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Optimization of Fermentation Conditions for Protein Production from *Prosopis africana* Seeds using Response Surface Methodology

Asiru, R. A.[1]* and Musbau, S.[2]

[1]Department of Biology, Faculty of Science, Northwest University, PMB 2331, Sokoto, Nigeria.
[2]Department of Microbiology, Faculty of Science, Yobe State University, PMB 1144, Damaturu, Yobe State, Nigeria.

*Corresponding author email: asirunet@gmail.com

Abstract

Due to the increasing incidence of protein malnutrition resulting from the high cost of animal protein in many African countries, including Nigeria, there has been a renewed interest in legume seeds as affordable sources of plant-based protein for human consumption. *Prosopis africana* seeds, an underutilized leguminous crop, are traditionally used as a condiment known as Okpehe. This study was designed to establish the optimum fermentation conditions for producing *Prosopis africana* seed–based vegetable protein condiment (Okpehe) using Response Surface Methodology (RSM) with four variables: fermentation duration (X_1), inoculum concentration (X_2), pH (X_3), and temperature (X_4). *Prosopis africana* seeds were fermented at various temperatures of 35°C, 40°C, 45°C and 50°C for four days (96 hours).The inoculums used for fermentation contained (7.5×10^8 cells/5ml^{-1} , 1.5×10^9 cells/10ml^{-1} and 2.3×10^9 cells/15ml^{-1} into 20g of boil unfermented *P. africana* seeds. The cell population was calibrated using McFarland standards (No1). The interaction effects of these four variables on the % protein yield were investigated using Central Composite Design (CCD) fractional design of experiments. From experimental data generated by the design of experiment (DOE), using MINITAB 17 software, optimum conditions were obtained at 40ºC, with inoculum (*Bacillus licheniformis*) concentration of 1.5×10^9 cells/10ml^{-1} with pH 7 at fermentation duration of 96 hours. The adequacy (R^2) of the models was highly satisfactory for the processes being 0.90. In conclusion, it was observed that protein content of fermented *Prosopis africana* seeds were increased during fermentation because, Protein had the highest yield of 44.52 % after 96 hours of fermentation at temperature of 40°C.

Keywords: *Prosopis africana*, fermentation duration, *Okpehe* and Response Surface Method.

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Introduction

Protein malnutrition is a major problem; in many countries in Africa including Nigeria. The high cost of animal protein has directed interest towards several proteins containing leguminous seed as potential sources of vegetable protein for human food. *Prosopis africana* (*African mesquite*) is one of the lesser common legume seed crops used as a food condiment called *Okpehe*. *Prosopis africana* tree (Figs. 1& 2) is of great economic value to man and animals. It fixes nitrogen to enrich the soil, generates hardy timbers, produce rich leaves and sugary pods used as feed stuffs to ruminants (Afolabi *et al*., 2018).

Mesquite seeds being an oil seed along with other oil seeds such as African locust bean *Parkia biglobosa*, melon seed, castor seeds and soybean are fermented to produce condiments. Traditional fermented condiments are rich in vegetable proteins and consumed by different ethnic groups in Nigeria. It is evident that these products played a major role in the food habit of communities in the rural regions, they serve not only as a nutritive non-meat protein supplements but as functional ingredients in prepared food.

The diets of Nigerians are mostly from roots, tubers and cereals. The low protein in these diets contributes to low nutrition security of the people (Karim and Adekunle, 2010). Soups are the main sources of protein and minerals and one of the ways to improve the diet has been to improve the nutrient content of soups.

