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RESEARCH ARTICLE

Screening Key Factors in Cassava-Based Bioethanol Production by Simultaneous Saccharification and Fermentation

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ABSTRACT

Simultaneous saccharification and fermentation (SSF) is considered an efficient approach for bioethanol production from starchy biomass, although its performance is often affected by multiple variables acting at different stages of the process. In the present study, Plackett–Burman experimental design was used as a screening method to evaluate factors affecting ethanol production from cassava flour using *Saccharomyces cerevisiae*. Eleven variables related to both liquefaction and SSF stages were examined, including temperature, pH, processing time, agitation, enzyme loading, nitrogen supplementation and inoculum level, arranged in twelve experimental runs. As the experimental design was performed without replication, the effect of each variable was evaluated based on the magnitude of standardized effects and interpreted using a Pareto chart. It was observed that not all variables contributed equally to ethanol production. Among the factors tested, SSF temperature showed the most dominant effect, followed by liquefaction pH and liquefaction time within the range studied. Other variables appeared to have a relatively smaller contribution under the present conditions. These results suggest that the use of Plackett–Burman design is suitable for initial screening of variables in the SSF system, particularly when dealing with multiple factors. The outcome of this study can be used as a basis for further optimization using an experimental design with replication to obtain a more reliable estimation of the effects of significant variables.

Keywords: Plackett-Burman screening, Nitrogen source, Process variables, Yeast fermentation, Starch hydrolysate

Introduction

Fossil fuels remain the primary global energy source, but their reserves are rapidly depleting due to increasing demand driven by population growth.[1] This situation highlights the need for alternative energy sources that are renewable and accessible.[2] The development of science and technology has encouraged extensive research on alternative fuels in many parts of the world. However, in addition to improving energy efficiency, the development of such fuels must also consider environmental impact and economic feasibility.[3,4] Biofuels, especially bioethanol, are considered one of the potential alternatives that can fulfil these requirements. Bioethanol is derived from biomass through biotechnological processes and can be utilized as fuel either in pure form or blended with gasoline, such as

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in E85, for flex-fuel vehicles and modified engines.[5] In addition, the use of bioethanol is associated with lower life-cycle CO_2 emissions compared to fossil fuels, and therefore may contribute to reducing the environmental burden.[6] In recent years, global bioethanol production has shown an increasing trend, particularly in regions such as the Americas, Europe, and Asia. This development is closely related to the implementation of renewable energy policies as well as the growing demand for alternative fuels.[7]

In Indonesia, agricultural feedstocks provide abundant raw materials for bioethanol production, in which molasses is currently the main substrate used.[8,9] However, the current production level is still insufficient to meet domestic blending targets. With the implementation of renewable fuel programs, including mandatory ethanol-gasoline blending, there is increasing interest in exploring alternative feedstocks such as cassava (*Manihot esculenta*) and other starch-based materials to support energy security as well as rural development. Cassava is considered one of the promising alternatives due to its high carbohydrate content and the availability of production surplus that has not been fully utilized for domestic consumption.[10,11] Therefore, the utilization of cassava for bioethanol production may provide additional value, not only for energy diversification but also for improving rural economic conditions. At the global level, bioethanol production continues to attract considerable attention, both in research and policy development, as part of renewable energy strategies. Various feedstocks and technological approaches have been explored to improve sustainability and to reduce dependence on fossil fuels.[12,13]

Bioethanol production from biomass can be carried out using different approaches for saccharification and fermentation. In Separate Hydrolysis and Fermentation (SHF), the enzymatic hydrolysis and fermentation processes are performed in separate reactors, allowing each step to be conducted under its respective optimum conditions. However, this approach may increase process complexity.[14] An alternative approach is Simultaneous Saccharification and Fermentation (SSF), in which both processes are carried out in a single reactor. This method can reduce the need for additional equipment, shorten processing time, and minimize product inhibition during fermentation.[15] Other approaches, such as Simultaneous Saccharification and Co-Fermentation (SSCF) and Pre-Simultaneous Saccharification and Fermentation (PSSF), have also been developed to improve sugar utilization, including pentose sugars, and to increase overall ethanol yield.[16] Recent studies have indicated that these integrated and multi-stage approaches play an important role in the development of biomass-based biorefinery systems, particularly in improving conversion efficiency when various types of feedstock are used.[17]

