APPLICATION OF RESPONSE SURFACE METHODOLOGY IN OPTIMIZING BIOETHANOL PRODUCTION FROM CALABASH (*Crescentia cujete*) SUBSTRATE USING *Saccharomyces cerevisiae.*

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Abstract

Aim:

The study employed the Response surface methodology (RSM) model to optimize ethanol production from Calabash (*Crescentia cujete*) pulp juice using *Saccharomyces cerevisiae*.

Study Design

The Calabash pulp was squeezed with muslin cloth, and vacuum filtered to clear solution before use. The clear juice was tested for reducing sugars using the Dinitrosalicylic acid (DNS) method. Twenty three runs (23), including 3 controls,of the fermentation was conducted at varying temperatures, pH, and volumes of inoculum. The process parameters (input variables): volumes of inoculum, temperature, and pH were subjected to response surface model, using the Central composite design (CCD).

Place and Duration of Study:

This study was carried out at the Environmental Microbiology Laboratory, University of Port Harcourt for six months.

Methodology:

Fermentation was done in conical flasks covered with cotton wool and foil in a stationary incubator for four days (96 hours). Active stock culture of *Saccharomyces cerevisiae* was used, with inoculum developed using Marcfaland's method. Samples were collected every 24 hours, centrifuged, filtered and analyzed for measurement of the output variables: reducing sugar, cell density and ethanol concentration.

Results:

The concentration of reducing sugars from Calabash pulp was 3.2 mg/ml. Results obtained also revealed that the fermentation can take place on a wide range of temperature; 25-40^{o}C . The optimal pH range for performance of *S.cerevisiae* for the fermentation process was pH 5.0-6.5. The optimum volume of inoculum was 5.5%v/v (ie 5.5 ml in 94.5ml juice). The optimized process using the RSM model gave 6.19% v/v bioethanol.

Control:

The bioethanol yield from Calabash substrate is reasonable considering the concentration of reducing sugars obtained from the juice and the duration of the fermentation.

Key words: Calabash juice, fermentation, optimization, Response surface methodology and bioethanol.

Introduction

Bioethanol is an energy source of the future(11).It is a fermentation product of various sugar sources including; sugar cane, corn, cassava, grasses and organic wastes, etc (9,17).

There is increased search for renewable and alternative energy sources. This is due to uncertainty in oil prices and effects of climate change due to the continuous use of fossil fuel (3, 17).

Bioethanol-petrol blends are available as 'gasohol'; E10 as 1:9 ethanol/petrol or 10% ethanol and 90% petrol (14,17).

Calabash (*Crescentia cujete*) is a lesser used plant in Nigeria. It is a perennial tree, producing flowers and fruits throughout the year. The fruits are round and large, with diameter of 12-14 cm (4;17).The pulp is not edible in Nigeria (19), and so was used as a substrate in this study. It is usually thrown away as waste, while the shell(gourd) was used as containers, musical instruments, in decoration by artists, for storing food materials, for fishing and processing of 'garri' and 'fura de nunu' (5,17).

Calabash pulp contains sugars which have been determined spectroscopicall(5,18).It was on this inference that we used the pulp for bioethanol production since it has been established that it contains fermentable sugars.

The experimental design used for the fermentation was the Response surface methodology (RSM). Fermentation lasted for 96 hours/ four days. This model helps to define the relationships between multiple variables at the same time (1,17). This methodology has been applied in a lot of chemical and biochemical processes inorder to get optimal yield in industrial processes (26). This optimization process is aimed at increasing the yield of the final product.

The Nigeria's biofuel policy of 10% (bioethanol) and 20% biodiesel has been gazette as Incentives No. 72 vol. 94 of June, 20, 2007. This target is expected to reach 2.0 billion litres by the year 2020(20,17).

The relevance of RSM in this study, and as part of efforts to meet up with this target; is to provide a good reference for future industrial production of bioethanol from Calabash juice. Calabash is a lesser used plant,that produces fruit all year round. Its use in bioethanol production would be sustainable. *Saccharomyces cerevisiae* used in this study is widely used in bioethanol production, and ferments a range of sugars to ethanol.There is need to explore other organic sources in the production of bioethanol.

Materials and Methods:

Isolation of the microbial strain

The yeast *Saccharomyces cerevisiae* was isolated from palm wine. Standard solid medium of composition: yeast extract10g/l, peptone 20g/l,glucose 20g/l, agar 15g/l, pH 6.8 was used. Glucose was filter sterilized and added after autoclaving the other ingredients (22,17).

Preparation of Calabash juice

Calabash was sourced from homes were they are used as hedges, and serve as shades from the sun. The pulp juices were squeezed out with muslin cloth and concentrated at 50^{o}Cfor 4 hours. Juices were further subjected to vacuum filtration using Whatman No 1 filter paper of dimension 12.5cm.It was sterilized at 121^{o}C and 15 psi for 10 minutes, allowed to cool before used for fermentation.

