Chapter 6

Statistical Optimization of Acetoin Production Using Corn Steep Liquor as a Low-Cost Nitrogen Source by *Bacillus Subtilis* CICC 10025

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http://dx.doi.org/10.5772/intechopen.79353

Abstract

This chapter investigates the effect of some selected nitrogen sources (corn steep liquor—CSL, yeast extract, and beef extract) on the bioproduction of a selected flavor—acetoin (AC) from glucose—with a view to optimize its production. The results revealed that by using a cheap nitrogen source, corn steep liquor, the yield of acetoin is similar to those of the extracts of yeast and beef. Furthermore, it was shown that by using the Box-Behnken design, the optimum parameters such as glucose concentration, corn steep liquor, and inoculum size to maximize the concentration of acetoin produced are 78.40 g/L, 15% w/v, and 2.70% v/v, respectively. The validated concentration of acetoin produced in a triplicate analysis, 10.70 g/L, was 0.06% less than the predicted value. The results of this study may encourage the development of cost-effective nutritional use of corn steep liquor for bioproduction of acetoin on an industrial scale.

Keywords: acetoin, bioproduction, corn steep liquor, statistical optimization, Box-Behnken design, response surface methodology

1. Introduction

The increasing trend in the consumption of healthy food and the growing demand of natural products by consumers coupled with the price margin between the synthetic and natural flavors have motivated the bioconversion of natural flavors like vanillin, acetoin (AC), etc. that is cost-effective and commercialized. Likewise, the new sustainable development goals of the

united nation, goal 3 and 12 stated the need for the good health and well-being of the consumer and to ensure the sustainable production and consumption patterns of food product. Acetoin (3-hydroxy-2-butanone or acetylmethylcarbinol) is widely used in the food industry as a flavor enhancer, giving a buttery taste [1]. It can also be used as a building block for various chemicals such as alkyl pyrazines, diacetyl, and acetyl butanediol [1, 2]. Currently, most of the commercial acetoin is produced by chemical synthesis from 2,3-butanedione and 2,3-butanediol. However, the use of such chemically derived acetoin is restricted to food and cosmetic industries because of safety concerns [3]. The production of acetoin by microbial fermentation has been reported to gain increasing interest due to its safety and environmental friendliness [3, 4]. Intensive efforts have been taken to enhance microbial production of acetoin, including screening of high-yield acetoin-producing strains [5, 6], metabolic engineering breeding [7–9], and fermentation optimization [9–11]. The price of chemically generated acetoin (racemic) is over $ 16.2/kg, and biologically generated dietary acetoin can be two times more expensive [12]. One of the chemicals that can use acetoin as a building block is 1,3-butadiene (BD), which has a worldwide market of 11 Mt./year [13]. However, acetoin is only a minor by-product of 2,3-butanediol production, and its accumulation usually needs complex and expensive growth factors. Apart from carbon, which acts as feedstock for most fermentations, nitrogen is another significant nutrition compositional element needed for the active microbial growth for the fermentation of most targeted bioactive compounds [14].

Studies have shown the potential of low-cost media and complex nitrogen sources like urea, peptone, yeast extract, beef extract, soya bean [14–16] for acetoin production but yet to ascertain effectively the optimized condition. Nitrogen source is one of the main contributors in the total material cost of commercial acetoin production. Hence, replacing a costly nitrogen source with less expensive ones for acetoin production may improve the economics of the process. Corn steep liquor (CSL) has been identified as a potential nitrogen source in biochemical industries and a good substitute for other expensive complex media. It is a major by-product found during cornstarch processing. it is a low-cost source of proteins (**Figure 1**), amino acids, minerals, vitamins, and trace elements and can be used as a rich and effective

