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Full Length Research Paper

Simultaneous effect of divalent cation in hydrolyzed cassava starch medium used by immobilized yeast for ethanol production

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Response surface methodology was adopted in a central composite design to optimize ethanol production from cassava starch hydrolysate medium. Starch hydrolyzate was prepared from TMS 98/0581, a genetically developed cassava mosaic disease-resistant variety. The yeast whole cell, *Saccharomyces pastorianus*, a lager brewing strain (726 x 10^6 cells/ml, 98.78% viability) and fungamyl and termamyl (α-amylase enzymes), used for the 120 h fermentation, were immobilized by entrapment in calcium alginate gel. Effects of three divalent cationic concentrations Mg^{2+}, Zn^{2+} and Ca^{2+} on ethanol yield were investigated at five variable combinations in 20 experimental runs in accordance with the experimental design. Maximum ethanol concentration of 12.53 %v/v was produced in the 96 h of fermentation when the divalent cationic combination was 64, 0.48 and 30 mg/l (Mg^{2+}, Zn^{2+} and Ca^{2+}), respectively. The study showed that effect of Zn^{2+} on ethanol yield was significantly ($P \leq 0.05$) quadratic.

Keywords: Ethanol, immobilization, *Saccharomyces pastorianus*, divalent cations, optimization, response surface methodology, cassava mosaic disease.

INTRODUCTION

Yeast fermentative growth in simple media and carbon skeleton requires adequate nitrogen (for protein synthesis), mineral salts (metal ions), one or more growth factors and molecular oxygen (Hough et al., 1982). Metal ions, especially divalent cations are necessary for the activation of several glycolytic enzymes and, in practical terms, if industrial media is deficient in them, the conversion of sugar to ethanol may be suppressed leading to slow or incomplete fermentation process (Walker et al., 2006). The uptake of these divalent cations into cells depends on the concentration of particular ions in the growth environment and on their bioavailability (Chandrasena et al., 1997).

To develop a process for the maximum production of ethanol, standardization of media and fermentation conditions is crucial (Ratnam et al., 2005). Optimization of the divalent cationic nutrients (Mg^{2+}, Zn^{2+}, Ca^{2+}) required by yeast for fermentation is therefore very important for maximizing the yield and productivity and minimizing the production costs. Response surface methodology (RSM) has been successfully applied to optimize alcoholic fermentation and other fermentation media (Chen, 1996; Chandrasena et al., 1997; Ambati and Ayyanna, 2001; Ratnam et al., 2003; Ratnam et al., 2005).

Ethanol production by immobilized yeast cells has been extensively investigated during the last few decades (Rakin et al., 2009). Immobilization of cells is very similar to the enzyme counterpart (Wang, 2008). For fermentation, immobilization of cells has been developed to eliminate inhibition caused by high concentration of substrate and product, thereby enhancing productivity and ethanol yield (Kourkoutas et al., 2004; Vullo and Wachsman, 2005; Baptista et al., 2006). According to Groboillot et al. (1994) the main advantages of the immobilization of yeast are the increase of ethanol yield and cellular stability, and a decrease of process expenses

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due to the ease of cell recovery and reutilization.

The aim of this study was to investigate the effect of divalent cation combinations in hydrolyzed cassava starch medium for ethanol production using immobilized yeast (*Saccharomyces pastorianus*). Bioavailability of these cations in an optimum combination is vital for successful ethanol production by directly influencing sugar metabolism by yeast in the 120 h fermentation period in this study.

MATERIALS AND METHODS

Raw materials

Cassava starch: The raw material for ethanol production was starch from cassava mosaic disease (CMD) resistant variety (TMS/0581) developed for food, feed and industrial use (Dixon et al., 2005). The CMD variety was obtained from National Root Crops Research Institute (NRCRI), Umudike, Nigeria and processed into starch according to the method described by the International Institute of tropical Agriculture (IITA, 2005). The starch constituted 100% particles that passed through a 425 µm sieve.

