OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF CASSAVA (*MANIHOT ESCULENTA*) STARCH CONTAINING CYANIDES

Hargono Hargono, Andri Cahyo Kumoro, Bakti Jos,
Aji Prasetyaningrum, Dani Puji Utomo

Department of Chemical Engineering, Faculty of Engineering,
Universitas Diponegoro, Semarang, 50275, Indonesia,
hargono@che.undip.ac.id (H.H.), andrewkomoro@che.undip.ac.id (A.C.K.),
baktijos@che.undip.ac.id (B.J.), ajiprasetyaningrum@che.undip.ac.id (A.P.),
danipujiutomo@che.undip.ac.id (D.P.U.)

Received 02 December 2024
Accepted 28 February 2025
DOI: 10.59957/jctm.v60.i3.2025.1

ABSTRACT

The bitter cassava plant (Manihot esculenta), commonly referred to as bitter cassava, is a wild species that thrives in forests and gardens. The tubers of bitter cassava contain cyanogenic compounds, which are toxic due to the presence of cyanide. Cyanide removal can be achieved through extraction using water or heating at a temperature of 50 ºC. Bitter cassava starch can be converted into reducing sugars through enzymatic processes. In this study, experiments were conducted using bitter cassava starch at concentrations of 100, 200, and 300 g L^{-1} (native), with added cyanide concentrations of 50, 100, and 150 mg kg^{-1} as inhibitors. The effects of these variables on reducing sugar production were analysed through enzymatic hydrolysis at 30ºC. The optimization of enzymatic hydrolysis in the presence of cyanide was performed using Response Surface Methodology (RSM). The study investigated the effects of starch concentration, cyanide concentration, and hydrolysis time on reducing sugar production. RSM has been proven to be an effective and reliable tool for optimizing the process and understanding the interactive effects of the three variables involved in cassava starch hydrolysis. A highly significant quadratic regression model (R^2 = 0.9900, $P < 0.0001$) was developed. The predicted optimal conditions for hydrolysis were identified as 278.5 g L^{-1} starch concentration, 51.4 mg kg^{-1} cyanide concentration, and a hydrolysis time of 14.8 h, resulting in a reducing sugar concentration of 63.498 g L^{-1}.

Keywords: enzymatic hydrolysis, optimization, cassava starch; reducing sugar, cyanide.

INTRODUCTION

Cassava (*Manihot esculenta*) is a tuberous plant that grows easily in tropical regions. Due to its high carbohydrate content, it serves as a staple food for millions of people in African, Latin American, and Asian countries. However, cassava contains two cyanogenic glucosides (CGs), lotaustralin and linamarin [1]. These compounds are toxic to humans, as their consumption can cause symptoms such as dizziness and nausea. Based on cyanide content, cassava is classified into two types: high-cyanide cassava (bitter cassava) and low-cyanide cassava (sweet cassava) [2]. *Manihot esculenta* is a prime example of sweet cassava, while *Manihot glaziovii* represents bitter cassava. Although *M. glaziovii* is rarely marketed as a food source, it has potential as a feedstock for ethanol production. Cassava tubers are a promising raw material for ethanol production due to their high starch and cellulose content, which can be hydrolysed and fermented into ethanol [3]. Cyanide (CN), however, has been reported as an inhibitor of enzymatic activity, thereby reducing the production of reducing sugars during hydrolysis [4]. It is also an example of an irreversible enzyme inhibitor.

The enzymatic hydrolysis of starch into reducing sugars can traditionally be performed by cooking a 30 % (weight per volume) starch slurry with thermostable α-amylase at temperatures exceeding 100ºC, maintaining the slurry at 90ºC for 1 - 3 h, and then cooling it to 60ºC with the addition of glucoamylase [5]. Alternatively, the use of granular starch hydrolysing enzyme (GSHE) allows the direct conversion of raw or granular starch into glucose and fermentable sugars at lower temperatures (30 - 50ºC). GSHE can directly hydrolyse raw starch granules without requiring gelatinization, significantly reducing the energy required for the hydrolysis process [6].

