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Response surface methodology and optimization of the processes for bioethanol production from Calabash (*Crescentia cujete*) Using *Cronobacter malonaticus*

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Abstract

Aim: Response surface methodology (RSM) model was applied to optimize ethanol production from Calabash (*Crescentia cujete*) pulp juice using *Cronobacter malonaticus*.

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 (23) runs, 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 in 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 *Cronobacter malonaticus* 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 28-32°C. The optimal pH range for performance of *C.malonaticus* for the fermentation process was pH 5.95-6.5. The optimum volume of inoculum was 10%v/v (i.e. 10 ml in 90 ml juice). The optimized process using the RSM model gave 5.08% v/v bioethanol, being the highest achieved at pH6.08 and 28^{o}C .

Conclusion: The bioethanol yield from Calabash substrate is reasonable considering that the bacterium used is not known for ethanol production. Also the concentration of reducing sugars in the substrate and the duration of fermentation could be responsible for the yield.

Keywords: Calabash juice; Fermentation; Optimization; Response Surface Methodology and Bioethanol

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1. Introduction

The world's current energy supply may be depleted because fossil fuels are non-renewable. This, amongst other reasons call for search into alternative energy sources. Such energy sources include: solar energy, nuclear energy, wind energy, hydropower, tidal energy and biofuels [1; 2]. Biofuels include biogas and bioethanol, mostly produced from plant and animal materials with the action of microorganisms [3; 2].Sugar sources and cellulosic materials such as sugar cane, corn, wheat, grasses and woody crops, respectively have been widely used in bioethanol production. It has been established that these materials have attendant problems such as interfering with food supply, and the pretreatment methods required [4; 5].

Bioethanol has high economic value as a solvent, a germicide, a beverage, an anti-freeze, a fuel, and a depressant [6]. Bioethanol can be used as a partial gasoline replacement in some countries of the world [1]. It contains 35% oxygen in its chemical molecule, useful in complete combustion of fuel and lowers emission of harmful gases in compression-ignition engines [3].

The substrate used in this study was fresh pulp juice from Calabash (*Crescentia cujete*) fruit. This fruit is underutilized, but has the rare potential of fruiting all through the year; with the characteristic large, round fruit of 12-14 cm in diameter [7]. It has been established that the fruit contains fermentable sugars [8; 9]; hence the basis for use in the production of bioethanol in this work.

The fermenting organism, *Cronobacter malonaticus* was recently identified from reclassification of *Enterobacter sakazaki*, a member of the *Enterobacter*iaceae family. *Cronobacter* species are primary inhabitants of plant materials, like their counterpart *Enterobacter* species, hence the isolation from rotten orange. The members of the genus are facultatively anaerobic, gram-negative, oxidase-negative, catalase positive, motile and produce acetoin (Voges-Proskauer positive and Methyl red test negative).They ferment D-glucose and other sugars yielding acid and gas; produce aldehydes, ketones, alcohols, sulfur compounds[10]. They metabolize glucose through the 2, 3-butanediol fermentative pathway [10].

The optimization of the process was done using the Response Surface Methodology (RSM) Model. This method compares relationships between multiple variables on the entire fermentation process at the same time. It has been employed in several chemical and biochemical processes to improve optimal yield in the industries [11; 12].

The RSM model used in this study will help provide a benchmark in production processes for future production of bioethanol from Calabash. Since the fruit is produced all year round, and is from a lesser used plant, it can form a sustainable substrate for bioethanol production. It is however necessary that other organic sources be studied for bioethanol production so as to meet our energy demand.

2. Material and methods

2.1. Preparation of the Calabash Juice

The Calabash fruit was sourced from the garden around homes. They were used as hedges, as well as shades from the sun in most traditional family settings. The pulp juice used in this study was formerly thrown away, while the shell or gourd served as material for containers to store food materials and for musical instrument amongst others. The pulp was squeezed to get the juice out with muslin cloth and heated on an electric plate at 50^{o}C for 4 hours to concentrate the juice. It was vacuum filtered using Whatman No 1 filter paper of 12.5cm dimension to get it clear. The resulting clear juice was sterilized 121^{o}C and 15 psi for 10 minutes and allowed to cool before fermentation was initiated.