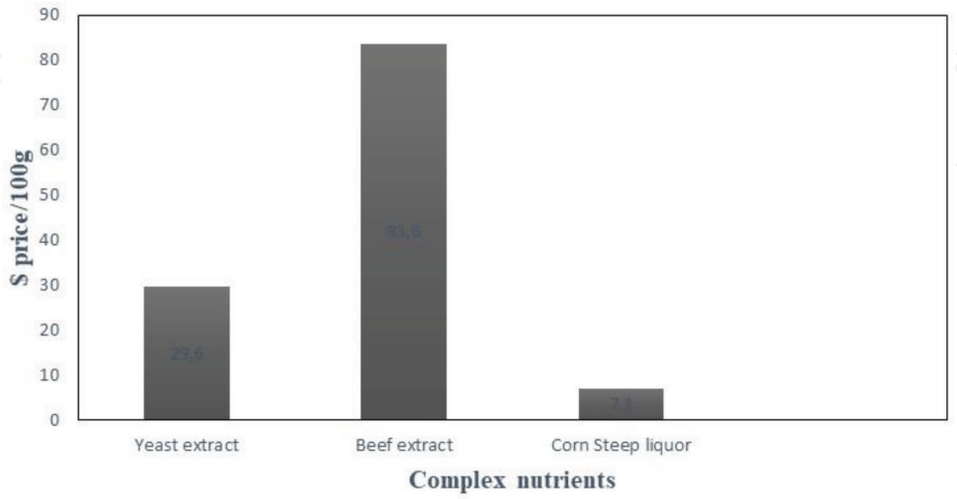

Figure 1. Graphical illustration of complex nitrogen nutrients and price margin.

nutritional substitute for expensive complex media like yeast extract, beef extract, and peptone in fermentations [17–20].

Studies have shown that CSL can be used as a nutrition supplement and a cheap nitrogen source for cellular growth in the production of some fermentation products [18, 20, 21]. Yang et al. [15] reported that when a high concentration of CSL was used in acetoin production, cell growth improved with a 3.69-fold increase in acetoin and an improved acetoin productivity [15]. Another author reported that a lower concentration of corn steep liquor had negative effects in the screening of medium components for acetoin production using a newly isolated *Paenibacillus polymyxa* CS 107 [2]. The work by Xu et al. [22] also confirms that CSL and yeast extract had a positive influence on acetoin when screened among other nitrogen sources. All these authors report differently on the optimized condition of CSL as nitrogen source and mostly combined its usage with other costly nutrients like yeast extract and beef extract. This necessitated the need for an optimizing tool like response surface methodology (RSM). Response surface methodology (RSM) is an empirical modeling technique used to establish the relationship between a set of controllable experimental factors and the observed results. RSM defines the effect of the independent variables, alone or in combination, in the processes. In addition to analyzing the effects of the independent variables, this methodology also generates a mathematical model [23]. The applicability of RSM to optimization studies has been demonstrated successfully [23–25]. This chapter focuses on the use of corn steep liquor as a cheap replacement for other expensive complex nutrients such that the CSL and other fermentation controlling parameters could be optimized, using RSM technique for acetoin production. The view of this study is to develop a low-cost fermentation medium that precludes redundant nutritional supplements and minimizes the costs associated with industrial acetoin fermentation.

2. Materials and methods

2.1. Microorganism and inoculum preparation

B. subtilis CICC 10025 purchased from the China Centre of Industrial Culture Collection was used in this study. It was maintained on agar slants with the following medium (g/L): glucose 10, beef extract 10, peptone 10, sodium chloride 5, and agar 16 at pH 7.0. The seed culture was prepared by growing the bacterium in 50 ml of the following medium in a 250-ml shake flask for 10 h with agitation of 150 rpm and temperature at 37°C: glucose 60 g/L, beef extract 10 g/L, peptone 10 g/L, yeast extract 10 g/L, and sodium chloride 5 g/L at pH 7.0 [16].

2.2. Medium composition for acetoin production

The fermentation medium used for this work was the significant optimized medium of Xiao et al. [16], which was then modified. The nutrients used comprised the following (g/L): glucose 150, K_2HP0_4 0.5 g, CH_3COONa 0.5 g, NaCl 5 g, and $MgSO_4.7H_2O$ 0.5 g, while 1 g/L of three different nitrogen sources were used in the preliminary experiment (corn steep liquor, yeast extract, and beef extract). The medium was adjusted to pH 7.0 and autoclaved at 121°C for 15 min. The flasks were incubated at 37°C with an orbital shaker at 150 rpm for 7 days.

2.3. Batch fermentation study

The preliminary experimental study of acetoin production was performed with 50 mL of glucose solution (100 g/L) measured into 250-mL Duran flask, and the growth effect of three complex nitrogen sources (yeast extract, beef extract, and corn steep liquor) were tested for acetoin fermentation nutrients. The pH of the medium was adjusted with 120 g/L of NaOH and 36.5 g/L of HCl buffer solutions. Subsequently, 5% volume fraction of inoculum size was added aseptically to the flask. The flasks were transferred into the environment-controlled incubator shaker (platform shaker, model: FSIM SP016) at 30°C and 150 rpm. Fermentation was performed for 168 h with 12-h sampling interval.

2.4. Experimental design by Box-Behnken Design

A three-level factor was employed to generate 17 experimental runs by considering the effect of glucose concentration (g/L), inoculum size (% v/v), and corn steep liquor (% w/v). The range and the levels of the independent variables investigated using the Box-Behnken experimental design (**Table 1**) were chosen based on variables previously reported to influence acetoin [11, 26]. The minimum, center point, and maximum levels of each variable were coded as −1, 0, and +1, respectively.

