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Bioethanol Production from an Underutilized Plant, Calabash (*Crescentia Cujete*) Using Co-Culture of Saccharomyces Cerevisiae and Cronobacter Malonaticus

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Response surface methodology (RSM) model was used to optimize ethanol production from calabash (*Crescentia cujete*) pulp juice using co-culture of *Saccharomyces cerevisiae* and *Cronobacter malonaticus.* 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 were 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). Fermentation was done in conical flasks covered with cotton wool and foil in a stationary incubator for four days (96 hours). Active co-culture of *Saccharomyces cerevisiae* and *Cronobacter malonaticus* was used, with inoculum developed using

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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. 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; 29-31.6^{0}C . The optimal pH range for performance of the co-culture for the fermentation process was pH range 7.9-8.0. 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 6.97% v/v bioethanol at 29^{o}C and pH 7.9. The bioethanol yield from Calabash substrate is reasonable with co-culture considering the concentration of reducing sugars obtained from the juice and the duration of the fermentation.

Keywords: Calabash juice; fermentation; optimization; response surface methodology and bioethanol.

1. INTRODUCTION

Bioethanol has gained attention as a biofuel for the future. It is a renewable and sustainable energy source from biomass. Biorefining of biomass is a viable way of producing biofuels, biochemicals and bioenergy. Bioethanol is one of the liquid biofuels estimated to have accumulated US$80 billion in revenue in 2020 [1] and can significantly reduce CO_2 emissions. This outcome is expected to impact transportation, driving 27% of the sector's demand by 2050 [1]. Fossil or petroleum-based fuels contribute to the rise in carbon dioxide (CO_2) level in the atmosphere responsible for global warming [2]. Global bioenergy consumption is estimated to increase in future, and is expected to supply up to 30% of global energy in 2050 [1].The US, Brazil and China are the world's major producers of bioethanol, using first generation feedstock [2]. Bioethanol is usually blended with gasoline, and can be used in existing motor engine [3]. The available ethanol-gasoline blends are 'gasohol': 'E10' as 1:9 ethanol/ petrol blend, or 10% ethanol and 90% petrol [4]. The Kyoto Protocol of 1997, implemented in 20005 set bilateral agreements amongst nations on the use of biofuels in fighting the effects of global warming. There is a dare need for such agreements considering the dwindling fossil fuels and fluctuating crude oil prices. The global oil price is estimated at $60 per barrel by 2030 [5]. The European Union in 2009 published a directive to its members to incorporate 20% renewable energy into its final energy consumption by 2020.It also includes a binding target directive to its members to include a minimum of 10% share of renewable energy sources in transport. The US proposes yearly biofuel blended capacity for fuel distributors to 36 billion gallons (136 billion liters) by 2022.That are to blend 20% biofuels to gasoline for road transport by 2022.Japan set a target to achieve 5% biofuel addition to transport energy, by producing 6 billion liters per year by 2030 [6]. Nigerian biofuel policy adopts the blending of 10% bioethanol (E10) and 20% biodiesel (B20) into the energy mix and is expected to reach 2 billion liters by 2020 and beyond [7]. The target feedstocks for ethanol production in Nigeria are sugar cane, cassava and sweet sorghum. This would mean reserving 1 million hectares of land, representing 3% of the 34 million hectares under cultivation [8].

Bioethanol is ethanol produced from biological materials; different sugar sources and cellulosic substrates [9]. These substrates are fermented using suitable microorganisms to ethanol. The substrates for the production of first generation bioethanol were food crops; sugarcane, corn, wheat, rice and sorghum. There were significant increases in prices of these commodities which constitute more than half of the world's carbohydrate sources, hence the need for sourcing other substrates for ethanol production [1]. Second generation biofuels were produced from lignocellulosic biomass, mainly agro wastes made of cellulose, hemicellulose and lignin.The interactions of the lignocellulosic subunits are not easily hydrolysed by microbial enzymes [1]. Chemical pretreatment methods adopted to release fermentable sugars are expensive, and may remain in residual amounts in the hydrolysate, affecting the overall fermentation by formation of inhibitors such as furfural and hydroxymethyl furfural [10]. More so, the traditional ethanol production from distillation of palm wine has dropped due to low yield occasioned by inefficient cooling of the distillate, and non- availability of palm wine itself. The crave for white collar jobs and rural-urban migration has made the art of palm wine tapping defunct, and the research into the use of Calabash pulp juices for bioethanol production is timely.

Calabash, an underutilized plant was used in this study. It produces fruits all through the year. The

fruit is round, 12 to 14cm in diameter with a smooth hard shell. It takes six-seven months to ripen and ultimately falls to the ground once ripe [11]. The pulp has a carbohydrate content of 18.61% and mean values of 59.86%, 25.09% and 18.24% for sucrose, fructose and galactose respectively [12]. These fermentable sugars no doubt when utilized by appropriate microorganisms will yield several commodity products as their metabolic materials such as ethanol, methanol and acetic acid, etc [13]. The choice for the substrate in this study was formed on these assumptions.