2.2. Isolation of the Microbial Strain

Cronobacter malonaticus was isolated from rotten orange. Glucose agar medium made up of the following was used: yeast extracts 10g/l, glucose 20g/l, agar 15g/l, KH_2PO_4 1g, $MgCl_2$ 1g/l, $(NH_4)_2SO_4$ 1g/l, and pH6.0 [2]. The Glucose was filter sterilized and added after other ingredients have been autoclaved.

2.3. Molecular Characterization of the Isolate, *Cronobacter malonaticus*:

The extraction of DNA and sequencing of 16S rRNA was done. Further phylogenetic analysis was carried out, and sequences were matched with National Biotechnology Information Center (NCBI) database using Blast N, and linked using Clustal X [12; 13].

2.4. Application of Response Surface Methodology (RSM).

Response surface methodology (RSM) involves a set of mathematical and statistical techniques used to develop functional relationships between a variable of interest referred to as the response/dependent/output variable(y), and a number of associated independent/input/control variables denoted by X1, X2, X3….Xn. This relationship can be represented by a polynomial model as follows:

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

Where X=(x1,x2,------xn), f'(x) is a vector function of ρ elements comprising of powers and cross products of powers x1,x2-----xn, reaching to a point denoted by d(≥1), β is a vector of unknown constant coefficients known as parameters, while ε is a random experimental error assumed to have a mean of zero.

The expression in equation (1) above is assumed to offer adequate representation of the response: f'(x) β denotes the mean response, which is the expected value of y.

It is expected that RSM would help achieve the following:

- Establish an approximate relationship between y and x1,x2 -----xn used to predict response values for given settings of the control variables
- Determine optimum settings of x1, x2----xn that result in the maximum (or minimum) response over a certain region of interest.

2.5. Fermentation of Calabash Pulp Juice

2.5.1. Experimental Design:

Response surface methodology (RSM) model was applied were a set of 23 replicates; including control was used at varying pH and volumes of inocula (Experiments 1-4). Fermentation of the Calabash juice was run in 250 ml capacity Erlenmeyer flasks containing 100ml each. Flasks were sterilized at 160^{o}C for 1 hour before use. The juice was inoculated with varying amounts of *Cronobacter malonaticus* inoculum, according to the RSM model, and covered with cotton wool and aluminum foil. They were incubated at temperatures 25^{o}C, 32.5^{o}C and 40^{o}C for 4 days (96 hours) in a stationary culture. Samples were collected every 24 hours to check for changes in reducing sugar concentrations; pH and cell density as fermentation was in place.

2.6. Determination of Reducing Sugars:

This was done by Dinitrosalicylic acid (DNS) method. A standard curve was created using Standard glucose solution, where the concentration of the unknown sample was derived in mg/ml. Two milliliter (2ml) of dinitrosalicylic acid (DNS) reagent was added to 1 ml of the sample in a clean test tube. The mixture was put in a boiling water bath for 5 minutes. After cooling, 7 ml distilled water was added, and the absorbance read at 540nm using blank as control

2.7. Recovery and Determination of Ethanol Concentration

Distillation method was used to recover the ethanol from the fermentation broth. The broth was poured into round-bottom flask attached to a distillation column surrounded by running water. The distillate was collected in a quick-fit flask at the other end of the distillation column. Temperature of the heating mantle was set at 78^{o}C.

Determination of ethanol concentration was done by the potassium dichromate method. Plotting of ethanol calibration curve was carried out using 20% absolute ethanol. Two milliliter (2ml) of acidified potassium dichromate solution was added to 5ml of the distillate and allowed to stand for color development. Absorbance was read at 588nm.The ethanol concentration of the distillate was derived from the calibration curve.

2.8. Quantitative Determination of Ethanol Concentration by Gas Chromatography (GC-FID).

Gas-chromatography flame-ionization detector (GC-FID) was run on the distillates to validate the qualitative and quantitative properties of bioethanol. This was done with the GC type: HP589011. The GC was connected to a computer running peak simple software version 2.8. Oven temperature was set initially at 40°C for 2minutes, 180°C final for 5 minutes at 15°C/min and then 300°C final at 20°C. Two micro liter (2µl) samples 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°C.