Figure 1: *Prosopis africana* tree

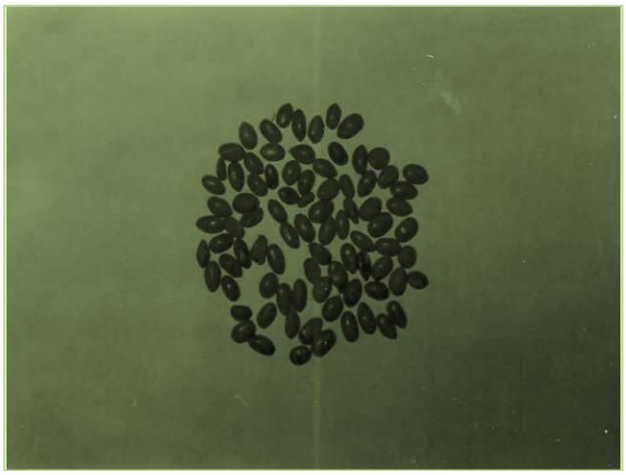

Figure 2: *Prosopis africana* seed

Design of experiments (DOE) can be defined as the systematic approach of determining the relationship between variables affecting a process and the output of that process. Design of experiments is an advanced statistical tool to study perfectly the effect of a large number of factors with a minimum effort in data collection (Salil and Nyoman, 2003). DOE investigates the effects of input variables on output variables at the same time. The design of experiments (DOE) is an effective procedure for planning experiments so that the data obtained from the experiment can be analyzed to yield good objective conclusions Ojewumi et *al*. (2016b). *Prosopis africana* has been used as condiment since ancient times not much is however documented of its nutritional benefits. This study was designed to establish optimum conditions for the fermentation of *Prosopis africana* seeds into vegetable protein based condiment (*Okpehe*) using Response Surface Methodology. Result of the study is expected to improve available data on the nutritional value of the *Prosopis africana* seeds.

Optimization of fermentation medium using RSM is very important compare to the conventional methods of optimizing i.e. "one-variable-at-a-time" was used frequently more than other approaches, but it is time-consuming and often fails to identify the optimal conditions because interactions among different variables are neglected (Krouse, 1999). During the process of screening the most suitable fermentation condition, some statistical techniques, such as fractional factorial designs, Taguchi method and Plackett-Burman design which can estimate the effects of many variables and determine the interaction of the factors (Peter, *et al.*, 2006; Pio and Macedo, 2008; Teng and Xu, 2008), also the response surface methodology (RSM) has some advantages which including less experiment numbers used for multiple factor experiment numbers, search for interaction among factors and finding of the most suitable condition and predicting response (Chang, *et al.*, 2006; Zhao, *et al.*, 2008; Alam, *et al.*, 2008; Chou, *et al.*, 2010). These statistical methods (RSM) were proved to be useful for developing the conditions to improve the production which were extensively used in the industries (Singh, *et al.*, 2009; Deepak, *et al.*, 2008; Gao, *et al.*, 2009; Ghosh and Hallenbeck, 2010; Goswami, *et al.*, 2009).

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Materials and Methods

Sources of Seeds and Authentication

Two hundred grams (200g) of *Prosopis africana* seeds were purchased from Brigade Market in Kano State of Nigeria. The *Prosopis* seeds were identified with a herbarium voucher number of BUKHAN 0193 at the Department of Plant Biology, Bayero University, Kano. *Prosopis africana* seeds were transported to the Department of Microbiology, Bayero University, in polythene bag for investigations.

Sources of Organisms

Bacillus licheniformis that were used as starter cultures were isolated from previous natural fermented *Prosopis africana* condiment sourced from Brigade Market as described by Omafurba et *al*. (2004). The cultures were preserved in an agar slant at 4(℃) until use.

Preparation of *Bacillus* inoculums (inoculums size)

Pure and discrete colonies of bacterium *(B. licheniformis)* was transferred in to sterile test tube containing 100 mL sterile normal saline and standardized until a turbidity equivalent to 0.5 Mcfarland standard were obtained which contained approximately 1.5 $\times 10^8$ cells/ml^{-1}. From 0.5 Mcfarland standard prepared, other concentrations were measured (5mL, 10mL and 15mL) which corresponds to (7.5×10^8 cells/5mL^{-1} , 1.5×10^9 cells/10mL^{-1} and 2.3×10^9 cells/15mL^{-1}) into 20g of boil unfermented *P. africana*seeds for fermentation.

Controlled fermentations of *P. africana* with inoculum

Fermentation processes of *P. africana* were set up using inoculums. The organisms *(B. licheniformis)* were inoculated into 20g of the boil unfermented seeds at each set up and were wrapped with sterile aluminum foil and placed in an incubator and incubated at adjusted temperature and pH.

Powdering blending of dried fermented seeds of *P. Africana* with inoculum

The dried fermented seeds of *P. Africana* were blended into powdered form using a sterile blender. Ten grams of the powdered condiment were packaged into small plastic containers with seals sterilized with 70%ethanol. The packaged condiment was stored at refrigeration temperature (9±2 (℃) for further use for optimization.

Experimental design for the fermentation conditions

Total number of run of experiment in CCD was thirty-one (31) and responses were generated as functions of four variables namely: X_1 as Time (fermentation duration), X_2 as inoculum concentration (g broth/g seed) X_3 as Temperature (°C) and X_4 as pH.