Previous studies have explored the enzymatic hydrolysis of various starch and flour types, such as cassava starch, corn flour, corn starch, and potato starch, using different enzyme combinations. These include double enzymes (α-amylase and glucoamylase), single enzymes (α-amylase), and mixtures of α-amylase and GSHE at high temperatures (80 - 140ºC) to produce glucose [4, 7 - 9]. However, the optimization of cassava starch hydrolysis in the presence of cyanide at low temperatures has not been extensively studied.

RSM is a mathematical and statistical approach based on experimental design. It is commonly employed to study the effects of independent variables on a dependent (response) variable. RSM is widely used to optimize processes involving multiple variables, as it predicts optimal operating conditions while minimizing the number of experiments required.

In this study, cassava starch was enzymatically hydrolysed into reducing sugars. RSM was applied to optimize the process by investigating the effects of three factors, each at three levels, and their interactions on the reducing sugar yield from the hydrolysis process.

EXPERIMENTAL

Potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$), 3,5-Dinitrosalicylic acid (DNS), Cyanide (as Potassium cyanide) produced by MERCK, NaOH (98 %, Merck), Na_2SO_3 (98.5 %, Merck), H_2SO_4 (98.5 %, Merck), sodium acetat buffer, glucose (99.5 %), were obtained from their approved distributor. Granular starch hydrolysing enzyme as Stargen™ 002, which is a mixture of α-amylase and glucoamylase was produced by Genencor (Palo Alto, USA). Its activity was 570 GAU g^{-1} and pH range stated by the producer was 4.0 - 4.5 [10].

Preparation of cassava starch

Cassava (*Manihot esculenta Crantz*), aged 10 months, was obtained from a commercial farm located in the Wonogiri district, Indonesia. The starch extraction procedure was carried out following the method described by Hargono et al. [11]. The physicochemical properties and cyanide content of the cassava used in this study were analysed using the same methodology employed by Hargono et al. in their previous research [11]. The extracted starch, serving as the substrate specimen, was stored in an airtight container at 4°C in the Bioprocess Laboratory, Department of Chemical Engineering, Faculty of Engineering, Universitas Diponegoro, Indonesia.

Enzymatic hydrolysis

Sweet cassava starch slurries with concentrations of 100, 200, and 300 g L^{-1} were used in this study. The starch slurry was adjusted to pH 4 using 50 mM sodium acetate buffer, and Stargen™ 002 (1.5 % by weight per volume) was added. Prior to enzyme addition, the mixture was pre-treated for 2 h at 48°C in a reciprocating water bath, as recommended by the GSHE manufacturer [10]. Subsequently, the slurry was cooled to room temperature (30±1°C) and incubated for 18 h. Samples were collected at 6 h intervals throughout the hydrolysis process for reducing sugar concentration analysis [12]. To investigate the inhibitory effect of cyanide, varying cyanide concentrations of 50, 100, and 150 mg kg^{-1} were introduced into the cassava starch slurry. The reducing sugar concentrations were then measured accordingly.

Reducing sugar assay

The reducing sugar value was analysed using the 3,5-dinitrosalicylic acid (DNS) method [12]. Before the samples were analysed by Spectrophotometer, they were centrifuged (100 Hz, 4ºC and 10 min) to obtain the filtrate then their absorbances were measured at a wavelength of 540 nm using an ultraviolet-visible (UV-Vis SPEKOL® 1500, Analytik Jena, Berlin, Germany).

Experimental design

The effects of starch concentration, cyanide concentration, and hydrolysis time on the enzymatic hydrolysis of cassava starch were investigated using Response Surface Methodology (RSM). The statistical model was developed based on Central Composite Design

(CCD) experiments [13]. Starch concentration (X_1), cyanide concentration (X_2), and hydrolysis time (X_3) were selected as independent variables, as presented in Table 1.

