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OPTIMIZATION OF SOLID STATE FERMENTATION CONDITIONS FOR CYANIDE CONTENT REDUCTION IN CASSAVA LEAVES USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Cassava leaves are a good source of protein. However, their use is limited because of the presence of cyanogenic glucosides. These require a further detoxification process in order to reduce the cyanide to a safe level prior to human consumption. The main objectives of this work are: (i) to demonstrate the effectiveness of solid-state fermentation using *Saccharomyces cerevisiae* on the cyanide content degradation of cassava leaves; and (ii) to optimize the independent variables for the minimum cyanide content level of cassava leaves by the application of response surface methodology (RSM). The various process parameters investigated for these purposes were sucrose concentration, urea concentration, moisture content, and fermentation time. The degradation of cyanide content was described by the quadratic model, which resulted in an excellent fit of the experimental data ($p < 0.01$). The statistical tests show that linear terms for sucrose concentration, urea concentration, moisture content and fermentation time had a significant effect on cyanide content ($p < 0.01$). Moreover, the interaction coefficients between sucrose concentration and fermentation time; urea concentration and moisture content; and nitrogen concentration and fermentation time were significant model terms ($p < 0.05$). A minimum cyanide content of 0.81 ppm was obtained at 1% (w/w) sucrose concentration, 0.5% (w/w) urea concentration, 60% (v/w) moisture content and with a fermentation time of 78 hours. The optimal level made a significant reduction in cyanide content of 97.96%, which is lower than the toxicity level suggested by the World Health Organization of 10 ppm.

Keywords: Cassava leaves; Cyanide content; Response surface methodology; Solid state fermentation

1. INTRODUCTION

With the growth in food consumption, the majority of people rely heavily on food crops as their primary food sources. Root crops, such as cassava, are grown in developing countries as a primary source of carbohydrates (Hawashi et al., 2018). This crop represents one of the primary sources of food for Indonesian people, along with other staples such as rice, sago and corn. Reports indicate a production rate of nearly 20 million tons per year, harvested from 1.93 million hectares (Agustian, 2016). Cultivation of cassava plants can take place even in marginal environmental conditions, due to their high drought tolerance, with an optimal yield of

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approximately 50% for leaves and 6% for roots upon plant maturity (Tewe & Lutaladio, 2004). Cassava leaves contain valuable protein and nutrients and are consumed in many countries, including Indonesia, Malaysia, the Congo, Madagascar and Nigeria (Latif & Muller, 2015). However, they contain both nutritive and non-nutritive compounds. Among the anti-nutrients, particular concern is paid to cyanide acid (HCN), whose concentration in fresh cassava leaves is much higher than the safe limit recommended by the World Health Organization (WHO) for human consumption (10 ppm). As an effect of consuming a high concentration of cyanide, HCN poses health problems to the human body. Such a condition is known as Konzo disease, an irreversible neurological disorder associated with cyanide consumption (Bradbury, 2006). Other long-term exposure to cyanogenic glycosides from eating cassava includes tropical ataxic neuropathy, neurological effects, and damages to goiter, and thyroid functions (WHO, 2008). In addition, HCN is an inhibitor of the oxidation processes occurring in the mitochondria, which can lead to chronic toxicity. Therefore, it is essential that the content of cyanide be reduced below 10 ppm to allow people to safely consume cassava (WHO, 1995).

Reduction of cyanide levels can also be made in cyanide-rich raw food sources such as cassava. Worldwide, the most common methods of cassava leaf processing include boiling and soaking in water, steaming, sun drying, and oven drying. These approaches aim to reduce the toxic compounds in the leaves for human consumption (Fasuyi, 2005). Cassava leaf processing is mainly based on the endogenous cassava enzyme (linamarase), which catalyzes the conversion of cyanide-containing compounds (linamarin) into acetone cyanohydrin, which either enzymatically or spontaneously decomposes into HCN and acetone (Montagnac et al., 2009). However, some methods (such as steaming and oven drying) have been proven to be ineffective for lowering the cyanide content in cassava leaves to the safe limit. Studies have shown that the fermentation of cassava leaves is a promising method for reducing cyanide content (Kobawila et al., 2005; Morales et al., 2018). These reports show respective reductions of at least 70% and 94.18% in cyanide content during fermentation. They further validate the preference for the fermentation technique over conventional methods. The SSF technique has several advantages, including high productivity and reduced processing time (Febrianti et al., 2017). However, reports indicating the efficiency of cassava leaf fermentation are quite scarce compared to those which investigate tubers.