Experiment 1: DAY 1

Cronobacter malonaticus

Run	pH	Temp (°C)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	5.5	25	13	0.433	3.224	2.476
2	6	32.5	10	0.654	3.259	2.488
3	6.5	25	7	0.383	3.199	2.659
4	6	32.5	7	0.630	2.961	2.391
5	6	32.5	10	0.622	3.320	2.999
6	6	32.5	10	0.728	3.168	5.125
7	6	32.5	10	0.531	3.479	2.889
8	6.5	40	13	0.353	3.004	0.605
9	6	25	10	0.361	3.163	2.695
10	6.5	32.5	10	0.582	3.608	4.323
11	6	32.5	13	0.513	3.335	2.439
12	6.5	40	7	0.343	3.042	1.456
13	5.5	40	7	0.255	2.931	1.055
14	6	40	10	0.412	2.961	1.115
15	5.5	32.5	10	0.526	3.297	4.663
16	6	32.5	10	0.518	3.289	7.312
17	6.5	25	13	0.352	3.179	2.002
18	6	32.5	10	0.528	3.055	3.448
19	5.5	25	7	0.365	3.204	2.185
20	5.5	40	13	0.313	2.923	0.411

DAY 2

Cronobacter malonaticus

Run	pH	Temp (°C)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	5.5	25	13	0.481	3.199	3.060
2	6	32.5	10	0.680	3.042	2.889
3	6.5	25	7	0.621	3.173	2.841
4	6	32.5	7	0.646	2.951	3.521
5	6	32.5	10	0.650	3.299	4.348
6	6	32.5	10	0.732	3.148	5.575
7	6	32.5	10	0.548	3.363	3.060
8	6.5	40	13	0.450	2.921	1.650
9	6	25	10	0.607	3.123	2.780
10	6.5	32.5	10	0.590	3.345	1.115
11	6	32.5	13	0.530	3.227	3.448
12	6.5	40	7	0.443	2.885	2.926
13	5.5	40	7	0.452	2.905	1.893
14	6	40	10	0.672	2.893	2.258
15	5.5	32.5	10	0.542	3.204	3.205
16	6	32.5	10	0.580	3.280	5.903
17	6.5	25	13	0.625	3.148	2.197
18	6	32.5	10	0.556	3.047	3.436
19	5.5	25	7	0.638	3.173	3.230
20	5.5	40	13	0.484	2.895	2.439

DAY 3

Cronobacter malonaticus

Run	pH	Temp (°C)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	5.5	25	13	0.531	2.948	5.089
2	6	32.5	10	0.702	3.778	3.728
3	6.5	25	7	0.746	3.100	6.085
4	6	32.5	7	0.650	3.138	3.800
5	6	32.5	10	0.656	3.118	4.699
6	6	32.5	10	0.738	2.865	5.915
7	6	32.5	10	0.556	3.133	3.582
8	6.5	40	13	0.665	2.415	1.395

9	6	25	10	0.674	3.133	4.530
10	6.5	32.5	10	0.604	3.279	2.318
11	6	32.5	13	0.562	3.113	3.947
12	6.5	40	7	0.655	2.379	2.671
13	5.5	40	7	0.673	2.596	1.723
14	6	40	10	0.674	2.582	2.002
15	5.5	32.5	10	0.568	3.082	3.655
16	6	32.5	10	0.588	3.103	6.158
17	6.5	25	13	0.643	3.158	4.165
18	6	32.5	10	0.562	3.022	3.800
19	5.5	25	7	0.645	3.034	4.894
20	5.5	40	13	0.624	2.592	2.318

DAY 4

Cronobacter malonaticus

Run	**pH**	**Temp (^oC)**	**Vol.(ml)**	**Cell Density (OD)**	**Reducing sugar (g/l)**	**Ethanol concentration (%v/v)**
1	5.5	25	13	0.601	2.268	3.011
2	6	32.5	10	0.704	3.381	3.849
3	6.5	25	7	0.799	2.294	4.250
4	6	32.5	7	0.656	3.118	4.019
5	6	32.5	10	0.660	2.849	4.894
6	6	32.5	10	0.742	3.123	6.085
7	6	32.5	10	0.561	3.224	3.995
8	6.5	40	13	0.870	2.061	1.395
9	6	25	10	0.681	2.271	4.274
10	6.5	32.5	10	0.608	2.865	2.561
11	6	32.5	13	0.571	3.027	4.092
12	6.5	40	7	0.862	2.341	2.524
13	5.5	40	7	0.851	1.990	1.832
14	6	40	10	0.861	1.965	1.966
15	5.5	32.5	10	0.580	3.052	4.044
16	6	32.5	10	0.602	3.072	6.498
17	6.5	25	13	0.744	2.172	3.558
18	6	32.5	10	0.570	2.991	4.092
19	5.5	25	7	0.754	2.106	4.724
20	5.5	40	13	0.865	2.084	2.233

3. Results

3.1. Identification of the Microbial Strain

Morphological and biochemical characteristics of the isolate were used for its identification (Table 1).