Statistical data analysis

The experimental data were analysed statistically using RSM to fit a second-order mathematical model generated by STATISTICA 10.0 (StatSoft, Inc.), and the optimal conditions were calculated using Design-Expert 10 (Stat-Ease, Inc.) [15, 16]. The response variables and independent variables were correlated through multiple regression to determine the coefficients of the polynomial model for each response [15]. The significance tests and analysis of variance (ANOVA) were employed to assess the quality of the model fit. The fitted second-order mathematical model used in this study is represented in Eq. (1).

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i<j}^{k} b_{ij} X_i X_j + e \quad (1)$$

where Y is the response factor (reducing sugar concentration), b_0 is the intercept value, b_i (i = 1, 2, …, k) is the first order model coefficient, b_{ij} is the interaction effect, b_{ii} represents the quadratic coefficients of X_i, and e is the random error [15].

The central values (zero levels) selected for the experimental design were 100 and 300 g L^{-1} for starch concentration, 50 and 150 mg kg^{-1} for cyanide concentration, and 6 and 18 h for hydrolysis time. These parameters were chosen based on prior studies that indicated their significant influence on the enzymatic hydrolysis of cassava starch. Reducing sugar was selected as the dependent variable for this study. A total of 27 experiments were conducted using Central Composite Design (CCD) with varying combinations of the independent variables, and the results are presented in Table 2 [14].

RESULT AND DISCUSSION

Optimization of enzymatic hydrolysis of cassava starch and Interaction between independent variables

Table 2 presents the experimental design matrix for the enzymatic hydrolysis of cassava starch. Natural values were used to describe the 27 sets of coded conditions, as shown in Table 2. The corresponding results of the enzymatic hydrolysis are also included. The objective of this section is to identify the optimal values for starch concentration (X_1), cyanide concentration (X_2), and hydrolysis time (X_3) that maximize the yield of reducing sugar. These three factors were optimized simultaneously, including the interaction effects between the variables. The experimental design matrix in Table 2 served as a guide for conducting the experimental runs. Multiple regression analysis was employed to estimate the coefficients of the empirical model described in Eq. (1). The resulting empirical mathematical model for reducing sugar concentration (Y) is provided in Eq. (2).

$$Y = 52.23 + 7.06X_1 - 3.24X_2 + 7.33X_3 + 0.24X_1X_2 + 1.01X_1X_3 + 0.28X_2X_3 + 1.46X_1^2 - 0.28X_2^2 - 7.01X_3^2 \quad (2)$$

where Y is reducing sugar concentrationin g L^{-1}.

A positive coefficient in the model indicates a synergistic effect, while a negative coefficient indicates an antagonistic effect [17, 18]. The Pareto chart, presented in Fig. 1, was used to visually assess the main and interaction effects of all factors on the response variable [19]. In this chart, effects are represented as bars, and those extending to the right of the vertical line at a 0.05 p-value threshold are statistically significant and should be included in the mathematical model [20].

According to the Pareto chart, the factors that significantly influence the enzymatic hydrolysis of cassava starch include hydrolysis time, starch

Table 1. Coded levels of the independent variables for the design of the experiment.

Variable	Symbol	Coded variable levels		
		-1	0	+1
Starch concentration, g L^{-1}	X_1	100	200	300
Cyanide concentration, mg kg^{-1}	X_2	50	100	150
Hydrolysis time, h	X_3	6	12	18

Table 2. Combinations of experimental factors and values of modelled responses.

