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OPTIMIZATION OF THE TWO-STAGE FERMENTATION PROCESS FOR *Antidesma bunius* (L.) Spreng. FRUIT TOWARD THE DEVELOPMENT OF PHENOLIC-RICH BEVERAGE

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ABSTRACT

Fermentation is a well-established food processing technique that has been used to improve the shelf life, organoleptic and nutritional properties of food products such as fruits and berries. In this study, the two-stage fermentation of the 'bignay' fruit [*Antidesma bunius* (L.) Spreng.], involving alcoholic and acetic acid fermentation, has been optimized through the aid of response surface methodology to produce a phenolic-rich fermented 'bignay' fruit functional beverage. The factors used for the process optimization of the two-stage fermentation of bignay were the duration of the acetic acid fermentation and the amount of fruit puree, while the response variables used were the total phenolic (TPC) and total flavonoid content (TFC). The resulting optimization process suggested a quadratic model design for both total phenolic and total flavonoid content. Furthermore, verification of the model, resulted in optimized conditions of 3.5 days of acetic acid fermentation using 75% fruit must. This optimized process was able to yield a maximum of 251.90 ± 0.30 GAE mg g^{-1} (TPC) and 249.01± 0.41 QE mg g^{-1} (TFC). The fermented 'bignay' juice using the optimized fermentation process had a significant increase in its TPC and TFC when compared to its unfermented counterpart. The optimization process of the two-stage fermentation can be applied for the development of phenolic-rich bignay beverages.

Keywords: Two-stage fermentation, phenolics, flavonoids, optimization, *Antidesma bunius* (L.) Spreng

INTRODUCTION

Fermentation is a food processing technique that has been adopted for centuries around the world, especially in developing nations. It involves an intentional conversion or modification of a substrate through activities of microorganisms to get a desired product. This is usually completed through microbial actions, which positively alter the appearance, flavor, functionalities, nutritional composition, color, and texture **(Adebo *et al.*, 2020)**. Fermentation has been reported to increase the bio-conversion of phenolic compounds from their linked or conjugated forms to their free ones, resulting in an increased concentration of phenolic components with greater antioxidant power **(Acosta-Estrada *et al.*, 2014)**.
In the two-stage fermentation method through alcoholic followed by acetic acid fermentation, recent studies have demonstrated that some of the phenolic compounds such as flavonoids were produced and enhanced **(Hata *et al.*, 2023; Xu *et al.*, 2022).** Among many known fermentation techniques, acetic acid fermentation is one of the most commonly used food processing techniques, in-sequence with ethanol fermentation, to prolong the shelf-life of starchy and sugary raw material and produce a vinegar-based product. This type of fermentation is an aerobic process that starts out after ethanol fermentation to which ethanol is oxidized to produce acetic acid. The fermentative process occurs in the presence of a microorganism commonly called as acetic acid bacteria (AAB). Aside from the production of acetic acid, noticeable production of other metabolites, such as phenols and other organic acids can be observed during and or after the fermentation process **(Zou *et al.*, 2017; Fonseca *et al.*, 2018; Chen *et al.*, 2020)**.
In the Philippines, acetic acid fermentation is only used to produce the common condiment-style vinegar, where the fermentation process is carried out naturally using the inherent microflora of the ingredients or by adding a small amount of unpasteurized vinegar from previously made vinegar. The usual raw material used in making vinegar are coconut or 'nipa' sap.
'Bignay' [*Antidesma bunius* (L.) Spreng.] is an indigenous fruit commonly found in the Philippines and other parts of Southeast Asia. This fruit is mostly known for its phenolic compounds such as organic acids and flavonoids **(Butkhup and Samappito, 2008).** Many have studied its phenolic compound contents which are most known for their antioxidant properties **(Belina-Aldemita *et al.*, 2013, Lheman *et al.*, 2021, Leyeza *et al.*, 2025).** The usual processing of 'bignay' in the Philippines is through alcohol fermentation to produce wine because of its high economic value. The nutritive value of wine is also increased due to the release of amino acids and other nutrients during fermentation **(Swami *et al*, 2014).** To date, there have been no reports of 'bignay' being processed into vinegar-based drinks or similar products which are shown to have anti-infective properties, antitumor activity, anti-glycemic effect, and can lower the risk for cardiovascular diseases **(Johnston and Gaas, 2006; Budak *et al.*, 2014)**. Thus, this study aims to provide a more controlled fermentation process by optimizing the two-stage fermentation of 'bignay', in order to develop a phenolic-rich fermented product which can be used for the production of a functional beverage.