A second-order mathematical equation, including all interaction terms, was used to calculate the predicted response:

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

where Y is response variable (acetoin 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, and b_{ii} represents the quadratic coefficients of X_i. X_i and X_j are the input variables that influence the response variable and *e* represents the random error.

2.5. Statistical analysis

The observed data were subjected to multiple regression analysis using Design-Expert versions 10.0 (Stat Ease Inc., Minneapolis, USA) to obtain the coefficients of the quadratic equation. The *F*-value and the probability *p*-value were used to appraise the significance of the model. The coefficient of determination (R^2) and adjusted R^2 was calculated to evaluate the performance of the regression equation. The behavior of the model in the experimental area

Factor	Unit	Symbols	Coded factors		
			0	−1	1
Glucose	(g/L)	X_1	50	100	150
CSL	(% w/v)	X_2	5	10	15
Inoculum size	(% v/v)	X_3	2	3.5	5

Table 1. Factors and their levels for Box-Behnken design.

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was investigated graphically. Statistical evaluation of the model was carried out using analysis of variance (ANOVA).

2.6. Analytical methods

2.6.1. Reducing sugar analysis

Reducing sugar concentration was analyzed using the dinitrosalicylic acid (DNS) method [27] and the results were expressed as glucose equivalent. To 1 mL of the supernatant, 3 mL of the DNS solution was added in the test tube and boiled for 15 min, cooled, and diluted appropriately after which the absorbance was measured at a wavelength of 540 nm using a UV–Visible Spectrometer (GBC Cintra 2020).

2.6.2. Biomass concentration determination

Dry cell weight (DCW) was obtained by centrifuging an aliquot of the sample followed by drying the cell pellet to a constant weight using an electric oven (Scientific, series 2000), in a pre-weighed centrifuge tube, at 105°C for approximately 24 h. It was later cooled in a desiccator. The biomass concentration was calculated on the basis of the volume of the fresh sample as the difference between the weight of the empty tube and the final weight of the tube plus the dried biomass after drying and cooling [28, 29].

2.6.3. Acetoin concentration determination

Acetoin was determined by the modified Voges-Proskauer (VP) reaction of Westerfield [30]. An aliquot of the sample solution was pipetted into a 25 cm^3 calibrated flask. A total of 2.5 cm^3 of 1-naphthol solution and 1.0 cm^3 of creatine solution were added. After adjusting the mixture to volume and shaking vigorously, the solution was kept at 30°C. The color intensity of the complex was determined by measuring the absorbance after 40 min at 530 nm using a UV–Visible Spectrometer of 2020 GBC Cintra model [31].

3. Results and discussion

3.1. Preliminary evaluation of complex nitrogen sources on AC production

The study in **Figure 2** shows that CSL supports rapid utilization of the reducing sugar (from 150 to 84 g/L) within the first 60 h of fermentation [32], the resultant acetoin and biomass growth being maximum during this period. The maximum biomass growth is found to be fairly constant at ~ 8 g/L until the 144 h when it starts to decline. Likewise, the maximum acetoin concentration is found to be ~ 7 g/L after 60 h of fermentation and then declines. The decline in the acetoin concentration could be attributed to the complete metabolism of glucose in the fermentation medium. Although the biomass concentration remained constant after the 60 h of fermentation, it can be assumed that the energy derived from reducing sugar metabolism was channeled toward cell maintenance since biomass growth remained constant and acetoin was still produced till the fermentation lapse. It has been shown previously that CSL is a rich source of proteins, amino acids, minerals, vitamins, and trace elements and can

be used as nutritional supplement [32]. The addition of CSL reduced the fermentation time and promoted the growth and fermentation of the strain.

On the other hand, when yeast extract was used as the nitrogen source in the fermentation media, as depicted in **Figure 2b**, after reducing 150 g/L of sugar, the results showed a rapid consumption of the reducing sugar when compared to **Figure 2a**. More than 97% of the reducing sugar was converted (during the first 84 h of fermentation), the acetoin concentration approached a maximum, and the biomass growth was constant at 8.6 and 7.7 g/L, respectively. The wide range of amino acids, peptides, vitamins, inorganic salts, and carbon in growth media of yeast extract supports the biomass growth and rapid sugar utilization [33].