Various process conditions such as moisture content, pH, inoculum size, fermentation time, concentration of nutrient supplementation and temperature can affect the microbial growth, enzyme production, and formation of the product during the fermentation process (Ezekiel & Aworh, 2013). The optimization processes using the "One Variable at One Time (OVAT)" technique (changing one single variable, while keeping others at constant levels) is an inefficient way of determining the interaction between the process variables as it involves high cost and requires various experiments to obtain the optimum levels (Braga et al., 2011; Hadiyat & Wahyudi, 2013). Recently, the application of RSM has attracted the attention of researchers working with fermentation to optimize process conditions and evaluate the correlation between independent variables and their responses (Istianah et al., 2018).

Yeast and lactic acid bacteria (LAB) are the most investigated microorganisms for the production of linamarase during cassava fermentation and the development of flavor. Yeast, such as *Saccharomyces cerevisiae*, has several advantages, including its availability, low cost, ability to secrete extracellular enzymes, non-pathogenic character, and widespread use in traditional fermentation, particularly in fermented foods (Oboh & Akindahunsi, 2003). Furthermore, *Saccharomyces cerevisiae* is able to use cyanogenic glucosides and their metabolites during food processing, making it one of the micro-organisms which is most involved in the cassava fermentation process (Lambri et al., 2013). Therefore, the objective of this work is to demonstrate the effectiveness of solid-state fermentation using *Saccharomyces*

cerevisiae in the reduction of cyanide in cassava leaves. Furthermore, the optimization of the independent variables (moisture content, incubation time and nutrient supplementation) to achieve a minimum cyanide content level in cassava leaves by employing response surface methodology (RSM), is also studied in detail.

2. EXPERIMENTAL PROCEDURE

2.1. Material

Freshly harvested cassava leaves were obtained from a cassava plantation in Menganti, Gresik. Raw leaves were chopped and dried at 40ºC in an oven for 48 hours, milled in a blender and subsequently sieved to obtain a granulometric fraction below 100 meshes.

2.2. Microorganism and Preparation of Inoculum

The *Saccharomyces cerevisiae* (InaCCY655) was obtained from the Research Center for Biology, Indonesian Institute of Sciences (LIPI). It was cultured in a 500 mL Erlenmeyer culture flask that contained 100 mL of PDB (Potato Dextrose Broth) as a cultivation medium for yeast and incubated in a shaking incubator at 200 rpm for 24 hours at 30ºC. The cell density of 1 (OD_{600nm}) was reached and was further used to inoculate the solid substrate.

2.3. Determination of Cyanide Content

The titration method described by SNI (2011) was used to determine the cyanide acid content in the fresh, fermented cassava leaves. 20 g of leaves were transferred to a digestive flask and 200 milliliters of distilled water added. Then, the digestive flask was placed on a heater to recover the cyanide acid as a distillate. The distillate was then collected in a conical flask containing 20 mL of 2.5% NaOH. 8 milliliters of NH_4OH solution and 5 milliliters of KI solution were then added to the mixture, which was titrated to 0.02 N $AgNO_3$. A blank titration experiment was also run. The cyanide acid content was calculated using the following equation from the amount of $AgNO_3$ used for titration:

$$HCN = \frac{(V_1 - V_2) \times N \times 27}{(V_3 \times W)} \quad (1)$$

where V_1, V_2, V_3, N and W are the blank titration volume, sample titration volume, distillate volume, AgNO3 normality and sample weight, respectively.