The uses of co-cultures in fermentation are reported to have several advantages over single culture. *Saccharomyces cerevisiae* has been documented as effective fermentation yeast, producing ethanol from sugar substrates, both as a single inoculum or in a mixture with other yeasts, as well as bacteria [14]. The fermentation was done by co-culture of two microorganisms; *Saccharomyces cerevisiae* and *Cronobacter malonaticus*. The choice of these microorganisms was based on reports by Akponah et al. [15] and Piyapong et al. [13] *Saccharomyces cerevisiae* was isolated from palm wine, while *Cronobacter malonaticus* was isolated from spoilt orange. *Saccharomyces cerevisiae* have been previously isolated from palm wine [16,17]. Yeasts breakdown sugars anaerobically or aerobically using glycolytic, tricarboxylic acid and pentose pathways that differ only in the initial basic steps of metabolism [18]. *Cronobacter malonaticus* ferments sugars through the 2,3-butanediol pathway [19].

The design of the experiment was based on the response surface methodology (RSM) model. The fermentation period lasted for four days (96 hours). RSM model helps compare relationships between multiple variables at the same time [18]. The three-level-three-factor central composite design was used. This implies that the input/independent/control variables or the fermentation parameters (pH, temperature, volume of inoculum) together have effects on the output/dependent/ response variables (sugar concentration, cell density and ethanol yield). It is an optimization process aimed at increasing the yield of the desired product, bioethanol. It is expected that this study would give results that could form the basis for future production of bioethanol from this lesser used fruit, Calabash.

Co-cultures are reported to give improved ethanol yields from substrates than single microorganisms [20]. The use of several combinations of ethanologenic microorganisms should be explored in different organic substrates to improve bioethanol yield.

This study was aimed at producing reasonable amounts of bioethanol from the pulp juices of an underutilized plant, Calabash using a co-culture of *Saccharomyces cerevisiae* and *Cronobacter malonaticus*.

2. MATERIALS AND METHODS

2.1 Sample Preparation

The underutilized Calabash fruit was obtained from homes in the villages where the plant serves as hedges and provides shade. The gourd or shell had traditional applications in the storage of grains, art work, and music instruments, amongst other uses [12].

The juice was obtained by squeezing the pulp using a muslin cloth and was subjected to flash heat at 50^{o}C for 4 hours to concentrate it. The resulting liquid was vacuum filtered, for clarity with the help of Whatman No 1 filter paper of 12.5cm diameter. The clear juice was sterilized at 121^{o}C and 15 psi for 10 minutes and left to cool to about 45^{o}C before being inoculated with the desired microbial culture [18].

2.2 Isolation of the Microbial Strains

Saccharomyces cerevisiae, the yeast used in this study was source from a natural source, palm wine.The culture was done on standard solid medium comprising of 10g/l yeast extract, 20g/l peptone, 20g/l glucose, 15g/l agar and pH 6.8. Glucose was filter sterilized and added after autoclaving the other ingredients [4].

Cronobacter malonaticus was sourced from decayed orange. The growth medium used for isolation was Glucose agar medium. The compostion is as follows: 10g/l yeast extract, 20g/l glucose, 15g/l agar, 1g/l KH_2PO_4 , 1g/l $MgCl_2$,1g/l $(NH_4)_2SO_4$, and pH6.0 [4]. The Glucose was filter sterilized and added after other ingredients have been autoclaved so that it is not denatured.

2.3 Molecular Characterization of the Isolates, *Cronobacter malonaticus* and *Saccahromyces cerevisiae*

The extraction of DNA and sequencing of 16S rRNA was done to authenticate the use of these

isolates in the study. 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 [21,4].

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 X_1,X_2,X_3….Xn. This relationship can be represented by a polynomial model as follows:

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

Where $X=(x_1,x_2,\text{------}x_n)$, f'(x) is a vector function of β elements comprising of powers and cross

products of powers $x_1,x_2\text{-----}x_n$, 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 (Wand et al. 2013).

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:

a) Establish an approximate relationship between y and x_1,x_2 $\text{-----}x_n$ used to predict response values for given settings of the control variables
b) Determine optimum settings of $x_1,x_2\text{----}x_n$ that result in the maximum (or minimum) response over a certain region of interest.

In equation (1), Y is the response variable, which for this study is reducing sugar, ethanol concentration or cell density.

ε is the random experimental error

f '(X) β is the function of interactions of the independent/control/input variables:

Temperature, pH and volume of inoculum X_1,X_2 and X_3.

Considering all the variables, we have the next expression:

$$Y=\beta 1X1+ \beta_2X_2+ \beta_3X_3+ \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{23}X_2X_3+ \beta_{11}X^2_1+ \beta_{22}X^2_2+ \beta_{33}X^2_3+ \varepsilon\text{---}2.$$

ε= intercept term; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are interactive coefficients; β_{11}, β_{22} and β_{33} are quadratic coefficients; and X_1, X_2 and X_3 are the independent variables.