Molecular Characterization of the isolate, *Saccharomyces cerevisiae*

DNA extraction and 16S rRNA sequencing was done on the isolate. Further phylogenetic analysis was done and sequences matched with National Biotechnology Information Center (NCBI) database using Blast N, and linked using Clustal X (23,13).

Fermentation of Calabash Juice

Experimental Design

The fermentation was run with the Calabash juice in 100 ml amounts, using 250 ml capacity Erlenmeyer flask, sterilized at 160^{o}C for 1 hour. The juice was inoculated with *Saccharomyces cerevisiae* in changeable amounts following the Response surface methodology model (RSM), and covered with cotton wool and aluminium foil. A set of 23 replicates was used, including controls, at varying pH and volumes of inocula. These were incubated at temperatures 25^{o}C, 32.5^{o}C and 40^{o}C, based on the RSM for 96 hours in a stationary culture. Samples were set aside after 24 hours to check the concentrations of reducing sugar, ethanol, pH changes and cell density as the fermentation progressed.

DAY 1

Saccharomyces cerevisiae

Run	pH	Temp(^{o}C)	Vol.(ml)	Cell Density(OD)	Reducing sugar(g/l)	Ethanol concentration (%v/v)
1	5.4	25	8	0.796	3.227	1.309
2	5.4	32.5	5.5	0.711	2.931	4.712
3	5.45	32.5	5.5	0.865	2.668	6.401
4	5.45	32.5	5.5	0.933	3.163	9.463
5	5.5	32.5	5.5	0.904	3.079	5.806
6	5.5	25	8	0.780	3.204	2.124
7	5.5	40	3	0.268	2.703	0.241
8	5.5	25	3	0.801	3.148	1.115
9	5.5	40	8	0.348	2.739	0.593
10	5.45	25	5.5	0.826	3.179	1.516
11	5.45	40	5.5	0.356	2.825	0.192
12	5.4	40	3	0.361	2.797	0.168
13	5.45	32.5	5.5	0.821	3.328	5.927
14	5.4	40	8	0.362	2.999	0.192
15	5.4	25	3	0.841	3.199	2.063
16	5.45	32.5	8	0.932	3.353	5.721
17	5.45	32.5	5.5	0.883	3.214	6.194
18	5.45	32.5	5.5	0.871	3.239	5.235
19	5.45	32.5	5.5	0.859	3.350	6.389
20	5.45	32.5	3	0.796	3.386	4.809

DAY 2

Saccharomyces cerevisiae

Run	pH	Temp (^oC)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	5.4	25	8	0.826	3.199	1.467
2	5.4	32.5	5.5	0.734	2.921	3.351
3	5.45	32.5	5.5	0.868	2.642	5.186
4	5.45	32.5	5.5	0.943	3.138	8.089
5	5.5	32.5	5.5	0.910	3.055	4.712
6	5.5	25	8	0.841	3.173	2.574
7	5.5	40	3	0.333	2.670	1.674
8	5.5	25	3	0.870	3.123	2.294
9	5.5	40	8	0.439	2.708	1.881
10	5.45	25	5.5	0.865	3.148	1.711
11	5.45	40	5.5	0.469	2.799	1.236
12	5.4	40	3	0.434	2.749	1.723
13	5.45	32.5	5.5	0.832	3.302	4.566
14	5.4	40	8	0.440	2.976	1.844
15	5.4	25	3	0.880	3.148	3.157
16	5.45	32.5	8	0.938	3.345	4.323
17	5.45	32.5	5.5	0.893	3.194	4.857
18	5.45	32.5	5.5	0.878	3.214	3.812
19	5.45	32.5	5.5	0.869	3.342	4.979
20	5.45	32.5	3	0.798	3.636	3.484

DAY 3

Saccharomyces cerevisiae

Run	pH	Temp (^oC)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	5.4	25	8	0.856	2.822	3.569
2	5.4	32.5	5.5	0.734	2.567	3.655
3	5.45	32.5	5.5	0.868	2.561	5.429
4	5.45	32.5	5.5	0.943	2.880	8.369
5	5.5	32.5	5.5	0.910	3.042	5.186
6	5.5	25	8	0.861	2.658	2.343
7	5.5	40	3	0.699	2.597	1.371
8	5.5	25	3	0.890	2.897	3.752
9	5.5	40	8	0.691	2.582	1.589
10	5.45	25	5.5	0.889	2.569	3.132
11	5.45	40	5.5	0.652	2.562	1.030
12	5.4	40	3	0.612	2.496	1.419
13	5.45	32.5	5.5	0.832	3.103	5.016
14	5.4	40	8	0.643	2.471	1.516
15	5.4	25	3	0.895	2.468	3.569
16	5.45	32.5	8	0.938	3.042	4.699
17	5.45	32.5	5.5	0.893	2.941	5.162
18	5.45	32.5	5.5	0.878	2.946	4.092
19	5.45	32.5	5.5	0.869	3.118	5.283
20	5.45	32.5	3	0.798	3.087	3.971