2.4. Solid-State Fermentation (SSF)

Fifty grams of dry cassava leaves were weighed in each 500 mL Erlenmeyer flask. Carbon sources at 1%, 2% and 3% (w/w) and nitrogen sources at 0.5%, 1% and 1.5% (w/w) were separately added to the substrate in the form of sucrose and urea. By adding 50, 75 and 150 milliliters of distilled water, the moisture content of the samples was adjusted to 45% (v/w), 60% (v/w) and 75% (v/w), respectively. The Erlenmeyer flasks containing the mixtures were sterilized at 121ºC for 10 minutes, then left to cool at 26ºC for 30 minutes. Subsequently, 14 mL (1.9×10^8 CFU/g) of inoculum was added to flasks. Cotton and aluminum foils were used to cover the flasks. The mixture was fermented at 30ºC for different periods of time (24, 60 and 96 hours) and cyanide analyses were conducted in duplicate for each experiment.

2.5. Experimental Design

The Box-Behnken factorial design under response surface methodology (RSM) was used to investigate the effect of the independent variables, namely sucrose concentration (%), urea concentration (%), moisture content (%) and fermentation time (hr) on the cyanide content of cassava leaves, as represented by the letters A, B, C and D respectively. The levels of the independent variables are given in Table 1. The variables were coded according to the following equations:

$$A = (Sucrose\ concentration - 2)/1 \quad (2)$$
$$B = (Urea\ concentration - 1)/0.5 \quad (3)$$
$$C = (Moisture\ content - 60)/15 \quad (4)$$
$$D = (Fermentation\ time - 60)/36 \quad (5)$$

Table 1 Levels of the independent variables used in RSM

Independent variable	Unit	Code	Actual value		
Sucrose concentration	%	A	1	2	3
Urea concentration	%	B	0.5	1	1.5
Moisture content	%	C	45	60	75
Fermentation time	hr	D	24	60	96

The number of runs for the experimental design was determined by Equation 6 (Mason et al., 2003):

$$N = 2^K + 2k + n_0 \quad (6)$$

where N is the number of experiments, k is the number of independent variables and n_0 is the number of central points.

In the experiments, k = 4 and n_0 = 5. Hence, the Box-Behnken design of RSM consisted of 29 experiments, with each independent variable at three levels. The second order polynomial regression equation was used to fit the experimental data, as shown in Equation 7. The data were analyzed by analysis of variance (ANOVA) to determine the F value, lack of fit, and the coefficient of determination (R^2) of the experimental model using Design-Expert software (trial version11.1.0.1).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD + \varepsilon \quad (7)$$

where Y is the response; β_0 is the equation constant; β_1, β_2, β_3 and β_4 are the linear coefficients; β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are the interaction coefficients; and ε is the error term.

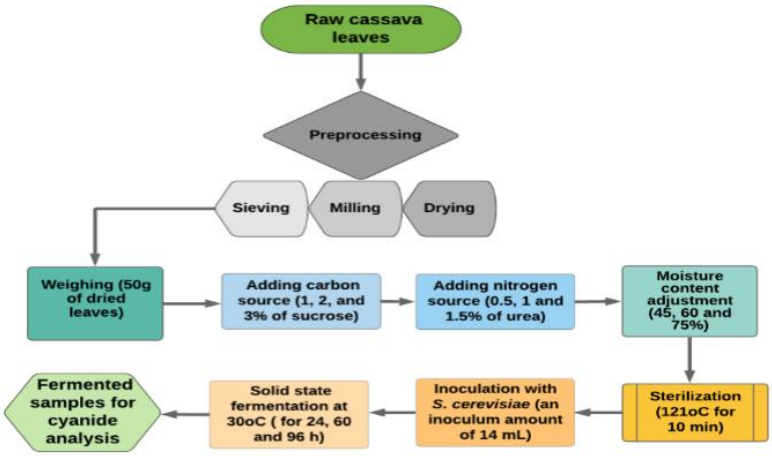


Figure 1 Schematic outline of the solid state fermentation process for cassava leaves