In **Figure 2c**, the extract was used in place of CSL and yeast extract as earlier discussed (**Figure 2a** and **b**). The beef extract is a mixture of peptides and amino acids, nucleotide fractions, organic acids, minerals, and some vitamins. Its function can, therefore, be described as complementing the nutritive properties of peptone by contributing minerals, phosphates, energy sources, and those essential factors missing from peptone [34]. The glucose was almost depleted after 96 h till the end of the fermentation lapse. It can be deduced from the study that the beef extract supports the utilization of glucose consumption as more than 98% of the glucose has been consumed within 96 h of fermentation.

To see the clear difference of the complex media earlier considered, the acetoin fermentation was done without the nitrogen sources (**Figure 2d**) but with the simple salts in the fermentation

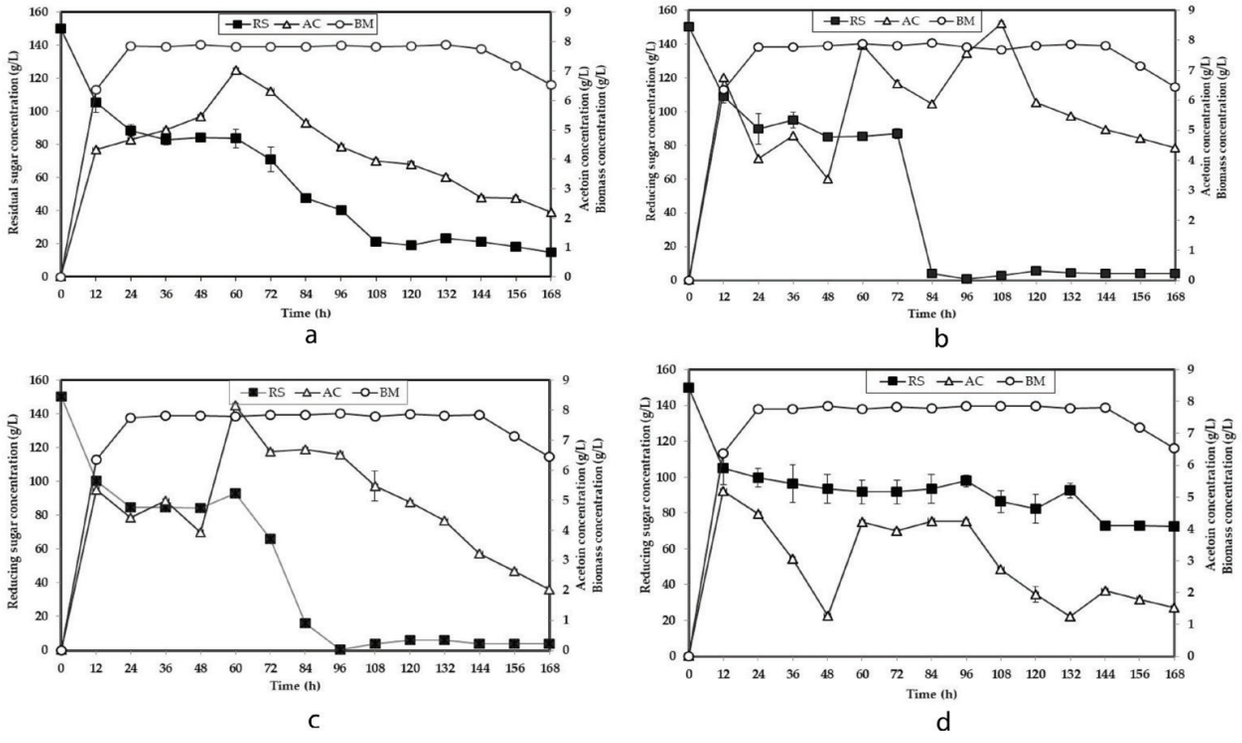

Figure 2. (a) Plots of AC fermentation using corn steep liquor as nitrogen source. (b) Plots of AC fermentation using yeast extract as nitrogen source. (c) Plots of AC fermentation using beef extract as nitrogen source. (d) Plots of AC fermentation using none of the complex media as the nitrogen source. (RS–reducing sugar; AC–acetoin; BM–biomass).

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media. A maximum acetoin of about 5.2 g/L was obtained during the first 12 h of the fermentation, after which there was no significant change in the biomass growth from that period until the lapse time of fermentation. About 50% (72.53 g/L) of sugar concentration was not consumed until the fermentation time lapse of 168 h, and the sluggish consumption of glucose could be traceable to the lack of rich nitrogen sources, which was needed as a nutritional supplement [28].