The concept of biorefinery places bioethanol production within a broader framework, in which biomass is processed and converted into multiple products, not only as fuel but also as other energy carriers and value-added compounds.[18] This approach is considered beneficial in improving the economic feasibility of the process, as various co-products such as biochemicals, bioelectricity, and biopolymers can be generated alongside bioethanol. In addition, the integration of these products may improve resource utilization and support the development of a circular bioeconomy.[19]

Among the available operational strategies, SSF is widely applied for starchy feedstocks such as cassava. In this process, enzymes, including glucoamylase, hydrolyze starch into fermentable sugars, which are then directly utilized by *Saccharomyces cerevisiae* during fermentation.[15] However, the performance of SSF is influenced by various biochemical and physicochemical factors, particularly nutrient availability and mineral composition. Several studies have reported that inorganic nitrogen sources, such as urea and ammonium salts, can support ethanol fermentation effectively and are considered more economical for large-scale applications.[20–22] In addition, certain minerals, including calcium, may play a role in improving yeast stress tolerance and fermentation performance.[20] The composition of nutrients in biomass hydrolysates may also vary depending on the type of feedstock and the pretreatment applied, and therefore supplementation is often required to maintain efficient fermentation.[23]

In recent years, process optimization studies have highlighted the limitations of the one-factor-at-a-time (OFAT) approach and suggested the use of statistical methods for screening influential variables in complex bioprocess systems.[24] Although these approaches have been widely applied in bioethanol production, systematic screening of both nutrient and physicochemical factors in cassava-based SSF is still limited. This is despite the availability of many optimization studies using methods such as response surface methodology (RSM) and OFAT. Therefore, a more structured screening approach is required prior to conducting detailed optimization, in order to identify the most relevant variables that significantly affect the process. The present study applies the Plackett-Burman design to determine key variables affecting bioethanol production during SSF using cassava flour and *S. cerevisiae*, providing a statistically grounded basis for optimization.

Materials and methods

Materials

The chemicals used in this study included alcohol dehydrogenase, ammonium sulfate, copper sulfate, distilled water, ethanol, glucose, glycine, hydrochloric acid, iodine, methylene blue, methyl red, *n*-hexane, NAD^+, oxalic acid, phenolphthalein, potassium dihydrogen phosphate, potassium ferricyanide, potassium iodide, potassium sulfate, semicarbazide hydrochloride, sodium carbonate, sodium hydroxide, soluble starch, sulfuric acid, and tetrasodium pyrophosphate. All chemicals were of analytical grade and obtained from Sigma-Aldrich (USA), unless otherwise specified. Bacteriological agar, peptone, and yeast extract were obtained from Oxoid; yeast nitrogen base from Difco; and Liquozyme Supra and Dextrozyme GA from Novozymes.

The cassava flour used in this study was prepared from the white cassava variety. The dried cassava chips were processed using a grinding machine to produce coarse cassava flour. The coarse flour was then sieved using a 60-mesh sieve to remove coarse fibers and to obtain a uniform particle size. Uniform particle size is essential to ensure consistent rates of starch liquefaction and hydrolysis into glucose.

Proximate analysis of feedstock

Proximate analysis was performed as described by Rima et al.[25] Crude protein was determined using the Kjeldahl method (conversion factor 6.25), total lipid content was measured using the Soxhlet method, and moisture and ash contents were determined by gravimetric methods. Total carbohydrate content was calculated by difference.

Yeast cell maintenance

The *S. cerevisiae* Summit strain, a proprietary industrial yeast strain, was provided by Dr. Anthony Heinrich (AB Mauri, Australia) and is not deposited in a public culture collection. It was maintained on agar slants prepared with yeast extract peptone (YEP) medium containing 0.5% yeast extract, 0.5% peptone, 0.3% ammonium sulfate, 0.3% potassium dihydrogen phosphate, 1% glucose, and 1.5% agar (w/v). Cultures were stored at 4°C and sub-cultured every six months.