3. RESULTS AND DISCUSSION

3.1. Effect of Solid State Fermentation on Cyanide Content

The experimental results of the cyanide content of fermented cassava leaves under different conditions are shown in Table 2. The experimental data suggest that the content is in the range of 1.06 to 15.77 ppm. The initial cyanide content in cassava leaves was 39.80 ppm, indicating that its reduction ranged from 97.3 to 60.4%. It was observed that the content was reduced in line with an increase in fermentation time. Indeed, the experimental results show that after 60 hours of fermentation, the concentration of the cyanide in the leaves was below 10 ppm, bringing it to a safe level for human consumption (WHO, 1995). These results agree with a study performed by Oboh and Akindahunsi (2003), which showed a marked decrease in the cyanide content of cassava products (cassava flour and gari) under solid-state fermentation with *Saccharomyces cerevisiae* after 72 hours of fermentation. Another work by Gunawan et al. (2015) reported that after fermentation with *Saccharomyces cerevisiae*, the cyanide content in cassava flour could be reduced by more than 88%. In our study, the results of the experiment demonstrated the effectiveness of the combination of pre-treatment followed by SSF with yeast to reduce cyanide content to safe levels for human consumption.

Table 2 Box-Behnken experimental design showing the experimental and predicted responses for cyanide content

Run No	Sucrose Concentration (%)	Urea Concentration (%)	Moisture Content (%)	Fermentation Time (hr)	Cyanide Cont. (Exp.) (ppm)	Cyanide Cont.(Predict.) (ppm)
1	2	0.5	60	96	1.06	1.02
2	1	1.5	60	60	2.72	2.51
3	2	1.5	45	60	4.23	4.52
4	3	1	60	96	2.15	2.37
5	1	1	60	96	1.22	1.44
6	2	1.5	75	60	2.37	2.62
7	3	0.5	60	60	4.35	4.42
8	2	1.5	60	96	1.14	1.01
9	1	0.5	60	60	1.88	2.00
10	2	1	60	60	2.28	2.29
11	3	1	60	24	15.77	15.67
12	2	1.5	60	24	13.26	13.32
13	2	1	45	96	1.96	2.01
14	3	1	75	60	4.37	4.61
15	2	1	75	96	1.29	0.96
16	1	1	60	24	11.31	11.20
17	2	1	60	60	2.25	2.28
18	2	1	45	24	13.44	13.63
19	2	0.5	45	60	3.14	3.01
20	2	1	60	60	2.11	2.14
21	3	1	45	60	6.40	6.24
22	1	1	45	60	3.28	3.30
23	2	1	60	60	2.49	2.28
24	1	1	75	60	2.18	2.37
25	3	1.5	60	60	5.77	5.50
26	2	0.5	60	24	11.59	11.74
27	2	1	60	60	2.27	2.28
28	2	0.5	75	60	2.76	2.59
29	2	1	75	24	12.56	12.38

3.2. Response Surface Analysis of Cyanide Content

In this study, the experimental data obtained from the RSM for the cyanide content were fitted with second-order regression models to study the interaction effect between the independent variables. An estimated regression model for cyanide content was expressed by Equation 8:

$$Y = +30.66 - 0.977A + 2.740B - 0.328C - 0.490D + 1.110A^2 + 0.875B^2 + 0.003C^2 + 0.0033D^2 + 0.290AB - 0.015AC - 0.0245AD - 0.049BC - 0.0220BD + 0.0097CD \quad (8)$$

Use of statistical parameters, such as the F value, lack of fit, and the determination coefficient R^2, are common practice in RSM studies, and provide useful information about the suitability of the experimental data for proposed models. The developed quadratic model was evaluated by using analysis of variance (ANOVA). As indicated by the obtained ANOVA results in Table 3, the model F-value of 582.51 implied that the model was significant at $p < 0.0001$; there was only a 0.01 percent chance that the F-value could occur due to noise. Moreover, the "lack of fit F value" of 4.73 implies that lack of fit was not significant. The non-significant "lack of fit F value" was acceptable, and therefore the suggested model was adequate to predict the response and interpret the effect of the variables on it (Silvestrini et al., 2013). The determination coefficient (R^2) was 0.9983, suggesting that 99.83% of the variation in cyanide content (ppm) could be explained by the developed model.