Enzymes

Fungamyl: This is purified fungal α-amylase produced from *Aspergillus oryzae*. This enzyme hydrolyzes 1, 4-α-glucosidic linkages in amylase and amylopectin, the two components of starch. *Termamyl*, an α-amylase isolated from *Bacillus licheniformis*, a soil bacterium. This enzyme hydrolyses 1, 4-α-glucosidic linkages in starch and possesses a high degree of heat stability. It is used for the continuous liquefaction of starch at temperatures of up to 105-110°C, breaking them rapidly to dextrins and oligosaccharides. *Amyloglucosidase* (AMG), an exo-1, 4-α-D-glucosidase (glucoamylase) was obtained from a selected strain of the fungus, *Aspergillus niger*. This enzyme hydrolyses 1, 4- and 1, 6-α-glucosidic linkages in liquefied starch in stepwise manner from the non-reducing end of the substrate molecules (Alais and Linden, 1999).

Yeasts: *S. pastorianus*, a lager brewing strain was used for the fermentation of cassava starch hydrolyzates. *S. pastorianus* is a hybrid organism of two yeast species- *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (Rainieri et al., 2006; Blieck et al., 2007; Dunn and Sherlock, 2008). It is thought that the combination of both parent species resulted in an organism able to out -compete other yeasts during the cold lager fermentations (Dunn and Sherlock, 2008). The enzymes and yeasts were gift from Champion Breweries Plc., Uyo, Nigeria.

Enzymatic hydrolysis of starch: A 250 g of cassava starch was mixed with distilled water at a weight ratio of 1:4 (IITA, 2005), stirred with a glass rod to obtain a uniform mixture (mash). The temperature of the mash was raised to 60°C at which the starch particles hydrate, swell and gelatinize, making them susceptible to enzymatic hydrolysis (Kumar et al., 1998). The mixture was then treated with enzymes in two steps: liquefaction and saccharification. The liquefaction was carried out at 90-95°C by adding 2 ml each of *fungamyl* and *termamyl* enzymes for 1 h. The liquefied mash was cooled to 60°C, 2 ml AMG added and heated to 75°C for saccharification. The hydrolysis was performed in flasks in a thermostatic water bath with a stirrer (TR24-A22BX, England).

Immobilization of *S. pastorianus* by entrapment in calcium alginate gel: A polymeric matrix was prepared using sodium alginate (NR 301054N, Hopkin and Williams Ltd., England). To obtain this, 300 ml of distilled water was pre-warmed at 60°C and 4.5 g of sodium alginate was added with continuous stirring until a homogenous solution was obtained (no clot) (Vullo and Wachsman, 2005). *S. pastorianus* (7.26×10^8 cells/ml, 98.78% viability) was weighed 11.25 g and mechanically suspended in the already prepared 1.5 % sodium alginate solution. An Erlenmeyer flask, 500 ml, was filled with the yeast suspension and then emptied by gravity, drop by drop, into 600 ml of 0.1 M $CaCl_2$ solution. The calcium cross-linking solution was agitated on a magnetic stirrer (STUART scientific magnetic stirrer SM 1, UK). Gel formation was achieved at room temperature as soon as the yeast-alginate drops came into direct contact with the calcium solution. Micro beads (0.8-1.0 mm diameter) were achieved with a 10 ml syringe/needle, to minimize the mass transfer resistance (Wang, 2008). The beads were fully harden in 15 min and were washed 4 times with distilled water to eliminate excess Ca^{2+} (Vullo and Wachsman, 2005). The drop volume was calibrated by passing distilled water through a diameter tube (10 ml syringe/needle) and weighing a fixed number of water droplets. The drop volume was 0.04 ml and all the suspension turned into immobilized yeast spheres with 10^7 cells per bead.

Ethanol fermentation of starch hydrolyzates: Starch hydrolyzates obtained by the 2-step hydrolysis of cassava starch were cooled, filtered to remove the trub and sterilized in an Oswald™ Autoclave steam sterilizer (JRIC 39, India). Ethanol fermentation by immobilized yeasts under anaerobic conditions was performed in 1l flasks with 500 ml of medium in laboratory temperature (20°C). Repeated batch fermentations were carried out with the same inoculum concentration and laboratory conditions, but different concentrations of divalent cation combinations for 5 days. The summary of ethanol production from cassava (CMD-resistant variety) TMS/0581is is given in Figure 1.