Molecular characterization technique used was gene sequencing which showed that the isolate has close evolutionary relationship to *Cronobacter malonaticus* (Fig 1).

Table 1 Morphological and Biochemical Characteristics of the isolate from rotten Orange

Test/ Attribute	**Remark**
Colonial Characteristics	Mucoid, creamish, umbonate elevation
Gram Reaction	_ Rod
Biochemical Tests	
Catalase	+
Indole	_
Urease	_
Oxidase	_
Fermentation Tests	
Glucose	AG
Fructose	AG
Sucrose	AG
Maltose	_
Lactose	_
Mannitol	AG
Microorganism	*Cronobacter malonaticus*

Key: + positive; −negative/ no fermentation; AG Acid/ Gas production

3.2. Concentration of Reducing Sugars

This was deduced from the calibration curve with the mean absorbance value gotten as 1.240 at 540nm. The equation from the standard curve is given as:

$y = 0.3955x - 0.0355$

Hence, Reducing sugar =3.22 mg/ml.

3.3. Determination of Ethanol Concentration

Ethanol concentrations were extrapolated from the calibration curve.

The equation was given as:

$y = 0.0823x + 0.0352$

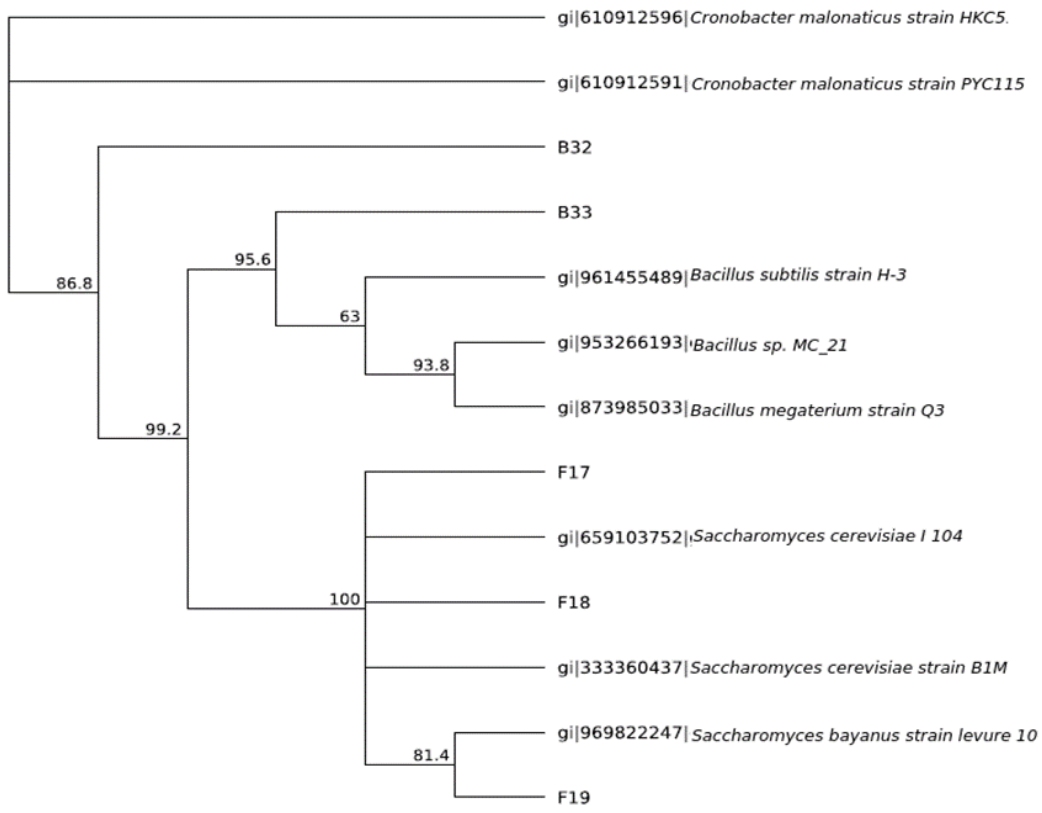

Figure 1 Evolutionary relationship amongst the bacterial isolates

3.4. Optimization of the Process Parameters using RSM

The optimization process yielded results as expressed in Tables 2 and 3, as well as Figures 2-4. The volume of inoculum was kept constant at 10%. Optimal temperature of performance ranged from 28-32oC, while pH was 5.95-6.5.The cell density during the four- day period increased from 0.57-0.66 on day 3, and slightly reduced to 0.63 on the fourth day(Fig. 2). The reducing sugar levels decreased steadily from 3.5g/l on day 1 to 2.9g/l on day 4(Fig. 3). Ethanol concentration of 5.08%v/v was recorded on day 3, with a desirability value of 0.9(Table 2).

Table 2 Optimized variables for Calabash Pulp Juice fermented using *Cronobacter malonaticus*

Time	pH	Temp. (°C)	Volume (ml)	Cell density (OD)	Reducing sugar (g/l)	Ethanol conc.(%v/v)	Desirability (d_i)
Day 1	6.50	32.0	10	0.5718	3.50	4.748	0.751 (75.1%)
Day 2	6.00	31.0	10	0.6368	3.2075	3.8362	0.823 (82.3%)
Day 3	6.08	28.0	10	0.6607	3.217	5.0819	0.911 (91.1%)
Day 4	5.95	28.6	10	0.6291	2.8672	4.8330	0.876 (87.6%)

Desirability values close to 1 depict higher probability of achieving optimal response. Also the coefficient of determination R^2 (goodness of fit) measures the level of variability of the response variable that the control variables could explain (Table 3). The values of R^2 lie between 0 and 1($0 \leq R^2 \leq +1$).The closer the R^2 value is to 1, the more predictive or reliable the model is.

Table 3 Goodness of fit (R^2) for Calabash juice fermented with *Cronobacter malonaticus.*

Fermentation Period	Model	Cell density	Reducing sugar	Ethanol concentration
Day 1	Quadratic	0.8417	0.6899	0.6583
Day 2	Quadratic	0.6186	0.6186	0.4790
Day 3	Quadratic	0.5518	0.5518	0.7175
Day 4	Quadratic	0.8605	0.7496	0.6857