DAY 4

Saccharomyces cerevisiae

Run	pH	Temp (^oC)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	5.4	25	8	0.882	2.314	0.666
2	5.4	32.5	5.5	0.760	2.061	3.776
3	5.45	32.5	5.5	0.892	2.142	5.550
4	5.45	32.5	5.5	0.962	2.415	8.515
5	5.5	32.5	5.5	0.932	2.612	5.332
6	5.5	25	8	0.916	2.147	0.848
7	5.5	40	3	0.892	2.339	1.286
8	5.5	25	3	0.911	2.266	1.784
9	5.5	40	8	0.791	2.066	1.479
10	5.45	25	5.5	0.939	1.935	1.128
11	5.45	40	5.5	0.780	2.288	0.994
12	5.4	40	3	0.762	2.192	1.383
13	5.45	32.5	5.5	0.846	3.027	5.186
14	5.4	40	8	0.780	2.061	1.443
15	5.4	25	3	0.955	1.871	0.787
16	5.45	32.5	8	0.962	2.976	5.064
17	5.45	32.5	5.5	0.902	2.890	5.332
18	5.45	32.5	5.5	0.898	2.926	4.554
19	5.45	32.5	5.5	0.892	3.045	5.526
20	5.45	32.5	3	0.832	3.027	4.092

Application of Response Surface Methodology (RSM)

The RSM establishes functional relationships between a variable of interest, known as the response/ dependent/ output variable (y) and a number of associated independent/input/control variables denoted by

$X_1, X_2,$……..Xn

The polynomial model of this form approximates such relationships thus:

$Y=f'(x)\beta + (\varepsilon)$ ----------(1)

Where

$X=(X_1, X_2$……..Xn), f(x) is a vector function of **p** elements consisting of powers and cross products of powers of X1, X_2 …….Xn up to a certain degree denoted by d(≥1), β is a vector of unknown constant coefficients referred to as parameters, and ε is a random experimental error assumed to have a mean of zero.

The equation (1) above is assumed to provide an adequate representation of the response. This implies that the quantity f(x)β represents the mean response, that is, the expected value of y.

Physicochemical Analysis of the Juice

Determination of Reducing Sugars

Dinitrosalicylic acid (DNS) method was used.

Standard glucose solution was prepared and a standard curve plotted to extrapolate the concentration of the unknown sample in mg/ml.

One milliliter (1 ml) of the sample was measured and 2 ml of dinitrosalicylic acid reagent(DNS) added in a clean test tube. It was placed in a boiling water bath for 5 minutes. It was left to cool and 7 ml of distilled water added. The absorbance was read at 540nm using blank as control.

Determination of ethanol concentration

The potassium dichromate method was used.

Ethanol calibration curve was plotted using 20% absolute ethanol. Five milliliter (5 ml) of the sample was taken and 2 ml of acidified potassium dichromate solution added; allowed for color development and absorbance read at 588 nm. The ethanol concentration of the sample was extrapolated from the calibration curve.

Recovery of ethanol

Ethanol was recovered from the fermentation broth by distillation. Fermentation broth was dispensed in a round- bottom flask fixed to a distillation column enclosed in a running tap water. A quick-fit flask at the other end was used to collect the distillate. Heating mantle had temperature adjusted to 78^0C.

Determination of ethanol concentration by gas chromatography (GC-FID)

To validate the qualitative and quantitative properties of bioethanol, gas –chromatography flame-ionization detector (GC-FID) was performed on the distillates from the test samples (25). Analysis was done using GC type: HP5890II. The GC was connected to a computer running peak simple software version 2.8. Oven temperature was set initially at 40^{o}C for 2 minutes, 180^{o}C final for 5 minutes at 15^{o}C/min and then 300^{o}C final at 20^{o}C.two microliter (2µl) sample was mixed with 5% Acetonitrile at the ratio of 1:1, was injected manually at time zero 0, using a 5 µl Hamilton syringe and temperature cycle was started. Ethanol regularly came out at retention time equivalent to 65^{o}C.

Results

Characterisation of the microbial strain

The isolate was identified using morphological and biochemical characteristics, as in Table 1.0.

Also, molecular characterization, using gene sequencing revealed the isolate to be close in evolutionary relationship to *Saccahromyces cerevisiae* as in Fig. 1.0.