Run	X_1	X_2	X_3	Reducing sugar concentration, g L^{-1}		Residual
				Observed value	Predicted value	
1	-1	-1	-1	36.74	36.77	-0.03
2	-1	-1	0	48.98	49.83	-0.85
3	-1	-1	+1	49.06	48.86	0.20
4	-1	0	-1	33.62	33.29	0.33
5	-1	0	0	46.10	46.62	-0.52
6	-1	0	+1	46.21	45.94	0.27
7	-1	+1	-1	31.18	29.25	1.93
8	-1	+1	0	41.97	42.86	-0.89
9	-1	+1	+1	42.01	42.45	-0.44
10	0	-1	-1	41.66	41.12	0.54
11	0	-1	0	55.20	55.19	0.01
12	0	-1	+1	55.82	55.23	0.59
13	0	0	-1	38.14	37.88	0.26
14	0	0	0	51.92	52.23	-0.31
15	0	0	+1	52.90	52.55	0.35
16	0	+1	-1	30.60	34.08	-3.48
17	0	+1	0	49.94	48.70	1.24
18	0	+1	+1	50.10	49.30	0.80
19	+1	-1	-1	47.85	48.39	-0.54
20	+1	-1	0	63.96	63.47	0.49
21	+1	-1	+1	64.12	64.53	-0.41
22	+1	0	-1	45.54	45.40	0.14
23	+1	0	0	60.93	60.75	0.18
24	+1	0	+1	61.38	62.08	-0.70
25	+1	+1	-1	42.68	41.83	0.85
26	+1	+1	0	58.10	57.46	0.64
27	+1	+1	+1	58.43	59.08	-0.65

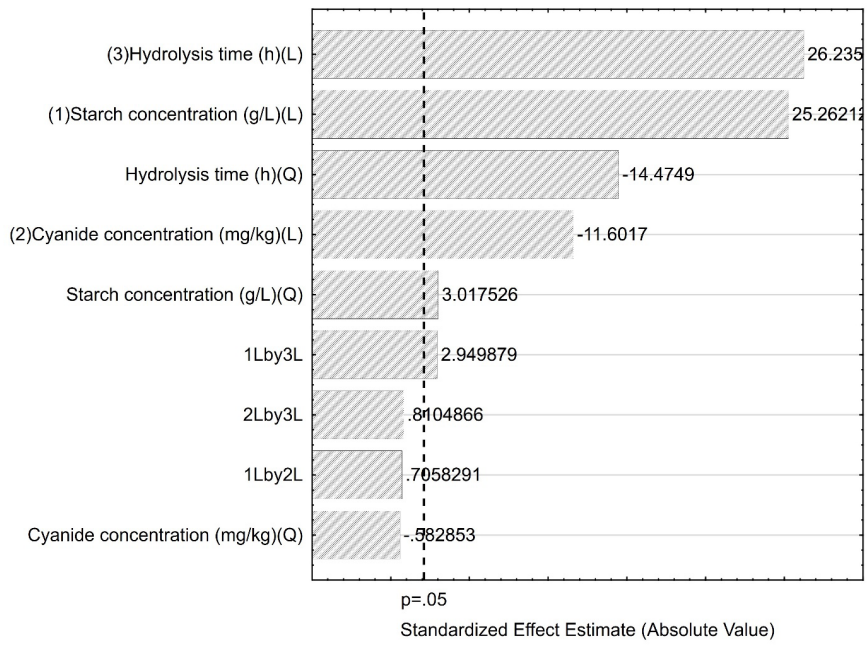

Fig. 1. Pareto chart of standardized effects on reducing sugar concentration.

concentration, the quadratic effect of hydrolysis time, cyanide concentration, the quadratic effect of starch concentration, and the interactive effect between hydrolysis time and starch concentration. Conversely, factors with less significant effects include the interaction between cyanide concentration and hydrolysis time, the interaction between starch concentration and cyanide concentration, and the quadratic effect of cyanide concentration.

The model confirmed that within the studied experimental range, hydrolysis time, starch concentration, the quadratic effect of starch concentration, and the interaction between hydrolysis time and starch concentration have a highly significant positive influence on reducing sugar concentration. This means that an increase in both incubation time and starch concentration generally result in higher reducing sugar yields. However, the quadratic effect of hydrolysis time has a highly significant inverse impact on reducing sugar concentration.