2.5 Fermentation of Calabash Pulp Juice

Experimental Design: Response surface methodology (RSM) and quadratic model were applied where a set of 23 replicates; including control was used at varying pH and volumes of inocula(Experiments,Days1-4) [22]. 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* and *Saccharomyces cerevisiae* inocula, according to the RSM model, and covered with cotton wool and aluminium 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 progress.

2.6 Determination of Reducing Sugars

The Dinitrosalicylic acid (DNS) method was adopted. A standard curve was generated using Standard glucose solution, where the concentration of the unknown sample was derived in mg/ml. A two milliliter (2ml) amount of the 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, and allowed to heat for 5 minutes. It was cooled and 7 ml distilled water was added. The absorbance then read at 540nm using blank as control [23].

2.7 Recovery and Determination of Ethanol Concentration

The ethanol from the fermentation broth was recovered using the simple distillation method. The broth was poured into a 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°C [24]. Determination of ethanol concentration was done by the potassium dichromate method. Ethanol calibration curve was determined using 20% absolute ethanol. Five milliliter (5ml) of the distillate was measured out and 2 ml of acidified potassium dichromate solution added. The solution was left to stand for color development. Absorbance was read at 588nm.The ethanol concentration of the distillate was derived from the calibration curve [24].

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 initially set to 40°C for 2minutes, then 180°C 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 (Upendra et al. 2013).

3. RESULTS

3.1 Identification of the Microbial Strain

The identification of the isolates was done with the help of their morphological and biochemical characteristics (Tables 1 and 2 respectively). The molecular characterization technique used was Gene sequencing was the molecular characterization method used and showed that the isolates have close evolutionary relationships to *Cronobacter malonaticus* and *Saccharomyces cerevisiae* (Fig.2) [21].

3.2 Concentration of Reducing Sugars

The concentration of reducing sugars was deduced from the calibration curve (Fig.1), with the mean absorbance value gotten as 1.240 at 540nm. The equation from the standard curve was given as:

$$y = 0.3955x - 0.0355$$

Hence, reducing sugar =3.22 mg/ml.

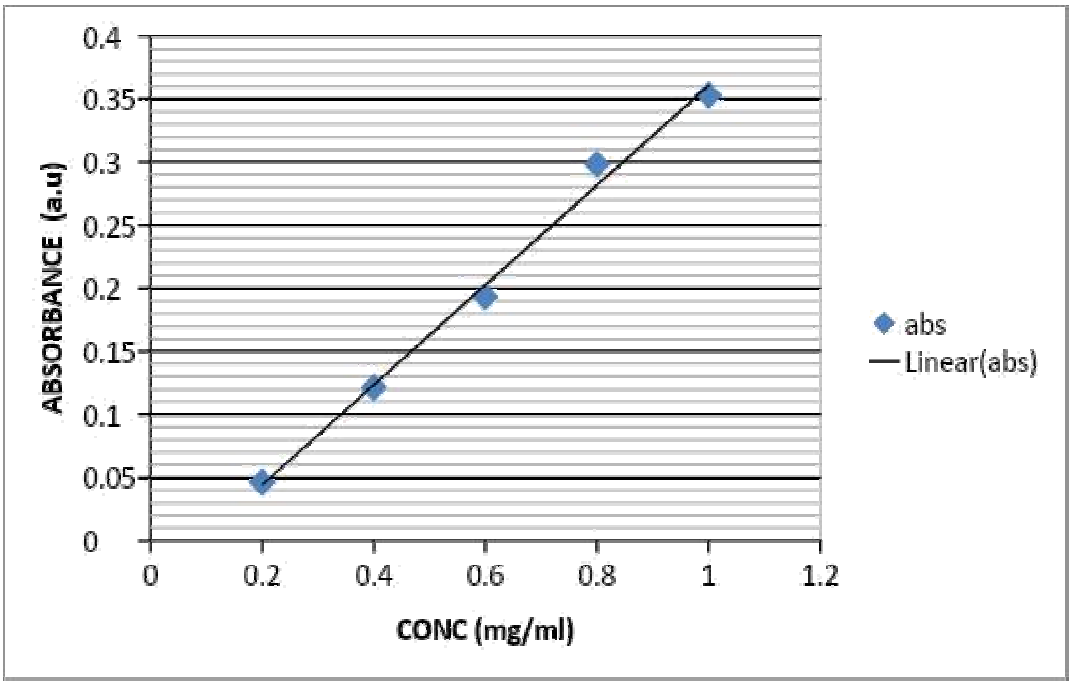


Fig. 1. Glucose calibration curve

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*

Table 2. Morphological and biochemical characteristics of isolate from palm wine

Test and 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

3.3 Determination of Ethanol Concentration

Ethanol concentrations were extrapolated from the calibration curve (Fig.2).

The equation was given as:

$$y = 0.0823x + 0.0352$$

3.4 Optimization of the Process Parameters using RSM

The optimization process yielded results as expressed in Tables 3 and 4, as well as Figs. 4-6. The volume of inoculum was kept constant at 10%. Optimal temperature of performance ranged from 28-32^{o}C, 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. 4). The reducing sugar levels decreased steadily from 3.5g/l on day 1 to 2.9g/l on day 4 (Fig. 5). Ethanol concentration of 5.08%v/v was recorded on day 3 (Fig 6), with a desirability value of 0.9 (Table 2).