3.2. Metabolism of corn steep liquor on AC production

It was observed in the preliminary evaluation of nitrogen sources discussed earlier (**Figure 2a–d**) that corn steep liquor produces acetoin at a short time interval when compared to other nitrogen sources. Therefore, the fermentation time interval of acetoin was reduced from the initial 168 h (**Figure 2a–d**) to 48 h (**Figure 3**) and sample taken at every 2-h interval, to investigate the biomass growth, utilization of glucose, and the acetoin accumulation when corn steep liquor was used as nitrogen source. The results show that the accumulated acetoin and the biomass growth were already at the peak of 7.03 and 7.83 g/L, respectively, in the first 36 h of fermentation (**Figure 3**). The rapid utilization of reducing sugar (from 150 to 66 g/L) within 36 h of fermentation was due to high amino acids and polypeptides, which are excellent sources of nitrogen in corn steep liquor and has been reported to support the growth of most microorganism [35]. CSL comprises a mixture of reducing sugars that contribute to the nutritional growth of the bacteria with a steady increase in biomass growth (7.7–7.8 g/L) from 18 to 46 h when it starts to decline [36]. It can be affirmed from the findings that corn steep liquor hastens fermentation of acetoin at short time interval and this makes large-scale production of acetoin cost-effective.

3.3. Optimization of AC production using response surface method

After the preliminary studies, RSM coupled with Box-Behnken design (BBD) was used for the optimization of the fermentation process with respect to glucose concentration, CSL

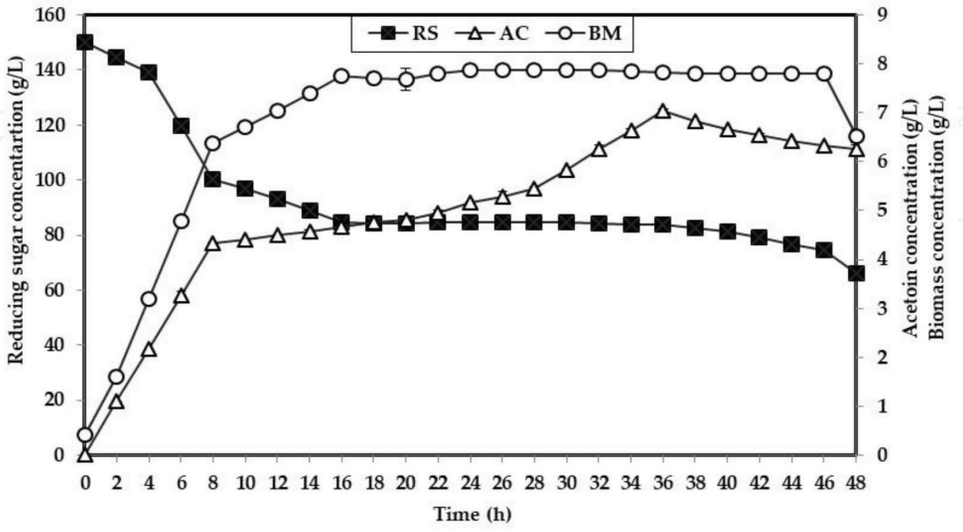

Figure 3. Acetoin growth profile using corn steep liquor at short fermentation time interval. RS–reducing sugar; AC–acetoin; BM–biomass.

concentration, and inoculum size with a view to maximize the AC production. **Table 2** shows the experimental conditions investigated together with the observed and predicted values. The data were fitted using the following second-order mathematical equation:

$$Y = 7.81 - 1.37\,X_1 + 2.56\,X_2 - 0.61\,X_3 - 0.17\,X_1X_2 - 0.57\,X_1X_3 - 1.02\,X_2X_3 - 1.25\,X_1^2 - 0.50\,X_2^2 - 1.09\,X_3^2 \quad (2)$$

where Y is the acetoin (AC) produced in g/L and X_1 is glucose concentration, X_2 is corn steep liquor, X_3 is Inoculum size. The residuals between the observed and predicted (**Table 2**) values in this work revealed good fit of the equation as shown by the parity graph which is a measure of agreement between the observed and predicted values (**Figure 4**). These observations implied that the model developed for the fermentation process adequately described the actual relationship among the selected factors.

The three-dimensional graph and contour plot, which is depicted in **Figure 5(a)** and **(b)** shows the relationship between corn steep liquor and glucose concentration when acetoin production is at the maximum (10.70 g/L). Also, the *p*-values of the model terms were significant at $p < 0.05$ (**Table 3**). Also, the observed low *p*-value of 0.0001 together with the corresponding *F*-value of 11.09 showed that the model obtained was significant. The *F*-value and *p*-value do not differentiate between negative and positive significant effects of each term in the model [37]. **Table 3** displays the test of significance and ANOVA of the regression equation results. The coefficient of determination (R^2) is used to assess the goodness of fit of the regression