Cyanide concentration exhibits a highly significant negative effect on reducing sugar concentration. Additionally, the quadratic effect of cyanide concentration, the interaction between cyanide concentration and hydrolysis time, and the interaction between cyanide concentration and starch concentration have a less significant negative impact on reducing sugar concentration. Therefore, as cyanide concentration increases, the yield of reducing sugar decreases.

Model validation and its statistical analysis

The validity of the fitted model was assessed through an F-test to evaluate its statistical significance. The results of the ANOVA for the response surface full quadratic model are presented in Table 3. The model was found to be highly statistically significant at a 95 % confidence level, with a high F-value of 187.77 and an extremely low probability (p-value < 0.0001). A lower P-value indicates greater significance relative to parameters with higher p-values [20].

The suitability of the model was determined based on the regression coefficient (R^2), which reflects the proportion of the total variation in the observed values explained by the model [20]. The regression coefficients, R^2 and R_{adj}^2, were calculated as 0.9900 and 0.9848, respectively. According to Guan and Yao, an R^2 value of at least 0.80 is required for a model to be considered a good fit [21]. The R^2 value of 0.9900 indicates that approximately 99.00 % of the variance in reducing sugar concentration is explained by the model (Eq. 2), with only 1.00 % remaining unexplained. This high R^2 value demonstrates the model has strong reliability and alignment with the experimental data.

As shown in Table 2, the experimental and predicted values of the response variable are in good agreement, confirming the adequacy of the regression model. The reducing sugar concentration obtained from the experiments ranged from 30.06 to 64.12 g L^{-1}, while the corresponding predicted values ranged from 29.25

Table 3. Analysis of variance (ANOVA) for the response of reducing sugar.

Source	Sum of squares	df	Mean square	F - value	p - value, Prob > F	
Model	2377.29	9	264.14	187.77	< 0.0001	significant
X_1	897.75	1	897.75	638.17	< 0.0001	
X_2	189.35	1	189.35	134.60	< 0.0001	
X_3	968.29	1	968.29	688.32	< 0.0001	
X_1X_2	0.70	1	0.70	0.50	0.4899	
X_1X_3	12.24	1	12.24	8.70	0.0090	
X_2X_3	0.92	1	0.92	0.66	0.4289	
X_1^2	12.81	1	12.81	9.11	0.0076	
X_2^2	0.48	1	0.48	0.34	0.5676	
X_3^2	294.75	1	294.75	209.52	<0.0001	
Residual	23.91	17	1.41			
Cor total	2401.20	26				

to 64.53 g L^{-1}. Additionally, the "Adeq Precision" value, which measures the signal-to-noise ratio, was found to be 48.877, significantly exceeding the desirable threshold of 4. This result indicates that the model provides an adequate signal and is reliable for exploring the design space.

The relationship between the experimental data and the predicted values for reducing sugar yield is illustrated in Fig. 2. The high correlation ($R^2 = 0.9900$) observed in Fig. 2 demonstrates a rational agreement between the experimental and predicted values. This confirms that the data fit the model well and provide a robust approximation of the response within the experimental range investigated.

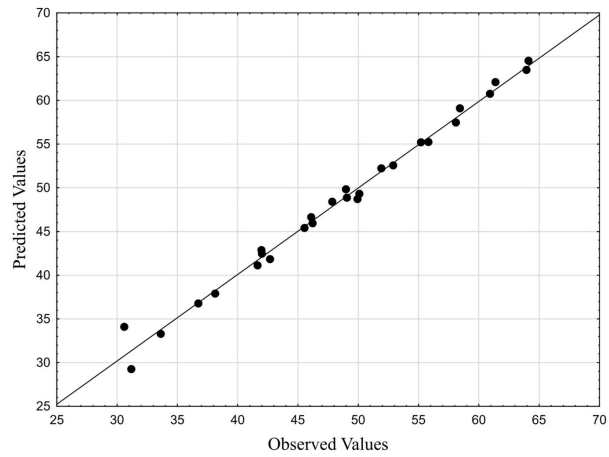

Fig. 2. Comparison of predicted and observed values of reducing sugar concentration produced.