Enzyme assay

Two enzymes were used in this study: α-amylase (Liquozyme Supra) and glucoamylase (Dextrozyme GA), both purchased from Novozymes. According to the manufacturer, Liquozyme Supra has an activity of 135 kilo Novo Unit (kNU), while Dextrozyme GA has an activity of 270 amyloglucosidase units per gram (AGU g^{-1}).

The α-amylase activity of Liquozyme Supra was determined using the iodine-starch assay described by Fuwa,[26] in which one unit of activity corresponds to a 10% reduction in the intensity of the iodine-amylose complex. Glucoamylase activity was determined based on the amount of reducing sugars released after incubation with soluble starch. Briefly, 0.2 mL of enzyme solution was added to 2% (w/v) soluble starch and incubated at 50 °C for 30 minutes. The reaction was stopped by boiling, and 75 μL of the hydrolysate was reacted with 750 μL of glucose oxidase reagent and incubated at 37°C for 5 minutes. Glucose concentration was determined using a standard calibration curve based on the Glucose Oxidase-Peroxidase Aminoantypyrine (GOD-PAP) method,[27] and enzyme activity was calculated accordingly. The Liquozyme Supra and Dextrozyme GA stock solutions had measured activities of 1.175×10^7 U mL^{-1} and 13,395 U mL^{-1}, respectively, and were diluted as required for enzyme activity assays and SSF experiments, see Table 1.

Table 1. Levels of factors in the present study using Plackett-Burman design.

No	Stage	Factors	Lower Limit (–1)	Upper Limit (+1)
1	Liquefaction	Temperature (°C)	80	90
2		pH	4	6
3		Time (hour)	1	2
4		Agitation speed (rpm)	250	350
5		α-Amylase (unit/g substrate)	10000	100000
6	Saccharification and	Temperature (°C)	30	50
7	Fermentation	Time (hour)	24	48
8		Agitation speed (rpm)	150	250
9		Urea Concentration (% w/v)	0.5	2
10		Glucoamylase (unit/ g substrate)	50	200
11		Inoculum OD	0.1	0.3

Table 2. Plackett-Burman design used in the present study.

	Liquefaction stage					Saccharification and Fermentation stage					
Run	Temperature (°C)	pH	Time (hours)	Agitation speed (rpm)	α-Amilase (unit/g substrate)	Temperature (°C)	Time (hours)	Agitation speed (rpm)	Urea (% w/v)	Glucoamilase (unit/g substrate)	OD of inoculum
1	90	4	2	350	10000	50	24	150	0.5	200	0.3
2	80	6	1	250	10000	50	48	250	0.5	200	0.3
3	90	6	2	250	100000	50	24	250	0.5	50	0.1
4	80	4	1	350	100000	50	24	250	2.0	50	0.3
5	80	6	2	350	10000	50	48	150	2.0	50	0.1
6	90	4	1	250	100000	50	48	150	2.0	200	0.1
7	90	4	2	250	10000	30	48	250	2.0	50	0.3
8	90	6	1	350	10000	30	24	250	2.0	200	0.1
9	90	6	1	350	100000	30	48	150	0.5	50	0.3
10	80	6	2	250	100000	30	24	150	2.0	200	0.3
11	80	4	2	350	100000	30	48	250	0.5	200	0.1
12	80	4	1	250	10000	30	24	150	0.5	50	0.1

Experimental design and statistical analysis

Previous studies on bioethanol production from starchy biomass have commonly used the OFAT approach or direct optimization using RSM, typically focusing on a limited number of variables such as temperature, pH, and enzyme loading.[28,29] While these approaches are useful for local optimization, they may not fully capture the relative importance of multiple interacting factors, especially under SSF conditions where both biochemical and physicochemical parameters are involved. In the present study, the Plackett–Burman design was applied as an initial screening step to evaluate a broader range of variables covering both liquefaction and SSF stages. This approach allows the identification of significant factors affecting ethanol yield in a relatively short number of experimental runs, prior to further optimization. Compared to previous studies on cassava-based SSF,[30] the present design also includes variables such as nutrient concentration and inoculum level in addition to commonly studied process parameters. This consideration is important, as yeast performance during SSF is closely related to nutrient availability. The range of each variable was determined based on previous reports, while still allowing sufficient variation to observe dominant effects under the conditions studied.