Table 3 Analysis of variance (ANOVA) for the quadratic model

Source	Sum of square	df	Mean square	F-value	P-value	Remarks
Model	551.61	14	39.47	582.51	< 0.0001	significant
A	21.92	1	21.92	323.54	< 0.0001	significant
B	1.85	1	1.85	27.28	< 0.0001	significant
C	3.99	1	3.99	58.89	< 0.0001	significant
D	398.02	1	398.02	5873.69	< 0.0001	significant
AB	0.0841	1	0.0841	1.24	0.2840	not significant
AC	0.2162	1	0.2162	3.19	0.0957	not significant
AD	3.12	1	3.12	45.97	< 0.0001	significant
BC	0.5476	1	0.5476	8.08	0.0130	significant
BD	0.6320	1	0.6320	9.33	0.0086	significant
CD	0.0110	1	0.0110	0.1627	0.6928	not significant
A^2	7.99	1	7.99	117.94	< 0.0001	significant
B^2	0.3104	1	0.3104	4.58	0.0504	not significant
C^2	3.02	1	3.02	44.59	< 0.0001	significant
D^2	118.75	1	118.75	1752.48	< 0.0001	significant
Lack of Fit	0.8747	10	0.0875	4.73	0.0739	not significant

R^2 = 0.9983; Adjusted R^2 = 0.9966; Predicted R^2 = 0.9907; Adequate precision = 78.54

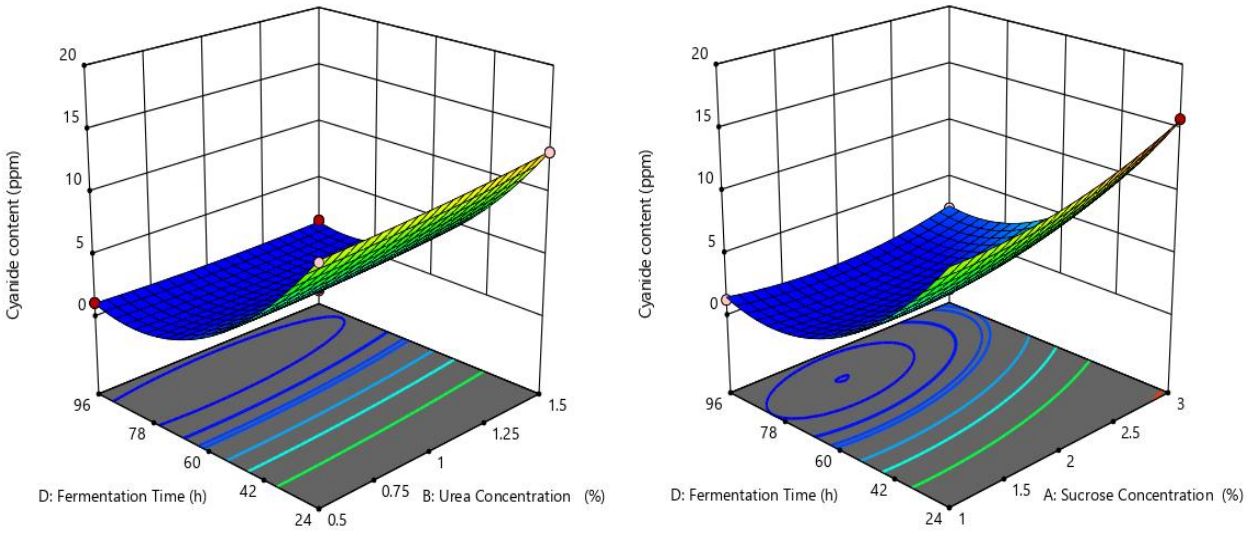
The predicted R^2 was 0.9907, implying that the developed model was able to explain 99.07% of the variability in predicting new observations (Silvestrini et al., 2013).