Interaction among factors influencing enzymatic hydrolysis of Cassava starch and reducing sugar concentration

The empirical predicted quadratic model for the response variable (reducing sugar concentration) in relation to the process variables (starch concentration, cyanide concentration, and hydrolysis time) is visualized through three-dimensional surface plots, as shown in Fig. 3. These plots facilitate the review of interactions among the variables and help determine the optimal conditions for maximizing reducing sugar concentration.

Fig. 3a illustrates the interaction between starch concentration and hydrolysis time. Based on the ANOVA

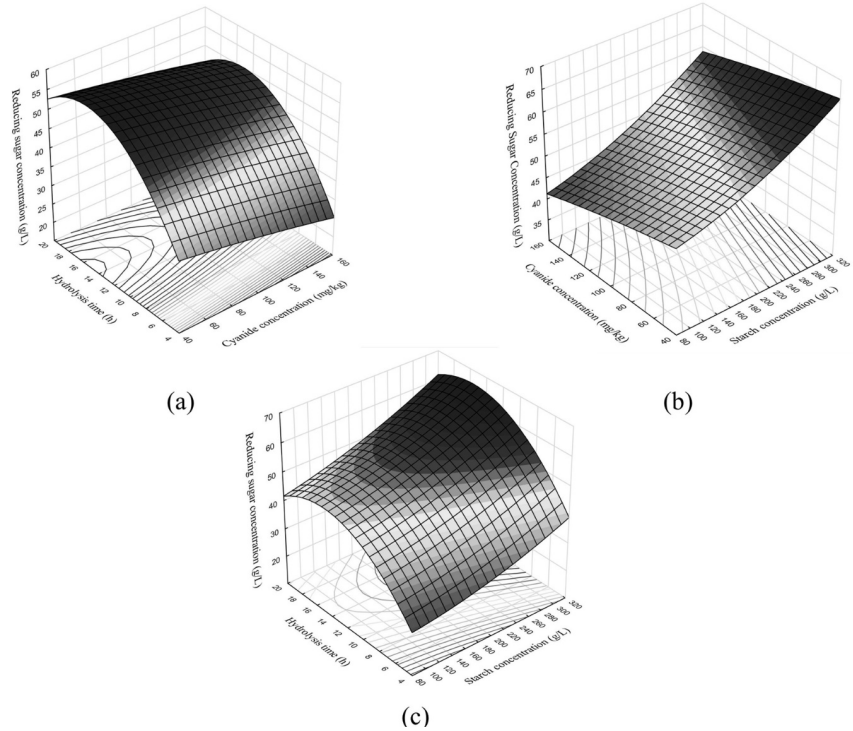

Fig. 3. 3D surface plots for the effect of interaction between (a) hydrolysis time, h and starch concentration, g L^{-1}, (b) cyanide concentration, mg kg^{-1} and starch concentration, g L^{-1}, (c) hydrolysis time, h and cyanide concentration, mg kg^{-1} on reducing sugar concentration.

analysis, this interaction is statistically significant, as it impacts the hydrolysis process. Reducing sugar is the product of a catalytic reaction involving starch and enzymes. Enzyme kinetics, particularly the catalytic efficiency, play a crucial role in this context. The curvature observed in the contour plot at the bottom of the graph indicates that hydrolysis time exhibits a pronounced quadratic effect, while starch concentration shows a linear effect on reducing sugar concentration.