To evaluate the variables affecting ethanol yield in the SSF process, a total of eleven factors were considered. These consisted of five variables related to the liquefaction step, namely temperature, pH, time, agitation, and α-amylase concentration, and six variables related to the SSF step, including temperature, time, agitation, nitrogen concentration, glucoamylase concentration, and inoculum level. The screening experiment was carried out using Plackett–Burman design, with the levels of each factor summarized in Table 1. The experimental design matrix and run order were generated using Minitab 20 software and are presented in Table 2. As the experimental design was performed without replication, an independent estimate of experimental error was not available, and therefore, formal statistical testing was not conducted. Instead, the relative importance of each factor was evaluated based on the magnitude and ranking of standardized effects using a Pareto chart analysis. This approach is in accordance with the primary objective of Plackett–Burman design as a screening method.[31]

Liquefaction

Liquefaction was carried out using cassava flour as the substrate. A slurry was prepared by dissolving 62.5 g of cassava flour in 250 mL of distilled water in an Erlenmeyer flask, resulting in a concentration of 25% (w/v). The mixture was then heated to either 80 or 90 °C, and the pH was adjusted to 4 or 6 according to the experimental design. After reaching the desired temperature and pH, α-amylase was added at concentrations of 1,000 or 10,000 U g^{-1} flour. The liquefaction process was subsequently performed for 1 or 2 h with agitation at 150 or 250 rpm, following the conditions specified in the experimental matrix.

Simultaneous saccharification and fermentation

Following liquefaction, the medium was cooled to either 30 or 50 °C, according to the experimental design. Inorganic nitrogen was then added in the form of urea to achieve final concentrations of 0.5% or 2% (w/v). Glucoamylase was subsequently added at 50 or 100 U g^{-1} flour. The medium was inoculated with *S. cerevisiae* Summit strain (industrial strain supplied

by AB Mauri, Australia; see Yeast Cell Maintenance section) to obtain initial optical densities (OD) of 0.1 or 0.3, as specified in Table 2. SSF was carried out for 24 or 48 h under agitation at 150 or 250 rpm, in accordance with the experimental matrix.

Ethanol determination

Ethanol concentration was determined using the alcohol dehydrogenase (ADH) assay, following the method described by Ishmayana et al. [32]

Results and discussion

Cassava flour characteristics

Based on the data presented in Table 3, the cassava flour used in the present study shows a higher protein content compared to the value reported by the Ministry of Health of Indonesia. This difference may be related to variations in analytical methods as well as the type of cassava cultivar used. A previous study by Widowati [33] reported that protein content in cassava flour from several Indonesian varieties ranged between 0.81% and 2.17%, depending on the cultivar. This indicates that genetic variation may have a significant influence on the nutritional composition of cassava flour.

The proximate analysis of fat content Table 3 showed values that are in agreement with the data reported by the Ministry of Health of Indonesia, with fat content around 0.4%. [34] In addition to fat, moisture and ash contents were also determined. Ash content represents the total mineral content in the sample and is obtained as the residue after combustion of organic components at high temperature. Moisture content was measured using the thermogravimetric method by heating the sample until a constant weight was achieved. This parameter is important in determining the appropriate storage condition of the material. In the present study, the moisture content of cassava flour was found to be 7.32%. According to the National Standardization Agency of Indonesia, cassava flour should have a moisture content below 12% to minimize microbial growth during storage. [35,36]

Table 3. Comparison of the proximate composition of cassava flour between the present study and data from the ministry of health of the republic of Indonesia.

	Composition (%)	
Component	Present study	Ministry of Health of Indonesia
Protein	11.88 ± 4.57	2.4
Fat	0.41 ± 0.02	0.4
Ash	1.45 ± 0.15	1.1
Moisture	7.32 ± 0.37	14.0
Carbohydrate	78.94 ± 4.77	83.1

These compositional characteristics are relevant for the SSF process, as protein and mineral contents may contribute to yeast nutrition as well as enzyme stability during fermentation. In addition, the relatively low moisture content may improve the stability of the raw material during storage and reduce the risk of microbial spoilage prior to processing.