Furthermore, the predicted R^2 of 0.9907 was in reasonable agreement with the adjusted R^2 of 0.9966; i.e., the difference was less than 0.2. The adequate precision value measures the signal-to-noise ratio, and in general any ratio greater than 4 is acceptable. Adequate precision of 78.54 indicates an adequate signal; i.e., the developed model can be used to navigate the design space. Statistical analysis of the experimental data (ANOVA) showed that all the individual terms of the model had a significant impact on cyanide content at $p < 0.0001$. Moreover, the quadratic coefficients of the three independent variables A^2, C^2 and D^2 had significant effects on cyanide content ($p < 0.0001$), and the interaction coefficients between sucrose concentration and

fermentation time (AD), urea concentration and moisture content (BC), and urea concentration and fermentation time (BD) were significant model terms.

For further investigation, three-dimensional plots of the response surface between two independent variables were used, while the two other independent variables were maintained at an optimal level in order to demonstrate the interactive effects of factors on the cyanide content of cassava leaves. Figure 2a shows the interactive effect of fermentation time and urea concentration on cyanide content. The lowest content of 1.06 ppm was obtained at 0.5% nitrogen concentration with 96 hours of fermentation, while the sucrose concentration and moisture content were at optimal levels of 2% and 60%, respectively. The effect of fermentation time and sucrose concentration on cyanide content is shown in Figure 2b. The minimum cyanide content (1.22 ppm) was observed at 1% sucrose concentration and 96 hours of fermentation. On the other hand, the maximum cyanide content (15.77 ppm) was obtained with 24 hours of incubation and 3% sucrose concentration.

From the response surface plots, it can be observed that cyanide content increased in line with an increase in the concentration of both urea and sucrose. The reduction in cyanide content also showed an increase with a further increase in fermentation time. Fermentation time has a significant effect on cyanide content. In general, it often determines the success of the fermentation process; longer fermentation times result in a greater reduction of cyanide content. Cyanide degradation was a result of linamarase activity during fermentation. Linamarase is an enzyme produced by microorganisms during fermentation, which effectively breaks down cyanogenic glucoside (Nwabueze & Odunsi, 2007). However, successful fermentation requires appropriate microbial activities, which are impacted by the quality and availability of carbon and nitrogen sources. Carbon and nitrogen are major components of vital biological structures (proteins, DNA/RNA, etc.) and as such carbon- and nitrogen-containing substrates are necessary to support microbial growth and proliferation (Gunawan et al., 2015).



(a) (b)

Figure 2 3D response surface plots showing interaction between variables: (a) Effect of fermentation time andconcentration of urea on cyanide content (concentration of sucrose 2%, moisture content 60%); (b) Effect of fermentation time and concentration of sucrose on cyanide content (moisture content 60%, concentration of urea 1%)