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-1m). The sample changer is a part of the sophisticated beer analyzing system of the Alcolyzer Plus. Ethanol concentration is displayed at 20°C.

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

A central composite rotatable response surface design for a three-

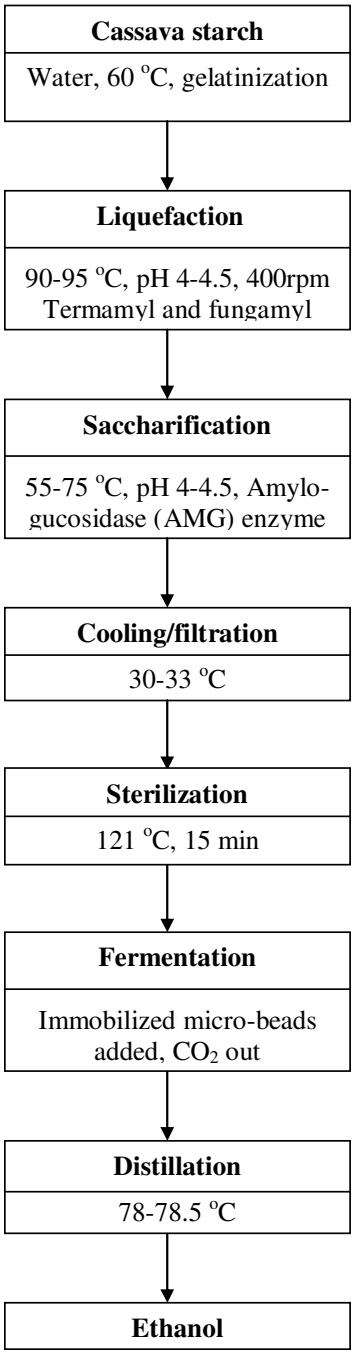

Figure 1. Flow chart for production of ethanol from cassava starch hydrolyzate using immobilized yeast cells.

variable, five level combinations coded -1, -1.682, 0, 1, 1.682 (Table 1) as modeled and used in literature (Nwabueze and Iwe, 2006; Nwabueze, 2007) was used for the optimization of the divalent cations for ethanol production from the 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 five levels of concentrations as shown in Table 1. A total of 20 experiments were employed for the optimization of the cations in fermentation.

Data analysis

Statistical analyses were carried out on the data obtained from the fermentations. The data were statistically regressed using Statgraphic Computer Software (STATISTICA) to test the significance of main and interactive effects of the cations (Nwabueze and Iwe, 2006; Nwabueze, 2007). Statistical significance was accepted at 5% probability levels ($P \leq 0.05$). 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 variable, 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+} and Ca^{2+}).
ε = random error.

RESULTS AND DISCUSSION

Response surface methodology is a sequential procedure with an objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. Using Central Composite Design, a total of 20 experiments with different of the divalent cations were performed. Responses were taken at 24 h interval until ethanol concentration dropped. Ethanol concentration at 0 to 120 h from the cassava starch hydrolyzates were as shown in Table 2. There was a general increase in the concentration from 0-120 h period of fermentation. Increase in ethanol concentration of fermented media have been reported by several authors including Balagopalan (1988)- cassava wort; Walker et al. (1996)- malt wort; Chandrasena et al. (1997)- molasses; Birch et al. (2003)- wine must; Vullo and Wachsman (2005) -synthetic media; Rakin et al. (2009)- corn meal hydrolyzates.

Effect of divalent cation on ethanol yield

Optimum alcohol production of 12.53%v/v was obtained from samples with divalent cation combinations of 64, 0.48 and 30 mg/l (Mg^{2+}, Zn^{2+} and Ca^{2+}), respectively at96 h period of fermentation. High concentration of Mg^{2+} and Zn^{2+} and low Ca^{2+} seems to favour ethanol production (Table 2). 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. This is in line with the report of Smith and Walker (2000). Mg^{2+} is also essential in nucleic acid synthesis and a cofactor of many enzymes in glycolysis while Zn^{2+} is an essential micronutrient and has stimulating effect in yeast metabolism. Alcohol production

Table 1. Independent variables in the central composite design.