Table 1.0: Morphological and Biochemical Characteristics of Isolate from palm wine

Test /Attribute	**Remark**
Colonial Characteristics	Smooth creamish
Cell shape	spherical
Gram Reaction	+
Fermentation Tests	
Glucose	AG
Fructose	AG
Sucrose	AG
Galactose	AG
Lactose	–
Mannitol	–
Microorganism	***Saccharomyces cerevisiae***

Key: **+ positive; − no fermentation; AG Acid/ gas production**

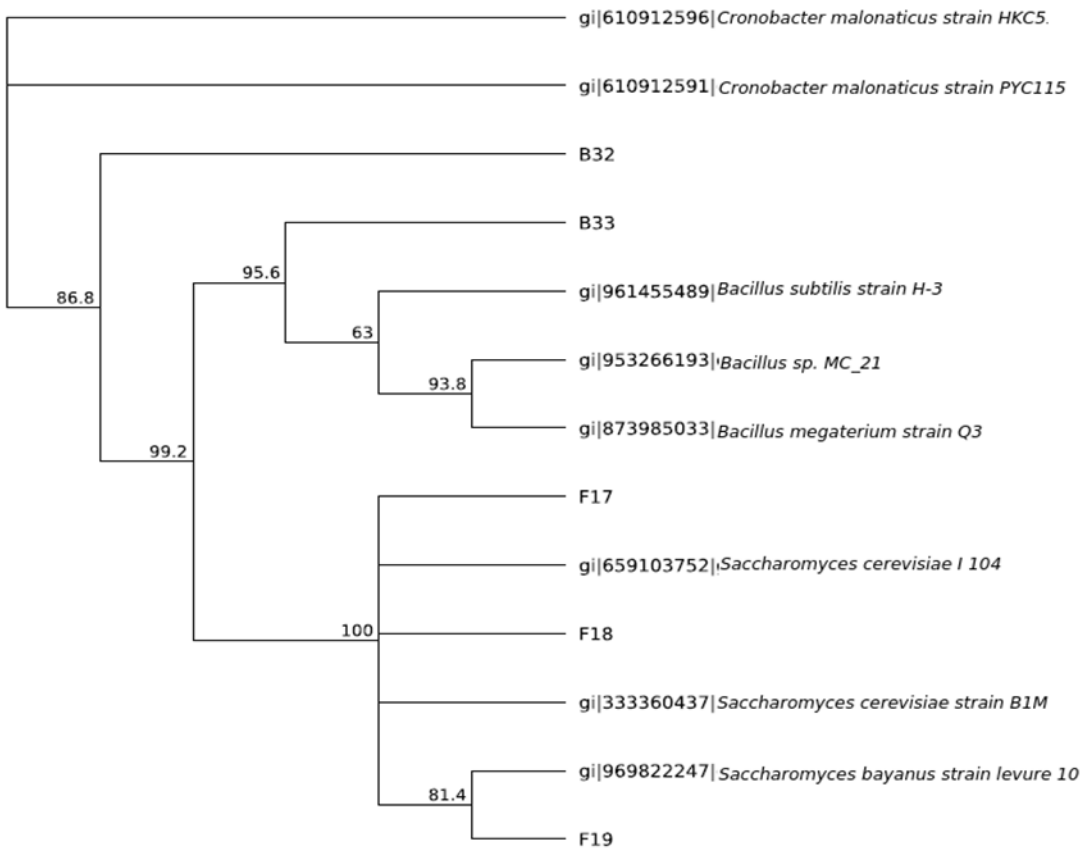


Fig 1.0 : Evolutionary relationship among the bacterial and yeast isolates from the study

Determination of reducing sugars

The concentration of the calabash juice was extrapolated from the calibration curve, using the mean absorbance value of 1.240 at 540 nm

Using the equation from the standard curve y=0.3955x-0.0355x

Reducing sugar = 3.22mg/ml.

Determination of ethanol concentration

Ethanol concentrations were extrapolated from the calibration curve.

Optimisation of process parameters using RSM

Table 2.0 *Saccharomyces cerevisae* – Optimized variables for Calabash pulp juice

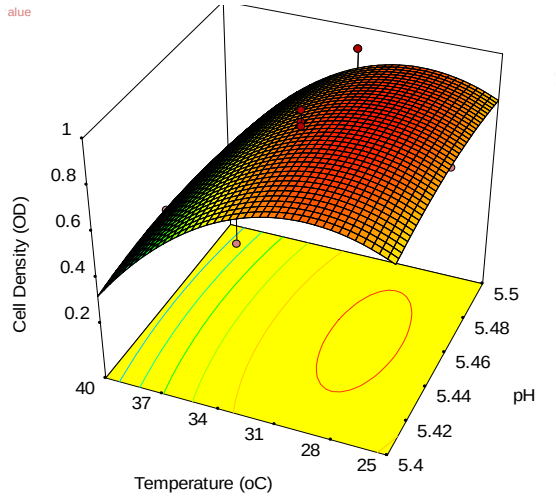
Time	pH	Temp. (^oC)	Volume (ml)	Cell density (OD)	Reducing sugar (g/l)	Ethanol conc. (%v/v)	Desirability (d_i)
Day 1	5.45	32.50	5.5	0.8646	3.1682	6.1941	1.00 (100.0%)
Day 2	5.50	31.40	5.5	0.8668	2.9440	4.8728	0.770 (77.0%)
Day 3	5.46	30.08	5.5	0.9016	2.9253	5.2018	0.792 (79.2%)
Day 4	5.47	31.16	5.5	0.9062	2.6651	5.3312	0.755 (75.5%)

