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Full Length Research Paper

Optimization of divalent cation in *Saccharomyces pastorianus* medium conditions for ethanol production

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Cassava starch fermentations were conducted in batch cultures to optimize the effect of divalent cations on ethanol production with *Saccharomyces pastorianus* using the central composite rotatable response surface design. Divalent cations used were magnesium (Mg^{2+}), zinc (Zn^{2+}) and calcium (Ca^{2+}). Maximum ethanol concentration of 11.12% v/v was obtained with cationic concentration combination of 64, 0.48 and 30 mg/l for Mg^{2+}, Zn^{2+} and Ca^{2+}, respectively, after 96 h of the fermentations. Minimum ethanol concentration of 7.53% (v/v) was obtained at a variable combination of 64, 0.48 and 76 mg/l for Mg^{2+}, Zn^{2+} and Ca^{2+}, respectively. Thus response surface methodology was used in a central composite design to optimize the process variables of Mg^{2+}, Zn^{2+}, Ca^{2+} in the fermentation medium, thereby increasing the ethanol production from 10.5% in the control to 11.12%. There were significant linear and quadratic effects of Zn^{2+} as well as a significant ($P \leq 0.05$) negative quadratic effect of Ca^{2+} on ethanol production, which are confirmed in the response surface plots.

Key words: Ethanol, *Saccharomyces pastorianus,* cassava starch, hydrolyzates, central composite design, response surface methodology.

INTRODUCTION

Ethanol is an important industrial chemical with emerging potential as a biofuel to replace fossil fuels (Rakin et al., 2009). It is one of the largest volume organic chemicals that are industrially produced (Ratnam et al., 2005). Ethanol can be produced by fermentation of sugars from agricultural products or waste materials (Rakin et al., 2009), basically those that contain starch, sugar, or cellulose (Kumar, et al., 1998). Worldwide production of ethanol by fermentation in 2003 reached 38 x 10^9 L per year. Biomass-derived fuels (biofuel) are carbon neutral, meaning that they do not add to the sum total of carbon dioxide in the atmosphere. Ethanol is increasingly being used as a substitute for fossil fuels in the transportation sector.

The importance of ethanol has prompted the use of adjuncts by various authors. Nwabueze and Uchendu (2009) working on African breadfruit (*Treculia africana*) seed as adjunct in ethanol production, reported values of 5.79, 6.39 and 6.10% of ethanol with defatted breadfruit, full fat breadfruit and maize, respectively. Under very high gravity (VHG) conditions, maximum ethanol levels were about 16.8% (v/v) and 11% (v/v) for media containing malted and unmalted milled sorghum grain, respectively (Bvochora et al., 2000). The authors carried out the fermentation for 96 h using malted and unmalted milled sorghum grain from sorghum cultivars DC-75 and SV-2. It was further observed that although fermentation did not occur to completion, levels of ethanol obtained under VHG conditions were 3 times higher than the levels obtained under normal fermentation conditions. Giovani et al. (2009) reported that addition of banana changed the concentration of all-malt wort or weight of the extract resulting to an increase in ethanol production, with approximately 0.4 g/g ethanol yield. Lakkana et al. (2009)

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reported that when sugarcane molasses was used as an adjunct, the juice under the same conditions gave the maximum ethanol concentration of 109.34 g/g.

In addition (Lakkana et al., 2009) reported that ammonium sulphate was not suitable for use as a nitrogen supplement in the sweet sorghum juice for ethanol production since it caused the reduction in ethanol concentration and yield for approximately 14% when compared to those of the unsupplemented juices. Any significant increase in ethanol yield by encouraging yeast functionality can be appreciated.

Metal ions play a role in the optimal functioning of yeasts which results in high ethanol yield. Among the cationic yeast nutrients, divalent cations such as magnesium, zinc and calcium, are involved in structural and enzymatic regulatory activities during growth and metabolism.