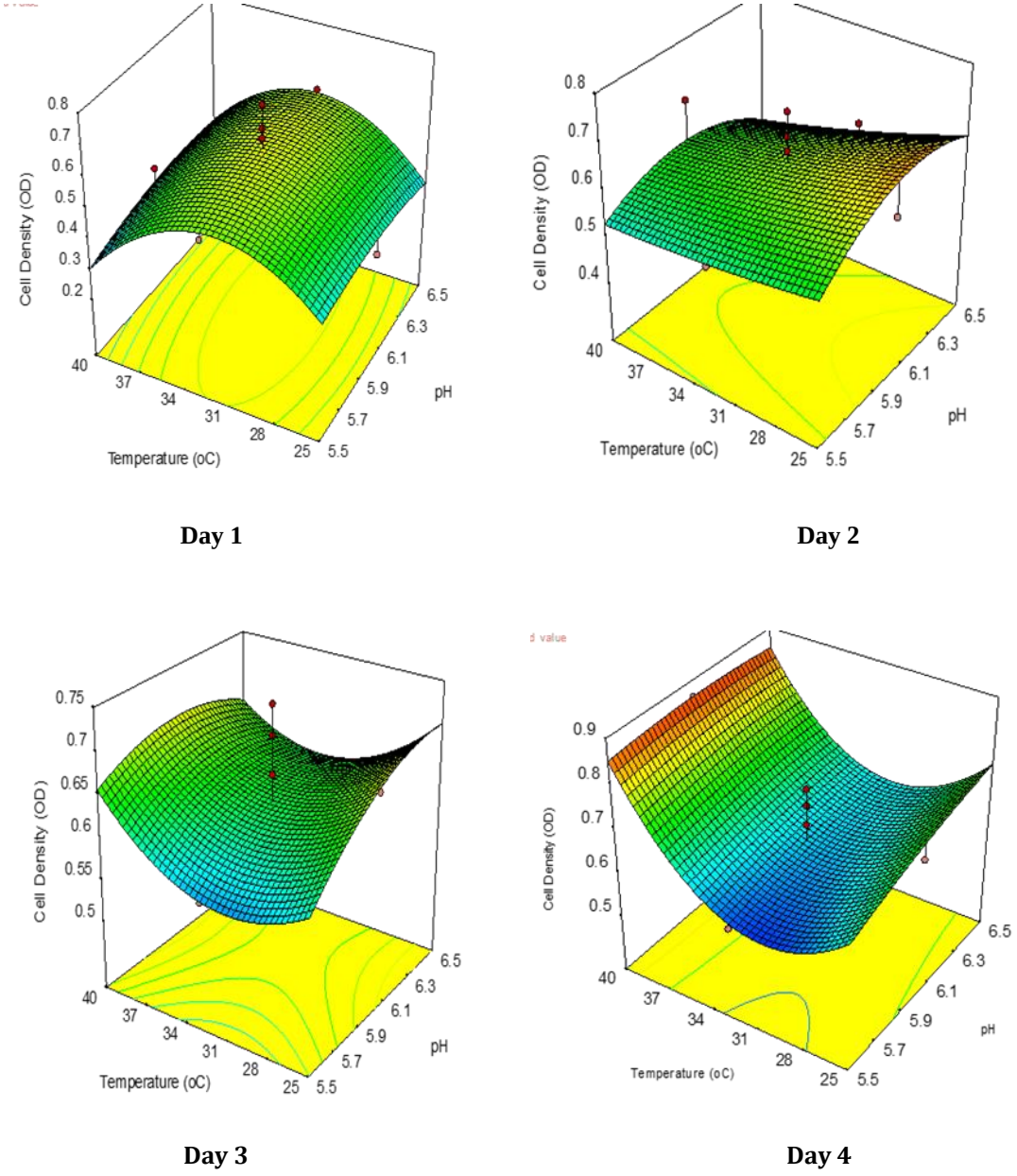

Figure 2 Response surface attributes of Cell density (OD) of *Cronobacter malonaticus* during Calabash fermentation for 4 days