The response variable (% Protein) was fitted by a second-order polynomials in order to correlate the design variables (X1, X2, X3, and X4) which is presented with the model below:

$Y = \alpha0 + \alpha1X1 + \alpha2X2 + \alpha3X3 + \alpha4X4 + \alpha1,1X1X1 + \alpha1,2X1X2 + \alpha1,3X1X3 \alpha1,4X1X4 + \alpha2,2X2X2 + \alpha2,3X2X3 + \alpha2,4X2X4 + \alpha3,3X3X3 + \alpha3,4X3X4 + \alpha4,4X4X4.$

The % Protein composition responses are represented by Y, which is associated with each factor level combinations. α0, α1, α2, α3, α1, 2 ….. α4, 4 are the regression coefficients. X1, X2, X3and X4 are the factors. X1X1, X1X2, X1X3, X1X4 X2X2, X2X3, X2X4, X3X3, X3X4 and X4X4 are the interactions of the variables.

Results and Discussion

Table 1 below shows the design of variables used with % Protein as the response. This is the Response surface model experimental values of the % Protein. This also shows the interactions between the four variables and expected number of experimental runs, similarly, Table 2 shows the design application for the process simulation, the % Protein yield for both experimental and predicted values. This table simulated the values of both experimental and predicted value and shows the percentage deviation. The experimental had close values with the predicted values giving an R^2 that is very close to 1(0.9029).

Table 3 described Analysis of Variance (ANOVA) for the Response Surface Regression. ANOVA was used to test statistical significance of the model. Table 3 also shows statistically the sum of square (SS), degree of freedom (DF), mean square (MS), F-value, p-value and ANOVA coefficients. The Fisher distribution (F-value and P-value) were used to determine the significance of the model. A large F-value and a small P-value implies that the models are adequate to predict the responses. R^2 (0.9029) indicate the reliability of the model. The closer the R^2 (0.9029) value to 1, the stronger and better the model prediction of the responses.

The optimization process using four (4) variables were shown in Table 4. This was used to design and generate a model to fit the experimental result. Below is the best fitted models obtained from the regression analysis.

Regression Equation (coded variables):

% protein yield = 178.2+6.9(X1)-0.76(X2)+16.3(X3)+ 6.75(X4)+2.912(X1(X1)0.0056(X2)(X2) -0.330(X3)(X3)-0.0598(X4)(X4)0.136(X1)(X2) + 0.221(X1)(X3)-0.255(X1)(X4- 0.014(X2)(X3)+0.0302(X2)(X4)-0.305(X3)(X4)

R-Sq. = 90.29%

Optimization of the percentage Protein yield

Figures 1-5 are response contour plots of Protein vs. (Inoc.conc. Day); Protein vs. (pH, Day), Protein vs (Temp., Day), Protein vs (Temp., Inoc.conc.) and protein vs (Temp., pH) respectively. With the aid of Minitab 17 software, the plots in figures 1-5 were analyzed to obtain the regression equation for predicting the protein composition for various values of X1, X2, X3 and X4. The result of the optimized regression equation shows that 4 days (96 hours) of fermentation, with Inoculums concentration of 1.5×10^9 cells/10mL^{-1} pH 7 and fermentation temperature of 40 °C predicts maximum yield of % Protein composition of 44.52 % which is slightly higher than predicted value of 36.171 % (Table 2).

The Contour plots below (Figures 3-7) show the predicted effect of process variables (X1, X2, X3, and X4) on % Protein as the response. The contour plot represents graphically the regression coefficient in equation form in order to obtain the optimum conditions of the variables within the design region.

The results of optimization using response surface methodology, the contour plots show the effect of process variables (X1, X2, X3, and X4) on % Protein as the response. Contour plots (Figures 3-7) represent graphically the regression coefficient in equation form in order to obtain the optimum conditions of the variables within the design region. The experimental results shows that on the fourth day with inoculums concentration of 1.5×10^9 cells/10mL^{-1}, Temperature: 40°C and pH; 7, highest yield of % protein was 44.52%, The protein composition also decreases with an increase in inoculums concentration. This observation is similar to the reports of (Ajala *et al.*, 2013, Soetan *et al.*, 2014). Modupe *et al.* (2017) reported three days (3 days) within temperature range of 28°C – 42°C. The statistical significance of this model was verified by variance analysis (ANOVA) using Minitab 17.0. As listed in Table 3, the high *F*-value and the low probability ($Pr>F<0.05$) indicated that the experimental model was in good agreement with the experimental results (Jo *et al.*, 2008).