Magnesium (Mg^{2+}) is essential for yeast growth, metabolism and fermentation. It is an essential cation in nucleic acid synthesis and a cofactor of more than 300 enzymes, including hexokinases, phosphofrutokinase, phosphoglycerate kinase, pyruvate kinase and enolase in glycolysis (Walker, 1994). Zinc (Zn^{2+}) is also an essential micronutrient for yeast. Zinc plays a major role in yeast fermentative metabolism not only because it is essential for aldolase and ethanol dehydrogenase activity, but also because it can stimulate uptake of maltose and maltotriose in brewing yeast cells, thereby augmenting fermentation rates (Walker et al., 2006). Calcium (Ca^{2+}) requirement for yeast growth is very low (Youatt, 1993; Walker, 1994). However, calcium ions, Ca^{2+}, are acknowledged to play a key role in the important process of flocculation in brewing fermentations. Ca^{2+} is known to protect the enzyme complex through its contribution to the buffer systems integrity. The presence of Ca^{2+} in fermentation media may also compete with essential divalent cations like Mg^{2+} (Walker et al., 1996) and cause growth inhibition at high concentrations (Salkutoglu and Slaughter, 1983).

Optimization of the cationic (Mg^{2+}, Zn^{2+}, Ca^{2+}) nutrients required by yeast for fermentation is therefore very important for maximizing the yield and productivity and minimizing the production cost. Optimization by the classical method: a single dimensional search involving changing one variable while fixing the others at a certain level is laborious and time consuming, especially when the number of variables is large (Ratnam et al., 2005). The solution lies with the application of response surface methodology (RSM), which economizes experimental points by compressing them to far less experimental data (Nwabueze, 2007). It relates product properties to process variables and then describes the interactions between them to give response changes magnitude and direction.

The objective of this research is to optimize the divalent cations (Mg^{2+}, Zn^{2+}, Ca^{2+}) concentrations in yeast medium fermentation of cassava starch hydrolyzates for ethanol production.

MATERIALS AND METHODS

Cassava

About 50 kg of tubers of TMS 98/0581, a genetically developed cassava mosaic disease (CMD) variety were obtained from the National Root Crops Research Institute (NRCRI), Umudike, Nigeria. TMS 98/0581 is one of the recently developed varieties grouped as best for industrial purpose, with high dry matter (Dixon et al., 2005).

Yeast

Saccharomyces pastorianus, a lager brewing strain, was obtained from Champion Breweries Plc., Uyo, Nigeria.

Enzymes

Termamyl, Fungamyl and Amyloglucosidase (AMG) were obtained from Champion Breweries Plc., Uyo, Nigeria.

Micronutrients

Salts of ammonium sulphate ($(HN_4)_2SO_4$) and the divalent cations: $MgSO_4.7H_2O$; $ZnSO_4.7H_2O$; $CaCl_2.6H_2O$, were also obtained from Champion Breweries Plc., Uyo, Nigeria.

Raw material preparation

About 50 kg of freshly harvested cassava tubers were peeled, washed and processed into starch according to the method descrybed by IITA (1990). The starch was dried to about 10% moisture content stored in low density polythene bag and sealed with an impulse sealer (300H, England) to prevent re-absorption of moisture.

Mashing process

Mashing is a process of enzymatic degradation (hydrolysis) of starch to fermentable sugars. The mash was prepared at a ratio of 1:4 (IITA, 2005). About 250 g of starch was dispersed in 1 L of distilled water, stirred with a glass rod to obtain a uniform mixture and heated. The heating was performed in a thermostatic water bath (TR24-A22BX CBOOO, England) with a stirrer. The temperature of the mash was raised to 60°C at which the particles of starch hydrate and swell and at higher temperature begin to gelatinize, making them susceptible to enzymatic hydrolysis (Kumar et al., 1998; Hough et al., 1982).

The mixture was then treated with enzymes in 2 steps, liquefaction and saccharification (Rakin et al., 2009). *2* ml of Fungamyl and Termamyl, α-amylase (endo-enzymes) enzymes were added to the mash and heated to 90 - 95°C for 20 min with continuous stirring. This is to liquefy the starch and break it down to dextrins and oligosaccharides. The liquefied mash was cooled to 60°C and 2 ml of amyloglucosidase (AMG) were added and heated to 75°C

Table 1. Central composite design for a three-variable, 5 level divalent cation combinations.

Independent process variables (mg/L)		Coded variable levels				
		Corner points		Centre point	Star points	
		-1.682	-1	0	+1	+1.682
X_1	Mg	35	64	107	150	179
X_2	Zn	0.24	0.30	0.39	0.48	0.54
X_3	Ca	14.31	30	53	76	91.69

Where -1.682 = lower corner point; -1 = higher corner point; 0 = centre point; +1 = lower star point; +1.682 = higher star point. The 3 variables (X_1 to X_3), 5 level combinations (- 1.682 to +1.682) produced 15 divalent cation combinations (3 x 5). The centre point (107, 0.39 and 53 mg/L) was replicated 5 times generating a total of 20 experimental runs.

with continuous stirring for 45 min for starch-saccharification. The temperature of the mash was brought down to 30°C and tested for complete saccharification.