Enzyme activities

In the present study, the α-amylase enzyme used was Liquozyme Supra (Novozymes), which is a thermostable enzyme produced by *Bacillus licheniformis*. The activity assay was conducted to determine the amount of enzyme required to hydrolyze 1 g of starch, so that the enzyme dosage in the sample could be calculated more precisely. In addition, the assay was also used to confirm that the enzyme still retained sufficient activity after storage. Although the product label stated an activity of 135 kNU, no standard assay procedure was provided. Therefore, the enzyme activity was determined using the Fuwa method.

The Fuwa method measures α-amylase activity based on the reduction of blue color intensity from the iodine–amylose complex, which decreases as starch is hydrolyzed by the enzyme. [37] In concentrated samples, enzyme activity was too high, leading to complete hydrolysis of starch and resulting in no detectable unhydrolyzed starch for colorimetric measurement. Therefore, activity could only be accurately detected at a 200,000-fold dilution. Absorbance was measured at 600 nm, where the intensity of the blue color reflects the remaining unhydrolyzed starch. A lower absorbance indicates greater enzymatic activity. The measured activity based on Fuwa assay of Liquozyme Supra was 1.175×10^7 U/mL.

In this study, the glucoamylase enzyme used was Dextrozyme GA (Novozyme), produced by *Aspergillus niger*. The glucoamylase activity assay aimed to determine the precise enzymatic activity applied in the SSF stage. The assay was conducted by measuring glucose released from starch hydrolysis using the GOD-PAP method. Glucose concentration in the hydrolysate was determined by measuring the absorbance at 500 nm using a spectrophotometer.

To enable accurate quantification, the enzyme stock solution was diluted 10,000-fold before being added to the soluble starch substrate. Without dilution, the enzymatic activity produced glucose concentrations exceeding the assay's linear detection range, making precise measurement impossible. Glucose levels in the diluted reaction were determined

using a standard calibration curve. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per minute under the assay conditions.[38]

According to the manufacturer, Dextrozyme GA has an activity of 270 AGU per gram, although the method used for this determination was not available in the present study. In this experiment, the activity of glucoamylase was measured at 13,395 U/mL, which is relatively close to the value reported by Chantika,[39] who obtained an activity of 14,727 U/mL. The slightly lower activity observed in this study may be associated with storage conditions. Prolonged storage of the enzyme under refrigeration may lead to a gradual loss of enzymatic activity over time.

In many previous studies, enzyme dosage is often determined based on activity values reported by the manufacturer without further verification under the experimental conditions used. In the present study, the activities of both α-amylase and glucoamylase were measured prior to their application. This allows a more accurate determination of enzyme loading and may improve the reproducibility of the SSF process. Such an approach may also reduce the uncertainty related to enzyme storage and handling, including possible loss of activity over time, which in turn may contribute to the variation in ethanol yield reported in different studies.

Significant factors influencing bioethanol yield identified by plackett-burman analysis

Previous studies on bioethanol production from sugary and starchy substrates have commonly applied the OFAT approach,[40] Taguchi designs,[41] or direct optimization using RSM,[28] generally focusing on a limited number of process variables. While these approaches are useful for optimizing specific conditions, they may not adequately describe the relative importance of multiple interacting variables, particularly during the early stage of process development under SSF conditions. In the present study, Plackett–Burman design was used as a screening approach to evaluate a broader range of biochemical and operational factors covering both liquefaction and SSF stages. This approach allows the identification of important variables before further optimization. A similar strategy has also been applied in other fermentation studies, where screening is conducted first to prioritize significant factors before applying more detailed optimization methods such as response surface analysis.

In the present study, the Plackett-Burman design was applied to identify factors that influence bioethanol production from cassava flour using *S. cerevisiae*. The variables examined include liquefaction temperature, pH, time, and agitation speed, as well as α-amylase concentration. In addition, variables in the SSF stage were also evaluated, including temperature, time, agitation speed, urea concentration, glucoamylase concentration, and the initial cell optical density in the fermentation medium.

Based on the Plackett-Burman design, the number of experimental runs should be arranged in multiples of four (4n), where n is an integer. In the present study, eleven factors were evaluated, and therefore twelve experimental runs were used (n = 3). The upper (+1) and lower (1) levels for each factor were determined based on preliminary experiments, as presented in Table 1. The experimental matrix was generated using Minitab 20 software, and the run order, together with the measured ethanol concentration as the response variable, is shown in Table 4.