Run	X_1 (g/L)	X_2 (% w/v)	X_3 (%v/v)	Observed AC (g/L)	Predicted AC (g/L)	Residuals
1	100 (0)	10 (0)	3.5 (0)	7.68	7.81	−0.13
2	150 (1)	10 (0)	2 (−1)	4.32	5.28	−0.96
3	150 (1)	5 (−1)	3.5 (0)	2.47	2.29	0.18
4	100 (0)	10 (0)	3.5 (0)	7.68	7.81	−0.13
5	100 (0)	5 (−1)	2 (−1)	3.66	3.24	0.42
6	100 (0)	15 (1)	2 (−1)	10.69	10.41	0.28
7	50 (−1)	10 (0)	2 (−1)	7.14	6.88	0.26
8	100 (0)	15 (1)	5 (1)	6.49	7.15	−0.66
9	50 (−1)	10 (0)	5 (1)	8.00	6.8	1.20
10	150 (1)	10 (0)	5 (1)	2.91	2.92	−0.01
11	100 (0)	10 (0)	3.5 (0)	7.68	7.81	−0.13
12	100 (0)	5 (−1)	5 (−1)	3.53	4.06	−0.53
13	150 (1)	15 (1)	3.5 (0)	7.88	7.08	0.80
14	50 (−1)	15 (1)	3.5 (0)	9.74	10.16	−0.42
15	100 (0)	10 (0)	3.5 (0)	7.70	7.80	−0.11
16	100 (0)	5 (−1)	3.5 (0)	5.72	4.74	0.98
17	50 (−1)	5 (−1)	3.5 (0)	3.65	4.69	−1.04

Table 2. BBD of three independent factors for AC production including the coded levels of each parameter.

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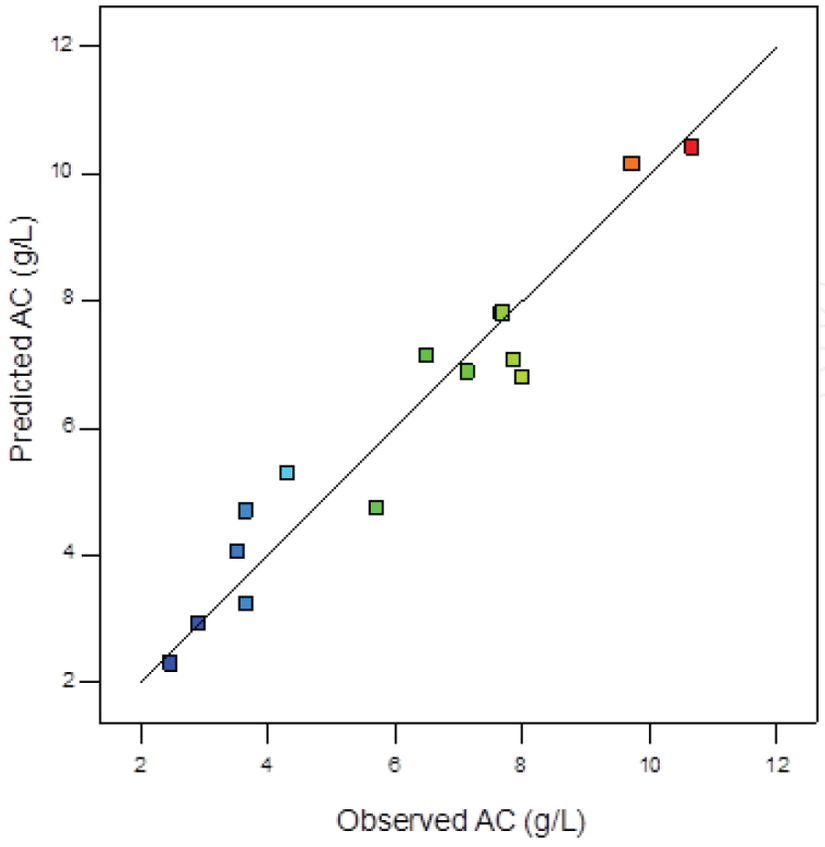

Figure 4. Parity plot of acetoin production.