Fermentation process

Fermentations were performed with *S. pastorianus*, a larger brewing strain (726 x 10^6 cells/ml, 98.78% viability). About 6 g was weighed, a starter inoculum prepared and inoculated into the sterile hydrolyzates (500 ml). To each medium were added divalent cations (Mg^{2+}, Zn^{2+}, Ca^{2+}) in combinations given in Table 1 and free yeast cells. The fermentors were swirled to obtain a uniform mixture. Batch fermentations were conducted in an anaerobic condition at laboratory temperature and the samples analyzed at 24 h interval. Fermentation continued until a maximum alcohol yield was obtained.

Analytical method

Ethanol concentrations of the fermenting hydrolyzates were determined using an Anton Paar GMBH Alcolyzer Plus (COM 1, Austria, Europe). The samples were drawn into a flask sealed, shaken and released to degas. The degassed samples were filtered through folded Whatman filter paper (1 Qualitative, 10 cm, England) and the funnels covered immediately with a watch glass. The samples were swirled very well (to bring back any condensation of ethanol into the solution) and 50 ml filled into the sample vial and placed into the magazine of the sample changer (SP-1 m). The sample changer is a part of the sophisticated beer analyzing system of the Alcolyzer Plus. Ethanol concentration was displayed at 20°C by the instrument after measurement.

Ethanol distillation

At the end of fermentation, the hydrolyzates were filtered for distillation (recovery of ethanol). A 100 ml distillation flask (Clearfit 34/36, England) was filled with the fermented sample, placed in an electric heater and connected to a Clearfit distillation apparatus (KSH 4/33, England) with thermometer. Ethanol was distilled off at the temperature of 78.5°C (Okwu and Eneboachi, 2002).

Experimental design for optimization

A central composite rotatable response surface design for a three-variable, 5 level combinations coded -1, -1.682, 0, 1, 1.682 (Table 1), as modeled and used in literature (Snedecor and Cochran, 1980; Nwabueze, 2007) was used for the optimization of the divalent cations for ethanol production from cassava starch hydrolyzates. Magnesium (X_1, mg/L), Zinc (X_2, mg/ L) and calcium (X_3, mg/L) were chosen as the independent variables at 5 levels of concentration as shown in Table 1. A total of 20 experimental runs were performed for the optimization of the cations for fermentation. This included 5 replicates at the centre point in the design which makes the reproducibility of data possible.

Statistical analysis

Data obtained from the fermentation experiments were statistically regressed using Stagraphic Computer Software (STATISTICA) to test the significance (accepted at 5% probability level) of main and interactive effects of the cations (Nwabueze, 2007). Three-dimensional response surface plots were made with MATLAB 7.1.0246 (R14) GIBSOFT software. The statistical design (multivariate regression analysis) with the model fitted to each set of data was as follows:

$$Y= \beta_0 + \beta_1X_1 +\beta_2X_2 +\beta_3X_3 +\beta_{11}X_1^2 +\beta_{22}X_2^2 +\beta_{33}X_3^2 +\beta_{12}X_1X_2 +\beta_{13}X_1X_3 +\beta_{23}X_2X_3 + \varepsilon \quad (1)$$

Where Y = dependent response variables (ethanol). $\beta_0 + \beta_1 \ldots \beta_{23}$ = estimated regression coefficients. X_1, X_2, X_3 = independent variables in the model (Mg^{2+}, Zn^{2+}, Ca^{2+}) and ε = random error.

RESULTS AND DISCUSSION

Table 2 shows a progressive increase in ethanol production which peaked at 96 h of the 120 h fermentation irrespective of divalent cation combinations. Highest ethanol production of 11.12% v/v was obtained from cationic variable combinations of 64, 0.48 and 30 mg/l (Mg^{2+}, Zn^{2+}, Ca^{2+}), respectively at 96 h fermentation. High concentration of Mg^{2+} and Zn^{2+} and low Ca^{2+} seems to favor alcohol production as shown in the experimental data. Yeast exhibits a high affinity for Mg^{2+} and increase in Mg^{2+} availability stimulates alcohol production. Thus, Mg^{2+} is essential for yeast growth, metabolism and fermentation (Walker et al., 1996; Smith and Walker, 2000).