The ANOVA also showed that the linear, quadratic and cross product terms between the variables revealed that there were obvious interactions among the four variables. The coefficient of determination (R^2= 90.29%) in the experimental model indicated a good agreement between experimental results and predictions (Guo, *et al.*, 2009). The adjusted determination coefficient (R^2= 81.80%) was also satisfactory to confirm the significance of the model.

T**able 1:** Design of the Variables with % Protein as the response

Run	Days (X_1)	Ino.Con.(X_2)	PH(X_3)	Temperature (X_4)	Response
1	4	10	7	40	44.52
2	2	10	7	40	25.49
3	2	20	7	40	17.65
4	1	15	6	35	8.45
5	2	10	7	40	17.89
6	1	15	6	45	8.95
7	1	5	6	45	7.23
8	1	15	8	45	7.67
9	2	10	7	30	13.74
10	2	10	7	40	11.63
11	2	10	7	40	11.62
12	3	5	6	45	26.22
13	3	15	8	35	29.53
14	3	15	6	35	25.67
15	2	10	9	40	11.98
16	2	0	7	40	15.42
17	3	5	8	45	20.12
18	1	15	8	35	10.42
19	3	15	6	45	23.21
20	1	5	8	35	10.87
21	0	10	7	40	12.96
22	2	10	7	40	13.31
23	2	10	7	50	8.49
24	1	5	6	35	8.89
25	3	5	6	35	27.85
26	2	10	7	40	21.32
27	2	10	7	40	16.56
28	1	5	8	45	7.53
29	3	15	8	45	22.83
30	2	10	5	40	19.56
31	3	5	8	35	36.98

Table 2: Compare Experimental value with Predicted value for % Protein Yield

Standard Order	X_1	X_2	X_3	X_4	Experimental Values	Predicted Values	& Deviation
1.	4	10	7	40	44.52	36.171	0.18753
2.	2	10	7	40	25.49	16.831	0.33970
3.	2	20	7	40	17.65	16.215	0.08130
4.	1	15	6	35	8.45	8.577	-0.01503
5.	2	10	7	40	17.89	16.831	0.05920
6.	1	15	6	45	8.95	14.103	-0.57575
7.	1	5	6	45	7.23	9.264	-0.28133
8.	1	15	8	45	7.67	7.795	-0.01630
9.	2	10	7	30	13.74	16.21	-0.17977
10.	2	10	7	40	11.63	16.831	-0.44721
11.	2	10	7	40	11.62	16.831	-0.44845
12.	3	5	6	45	26.22	23.190	0.11556
13.	3	15	8	35	29.53	29.325	0.00694
14.	3	15	6	35	25.67	24.878	0.03085
15.	2	10	9	40	11.98	16.608	-0.38631
16.	2	0	7	40	15.42	18.222	-0.18171
17.	3	5	8	45	20.12	21.822	-0.08459
18.	1	15	8	35	10.42	10.255	0,01584
19.	3	15	6	45	23.21	23.419	-0.00901
20.	1	5	8	35	10.87	12.490	-0.12970
21.	0	10	7	40	12.96	9.155	0.29360
22.	2	10	7	40	13.31	16.831	-0.26454
23.	2	10	7	50	8.49	7.387	0.12992
24.	1	5	6	35	8.89	8.643	0.02778
25.	3	5	6	35	27.85	29.554	-0.06119
26.	2	10	7	40	21.32	16.831	0.21055
27.	2	10	7	40	16.56	16.831	-0.01637
28.	1	5	8	45	7.53	5.126	0.31926
29.	3	15	8	45	22.83	19.88	0.12917
30.	2	10	5	40	19.56	16.298	0.16677
31.	3	5	8	35	36.98	42.152	-0.13986
Average value					**12064.31**		-0.04