Optimisation of the process parameters are further illustrated in Figures 2-4.The volume of inoculum was kept constant at 5.5%. Temperature ranged from 25- 40^{o}C, and pH from 5.4-5.5.The cell density during the 4- day period of the fermentation increased from 0.86- 0.91(Fig. 2.0). The reducing sugar levels reduced progressively after four days,from 3.2 g/l -2.6g/l; showing that *S. cerevisiae* was metabolizing the sugars present in the Calabash juice(Fig 3.0).A higher ethanol concentration of 6.19%v/v was recorded on Day 1 of the fermentation(Fig. 4.0). Desirability value of 1.0 was also recorded on Day 1, as against 0.77, 0.79 and 0.76 for other days, respectively(Fig 5.0). Desirability values closer to 1, relate to the higher probability of getting optimal response.

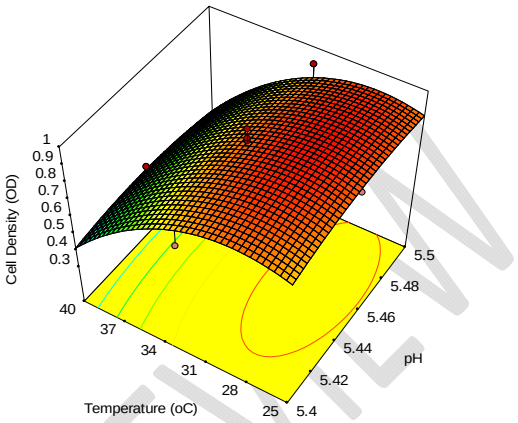
Table 3.0 Goodness of fit or Coefficient of determination (R^2) for *Saccharomyces cerevisiae* for Calabash

Fermentation Period	Model	Cell density	Reducing sugar	Ethanol concentration
Day 1	Quadratic	0.9631	0.6537	0.9010
Day 2	Quadratic	0.9588	0.6683	0.7080
Day 3	Quadratic	0.8742	0.7078	0.7538
Day 4	Quadratic	0.6232	0.7245	0.8612

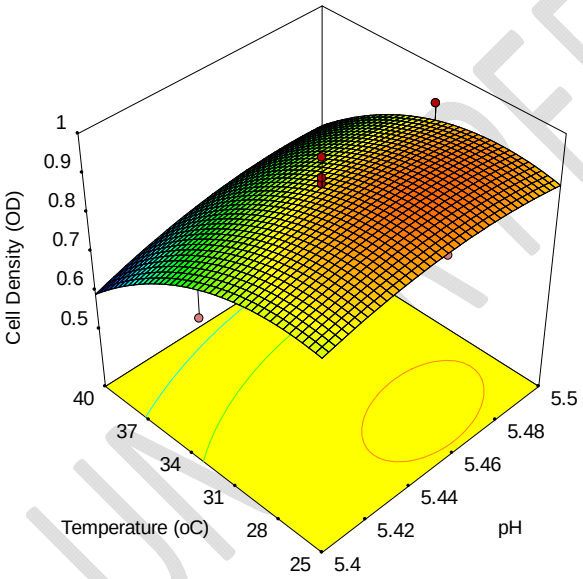
The coefficient of determination R^2(goodness of fit), measures the degree of variability in the response variable that could be explained by the control variables. R^2 lies between 0 and 1 ($0 \leq R^2 \leq +1$).The closer the R^2 value to 1, the more reliable or predictive is the model.



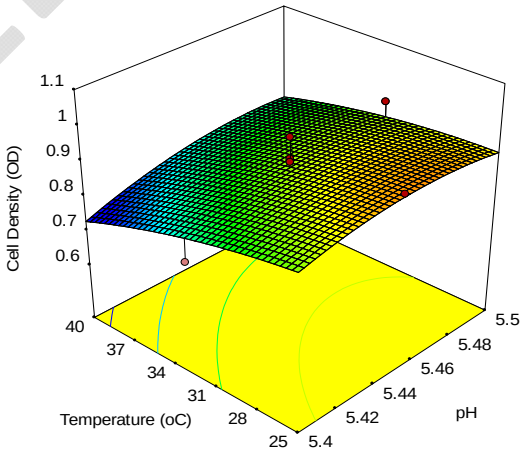
Day 1



Day 2



Day 3



Day 4

Fig.2.0: Response surface attributes for Cell density for *Saccharomyces cerevisiae* for Calabash for 4 days