Table 2. Effect of the divalent cations concentration combinations on the ethanol production of the fermenting cassava starch hydrolyzates.

S/N	Variables			Responses (ethanol %, v/v)					
	X_1 (Mg)	X_2 (Zn)	X_3 (Ca)	0	24	48	72	96	120
1	64	0.30	30	0.24	2.09	5.16	9.96	10.50	10.42
2	64	0.30	76	0.25	2.58	5.82	9.60	10.88	10.86
3	64	0.48	30	0.26	3.02	6.54	9.96	11.12	11.10
4	64	0.48	76	0.23	2.00	5.06	7.34	7.53	7.50
5	150	0.30	30	0.25	2.98	6.48	9.72	11.04	11.00
6	150	0.30	76	0.26	2.51	5.83	9.98	10.98	10.96
7	150	0.48	30	0.24	2.37	6.08	9.76	10.96	10.92
8	150	0.48	76	0.25	2.31	5.66	9.49	10.80	10.76
9	179	0.39	53	0.24	2.51	5.80	9.58	10.89	10.86
10	35	0.39	53	0.25	2.30	5.67	9.39	10.70	10.67
11	107	0.54	53	0.25	3.00	6.01	9.68	10.93	10.90
12	107	0.24	53	0.24	2.34	5.08	8.84	9.42	9.40
13	107	0.39	91.69	0.24	2.50	5.86	9.39	10.45	10.42
14	107	0.39	14.31	0.23	2.04	5.27	7.36	7.98	7.93
15	107	0.39	53	0.26	2.02	5.38	9.25	10.42	10.40
16	107	0.39	53	0.26	2.04	5.39	9.27	10.40	10.39
17	107	0.39	53	0.27	2.03	5.38	9.28	10.41	10.40
18	107	0.39	53	0.26	2.05	5.38	9.26	10.40	10.38
19	107	0.39	53	0.26	2.02	5.39	9.25	10.42	10.40
20	107	0.39	53	0.25	2.04	5.38	9.26	10.41	10.40
C	-	-	-	0.24	2.98	5.42	8.24	10.25	10.22

Where C = control medium without cation combinations.

Zn^{2+} is an essential micronutrient and has stimu-lating effect in yeast metabolism. The control medium without divalent cations had a maximum ethanol produc-tion of 10.25% at the end of the same 96 h period of fermentation.

Alcohol production increased with high concentrations of Zn^{2+} (0.30 - 0.48 mg/L). Desmartez (1993) stated that 0.45 mg/L concentration of Zn^{2+} promoted fermentation and consequently alcohol production. Ca^{2+} requirement for yeast growth, metabolism and alcohol production are low (30-76 mg/L) in this work as Walker (1994) and Youatt (1993) also observed in their research.

Minimum ethanol production was 7.53 from the cationic concentration combinations of 64, 0.48 and 76 mg/L for Mg^{2+}, Zn^{2+} and Ca^{2+}, respectively, at the end of the 96 h period of fermentation. It was observed that in the opti-mum ethanol production, concentration of Ca^{2+} was higher than Mg^{2+}, Ca^{2+} exhibited its inhibitory/antagonistic effect on Mg^{2+} and consequently on the Mg-dependent processes and yeast growth (Walker et al., 1996). And in very low concentrations, Ca^{2+} cannot successfully buffer the fermenting medium. Ca^{2+} is known to protect the enzyme complex through its contribution to the buffer systems integrity (Walker, 2000). Walker et al. (1996) showed that by altering the Mg^{2+} and Ca^{2+} ratio in favor of Mg^{2+}, alcohol production by yeast increased. However, it is interesting to note that the main effect of Ca^{2+} was not significant for a high level of Mg^{2+} in the fermentation medium, which indicates that yeast has a higher affinity for Mg^{2+} than for Ca^{2+}. This finding supports the views of Saltokoglu and Slaughter (1983), Walker et al. (1996) and Chandrasena et al. (1997).