Desirability values close to 1 depict higher probability of achieving optimal response (Table 3). 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 4). The R^2 values for reducing sugar were 0.7415, 0.7491, 0.7638 and 0.6567 (i.e 74.15%, 74.91%, 76.38% and 65.67%) respectively for the four days. This means that a greater percentage of the experimental data were relevant and only a few percentages of the total variations were not explained by the model. 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.

Experiments:

Day 1

Co-culture

Run	pH	Temp (^{o}C)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	8	40	8	1.536	0.650	7.130
2	3.5	40	3	1.300	1.123	5.891
3	8	25	8	1.716	0.711	7.142
4	5.75	32.5	5.5	1.729	0.799	6.109
5	3.5	32.5	5.5	1.735	1.282	6.194
6	5.75	32.5	5.5	1.747	0.908	8.430
7	5.75	32.5	3	1.738	0.900	6.753
8	5.75	32.5	5.5	1.729	0.880	6.255
9	8	25	3	1.759	0.847	7.033
10	3.5	40	8	1.647	1.049	5.040
11	5.75	25	5.5	1.766	1.363	5.818
12	5.75	32.5	5.5	1.726	0.837	5.866
13	8	40	3	1.500	0.807	7.944
14	5.75	32.5	5.5	1.716	0.847	6.158
15	5.75	32.5	8	1.730	1.179	6.619
16	5.75	40	5.5	1.576	0.807	6.887
17	5.75	32.5	5.5	1.765	0.804	7.932
18	3.5	25	3	1.774	1.318	6.182
19	8	32.5	5.5	1.710	0.673	7.288
20	3.5	25	8	1.775	1.201	5.295

Day 2

Co-culture

Run	pH	Temp (^{o}C)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	8	40	8	1.710	0.895	7.932
2	3.5	40	3	1.739	1.348	7.324
3	8	25	8	1.706	0.837	8.236
4	5.75	32.5	5.5	1.694	1.075	6.845
5	3.5	32.5	5.5	1.694	1.457	6.741
6	5.75	32.5	5.5	1.697	1.007	8.576
7	5.75	32.5	3	1.692	0.936	6.838
8	5.75	32.5	5.5	1.688	0.908	6.814
9	8	25	3	1.792	1.309	8.843
10	3.5	40	8	1.701	1.436	6.996
11	5.75	25	5.5	1.710	1.414	7.057
12	5.75	32.5	5.5	1.686	0.943	6.656
13	8	40	3	1.719	1.146	8.126
14	5.75	32.5	5.5	1.682	1.007	6.510
15	5.75	32.5	8	1.701	1.492	6.802
16	5.75	40	5.5	1.712	1.069	7.519
17	5.75	32.5	5.5	1.670	1.115	7.944
18	3.5	25	3	1.713	1.505	7.337
19	8	32.5	5.5	1.719	0.863	7.434
20	3.5	25	8	1.746	1.482	7.094

Day 3

Co-culture

Run	pH	Temp (^{o}C)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration (%v/v)
1	8	40	8	1.831	0.951	9.001
2	3.5	40	3	1.856	1.049	8.297
3	8	25	8	1.804	0.584	11.055
4	5.75	32.5	5.5	1.830	0.845	7.203
5	3.5	32.5	5.5	1.835	1.065	6.741
6	5.75	32.5	5.5	1.820	0.696	8.965
7	5.75	32.5	3	1.834	0.797	8.467
8	5.75	32.5	5.5	1.825	0.622	7.640
9	8	25	3	1.873	0.574	14.688
10	3.5	40	8	1.828	1.128	8.418
11	5.75	25	5.5	1.850	0.954	7.300
12	5.75	32.5	5.5	1.814	0.597	7.324
13	8	40	3	1.820	0.825	9.560
14	5.75	32.5	5.5	1.811	0.615	7.470
15	5.75	32.5	8	1.811	0.721	7.749
16	5.75	40	5.5	1.870	0.911	9.269
17	5.75	32.5	5.5	1.858	1.133	8.357
18	3.5	25	3	1.840	1.034	7.081
19	8	32.5	5.5	1.801	0.782	8.114
20	3.5	25	8	1.926	1.105	6.972

Day 4

Co-culture

Run	pH	Temp (^{o}C)	Vol.(ml)	Cell Density (OD)	Reducing sugar (g/l)	Ethanol concentration(%v/v)
1	8	40	8	1.841	0.963	8.467
2	3.5	40	3	1.857	1.019	8.224
3	8	25	8	1.899	0.471	11.273
4	5.75	32.5	5.5	1.812	0.746	5.903
5	3.5	32.5	5.5	1.816	1.105	5.563
6	5.75	32.5	5.5	1.804	0.875	6.729
7	5.75	32.5	3	1.816	0.756	6.037
8	5.75	32.5	5.5	1.807	0.804	5.344
9	8	25	3	1.884	0.529	17.337
10	3.5	40	8	1.845	0.976	8.187
11	5.75	25	5.5	1.894	0.948	8.467
12	5.75	32.5	5.5	1.809	0.746	5.319
13	8	40	3	1.824	0.849	9.196
14	5.75	32.5	5.5	1.793	0.706	5.623
15	5.75	32.5	8	1.795	0.678	5.829
16	5.75	40	5.5	1.849	1.024	8.102
17	5.75	32.5	5.5	1.814	0.619	6.559
18	3.5	25	3	1.908	0.873	7.543
19	8	32.5	5.5	1.809	0.670	5.806
20	3.5	25	8	1.898	1.069	7.349