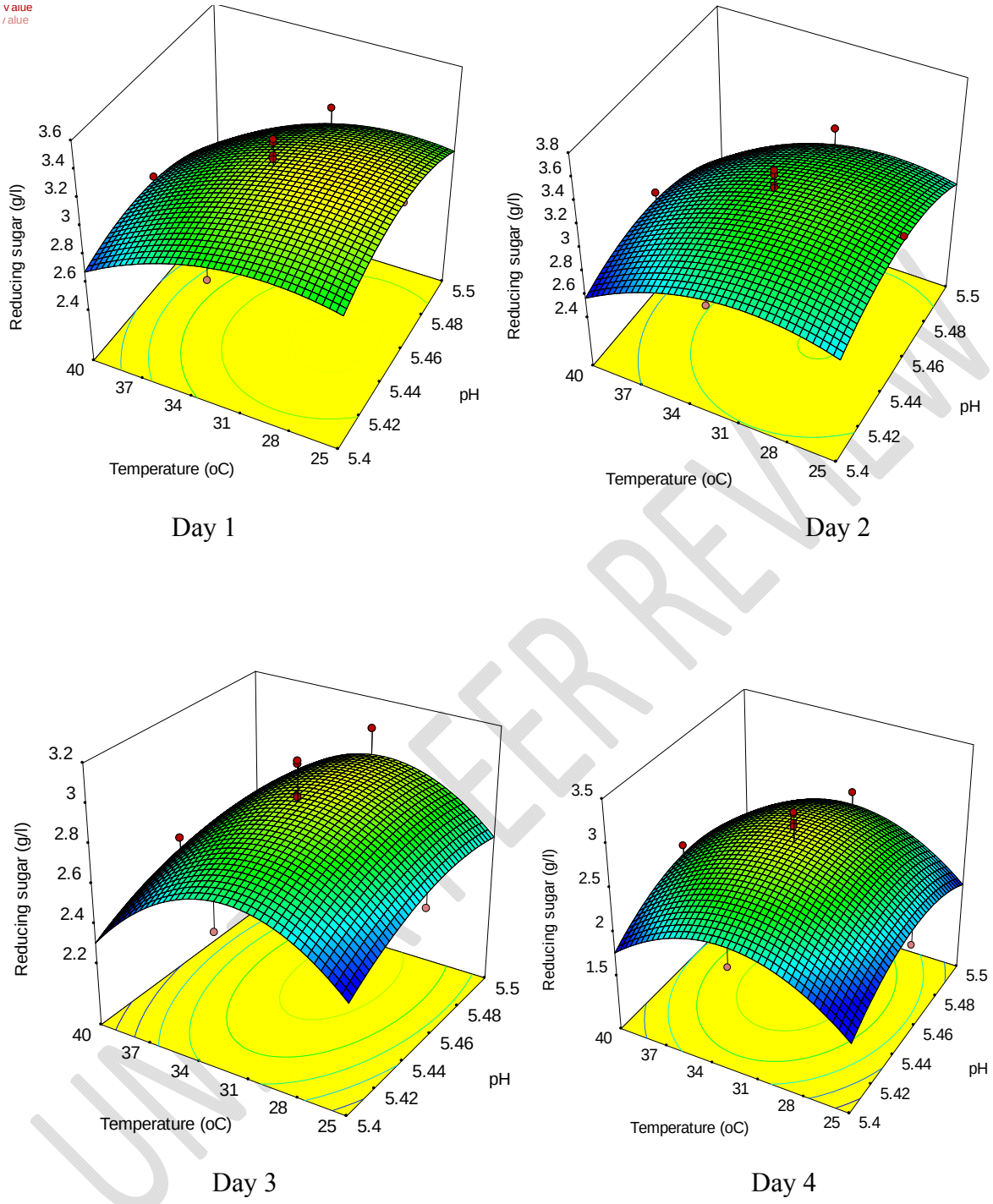
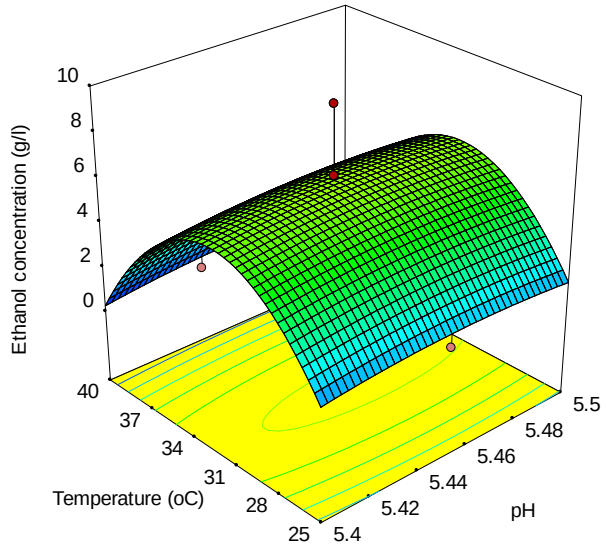
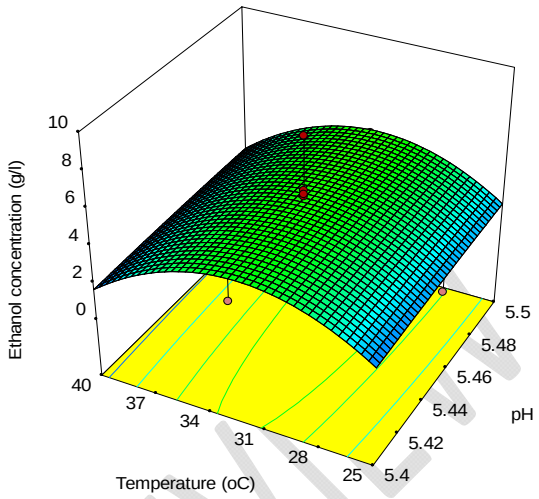