The estimated regression coefficients (Table 3), show-ed a significant ($P \leq 0.05$) negative quadratic effect of Ca^{2+} on ethanol. This is probably because Ca^{2+} antago-nizes uptake of Mg^{2+} and blocks essential Mg-dependent metabolic processes, thus, causing growth inhibition at high concentrations (Saltokoglu and Slaughter, 1983; Walker et al., 1996). The resultant polynomial equation after removing the non-significant terms is:

$$E = 0.23741 - 0.00001\ X_3^2 \quad (2)$$

Where E = ethanol; X_3 = Ca^{2+} and X_3^2 = quadratic order.

The response surface plot of interaction of Mg^{2+} and Ca^{2+} at 0 h period of fermentation showed a significant ($P \leq 0.05$) quadratic effect of Ca^{2+} on ethanol concentration

Table 3. Estimated regression coefficient for ethanol produced from fermented cassava starch hydrolyzates at 0 h.

Source	Coefficient	Standard error	df	*P*-value
Regression on constant	0.23741	0.01043		
X_1	-0.00019	0.00043	1	0.6706
X_2	0.03893	0.14941	1	0.7992
X_3	0.00085	0.00082	1	0.3234
X_1X_1	-0.000001	0.000001	1	0.3849
X_1X_2	0.00025	0.00084	1	0.7685
X_1X_3	0.000007	0.000004	1	0.0831
X_2X_2	-0.04202	0.26779	1	0.8782
X_2X_3	-0.00086	0.00161	1	0.6060
X_3X_3	-0.00001	0.000005	1	0.0446
R^2	0.4895			

Where X_1 = Mg^{2+}, X_2 = Zn^{2+} and X_3^{2+} = Ca^{2+}; X_1, X_2 and X_3= linear orders; X_1X_1, X_2X_2 and X_3X_3 =quadratic orders and X_1X_2, X_1X_3, X_2X_3 = interaction orders.

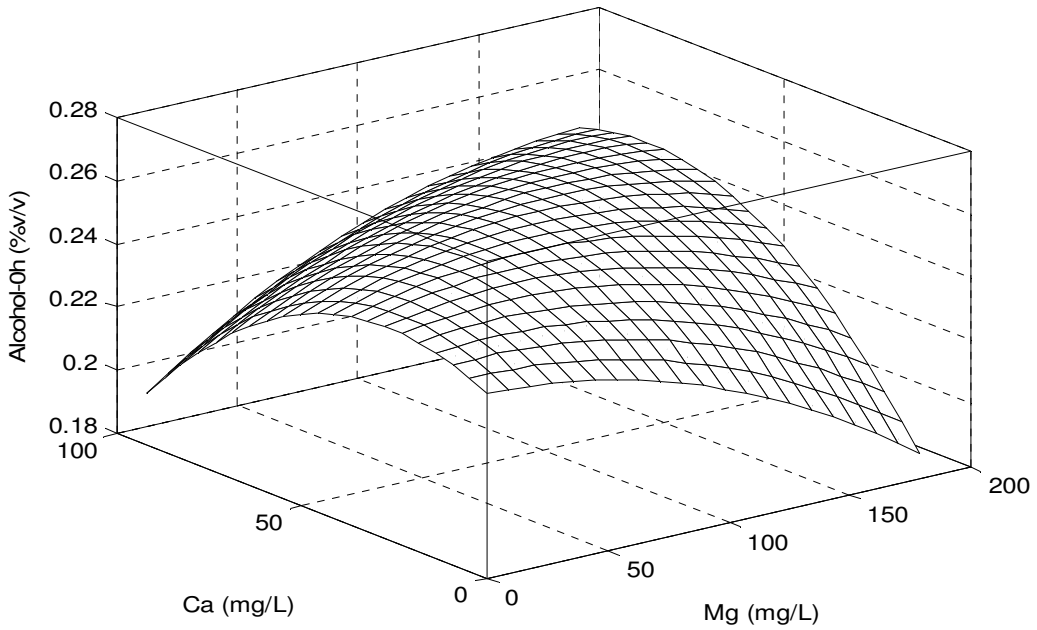

Figure 1. Response surface plot for ethanol at 0 h using Mg and Ca as process variables.

(Figure 1). The multiple regression model developed from the data explained 48.95% of the variation at this period of fermentation.

At 24 h, there was a significant linear and quadratic effect of Zn^{2+} on ethanol production (Table 4). This implies that Zn^{2+} is essential for fermentative metabolism and consequently alcohol production. Similar effect was reported by Chandrasena et al. (1997). Walker et al. (2006) also reported the importance of Zn^{2+} and its stimulatory effect on the rate of fermentation and alcohol production. The resultant polynomial after removing the non significant (P > 0.05) terms becomes:

$$E = 3.00168 - 9.60716X_2 + 24.69284X_2^2 \quad (3)$$

Where E = ethanol; X_2 = Zn^{2+}; X_2 = linear order and X_2^2 = quadratic order.