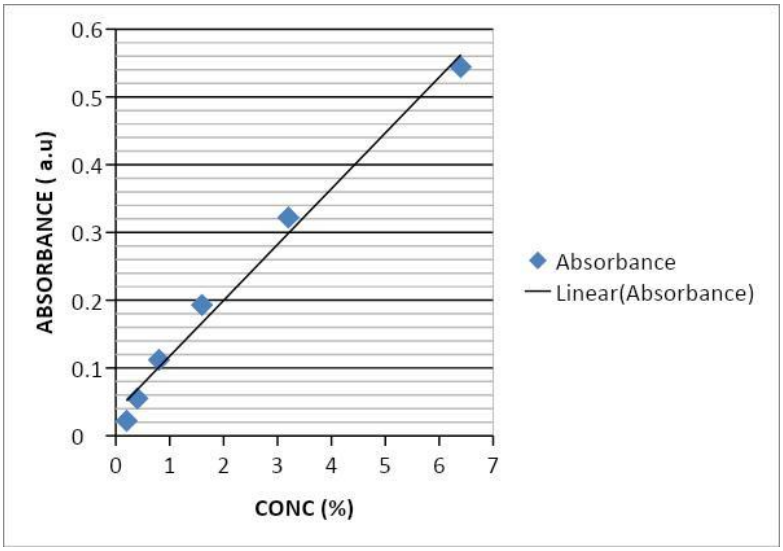


Fig . 2. Ethanol calibration curve

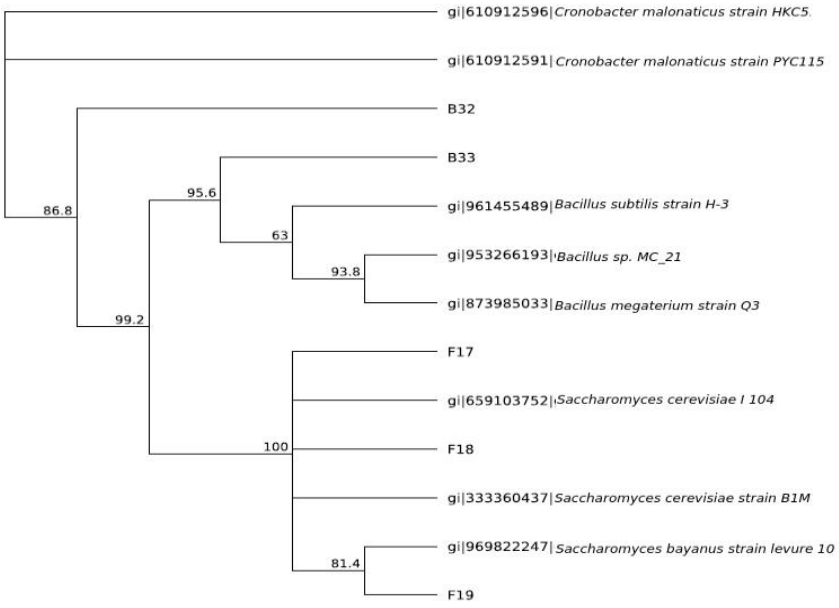


Fig. 3. Evolutionary relationships amongst the bacterial and yeast isolates

Table 3. Co-culture – Optimized variables for Calabash pulp juice

Time	pH	Temp. (^{o}C)	Volume (ml)	Cell density (OD)	Reducing sugar (g/l)	Ethanol conc. (%v/v)	Desirability (d_i)
Day 1	8.0	31.03	5.5	0.7661	3.4560	6.3121	0.860 (86.0%)
Day 2	8.0	29.10	5.5	0.7906	3.3179	5.2214	0.806 (80.6%)
Day 3	7.9	29.00	5.5	0.8237	3.1950	6.9781	1.00 (100.0%)
Day 4	8.0	31.60	5.5	0.7952	2.9855	5.9152	0.773 (77.3%)

Table 4. Goodness of fit (R^2) for co-culture for calabash pulp

Fermentation Period	Model	Cell density	Reducing sugar	Ethanol concentration
Day 1	Quadratic	0.8420	0.7415	0.7619
Day 2	Quadratic	0.8239	0.7491	0.6817
Day 3	Quadratic	0.7163	0.7638	0.8408
Day 4	Quadratic	0.7420	0.6567	0.8670