Because the Plackett-Burman design was conducted without replication, no independent estimate of experimental error was available. Consequently, formal hypothesis testing using ANOVA was not statistically

Table 4. Experimental runs of the Plackett-Burman design and corresponding ethanol concentrations measured as the response variable.

	Liquefaction stage					Saccharification and Fermentation stage						
Run	Temperature (°C)	pH	Time (hours)	Agitation speed (rpm)	α-Amilase (unit/g substrate)	Temperature (°C)	Time (hours)	Agitation speed (rpm)	Urea (% w/v)	Glucoamilase (unit/g substrate)	OD of inoculum	Ethanol Produced (% v/v)
1	90	4	2	350	10000	50	24	150	0.5	200	0.3	1.63
2	80	6	1	250	10000	50	48	250	0.5	200	0.3	3.33
3	90	6	2	250	100000	50	24	250	0.5	50	0.1	3.35
4	80	4	1	350	100000	50	24	250	2.0	50	0.3	2.15
5	80	6	2	350	10000	50	48	150	2.0	50	0.1	4.08
6	90	4	1	250	100000	50	48	150	2.0	200	0.1	1.7
7	90	4	2	250	10000	30	48	250	2.0	50	0.3	9.4
8	90	6	1	350	10000	30	24	250	2.0	200	0.1	7.57
9	90	6	1	350	100000	30	48	150	0.5	50	0.3	8.85
10	80	6	2	250	100000	30	24	150	2.0	200	0.3	9.07
11	80	4	2	350	100000	30	48	250	0.5	200	0.1	7.83
12	80	4	1	250	10000	30	24	150	0.5	50	0.1	6.53

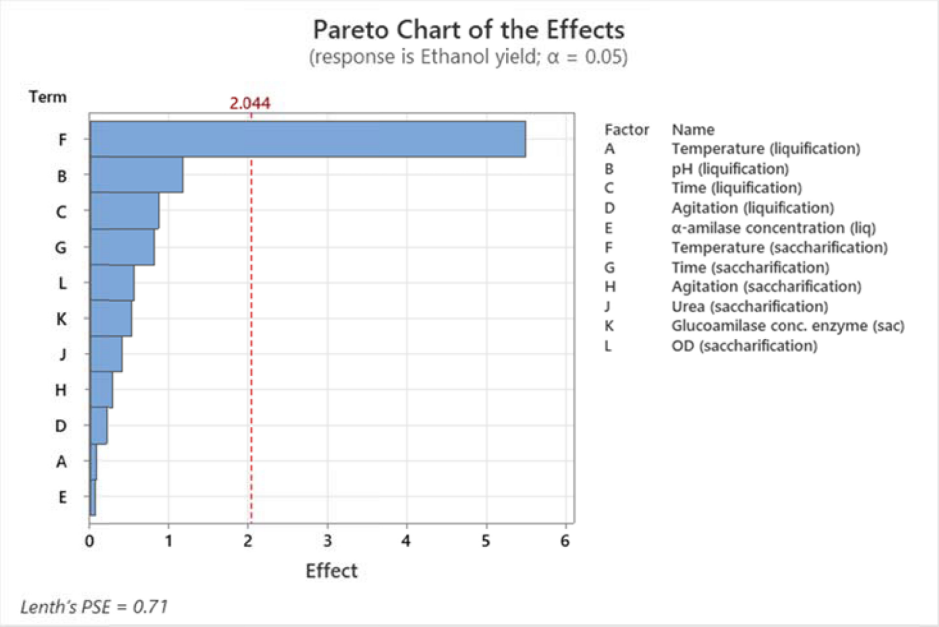

Fig. 1. Pareto chart of factors affecting ethanol yield using the SSF process, based on eleven variables evaluated with the Plackett–Burman design. Factors are ranked according to the magnitude of their standardized effects, with larger effects indicating greater influence in this screening analysis.

appropriate. Therefore, factor effects were evaluated based on the magnitude and ranking of standardized effects using a Pareto chart analysis rather than inferential statistical significance. This approach is consistent with the primary objective of Plackett-Burman designs, which is to screen for influential variables and reduce dimensionality prior to more rigorous optimization studies. [31]

Based on the Pareto chart analysis Fig. 1, SSF temperature, liquefaction pH, and liquefaction time exhibited the largest standardized effects on ethanol yield and were identified as the most influential factors within the experimental domain investigated. These variables were therefore prioritized for further discussion and are proposed as key candidates for subsequent optimization studies.