MATERIAL AND METHODS

Preparation of 'bignay' fruits

'Bignay' fruits, at the fully matured stage, were harvested in Los Baños, Laguna, Philippines. The fruits were cleaned, sorted, deseeded and mashed before homogenizing with distilled water using an a commercial blender (Osterizer). Afterwards, its total soluble solids and pH were adjusted to 20°Brix and 4.5, respectively.

Microorganisms used for fermentation

One percent (v/v) of pure cultures of 10^6 CFU ml^{-1} of *Saccharomyces cerevisiae* BIOTECH 2055 and 10^6 CFU ml^{-1} of the *Acetobacter aceti* ATCC 159973 were used for the alcoholic followed by acetic acid fermentation, respectively. Potato dextrose agar (PDA) or nutrient broth (NB) (Biokar Diagnostics, France) and acetobacter broth mannitol agar/broth (ABM agar/broth) (Sigma-Aldrich, Germany) were used as the general growth medium, respectively. Estimation of the total CFU of the microorganisms for inoculation was done through the OD_{600} spectrophotometric method.

Optimization of two-stage fermentation process

The optimization process was done using Response Surface Methodology in a central composite design for two factorial experiment. The optimization parameters were based on the method of **Jung and Woo (2016)** for grape wine-vinegar processing. After screening for the optimizable conditions, the variables considered were the amount of fruit puree and duration of acetic acid fermentation. The incubation for both alcoholic and acetic acid fermentation processes were at 30±3°C. The response parameters were the maximum total phenolic (TPC) and total flavonoid content (TFC). The optimization and validation process was done

using Minitab version 18. The entire optimization fermentation process can also be seen in Figure 1.

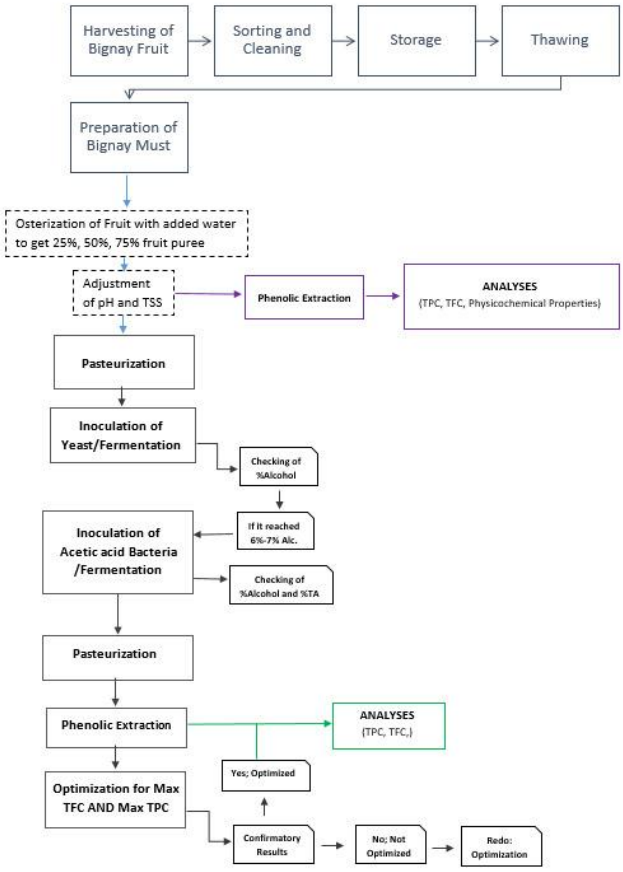

Figure 1 Schematic diagram for the optimization of 'bignay' juice fermentation process and analysis of physicochemical and biological properties.

Extraction of phenolic compounds

The extraction of phenolic compounds was done based on the method of **de Souza *et al.* (2014)** with some modifications. The sample was mixed with a solution of methanol: distilled water: glacial acetic acid with the corresponding ratio of 50:50:1. After which, the solution was agitated for 1 hour at room temperature. Then, the samples were centrifuged for 15 mins. The supernatant was collected and stored in an amber glass bottle and placed inside a refrigerator at 4°C until analysis.