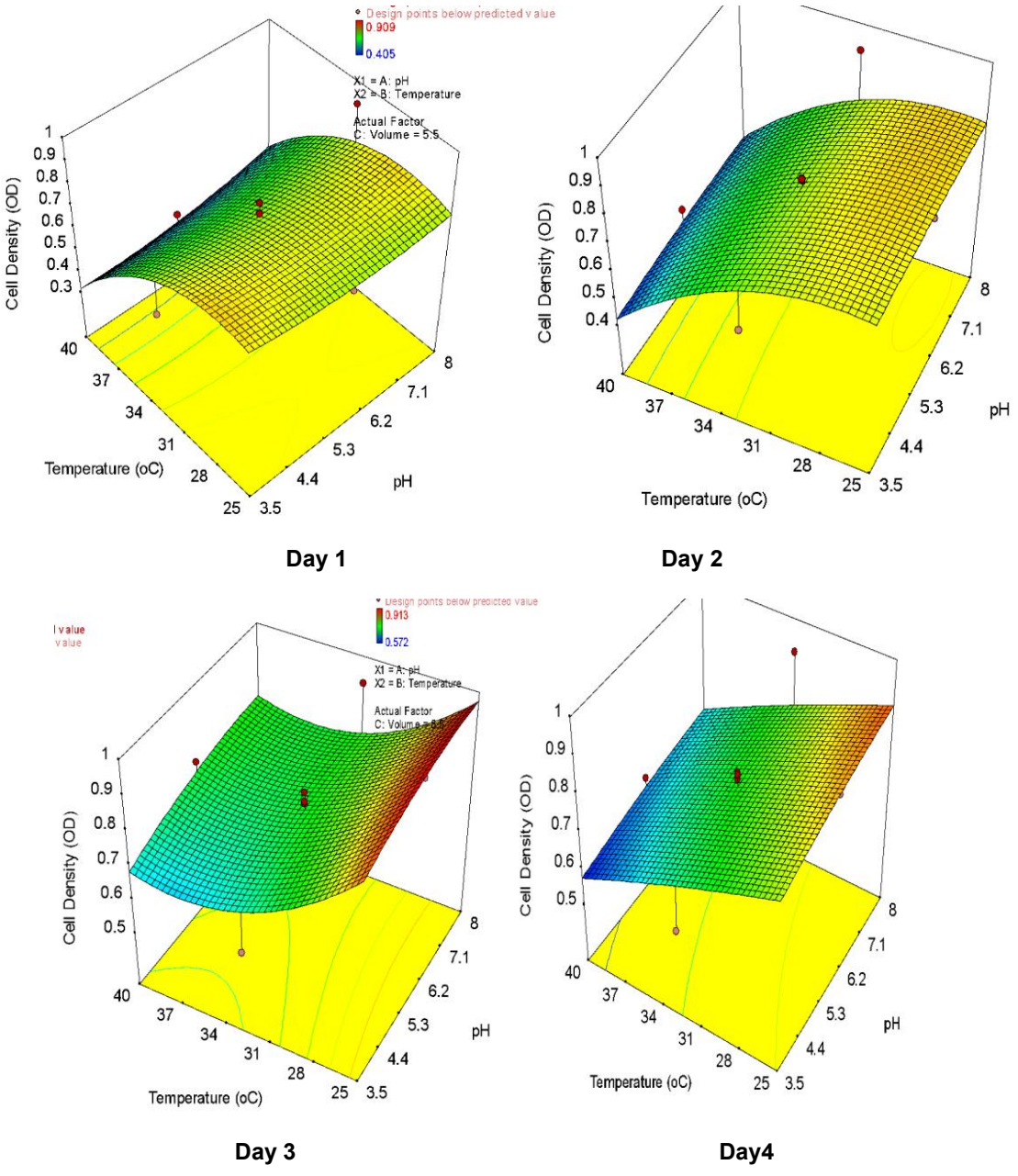


Fig. 4. Response surface of cell density (OD) for co-culture of calabash for 4 days