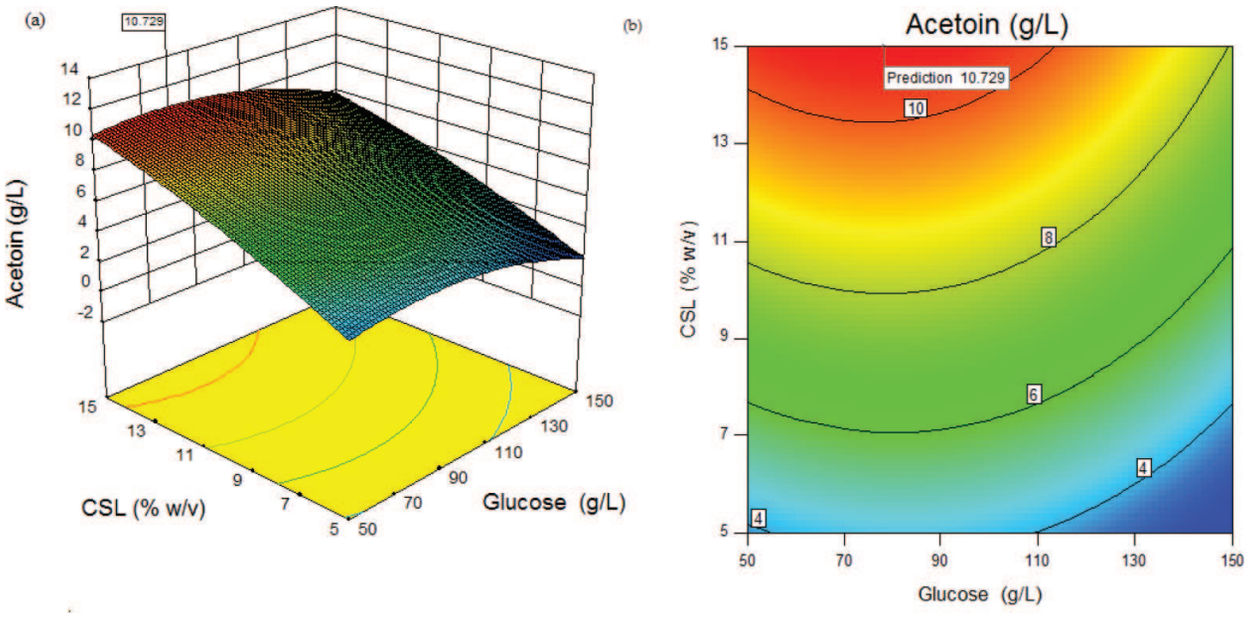

Figure 5. Contour and response surface plots. (a) The response surface plot and (b) contour plot showing the effects of corn steep liquor and glucose on acetoin production.

equation. R^2 of 0.930 of the model demonstrated a good correlation between the observed and predicted values. It showed that 93% sample variation for AC produced is attributable to the independent factors and just 0.70% of the total variations is not described by the model

Factor	Sum of squares	df	Mean square	*F*-value	*p*-value
X_1 (Glucose)	14.99	1	14.99	16.51	0.0048
X_1^2	6.52	1	6.52	7.18	0.0315
X_2 (CSL)	57.85	1	57.85	63.73	0.0001
X_2^2	1.06	1	1.06	1.17	0.3160
X_3 (Inoculum size)	2.98	1	2.98	3.28	0.1131
X_3^2	4.98	1	4.98	5.48	0.0517
X_1X_2	0.12	1	0.12	0.13	0.7317
X_1X_3	1.29	1	1.29	1.42	0.2724
X_2X_3	4.14	1	4.14	4.56	0.0701
ANOVA					
Model	90.63	9	10.07	11.09	0.0022
Error	3.000E-004	3	1.000E-004		
Total sum of squares	96.99	16			
$R^2 = 0.93$, Adjusted $R^2 = 0.90$					

Table 3. Test of significance for every regression coefficient and ANOVA.

[28, 38]. The adjusted R^2 of 0.90 proved that the model was significant. It has been suggested that R^2 should be less or equal to 80% for the good fit of a model [39].

3.4. Model validation

The optimum values of the three factors selected for the fermentation process were obtained by solving Eq. (2) using the Design-Expert software package (version 10.0). The optimal condition was statistically predicted as glucose concentration of 78.40 g/L, CSL of 15.00% w/v, and inoculum size of 2.70% v/v. Under this condition, the AC concentration predicted was 10.73 g/L. In order to validate the model, the optimal condition values were applied to three independent experimental replicates and the average value of AC produced was 10.70 ± 0.1 g/L. The correlations between predicted and experimental values after optimization infer the validity of the response model and the existence of an optimum point [40]. The bar chart (**Figure 6**) is a graphical view for each optimal solution showing the desirability of every dependent and independent factor with combined value. Independent factors are shown with red bars, while the dependent response and combined values are displayed in blue. The desirability result is accurate as it falls within the acceptable value ranging between 0.8 and 1 [41]. In **Table 4**, variance inflation factor (VIF) obtained showed that the center points are orthogonal to all other factors in the model. The 95% confidence interval (CI) bounds showing high and low help to hypothesize that there is 95% probability of including the right predicted responses by the model, and there is only 5% chance that the observed value lies either below or above the level of confidence limits. The coefficient estimate shows the confidence interval