Table 3: Analysis of variance (ANOVA) for the Response Surface Regression

Sources of variation	DF	SS	MS	F-Value	P-Value
Model	14	2273.25	162.37	10.63	0.000
Linear	4	1848.01	462.00	30.24	0.000
Days (X_1)	1	1759.94	1759.94	115.20	0.000
Inoc.conc.(X_2)	1	0.84	0.84	0.06	0.817
pH (X_3)	1	1.34	1.34	0.09	0.771
Temp.(X_4)	1	85.88	85.88	5.62	0.031
Square	4	344.61	86.15	5.64	0.005
X^2_1	1	242.54	242.54	15.88	0.001
X^2_2	1	0.55	0.55	0.04	0.852
X^2_3	1	3.12	3.12	0.20	0.658
X^2_4	1	63.82	63.82	4.18	0.058
2 Way interaction	6	80.63	13.44	0.88	0.531
X_1X_2	1	7.43	7.43	0.49	0.496
X_1X_3	1	0.78	0.78	0.05	0.824
X_1X_4	1	26.01	26.01	1.70	0.210
X_2X_3	1	0.08	0.08	0.01	0.943
X_2X_4	1	9.12	9.12	0.60	0.451
X_3X_4	1	37.21	37.21	2.44	0.138
Lack of fit	10	81.52	8.15	0.30	0.955

Model Summary

Std	R-sq	R-sq(adj)	R-sq(pred)
3.90868	90.29%	81.80%	72.54%

Table 4: Optimization of Various variables

Days(X_1)	Ino.conc. (X_2)	pH(X_3)	Temp(X_4)	Protein (%)	X_1	X_2	X_3	X_4	X_1X_1	X_1X_2	X_1X_3	X_1X_4	X_2X_2	X_2X_3	X_2X_4	X_3X_3	X_3X_4	X_4X_4
4	10	7	40	44.52	4	10	7	40	16	40	28	160	100	70	400	49	280	1600
2	10	7	40	25.49	2	10	7	40	4	20	14	80	100	70	400	49	280	1600
2	20	7	40	17.65	2	20	7	40	4	40	14	80	400	140	800	49	280	1600
1	15	6	35	8.45	1	15	6	35	1	15	6	35	225	90	525	36	210	1225
2	10	7	40	17.89	2	10	7	40	4	20	14	80	100	70	400	49	280	1600
1	15	6	45	8.95	1	15	6	45	1	15	6	45	225	90	675	36	270	2025
1	5	6	45	7.23	1	5	6	45	1	5	6	45	26	30	225	36	270	2025
1	15	8	45	7.67	1	15	8	45	1	15	8	45	225	120	675	64	360	2025
2	10	7	30	13.74	2	10	7	30	4	20	14	60	100	70	300	49	210	900
2	10	7	40	11.63	2	10	7	40	4	20	14	80	100	70	400	49	280	1600
2	10	7	40	11.62	2	10	7	40	4	20	14	80	100	70	400	49	280	1600
3	5	6	45	26.22	3	5	6	45	9	15	18	135	25	30	225	36	270	2025
3	15	8	35	29.53	3	15	8	35	9	45	24	105	225	120	525	64	280	1225
3	15	6	35	25.67	3	15	6	35	9	45	18	105	225	90	525	36	210	1225
2	10	9	40	11.98	2	10	9	40	4	20	18	80	100	90	400	81	360	1600
2	0	7	40	15.42	2	0	7	40	4	0	14	80	0	0	0	49	280	1600
3	5	8	45	20.12	3	5	8	45	9	15	24	135	25	40	225	64	360	2025
1	15	8	35	10.42	1	15	8	35	1	15	8	35	225	120	525	64	280	1225
3	15	6	45	23.21	3	15	6	45	9	45	18	135	225	90	675	36	270	2025
1	5	8	35	10.87	1	5	8	35	1	5	8	35	25	40	175	64	280	1225
0	10	7	40	12.96	0	10	7	40	0	0	0	0	100	70	400	49	280	1600
2	10	7	40	13.31	2	10	7	40	4	20	14	80	100	70	400	49	280	1600
2	10	7	50	8.49	2	10	7	50	4	20	14	100	100	70	500	49	350	2500
1	5	6	35	8.89	1	5	6	35	1	5	6	35	25	30	175	36	210	1225
3	5	6	35	27.85	3	5	6	35	9	15	18	105	25	30	175	36	210	1225
2	10	7	40	21.32	2	10	7	40	4	20	14	80	100	70	400	49	280	1600
2	10	7	40	16.56	2	10	7	40	4	20	14	80	100	70	400	49	280	1600
1	5	8	45	7.53	1	5	8	45	1	5	8	45	25	40	225	64	360	2025
3	15	8	45	22.83	3	15	8	45	9	45	24	135	225	120	675	64	360	2025
2	10	5	40	19.56	2	10	5	40	4	20	10	80	100	50	400	25	200	1600
3	5	8	35	36.98	3	5	8	35	9	15	24	105	25	40	175	64	280	1225