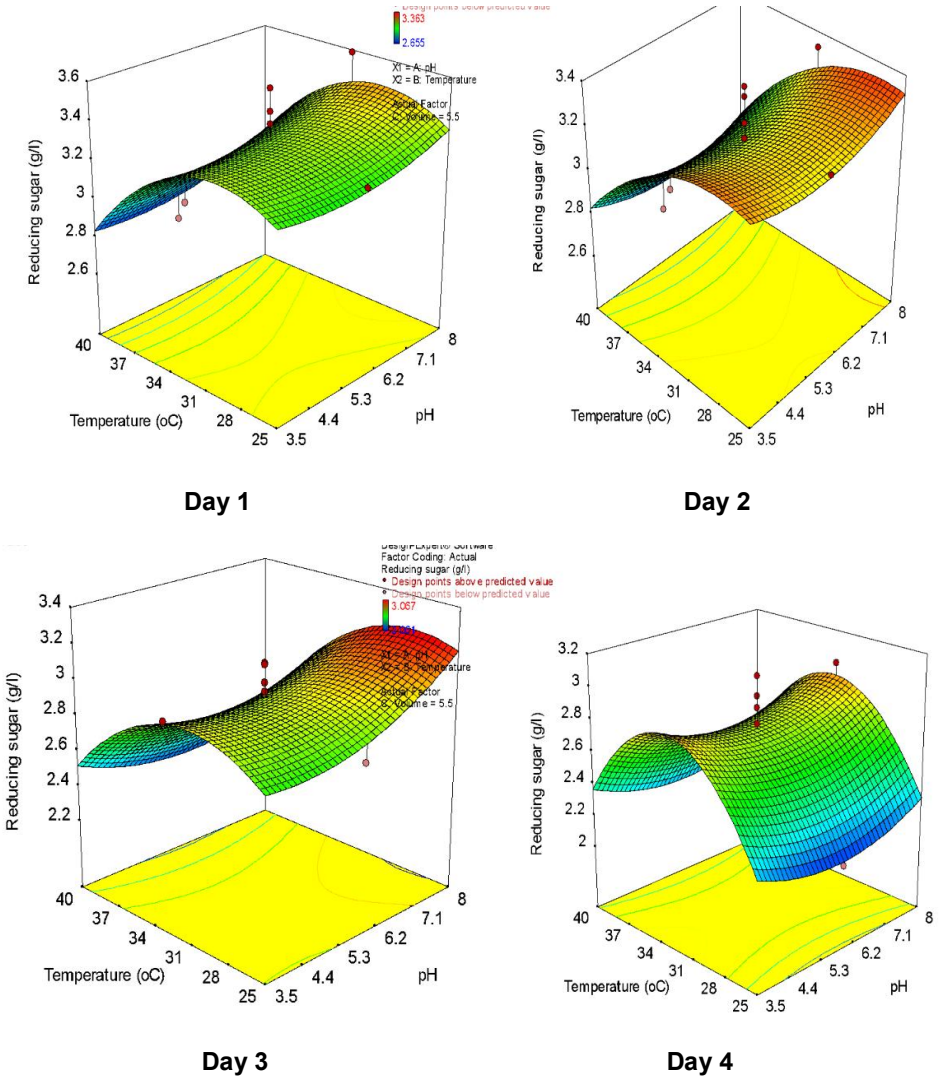
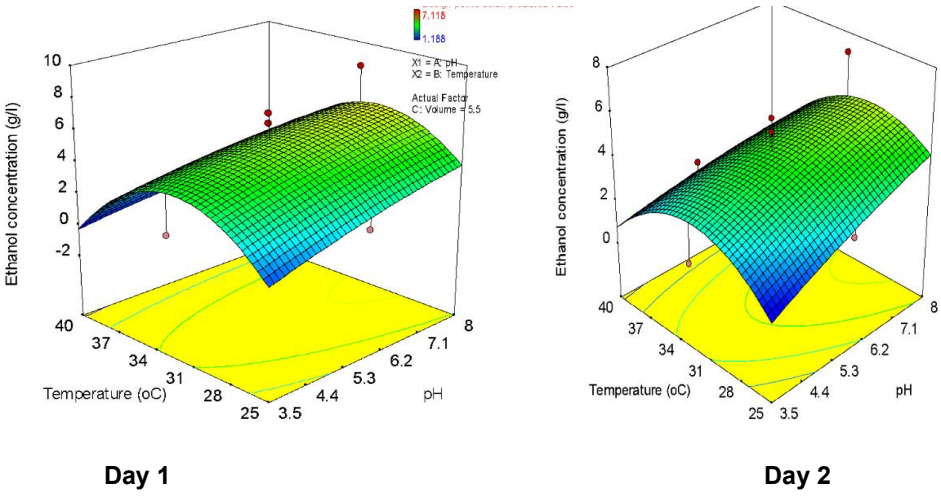


Fig. 5. Response surface attributes of reducing sugar for Co- culture of calabash for 4 days



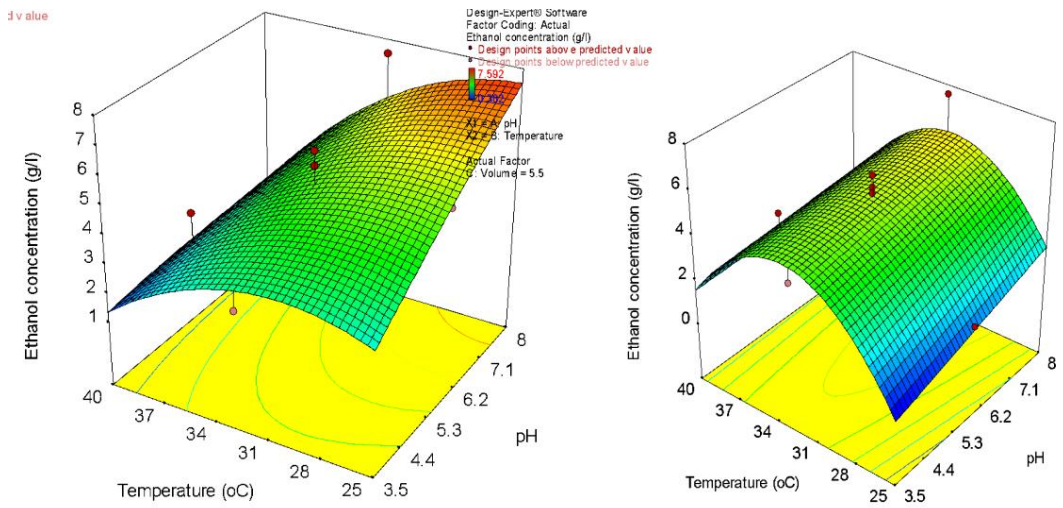


Fig. 6. Response surface attributes of Ethanol concentration (v/v %) for co-culture of calabash for 4 days