Independent process variables (mg/l)		Coded variable levels				
		Corner points		Central point	Star points	
		-1.682	-1	0	+1	+1.682
X_1	Mg^{+2}	35	64	107	150	179
X_2	Zn^{+2}	0.24	0.30	0.39	0.48	0.54
X_3	Ca^{+2}	14.31	30	53	76	91.69

Table 2. Effect of the independent variables (X_1, X_2 and X_3) on the ethanol of the fermenting hydrolyzates during fermentation.

S/N	Variables			Responses (Alcohol - %v/v) Period of fermentation (h)					
	X_1	X_2	X_3						
	Mg	Zn	Ca	0	24	48	72	96	120
1	64	0.30	30	0.17	5.81	9.66	11.11	11.08	-
2	64	0.30	76	0.13	5.30	10.58	12.26	12.23	-
3	64	0.48	30	0.17	6.04	11.03	12.50	12.53	12.33
4	64	0.48	76	0.17	6.20	8.14	8.72	9.21	9.19
5	150	0.30	30	0.18	5.70	10.74	12.45	12.44	-
6	150	0.30	76	0.19	6.77	11.91	12.38	12.33	-
7	150	0.48	30	0.19	5.77	11.33	12.37	12.33	-
8	150	0.48	76	0.19	5.22	8.65	11.43	11.46	11.42
9	179	0.39	53	0.19	6.02	10.87	12.26	12.22	-
10	35	0.39	53	0.13	6.23	11.00	11.10	11.14	11.03
11	107	0.54	53	0.18	6.09	11.01	12.34	12.36	12.20
12	107	0.24	53	0.12	5.99	10.09	10.23	10.35	10.11
13	107	0.39	91.69	0.15	6.78	10.57	11.02	11.08	10.98
14	107	0.39	14.31	0.13	5.72	9.75	9.98	9.92	9.82
15	107	0.39	53	0.19	6.45	10.33	10.97	11.01	10.96
16	107	0.39	53	0.15	6.28	10.27	11.00	10.94	-
17	107	0.39	53	0.18	6.02	10.29	11.01	11.02	10.94
18	107	0.39	53	0.18	6.04	10.31	11.01	10.98	-
19	107	0.39	53	0.18	6.12	10.30	11.01	10.97	-
20	107	0.39	53	0.19	6.20	10.30	11.02	10.98	-
Control				0.04	4.82	7.88	9.74	10.07	10.06

Control: Cassava starch hydrolyzate medium containing immobilized *Saccharomyces pastorianus* without divalent cations.

increased with high concentrations of Zn^{2+} (0.30-0.48 mg/l). Similar results have been reported by Densky et al. (1966) in brewing wort using ale yeast showed stimulating effect of Zn^{2+} at levels of 0.1-1 mg/l. Desmartez (1993) showed that 0.45 mg/l concentration of Zn^{2+} promoted fermentation and consequently alcohol production. Ca^{2+} requirement for yeast growth, metabo-lism and alcohol production are low (30-76 mg/l). The same trends have been reported by Walker (1994) and Youatt (1993).

Minimum alcohol production was 9.21%v/v from 64, 0.48, 76 mg/l (Mg^{2+}, Zn^{2+} and Ca^{2+}), respectively at 96 h period of fermentation. It was observed that where Ca^{2+} was higher than Mg^{2+}, Ca^{2+} exhibited its inhibitory/antagonistic effect on Mg^{2+}, consequently, the Mg-dependent processes and yeast growth (Walker et al., 1996). Walker et al. (1996) showed that by altering the Mg^{2+} and Ca^{2+} ratio in favour 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 Walker et al. (1996) and Chandrasena et al. (1997).

The estimated regression coefficients for ethanol at 0 h

Table 3. Estimated regression coefficient for ethanol at 0 h of fermentation using immobilized yeast cells and the variables ($X_1 = Mg^{2+}$, $X_2 = Zn^{2+}$,, $X_3 = Ca^{2+}$).