Analysis of the physicochemical properties of fermented 'bignay' juice

The alcohol content during the alcoholic and acetic acid fermentation of the 'bignay' fruit, was measured adapting the method of **Aldemita *et al.* (2013)**. Forty milliliters of sample and 20 mL of distilled water were placed in a round bottom flask. Distillation was done until 30 mL of distillate was collected in a 50-mL graduated cylinder. The distillate was made up to a volume of 40 mL using distilled water. The solution was cooled to 20°C. Percent alcohol was read directly using a hydrometer (Brannan, RS Philippines). The pH measurements were made using a digital pH meter (Ohaus Aquasearcher AB33PH-8). The total soluble solids of the sample was also determined by using a hand-held refractometer (Optika HR-130N) and was expressed as °Brix. In determining the total titratable acidity of the 'bignay' juice, 5 mL of the sample were titrated with 0.1N NaOH until pH of 8.2 was reached. The following formula was used:

TA (%)= ((0.1N NaOH x vol.of NaOH used x 60.052))/(weight of sample) x 100

where, 60.052g/mol = molar mass of acetic acid

Total phenolic content analysis

The total phenolic contents of the fermented bignay extract were estimated using a modified version of the Folin-Ciocalteau colorimetric method as used by **Sartagoda *et al.* (2021)**. In a 96-well microplate, 10 uL of sample extracts was combined with 90 uL of triple distilled water. Then, 10 uL of 10% Folin–Ciocalteau (Sigma-Aldrich, Singapore) reagent was added. After incubating for 5 mins at room temperature, 100 uL of 7% (m/v) Na_2CO_3 (Sigma-Aldrich, Singapore) and 40 uL triple distilled water was added and the solution was allowed to stand at room temperature in the dark for two hours. The absorbance was read at 630 nm using a Biobase microplate reader. The total phenolic contents of the samples were calculated based on the standard curve of gallic acid (Sigma-Aldrich, Germany). Results were expressed as milligrams of gallic acid equivalent (GAE) per gram of sample.

Total flavonoid content analysis

The total flavonoid content of the bignay extract was measured using a modified colorimetric method by **Li *et al.* (2013)** and **Sartagoda *et al.* (2021)**. In a 1.5 ml Eppendorf tube, 10 uL of the sample extract were mixed with 400 uL triple distilled water and 30 uL of 5% $NaNO_2$ (Sigma-Aldrich, Singapore). After 5 mins, 30 uL of 10% $AlCl_3 \cdot 6H_2O$ (Sigma-Aldrich, Singapore) solution was added and allowed to stand for 6 minutes. Then, 200 uL of 1 M NaOH was added to the mixture and immediately diluted by adding 330 uL of triple distilled water. The absorbance of the sample solution was measured at 510 nm using a microplate reader. The total flavonoid content were determined from the standard curve of quercetin (Sigma-Aldrich, Germany) and expressed as milligrams of quercetin equivalents (QE) per gram of sample.

Statistical Analysis

All analyses were performed in triplicates and results were expressed as mean ± standard deviation. T-test was performed to determine significant difference between treatment means for the total phytochemical contents at $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of two-stage fermentation

The Central Composite design (CCD) is based on a two-level factorial design with the addition of 2k where k is the number of independent variables, star points between the axes and repeated points at the centroid. In this study, XA is reported as % puree and XB is the acetic acid fermentation time, with total phenolic content (TPC) and total flavonoid content (TFC) as response variables Y1 and Y2, respectively. Table 1 shows the combination of variables XA and XB using CCD and their corresponding response variables.

Table 1 Two-factor central composite design: independent (Xn) and response (Yn) for the optimization of acetic acid fermentation of 'bignay' juice.

RUN ORDER	% FRUIT PUREE (A)	ACETIC ACID FERMENTATION (B)	Total Phenolic Content (mg GAE g^{-1} sample)	Total Flavonoid Content (mg Quercetin g^{-1} sample)
1	50.0000	12.0000	153.412 ± 0.05	100.995 ± 0.05
2	50.0000	20.4853	127.322± 0.01	51.775 ± 0.01
3	50.0000	3.5147	230.024± 0.01	186.940 ± 0.03
4	50.0000	12.0000	154.188± 0.05	100.540 ± 0.15
5	25.0000	18.0000	165.468± 0.01	49.768 ± 0.32
6	50.0000	12.0000	152.337± 0.02	98.477 ± 0.11
7	25.0000	6.0000	223.064± 0.06	131.284 ± 0.95
8	75.0000	18.0000	171.544± 0.03	115.481 ± 0.44
9	50.0000	12.0000	156.043± 0.01	90.587 ± 0.10
10	14.6447	12.0000	153.977± 0.01	47.590 ± 0.18
11	85.3553	12.0000	190.266± 0.04	135.168 ± 0.11
12	75.0000	6.0000	233.190± 0.01	199.435 ± 0.42
13	50.0000	12.0000	164.238± 0.01	92.379 ± 0.04