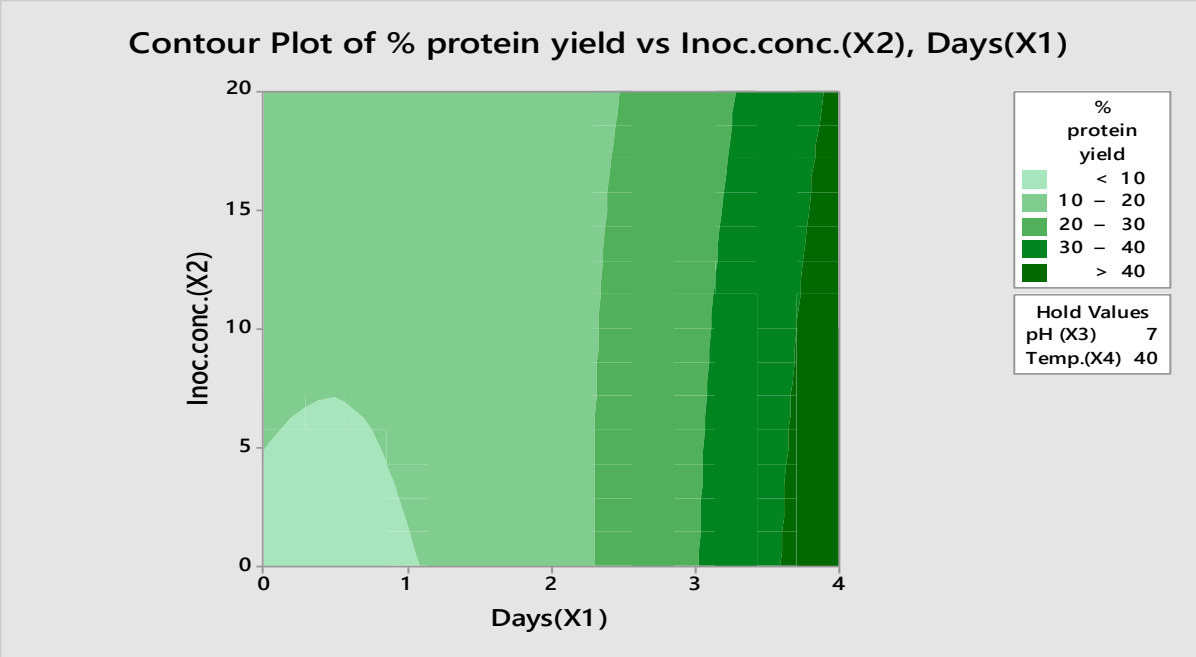

Figure 3: Contour plot of % Protein content yield vs Inoculum concentration and fermentation duration at fixed temperature and pH