The response surface plot of the interaction between Zn^{2+} and Ca^{2+} shows the quadratic effect of Zn^{2+} on ethanol

Table 4. Estimated regression coefficient for ethanol produced from fermented cassava starch hydrolyzates at 24 h.

Source	Coefficient	Standard error	df	*P*-value
Regression on constant	3.00168	0.28954		
X_1	0.00603	0.01205	1	0.6266
X_2	-9.60716	4.14761	1	0.0408
X_3	0.01640	0.02283	1	0.4877
X_1X_1	0.00007	0.00004	1	0.1338
X_1X_2	-0.04566	0.02345	1	0.0774
X_1X_3	-0.00001	0.00010	1	0.8984
X_2X_2	24.69284	7.43374	1	0.0068
X_2X_3	-0.07829	0.04474	1	0.1080
X_3X_3	0.00014	0.00014	1	0.3467
R^2	0.6492			

Where X_1 = Mg^{2+}, X_2 = Zn^{2+} and X_3^{2+} = Ca^{2+}; X_1, X_2 and X_3 = linear orders; X_1X_1, X_2X_2 and X_3X_3 = quadratic orders and X_1X_2, X_1X_3, X_2X_3 = interaction orders.

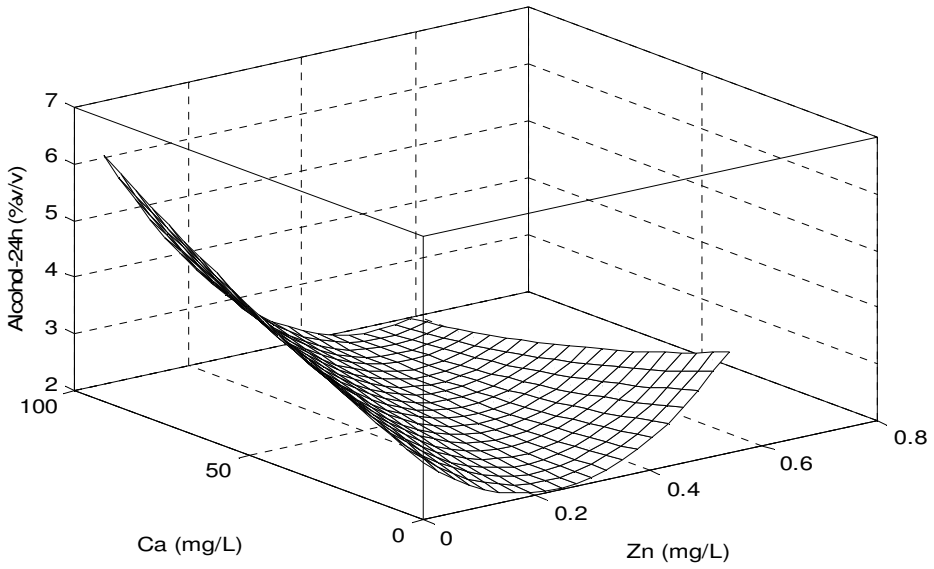

Figure 2. Response surface plot for ethanol at 24 h using Zn and Ca as process variables.

concentration (Figure 2). The multiple regression model developed from the data explained a variation of 64.92% at this period of fermentation.

Conclusion

A progressive increase in ethanol production using *S. pastorianus* peaked at 96 h of the 120 h fermentation irrespective of divalent cationic combinations. At the end of 96 h fermentation the control (no cation) recorded a maximum ethanol value of 10.25% while the experiential media recorded a range of 7.53% (4, 64 and 0.48) to 11.12% (64, 0.48 and 30 mg/L) for Mg^{2+}, Zn^{2+} and Ca^{2+} combinations, respectively. The response surface methodology took into consideration, effects of each cation as well as their interactions which conventional process methods lack, in determining appropriate divalent cation combination for maximum ethanol production in cassava hydrolyzate medium. Interaction of high concentrations of Zn^{2+} and Mg^{2+} favored ethanol production while interaction of high concentrations of Ca^{2+} and Mg^{2+} produced minimum ethanol at the end of the fermentation.

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