Response surface models for total phenolic and total flavonoid content

The model used for TPC and TFC was quadratic. This model was selected among linear, two-factor interaction, and quadratic. Table 2 displays the analysis of variance of the surface quadratic model for response parameters TPC and TFC, respectively. The model F-values of 16.00 and 41.30 for TPC and TFC, respectively, imply that the model is significant. The "lack of fit" F-values of 21.05 and 11.39 for TPC and TFC, imply that the lack-of-fit is not significant relative to the pure error. "Lack of fit" measures error caused by a deficiency in the model such as interaction terms or quadratic terms. Meanwhile, pure error reflects the variability of responses within each treatment. The p-value of lack of fit is 0.07 and 0.06 for TPC and TFC, respectively, which indicates that there is only a 7.00% and 6.00% chance that a "lack of fit" F-value this large could occur due to noise. A non-significant lack of fit is desirable since it indicates a good fit for the model.

Table 2 Summary of the analysis of variance of the quadratic response surface model for total phenolic and total flavonoid content at $p<0.05$.

SOURCE OF VARIATION	TOTAL PHENOLIC CONTENT					TOTAL FLAVONOID CONTENT				
	DF	Adj SS	Adj MS	F-value	P-value Prob > F	DF	Adj SS	Adj MS	F-value	P-value Prob > F
Model	5	176	352	16.00	0.00	5	25	51	41.30	0.000
Error	7	154	220			7	88	125		
Lack-of-Fit	3	145	483	21.05	0.07	3	788	262.9	11.39	0.06
Pure Error	4	918	229			4	92.3	23.1		
R-square			0.9195					0.9672		
Adjusted R-square			0.8620					0.9438		

The resulting R-square values for the TPC and TFC response surface models are 0.9195 and 0.9672, respectively. This means the models can explain 91.95% and 96.72% of the response value changes for their respective response surface models. The predicted R-square of 0.9195 for the TPC response surface model is in reasonable agreement with its adjusted R-square of 0.8620, because the difference is less than 0.2. It is also the same with the TFC response surface model, where the predicted R-square of 0.9672 is in reasonable agreement with the adjusted R-square of 0.9438, because the difference is also less than 0.2.

The following response surface models were fitted to the response variable (TPC and TFC) with two independent variables (XA and XB) which generated the following equations:

The model's equation in terms of coded factors for TPC and TFC:

TPC = + 24368 + 3042A – 12404B + 4788 A2 + 7091 B2 - 643 AB

TFC = +96.6 + 32.21A -44.58 B + 2.05A2 + 16.04 B2- 0.61 AB

where: A = Fruit Puree; and B = Duration of Acetic Acid Fermentation

Graphical explanation of the response model

The relationship between the independent variables and response parameters can be illustrated as graphs such as surface plots and contour plots. The resulting 3D response surface plot and 2D contour plot models which were generated from the optimization process are shown in Figure 4. It illustrates the interaction between the amount of TPC and TFC that can be generated given a set amount of fruit puree and duration of acetic acid fermentation in days.