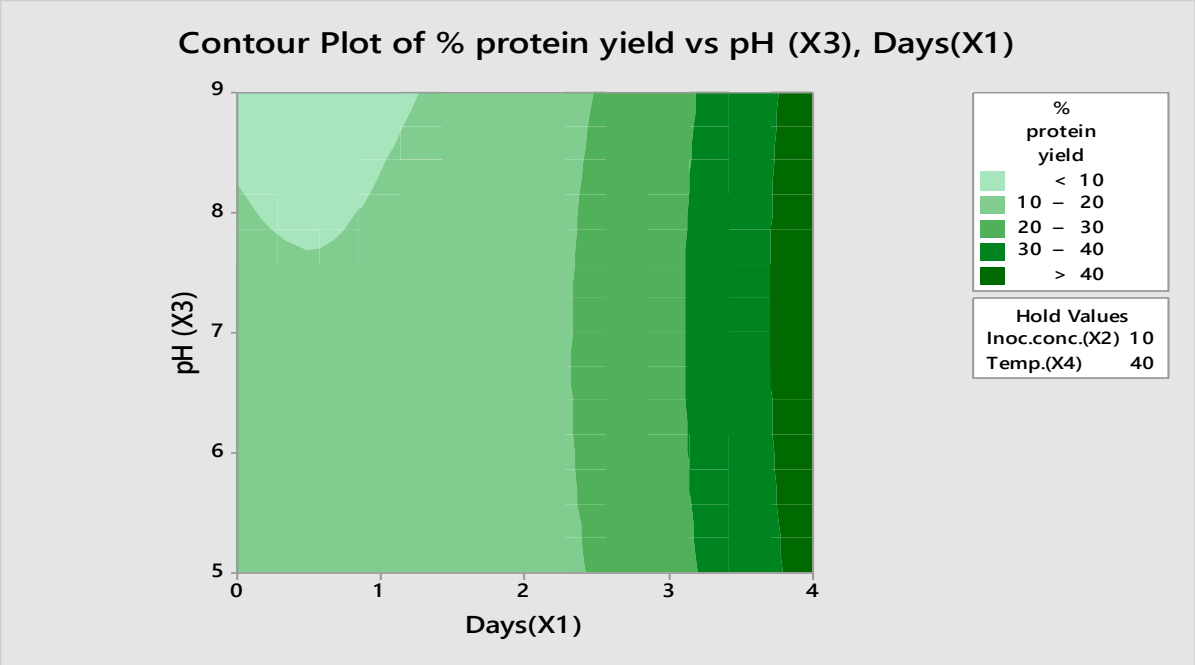

Figure 4: Contour plot of % Protein content yield vs pH and fermentation duration at fixed temperature and inoculum concentration

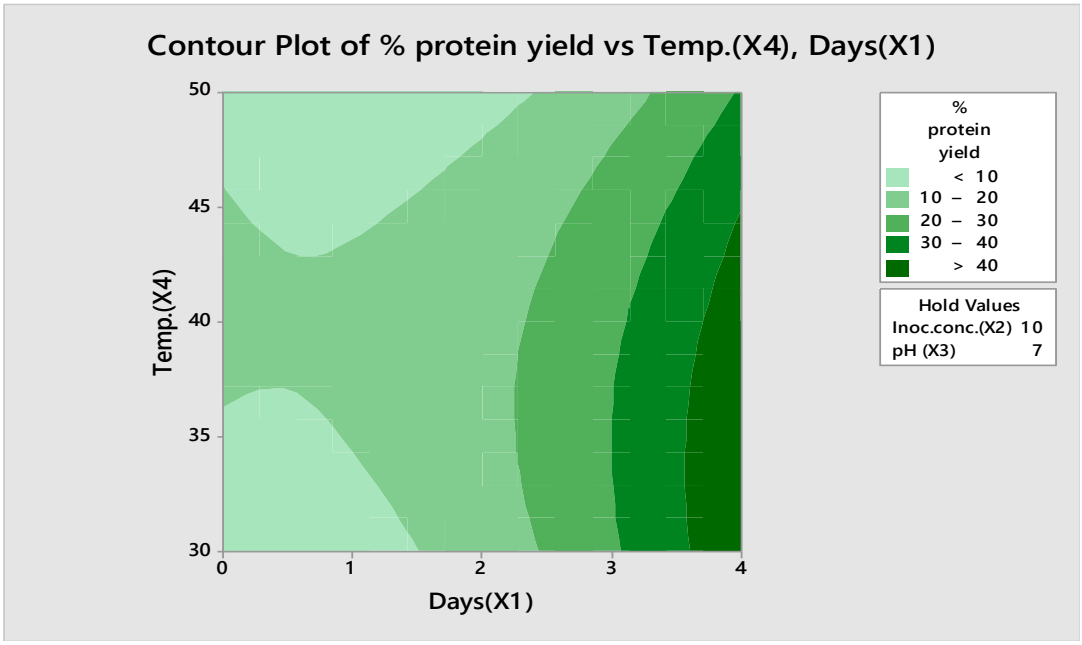

Figure 5: Contour plot of % Protein content yield vs Temp. and fermentation duration at fixed inoculums concentration and pH