Source	Coefficient	Standard error	df	*P*-value
Regression on constant	**21.41241**	**1.55217**		
X_1	0.00095	0.00998	1	0.9262
X_2	-11.82806	5.68796	1	0.0643
X_3	0.01962	0.01834	1	0.3098
X_1X_1	0.00001	0.00003	1	0.6924
X_1X_2	-0.00323	0.01784	1	0.8599
X_1X_3	-0.00002	0.00007	1	0.7780
X_2X_2	19.49185	6.44050	1	0.0128
X_2X_3	-0.03382	0.03335	1	0.3346
X_3X_3	-0.00005	0.00010	1	0.6391
R^2	0.6181			

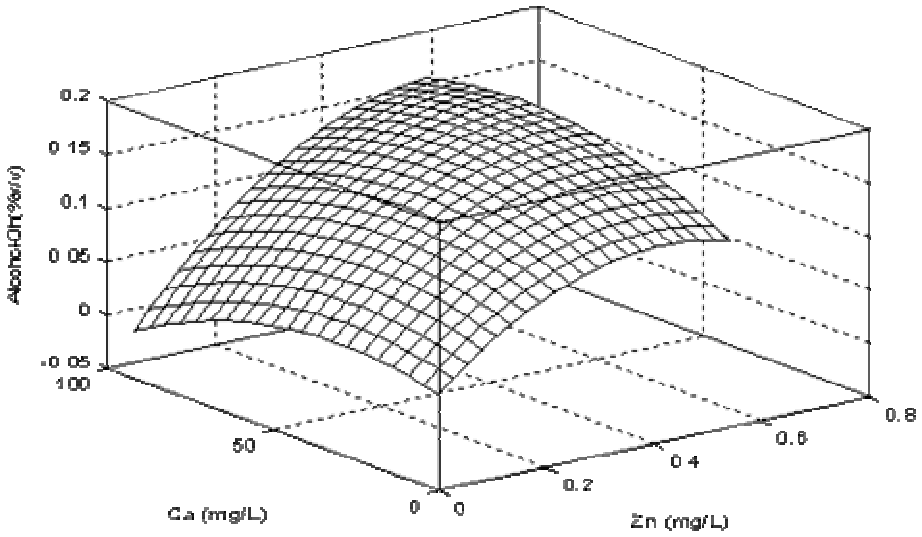

Figure 2. Response surface plot for ethanol at 0 h using Zn and Ca as process Variables.

fermentation are shown in Table 3. There was a significant ($P \leq 0.05$) quadratic effect of Zn^{2+} (X_2) on the alcohol production. Zn^{2+} is essential for yeast growth and fermentative metabolism. The same has been reported by Chandrasena et al. (1997) and Walker et al. (2006). The response surface plot (Figure 2) of the interaction between Zn^{2+} and Ca^{2+} confirms the quadratic effect of Zn^{2+} on ethanol yield. The multiple regression model developed from the data explained a variation of 61.81% at this period, and the resultant polynomial after removing the insignificant ($P>0.05$) terms becomes:

$$E = 21.41241 + 19.49185\ X_2^2 \quad (2)$$

Where E = ethanol; X_2^2 = quadratic order effect of Zn^{2+} on ethanol.

Conclusion

In this study, the response surface methodology was adopted in a central composite design to optimize ethanol production from cassava starch hydrolysate medium. *S. pastorianus* used for the 120 h fermentation was immobilized by entrapment in calcium alginate gel. Effects of three divalent cation (Mg^{2+}, Zn^{2+} and Ca^{2+}) combinations on ethanol production were investigated at five variable levels in 20 experimental runs in accordance with the experimental design. Maximum ethanol yield of 12.53% v/v was produced in the 96 h of fermentation when the

divalent cationic combination was 64, 0.48 and 30 mg/l (Mg^{2+}, Zn^{2+} and Ca^{2+}), respectively. Effect of Zn^{2+} on the ethanol production was quadratically significant ($P \leq 0.05$).

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