Fig.3.0: Response surface attribute for reducing sugar for *Saccharomyces cerevisiae* for Calabash for 4 days



Day 1



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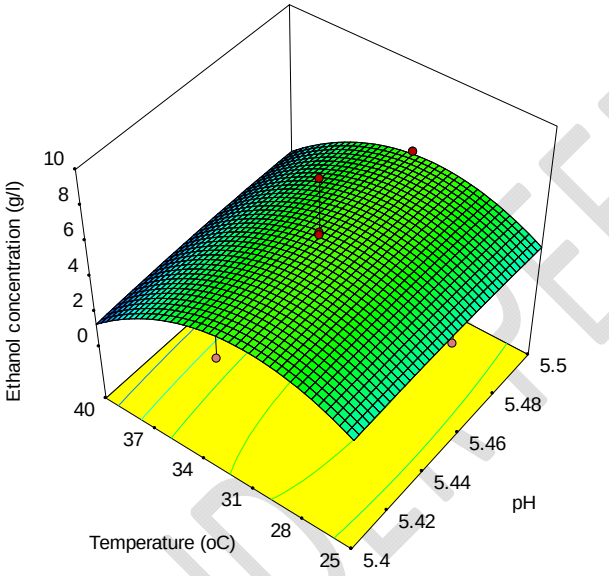
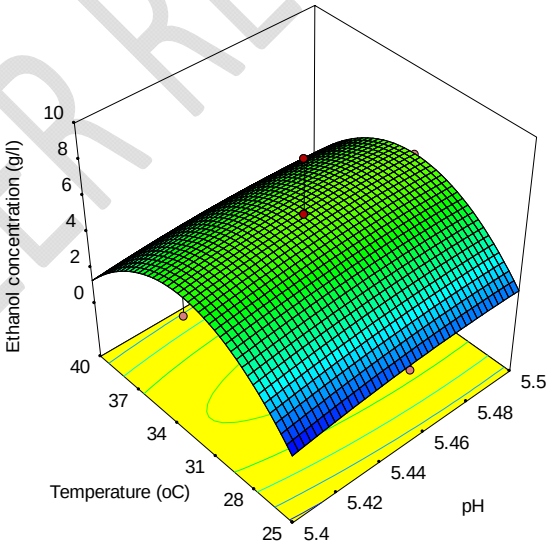
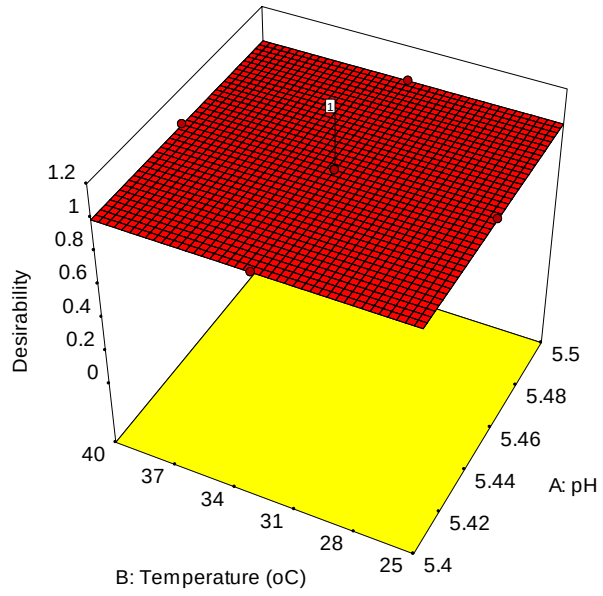