Fig. 3b demonstrates the interaction between starch concentration and cyanide concentration. As indicated in Table 3 (ANOVA analysis), the interaction between these parameters has a very high probability value, suggesting it does not significantly influence reducing sugar concentration. Although reducing sugar is produced during the hydrolysis process, cyanide does not directly participate in the hydrolysis reaction. Instead, cyanide interacts with the enzyme, inhibiting its activity [4]. This lack of direct interaction between cyanide and starch results in the observed insignificance of this interaction. The plot in Fig. 3b reveals that cyanide negatively affects the hydrolysis process in a linear manner. Increasing cyanide concentration leads to a decrease in reducing sugar concentration, as cyanide acts as an inhibitor during starch hydrolysis [4].

Fig. 3c depicts the interaction between cyanide concentration and hydrolysis time. According to the ANOVA analysis, this interaction is not statistically significant, as indicated by the high probability value in Table 3. The hydrolysis process is inhibited in the presence of cyanide, confirming that cyanide serves as an inhibitor for cassava starch hydrolysis. The optimal conditions for enzymatic hydrolysis were calculated using Design Expert 10 (Stat-Ease, Inc.). The optimization results indicated a maximum reducing sugar concentration of 63.498 g L^{-1}, achieved with a starch concentration of 278.5 g L^{-1}, cyanide concentration of 51.4 mg kg^{-1}, and a hydrolysis time of 14.8 h.

CONCLUSIONS

Response Surface Methodology (RSM) has been confirmed as an effective and reliable tool for optimizing and analysing the interactive effects of key process variables involved in cassava starch hydrolysis. A highly significant regression quadratic model ($R^2 = 0.9900$, $p < 0.0001$) was developed, demonstrating its applicability for this purpose. Hydrolysis time, starch concentration, cyanide concentration, the quadratic effects of hydrolysis time and starch concentration, and the interaction between hydrolysis time and starch concentration were identified as the most significant factors influencing the process. On the other hand, the interactions between cyanide concentration and hydrolysis time, between substrate concentration and cyanide concentration, and the quadratic effect of cyanide concentration were found to have less significant impacts. The predicted optimal hydrolysis conditions were achieved with a starch concentration of 278.5 g L^{-1}, a cyanide concentration of 51.4 mg kg^{-1}, and a hydrolysis time of 14.8 h.

Acknowledgments

The authors would like to thanks Diponegoro University for the finacial support.

Authors' contributions: *H.H.: Experimental work, Writing, Project management and funding acquisition; A.C.K.: Review, Editing; B.J.: Conceptualization, Design of the research, A.P.: Conceptualization, Writing; D.P.U.: Editing of the manuscript, Writing.*

REFERENCES

1. A. Burns, R. Gleadow, J. Cliff, A. Zacarias, T. Cavagnaro, Cassava: The Drought, War and Famine Crop in a Changing World, Sustainability**,** 2, 11, 2010, 3572-3607.
2. M. Lambri, M.D. Fumi, A.D.M. de Faveri, Improved Processing Methods to Reduce the Total Cyanide Content of Cassava Roots from Burundi, African J. Biotechnol., 12 19, 2013, 2685-2691.
3. A.P. Moshi, C.F. Crespo, M. Badshah, K.M. Hosea, A.M. Mshandete, B. Mattiasson, (2014). Characterisation and Evaluation of a Novel Feedstock, Manihot glaziovii, Muell. Arg, for Production of Bioenergy Carriers: Bioethanol and Biogas, Biores. Technol., 172, 2014, 58-67.
4. H. Hargono, A.C. Kumoro, B. Jos, Inhibitory Effects of Cyanide on the Activity of Granular Starch Hydrolyzing Enzyme (GSHE) during Hydrolysis of Cassava (Manihot esculenta Crantz) Starch, Periodica Polytech. Chem. Eng., 63, 1, 2019, 11-17.
5. H.H. Hyun, J.G. Zeikus, Regulation and genetic