It should be noted that the Plackett-Burman design only estimates main effects and does not account for possible interactions between factors. This limitation becomes more apparent when the design is carried out without replication, where some degree of confounding between variables may occur. Therefore, the results obtained in the present study should be considered as preliminary screening rather than definitive statistical conclusions. Further studies using a replicated factorial design or RSM are required to evaluate interaction effects, estimate experimental error, and determine the optimal levels of the significant variables identified in this study.

The dominant effect of SSF temperature observed in the present study is in agreement with previous reports on bioethanol production, where temperature plays an important role in both enzymatic saccharification and yeast metabolic activity. It is known that *S. cerevisiae*, as a mesophilic microorganism, produces ethanol optimally within a certain temperature range, while performance tends to decrease when the temperature exceeds this range. [42] A similar trend was also observed for liquefaction pH. Previous studies have shown that maintaining appropriate pH conditions is important for both α-amylase activity and yeast fermentation performance. When the pH is not within the optimal range, enzyme activity may decrease, resulting in lower availability of fermentable sugars and consequently reduced ethanol production. [43]

The identification of liquefaction time as an influential factor in the present study is somewhat different from several previous optimization studies, where this parameter is often kept constant. This observation suggests that certain upstream processing conditions, such as liquefaction duration, may have a more important role than previously assumed. This also highlights the advantage of using a screening-based experimental design, which allows the evaluation of multiple variables simultaneously. In such an approach, factors that are usually fixed in conventional optimization studies can be identified

as significant and should be considered in further process development.

Consistent with the Pareto chart results Fig. 1, factor F (SSF temperature), factor B (liquefaction pH), and factor C (liquefaction time) showed the highest standardized effects on ethanol production from cassava flour by *S. cerevisiae*. Among these variables, temperature appears to have a particularly important role, as it influences both yeast metabolic activity and the catalytic activity of α-amylase and glucoamylase. In addition, temperature may also affect ethanol production indirectly through evaporation at higher temperatures. Giyad et al.[44] reported that ethanol evaporation constants increase from 0.0216 to 0.44 $mm^2\ s^{-1}$ when the temperature is increased from 25 to 75 °C. This indicates that higher temperatures may lead to ethanol loss during the process. Fermentation rate generally increases with temperature up to an optimum range, which for *S. cerevisiae* is typically within 20–30 °C. This microorganism can tolerate a relatively wide mesophilic range, with an upper growth limit around 45 °C. However, when the temperature exceeds this range, heat stress may occur and negatively affect yeast viability and fermentation performance.[45,46]

Liquefaction pH was identified as the second most influential factor. *S. cerevisiae* generally exhibits optimal fermentative activity around pH 5, with a growth tolerance range of approximately pH 3-6; values below pH 3 substantially impair yeast metabolism. Shirvanyan[47] reported maximal biomass production at pH 5 compared with pH 6.5 across multiple strains, while Hashem[48] demonstrated reduced fermentative efficiency at pH 3.75 and increased by-product formation, such as glycerol, under more alkaline conditions. At extremely low pH values (approximately 2.5), *S. cerevisiae* activates stress-response pathways, consistent with reduced metabolic efficiency. Maintaining a moderately acidic environment (pH 4-5) therefore supports metabolic selectivity and process stability during SSF.

Previous studies on starch-based bioethanol production under SSF conditions have generally identified temperature, pH, and enzymatic parameters as important factors affecting fermentation efficiency. However, the dominant variables reported may differ depending on the type of feedstock, microbial strain, and experimental conditions used.[49,50] Many studies on cassava and other starch-based materials apply direct optimization using OFAT or RSM, with primary focus on SSF temperature and enzyme loading.[51,52] In the present study, a screening approach was used to evaluate a wider range of biochemical and physicochemical variables covering both liquefaction and SSF stages. The identification of SSF temperature and liquefaction pH as important factors is consistent with previous reports. However, the influence of liquefaction time observed in this study indicates that upstream processing conditions may also play a significant role and should not be overlooked. This finding highlights the importance of preliminary screening in identifying relevant variables prior to further optimization using multivariate approaches.