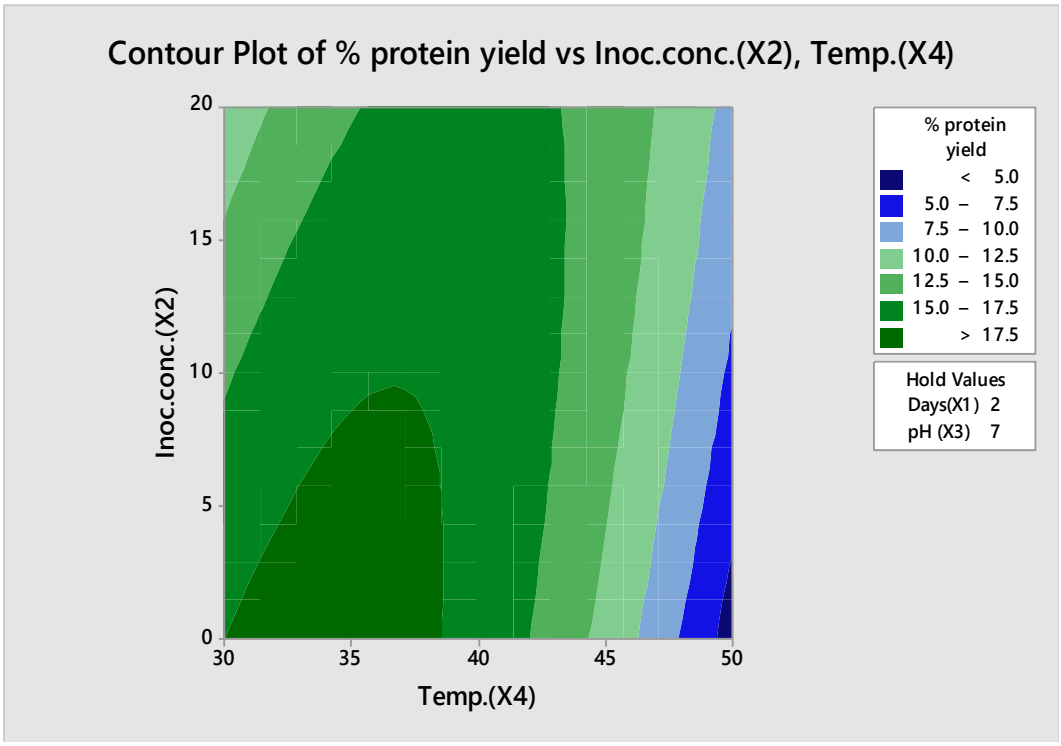

Figure 6: Contour plot of % Protein content yield vs Temp. and inoculums concentration at fixed pH and fermentation duration

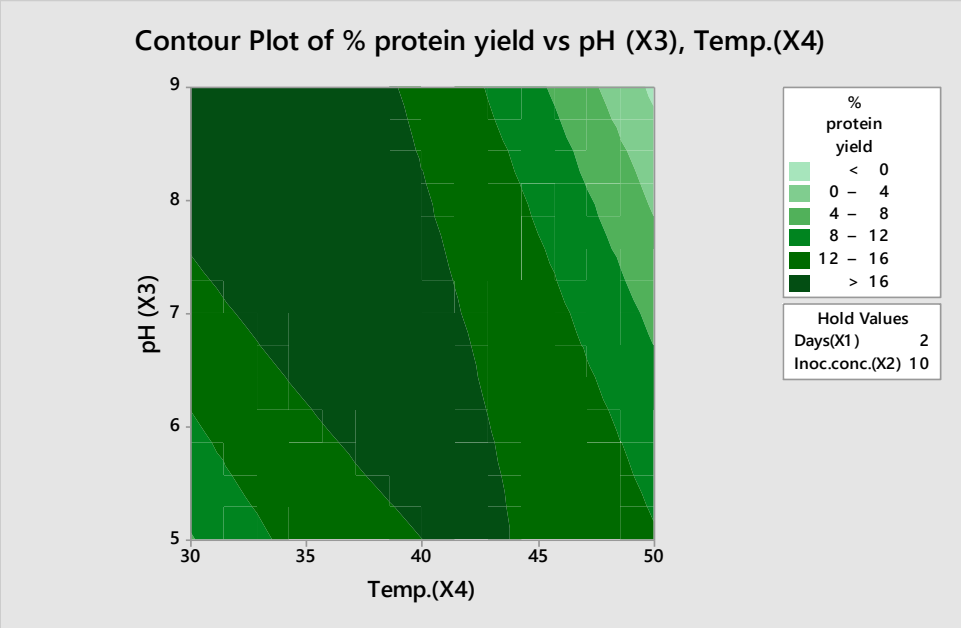

Figure 7: Contour plot of % Protein content yield vs Temp. and pH at fixed fermentation duration and inoculums concentration

Conclusion

This work concluded that a maximum yield of 44.52 % protein content was achieved through the fermentation of *Prosopis africana* seeds using *B. Licheniformis* at the optimum conditions of 4 days of fermentation, with inoculums concentration of 1.5×10^9 cells/$10mL^{-1}$, pH; 7 and 40ºC operating temperature using Response Surface Methodology.

Recommendations

Further studies should be carried out to investigate mineral, vitamin and amino acid content of fermented and unfermented *Prosopis africana* seeds.

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