enhancement of beta-amylase production in Clostridium thermosulfurogenes, J. Bacteriol., 164, 3, 1985, 1162-1170.
6. G.H. Robertson, D.W. Wong, C.C. Lee, K. Wagschal, M.R. Smith, W.J. Orts, Native orraw starch digestion: a key step in energy efficient biorefining of grain, J. Agric. Food Chem., 54, 2, 2006, 353-365.
7. A. Kosugi, A. Kondo, M. Ueda, Y. Murata, P. Vaithanomsat, W. Thanapase, Y. Mori, Production of Ethanol from Cassava Pulp via Fermentation with Surface-engineered Yeast Strain Displaying Glucoamylase, Renewable Energy, 34, 5, 2009, 1354-1358.
8. F. Taylor, F., M. Marquez, D.B. Johston, N.M. Goldberg, K.B. Hicks, Continous high solid corn liquefaction and fermentation with stripping of ethanol, Biores. Technol., 101, 12, 2010, 4403-4408.
9. S. Shanavas, S., G. Padmaja, S.N. Moorthy, M.S. Sajeev, J.T. Sheriff, Process Optimization for Bioethanol Production from Cassava Starch Using Novel Eco-Friendly Enzymes, Biomass Bioenergy, 35, 2, 2010, 901-909.
10. Genencor, "STARGEN™ 002: Granular Starch Hydrolyzing Enzyme for Ethanol Production", 2009.
11. H. Hargono, B. Jos, A.C. Kumoro, Kinetics of the Enzymatic Hydrolysis of Sweet Cassava Starch and Bitter Cassava Flour and Gadung (Dioscorea hispida Dennst) Flour at Low Temperature, Bull. Chem. React. Eng. Catal., 12, 2, 2017, 256-262.
12. G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem., 31, 3, 1959, 426-428.
13. G.E.P. Box, D.W. Behnken, Some New Three-Level Designs for the Study of Quantitative Variables, Technometrics, 2, 1960, 455-475.
14. U.D. Miljic, V.S. Puskas, Influence of fermentation conditions on production of plum (Prunus domestica L) wine: A response surface methodology approach, Hemijska industrija, 68, 2, 2014, 199-206.
15. E. Betiku, O.O. Akindolani, A.D. Ismaila, Enzymatic Hydrolysis Optimization of Sweet Potato (Ipomoea batatas) Peel Using A Statistical Approach, Brazilian J. Chem. Eng., 30, 3, 2013, 467-476.
16. A.H. Sebayang, M.H. Hassan, H.C. Ong, S. Dharma, A.S. Silitonga, F. Kusumo, T.M.I. Mahlia., A.H. Bahar, Optimization of reducing sugar production from Manihot glaziovii starch using response surface methodology, Energies, 10, 1, 2017, 35-48.
17. N.S. El-Gendy, H.R. Madian, S.S.A. Amr, Design and Optimization of a Process for Sugarcane Molasses Fermentation by Saccharomyces cerevisiae Using Response Surface Methodology, Int. J. Microbiol., 1, 2013, 815631, 1-9.
18. N. Eslahi, F. Dadashian, N.H. Nejad, Optimization of enzymatic hydrolysis of wool fibers for nanoparticles production using response surface methodology, Adv. Powder Technol., 24, 1, 2013, 416-426.
19. M. Alkiayat, A Practical Guide to Creating a Pareto Chart as a Quality Improvement Tool. Global J. Quality Safety Healthcare, 4, 2, 2021, 83-84.
20. A. Chittaranjan, The P Value and Statistical Significance: Misunderstandings, Explanations, Challenges, and Alternatives, Indian Psychiatric Society - South Zonal Branch, 41, 3, 2019, 210-215.
21. X. Guan, H. Yao, Optimization of viscozyme Lassisted extraction of oat bran protein using response surface methodology, Food Chem., 106, 2008, 345-351.