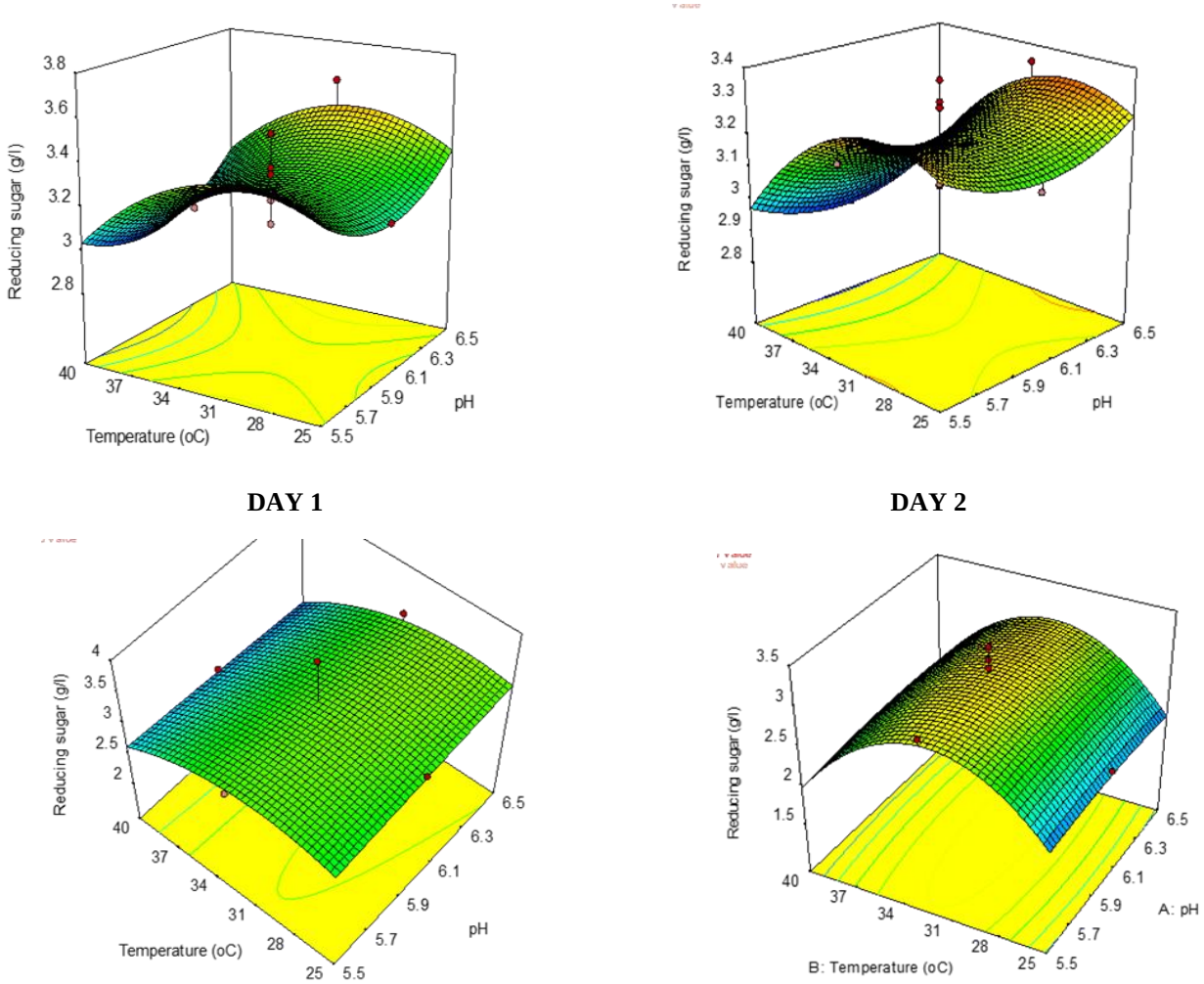

Figure 3 Response surface attributes of reducing sugar from Calabash using *Cronobacter malonaticus* for 4 days

