial starch. These low concentrations are favorable, as acetic acid levels above 10.00 g/L have been shown to significantly inhibit ethanol production (Erdei et al., 2010).

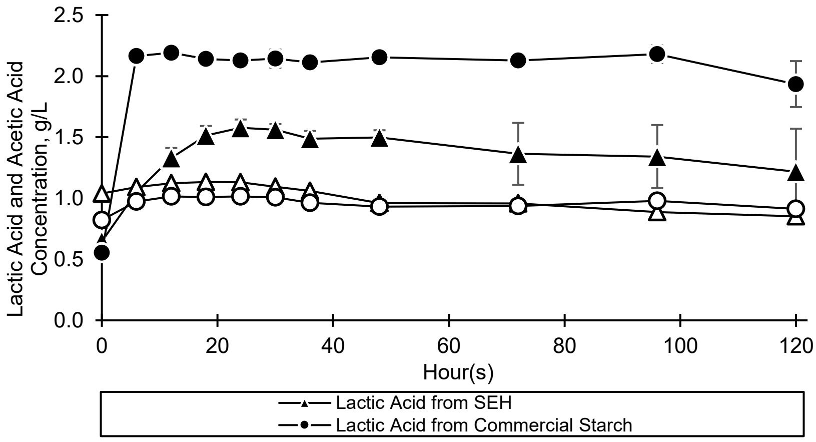

Figure 3: Time course of lactic acid and acetic acid production, as determined via HPLC. Note: The data points represent the average of three independent experiments (n=3)

Statistical analyses (Table 1) confirmed significant differences in the initial concentrations of glucose, lactic acid, and acetic acid between the two feedstocks, with all corresponding p-values < 0.05. However, no statistically significant difference was observed in the ethanol yield between SEH and commercial starch, as indicated by a p-value > 0.05. These results demonstrate that ethanol production from SEH closely parallels that of commercial starch, suggesting that SEH is a viable and comparable alternative substrate for bioethanol production via SSF.

Table 1: Summary of univariate analysis of variance for SSF initial and final products

Aspects	Sago Effluent Hydrolysate (g/l)	Commercial Starch (g/l)	R^2	p-value
Initial glucose	14.50 ± 0.62	17.66 ± 0.33	0.938	0.001
Lactic acid	1.58 ± 0.08	2.19 ± 0.02	0.988	0.000
Acetic acid	1.13 ± 0.06	1.01 ± 0.03	0.670	0.046
Ethanol yield	8.39 ± 0.44	8.88 ± 0.07	0.304	0.257

4. Conclusions

Ethanol production from carbohydrate-rich sago effluent hydrolysate (SEH) represents a promising strategy to mitigate fossil fuel dependence and reduce environmental pollution. SEH is an inexpensive, abundant, and readily available resource, providing a significant advantage over conventional substrates. In the present study, SEH subjected to simultaneous saccharification and fermentation (SSF) using amylolytic enzymes and *S. cerevisiae* yielded 8.39 ± 0.20 g/L of ethanol, corresponding to 84.89 ± 4.56 % of the theoretical ethanol yield (TEY) within 12 h. This performance was comparable to ethanol production from commercial starch, supporting the potential of SEH as an effective and sustainable feedstock for bioethanol production via SSF.

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