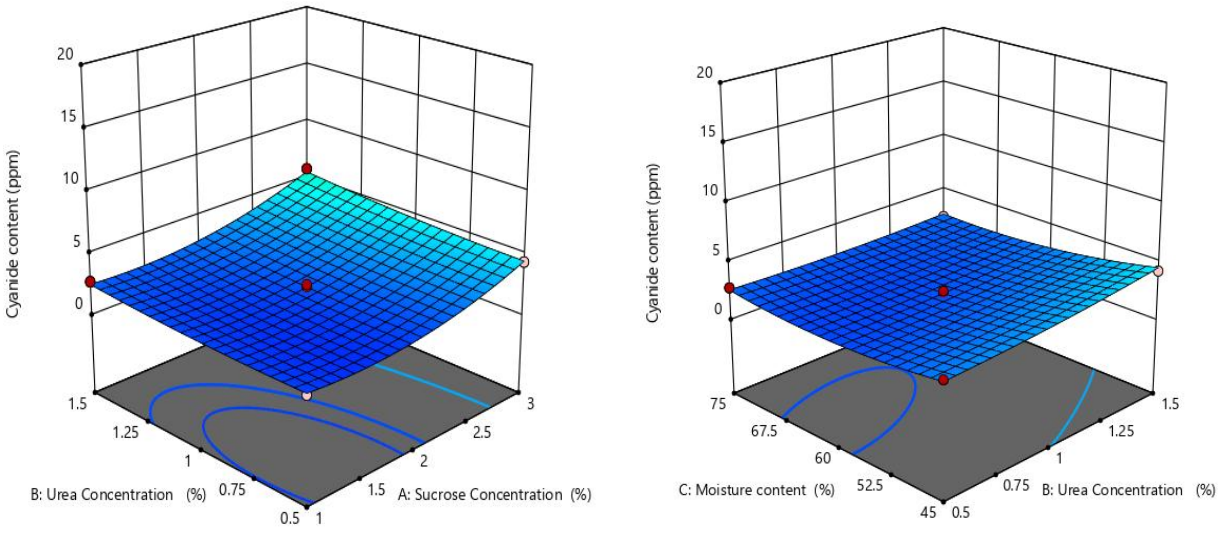


Figure 3 3D response surface plots showing interaction between variables: (a) Effect of urea and sucrose concentrations on cyanide content (moisture content 60%, fermentation time 60 h); (b) Effect of moisture content and concentration of urea on cyanide content (concentration of sucrose 2%, fermentation time 60 h)

Figure 3a shows that the content of cyanide was higher at both lower and higher concentrations of urea and sucrose. Therefore, the interaction between the concentration of urea and that of sucrose had a non-significant effect on the content of cyanide ($P > 0.05$). Figure 3b shows the effect of moisture content and urea concentration on cyanide content. The minimum cyanide content of 2.27 was observed at 60% moisture content and 1% urea concentration, with an optimal level of sucrose concentration of 2% and a fermentation time of 60 hours. Low moisture content had a slight effect on cyanide content. According to Grover et al. (2013), for an efficient SSF system, the required moisture content should range between 60 and 80%. In our experiments, the minimum cyanide content was observed in fermented leaves with 60% moisture content, which is in accordance with other studies.

Moreover, the experimental results were very similar to the predicted cyanide content (Table 2). The diagnostic plots conclude that the model fulfilled the assumptions of the variance analysis and reflects the precision and implementation of the response surface methodology to optimize the process of reducing the cyanide content of cassava leaves to the optimal level.

3.3. Optimal SSF Conditions and Model Validation

Optimal process conditions were identified by the numerical method of optimization using Design-Expert software. The predicted optimal process conditions for minimizing cyanide content were 1% w/w sucrose concentration, 0.5% w/w urea concentration, 60% v/w moisture content, and with a fermentation time of 78 hours. For validation of the developed model, two experiments were performed and the observed results were compared with the predicted results given by the Box-Behnken design (BBD). The experimental cyanide content (0.81 ppm) in optimized conditions was comparable to the predicted cyanide content (0.80 ppm), which gives a < 1.3% error. Under optimal conditions, 97.96% of the total cyanide was removed.

The application of response surface methodology (RSM) under BBD has been shown to be useful in the evaluation and optimization of the process parameters used in the reduction of cyanide content in bamboo shoots (Rana et al., 2012). Thus, the experimental SSF system generated results consistent with those predicted by the model, indicating a reliable model for predicting optimal conditions for achieving minimum cyanide content by solid-state

fermentation.

4. CONCLUSION

The research has investigated the effect of solid state fermentation using *Saccharomyces cerevisiae* on the removal of cyanide content from cassava leaves. The study has shown that response surface methodology (RSM) was a high-performance technique for optimization of the process conditions for minimizing cyanide content in fermented cassava leaves through solid-state fermentation. The optimal process condition was obtained at 1% (w/w) sucrose concentration, 0.5% (w/w) urea concentration and 60% (v/w) moisture content, with a fermentation time of 78 hours. It was observed that an exponential decrease in cyanide content over time can lead to satisfactory detoxification in cassava leaves, with cyanide concentration falling to levels lower than 10 ppm after 60 hours of fermentation, and thus providing a safe and healthy food source.

5. ACKNOWLEDGEMENT

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