4. DISCUSSION

Mixed culture fermentation of Calabash juice was carried out using *S. cerevisiae* and *C.malonaticus* in the ratio of 1:1 inoculum size. The use of mixed cultures in fermentation have been reported to shorten the fermentation time, reduce fermentation losses and increase yields [14].They also enable the utilization of cheap and impure substrates, provide remarkable stable association, as well as complement each other and work for the exclusion of unwanted microorganisms. This is also seen in yogurt production with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [25]. Rahmadhani et al. [26] reported better sugar consumption rate at 1:1 inoculum ratio,as well as no antagonist interactions of co-culture of ethanologenic yeasts. In corroboration to these reports, Calabash pulp juice used as the substrate in this study is cheap and sourced from the environment where it does not compete with the food supply chain. Mixed cultures also have another characteristic of being able to ferment substrates containing several different sugars [27]. Ejelonu et al. [12] reported that Calabash juice contains sugars such as sucrose, fructose and galactose.

The reducing sugar concentration of Calabash juice determined by DNS method was 3.22mg/ml. This result is lower than reports from Girisha et al. (2014) that reducing sugar contents of *Citrus sinensis*, *Citrus limetta* and *Ananas comosus* were 17mg/ml, 21mg/ml and 20mg/ml respectively. Calabash juice however has reducing sugar content higher than 1.24mg/ml gotten from sugar beet pulp [9] and 0.63 mg/ml reported by Itelima et al. (2013). It is however in line with reports from Ayele [28] with glucose concentrations ranging from

1.3-6.3g/l. The choice of the substrate in this study is in line with these reports; and further justifications based on facts that reducing sugars have been easily metabolized by several genera of microorganisms to industrial products such as biofuels [29].

Optimal ethanol concentration of 6.97% v/v was realized at 29^{o}C, pH 7.9 and 5.5% volume of inoculum after 4- days' fermentation period. This concentration is higher than 6.19% v/v (at pH 5.45 and 32.5^{o}C) and 5.08%v/v (at pH 6.08 and 28^{o}C) recorded respectively using *S.cerevisiae* and *C. malonaticus* alone on Calabash juice in our reports [4,30]. Optimum temperature and pH values are necessary for microbial growth and ethanol production [22]. Low or high pH values are known to cause chemical stress on yeast cells and affect their growth [22,8]. Also as temperature and time increases from the optimal value, the yield of ethanol decreases [8]. There was an agreement with this work as is seen in our report from day 3 to day 4 (Table 3), where there was increase in temperature from 29^{o}C to 31.6^{o}C; with the corresponding decrease in ethanol concentration to 5.915% v/v .More so, the result from our study is higher than 0.33%v/v (0.59g/g ethanol/reducing sugar) from co-culture

of *Zymomonas mobilis* and *Pichia* stipitis reported by Dewi et al. [31]. An earlier work by Chen [32], reported a yield of 0.49-0.50 g ethanol/g substrate using *Z.mobilis* and *P.stipitis* in a co-culture. These values are however; lower than 10.08% v/v reported from co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* using 0.63mg/ml reducing sugar in a fermentation that lasted for 7-days [33]. Also, Sopandi and Wardah [34] reported 8.52% ethanol using co-culture of *S.cerevisiae* and *Candida tropicalis*.

The range of optimal conditions at which improved yield of bioethanol could be produced from Calabash as revealed by RSM using co-culture of *S.cerevisiae* and *C.malonaticus* are pH 7.9-8.0; 5.55% volume of inoculum and temperature 29-31.6^{o}C (Table 3). The process parameters reported from this study agree with those of Hossain et al. (2014).

The Gas Chromatography-flame-ionization detector (GC-FID) analysis of the distillates from the fermentation broth gave concentrations of ethanol as 1.13mg/l, 1.21 mg/l and 1.37 mg/l respectively from fermentation temperatures of 32.5^{o}C, 40^{o}C, and 25^{o}C. Ire et al. [35] reported 19.08g/l ethanol concentration from GC-MS, amongst other products, from co-culture using *Bacillus cereus* and *Bacillus thuringiensis*.

5. CONCLUSION

The use of co-culture in fermentation of calabash juice in this report justified the aim of this research, which was to produce ethanol using co-culture of *S.cerevisiae* and *C.malonaticus* as there was yield greater than when the organisms were used singularly. It is a viable venture having used a renewable substrate, as well as indigenous fermenting microorganisms. More research into similar courses would help in the quest for renewable and alternative energy sources to help alleviate the crisis in the energy sector.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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