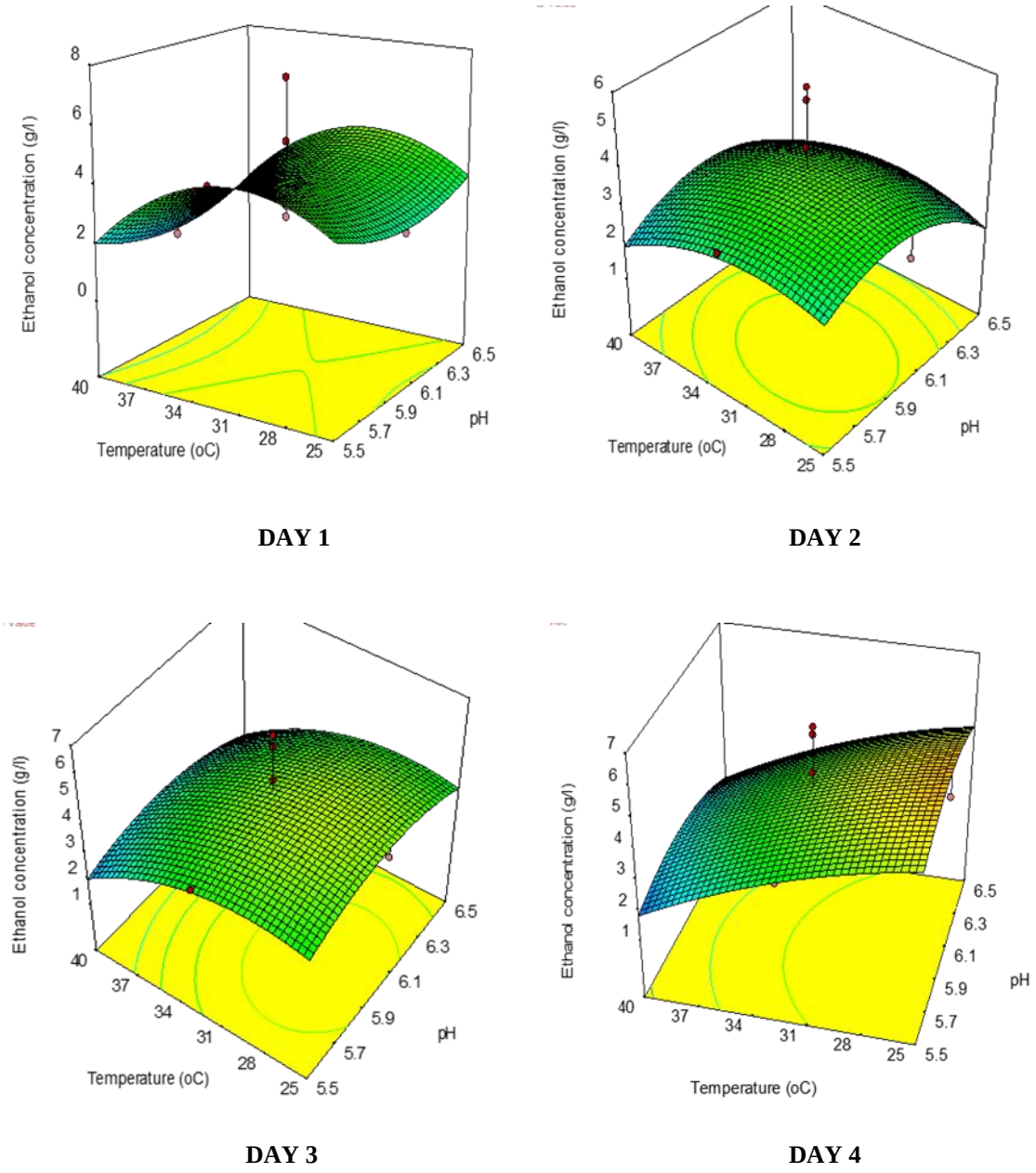

Figure 4 Response surface of ethanol concentration from Calabash using *Cronobacter malonaticus* for 4 days**.**

4. Discussion

The reducing sugar concentration determined from Calabash juice using glucose calibration curve was 3.22mg/ml. Free sugar containing juices have been adopted in bioethanol production because pretreatment is not required[14]. Initial sugar concentration is reported to have direct effect on fermentation rate and microbial cells .Inoculum size also affects sugar consumption rate and hence ethanol productivity [14; 15]. Carbohydrate content of Calabash pulp was 87.62%, and can be hydrolysed to small units (free sugar) for ethanol production [2].

Cronobacter malonaticus , the fermenting microorganism is a member of the *Enterobacter*iaceae family formerly in the genus *Enterobacter*.It was isolated from rotten orange and characterized. The use of this organism agrees with reports from[14] that most bacteria have been studied for bioethanol production from sugar containing juices. They are known to produce alcohols, aldehydes and other commodity products from glucose via the 2,3-butanediol fermentative pathway[10;16].

The average bioethanol concentration produced for the 4-day period from the 20 runs using the RSM model was 6.47% v/v. The optimization process also revealed maximum ethanol concentration of 5.08%v/v at 28^{o}C, pH 6.08 and 10% volume of inoculum.

However, Dates juices fermented at 30oC at natural pH gave maximum concentration of 25%v/v [14]. [17] Reported that fermented Citrus peel wastes gave 0.33%v/v ethanol. [18] Said that fermented corn cobs with co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* produces 10.08%v/v ethanol from 0.63mg/ml reducing sugars after a 7-day fermentation period.