Conclusion

Cassava flour can be considered a suitable substrate for bioethanol production due to its carbohydrate composition and its ability to be effectively converted under SSF conditions. In the present study, Plackett–Burman design was applied to screen variables in both liquefaction and SSF stages, and several factors were identified to influence ethanol yield. Among the variables studied, SSF temperature, liquefaction pH, and liquefaction time showed the most significant effects. Temperature was found to have the strongest influence, which is likely related to its role in controlling enzyme activity as well as the metabolic activity of *S. cerevisiae*. This observation is in agreement with previous studies on starch-based SSF systems.

Compared with studies that directly apply OFAT or RSM, this work highlights the importance of preliminary variable screening when multiple interacting parameters are involved. However, the Plackett–Burman design evaluates only main effects and does not account for factor interactions, and the experiments were conducted at laboratory scale using a single yeast strain. Future studies should therefore focus on multivariate optimization of the significant factors identified and validation under different operational and scale-up conditions.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images that are not ours have been

included with the necessary permission for republication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at Universitas Padjadjaran, Sumedang, Indonesia.

Authors' contributions statement

S.I. and U.M.S.S. conceptualized and designed the study. A.R.P.A., S.D.R.A., and D.T.H. performed the experiments. S.I., M.F., and F.S.N. conducted data analysis and statistical evaluation. S.I. and D.T.H. wrote the initial draft of the manuscript. I.K. and R.A. critically reviewed the manuscript and provided significant revisions for improvement. All authors read and approved the final manuscript.

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التحري عن العوامل الرئيسة في إنتاج الإيثانول الحيوي المعتمد على الكسافا باستخدام التحلل السكري والتخمير المتزامنين

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الخلاصة

يُعدّ التحلل السكري والتخمير المتزامنان (Simultaneous Saccharification and Fermentation, SSF) من الأساليب الفعّالة لإنتاج الإيثانول الحيوي من الكتلة الحيوية النشوية، إلا أن كفاءتهما تعتمد على تفاعل عدة متغيرات تشغيلية عبر مراحل مختلفة من العملية. في هذه الدراسة، استُخدم تصميم بلاكِت–بورمان التجريبي بوصفه أداة تحرٍّ أولية لتحديد العوامل المؤثرة في إنتاج الإيثانول الحيوي من دقيق الكسافا باستخدام خميرة Saccharomyces cerevisiae. تم تقييم أحد عشر متغيرًا مرتبطًا بمرحلة التسييل ومرحلة التحلل السكري والتخمير المتزامنين، شملت درجة الحرارة، والرقم الهيدروجيني، وزمن المعالجة، وسرعة التحريك، وحمل الإنزيمات، وإضافة النيتروجين، ومستوى التلقيح، وذلك من خلال اثنتي عشرة تجربة. ونظرًا لتنفيذ التصميم دون تكرار، تم تقييم أهمية العوامل بالاعتماد على مقدار التأثيرات المعيارية باستخدام تحليل مخطط باريتو. أظهرت النتائج أن درجة الحرارة خلال مرحلة التحلل السكري والتخمير المتزامنين، والرقم الهيدروجيني أثناء التسييل، وزمن التسييل كانت أكثر العوامل تأثيرًا في مردود الإيثانول ضمن نطاقات الدراسة، مع بروز درجة الحرارة خلال مرحلة SSF بوصفها العامل الأكثر هيمنة. وتُبرز هذه النتائج فاعلية تصميم بلاكِت–بورمان كأداة مناسبة للتحرّي الأولي عن المتغيرات في أنظمة SSF المعقّدة، كما توفر أساسًا منهجيًا واضحًا لإجراء دراسات تحسين لاحقة مُكرّرة لإنتاج الإيثانول الحيوي المعتمد على الكسافا.

الكلمات المفتاحية: إنتاج الإيثانول الحيوي، دقيق الكسافا، تصميم Plackett-Burman، التسكير والتخمر المتزامن (SSF)، خميرة Saccharomyces cerevisiae.