Day 3

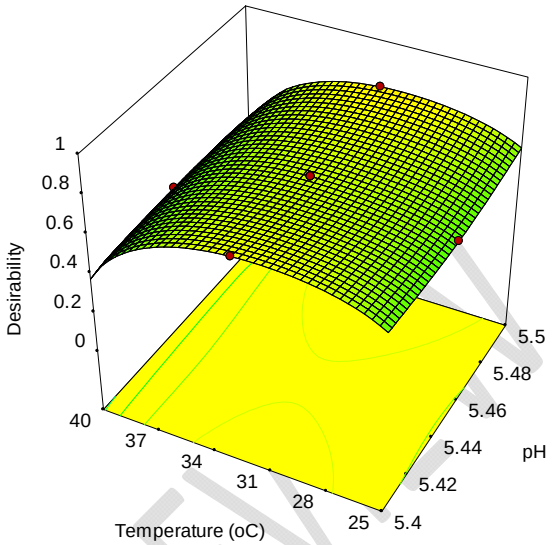


Day 4

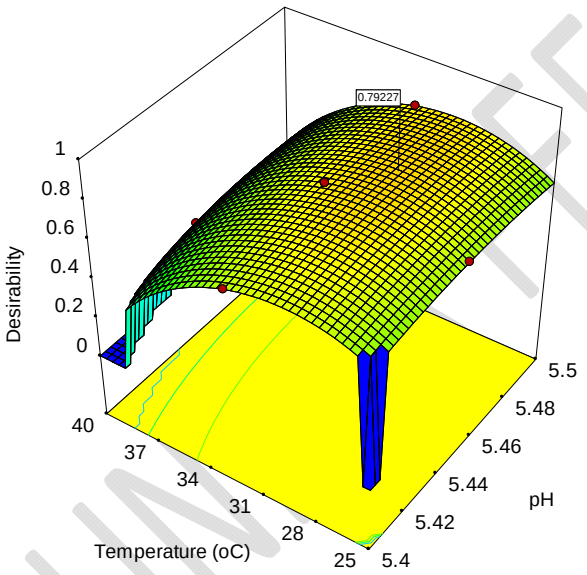
Fig.4.0: Response surface attributes of Ethanol concentration for *Saccharomyces cerevisiae* for calabash for 4 days



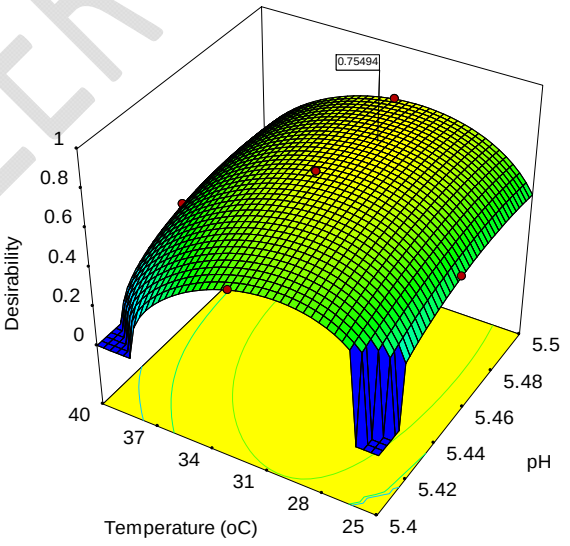
Day 1



Day 2



Day 3



Day 4

Fig.5.0: Response surface of desirability for optimized fermentation process for *S.cerevisiae* for Calabash for 4 days

Discussion

The reducing sugar content of Calabash juice determined using glucose calibration curve was 3.22mg/ml. The choice of substrate in this study was in line with reports by (8). He said that reducing sugars such as glucose and other hexoses and pentoses are easily metabolised by several genera of microorganisms to industrial products such as biofuels.

Saccharomyces cerevisiae has widely been used in bioethanol production from carbohydrate sources;and in the food industry(16,27).This yeast was isolated from natural source and characterised using molecular characteristics and gene sequencing. This was to establish the credibility of the fermentation, and the entire process.

Calabash pulp used in this study has carbohydrate content of 87.62%, hence can be hydrolysed to smaller units for bioethanol production (17).

The average concentration of bioethanol produced after 4 days from calabash pulp juice using *S. cerevisiae* was 8.60%v/v. The optimization process by RSM revealed maximum ethanol concentration of 6.19%v/v. Bioethanol production from corn cobs using co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* yielded 10.08% v/v from 0.63 mg/ml reducing sugar concentration, after 7 days(12).(10) reported that 0.33%v/v ethanol was produced from citrus peel wastes.

Also optimisation of fermentation with *S.cerevisiae* on fruit pulp substrate yielded 63g/l ethanol (24). The average yield from guinea corn and millet husks were 26.83g/l and 18.3g/l respectively, representing concentrations of 67.7 and 63.8% respectively too (21).

The application of RSM was to show the optimal conditions for the improved yield of bioethanol from the substrate. *Sacchromyces cerevisiae* had optimal performance at pH 5.4, temperature 25-28^{o}C and 5.5% volume of inoculum. These characteristics corroborate reports by (27), that *S.cerevisiae* has low nutrient requirement, resistance to high ethanol concentrations, tolerance to pH, and general robustness.

GC-FID analysis of distillates from *S.cerevisiae* fermentation revealed a concentration of 1.25mg/l at 32.5^{o}C.This was greater than that for the control sample. (3) reported values of 4.38g/l ethanol from fruit rinds by GC- analysis using *S.cerevisiae*.

Conclusion:

The fermenting organism used in this study,*Saccharomyces cerevisiae* isolated from palm wine was able to ferment Calabash juice to bioethanol. Response surface methodology (RSM) model was employed for the optimisation of the process parameters; and this revealed that at a particular volume of inoculum, the fermentation process could take place on a range of temperatures, and pH. This fermentation procedure is economically feasible because the substrates are wastes, and the fermenting strain was sourced from the environment.

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