Sugarcane juice without supplementation gave 11.0g/l ethanol, whereas addition of yeast extract, thiamine and micronutrients produced 39.4-42.1 g/l ethanol [14]. Optimisation of the process parameters help improve yield [19]. Production of ethanol from *Manihot glaziovi* using *S. cerevisiae*, after enzyme hydrolysis gave 0.45g ethanol/g reducing sugar, equivalent to 88.61% theoretical yield[20]. Response surface methodology revealed the optimal conditions for improved yield of bioethanol from Calabash substrate using *Cronobacter malonaticus* as follows: pH 5.95-6.5; temperature 28-32^{o}C and volume of inoculum 10%. This is in line with reports from [14].GC-FID analysis of distillates from *C.malonaticus* fermentation gave a concentration of 1.1 mg/l at 32.5^{o}C, greater than value from control sample. This was done to further authenticate the result obtained in the study. Saccharomyces cerevisiae when used on fruit rinds gave a value of 4.38g/l ethanol from GC analysis [1].

5. Conclusion

Cronobacter malonaticus was able to grow and convert Calabash juice to bioethanol. It was isolated from rotten orange, a plant material. The process parameters were optimized using the Response surface methodology and gave results to ascertain that the process can take place on a range of temperatures and pH, when the volume of inoculum is kept constant. The substrate and the inoculum were sourced locally from the environment making it a cheap process to help address our growing energy problems and aid environmental protection. More so, there was no pretreatment of the substrate, which is an expensive venture.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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