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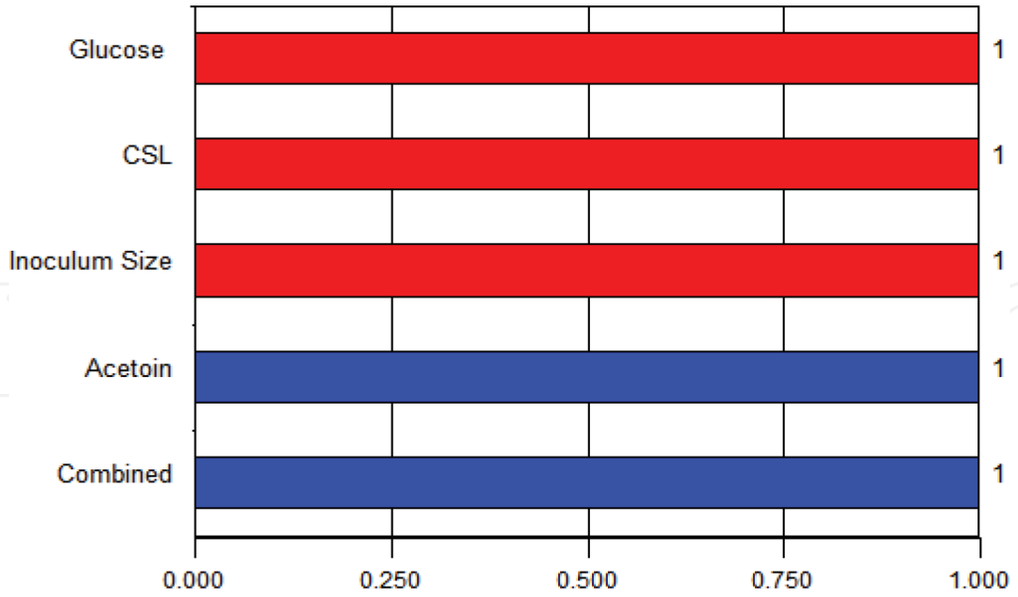

Figure 6. Numerical optimization bar graph of AC production.

Factor	Coefficient estimate	df	Standard error	95% CI low	95% CI high	VIF
Intercept	7.81	1	0.47	6.71	8.91	
X_1 (Glucose)	−1.37	1	0.34	−2.17	−0.57	1.00
X_2 (Corn steep liquor)	2.56	1	0.32	1.80	3.32	1.02
X_3 (Inoculum size)	−0.61	1	0.34	−1.41	0.19	1.00
X_1X_2	−0.17	1	0.48	−1.30	0.96	1.00
X_1X_3	−0.57	1	0.48	−1.69	0.56	1.00
X_2X_3	−1.02	1	0.48	−2.14	0.11	1.00
X_1^2	−1.25	1	0.47	−2.35	−0.15	1.01
X_2^2	−0.50	1	0.47	−1.60	0.60	1.01
X_3^2	−1.09	1	0.47	−2.19	0.011	1.01

df: degree of freedom; VIF: variance inflation factor; CI: confidence interval.

Table 4. Regression coefficients and significance of response surface quadratic.

around the model coefficient and the standard error less than 1 shows the statistical accuracy of the predicted responses (**Table 4**).

4. Conclusion

The feasibility of corn steep liquor to replace yeast extract and beef extract, which is an expensive nutrient source in acetoin fermentation, was investigated. Corn steep liquor—a low-cost nitrogen

source—competes with other complex nutrients (yeast extract and beef extract) for acetoin production and statistical optimization was carried out in batch fermentation. The model that best described the AC fermentation process was a quadratic model with R^2 of 0.930. The most significant positive factor for the process was glucose concentration and corn steep liquor, while inoculum size was an insignificant factor in the AC fermentation. Optimal condition predicted for the three independent factors were a glucose concentration of 78.40 g/L, CSL of 15% w/v, and inoculum size of 2.70% v/v, which were validated experimentally with AC concentration of 10.70 ± 0.1 g/L. Based on study results, it can be concluded that the optimization methodologies developed were effective in ascertaining the amount of CSL required for commercial acetoin production, and it reduced the cost, time, and effort associated with experimental techniques.

Acknowledgements

The authors wish to thank the National Research Foundation (NRF) and Cape Peninsula University of Technology (CPUT) for providing the running cost of this research. AE Taiwo acknowledges NRF in partnership with the World Academy of Sciences (TWAS)—NRF-TWAS—for the award of his doctoral scholarship and Landmark University Nigeria for the staff development program.

Conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

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