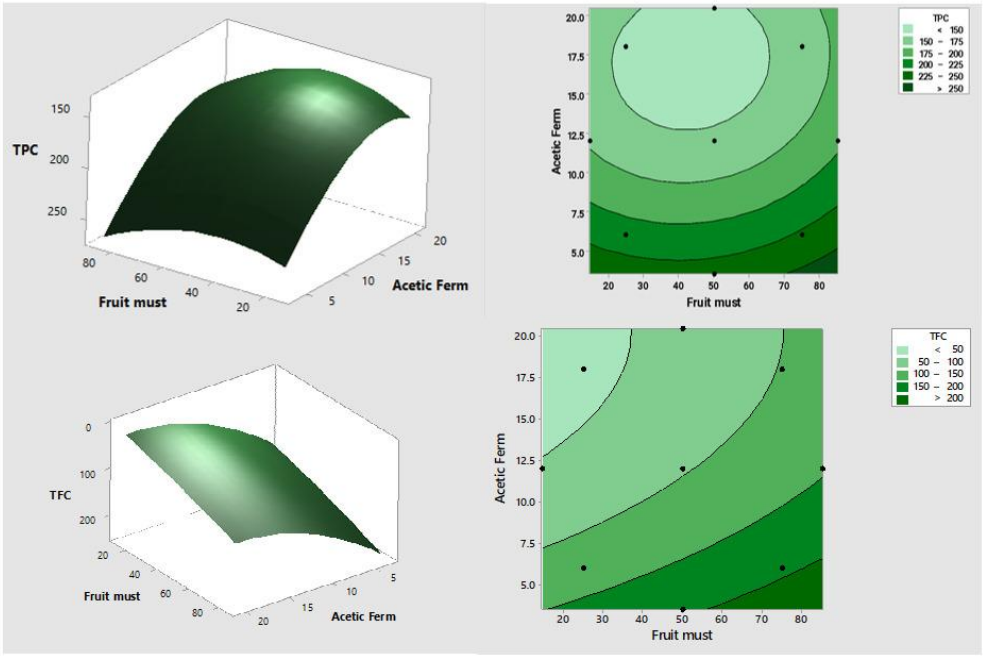

Figure 2 Response surface plots for the total phenolic and total flavonoid content as a function of amount of 'bignay' puree and fermentation period.

The darker regions seen in the response surface plot are the areas for the suggested optimum range for the maximum amounts of TPC and TFC that can be generated given a set amount of fruit puree and the duration of acetic acid fermentation. The plot also depicts that the greater the fruit must used in the fermentation process is and the closer the fermentation time is to 5 days, the higher would be the resulting total phenolic content and total flavonoid content of the fermented bignay. This can also be visualized using the contour plot, which is a 2D version of the response surface plot. This provides strong evidence of how responses change as the variables are adjusted.

Optimization analysis and confirmation of the response model

The optimization process was implemented after the analysis of surface models for responses TPC and TFC to find the optimum value for responses under constraints. The criteria used for the optimization process are presented in Table 3.

Table 3 Criteria used for the optimization process for the fermentation of 'bignay' juice.

NAME	GOAL	LOWER LIMIT	UPPER LIMIT	IMPORTANCE	PREDICTED VALUES
% Fruit Puree	within range	25	75	1	75
Fermentation time	within range	3.5147	20.4853	1	3.5147
Total phenolic content	maximize	127.322	233.190	1	250.459
Total flavonoid content	maximize	47.590	199.435	1	236.836

The goal of % fruit puree and duration of acetic acid fermentation was set "within range" in which % puree varies from 25% to 75%, and fermentation time varies from 3.51 to 20.49 days. On the other hand, the goal for TPC and TFC is set to "maximize." The lower limit for TPC is at 127.32 mg gallic acid equivalents (GAE) per gram of sample while the higher limit is at 233.19 mg GAE g-1. Similarly, the lower limit for TFC is at 47.59 mg quercetin equivalents (QE) g-1 while the higher limit is 199.43 mg QE g-1.

The priorities of each response could be changed to achieve the goal of the study. The important values vary from 1 to 5 which corresponds to the lowest priority and the highest priority, respectively. All importance was set to 1 in this study. This means that no goal is more important than others.

Based on the set criteria, different solutions of fermented 'bignay' were generated, and the one with highest desirability was selected. The selected solution for the two-stage fermentation of 'bignay' uses 75% fruit puree and 3.5 days of acetic acid fermentation with a desirability of 1.00.

The confirmation process was carried out to confirm the validity of the models. The prediction intervals (PI) were also generated, which include "PI low" and "PI high". PI values are useful in expecting the values of the response of the confirmation tests. If the data means are within the range of "PI low" and "PI high", the models are verified. The resulting mean values for TPC and TFC, as shown in Table 4, were 251.90 mg GAE g-1 sample and 249.01 mg quercetin g-1 sample, respectively. The resulting values for the TPC and TFC from the validation of the model were within the acceptance range. This suggests that the empirical models are validated.

Table 4 Confirmation for the optimized fermentation of 'bignay' juice based on the response parameters total phenolic and total flavonoid contents.

PARAMETERS	VALUES	
Fruit Puree	75:25	
Acetic acid fermentation duration	3.5147 days	
Predicted:	95% PI low	95% PI high
TPC (mg GAE g $^{-1}$)	235.228	265.69
TFC (mg QE g $^{-1}$)	197.091	256.78
Actual:	Mean Value	Error Rates
TPC (mg GAE g $^{-1}$)	251.898 ± 0.30	0.57 %
TFC (mg QE g $^{-1}$)	249.013 ± 0.15	5.14 %
Desirability of model	1.00	

The robustness of the model could also be explained through the calculated error rates. The robustness of the models signifies how accurate the model based on the generated experimental values from the model. Robustness of models based on the error rates can vary from model to model, and for fermentation processes it can range from 5% to 10% **(Alemneh *et al.*, 2023, Uslu *et al.*, 2022).** The lower the error rate values are, the more accurate the model can predict. With an error rate of 0.57% and 5.14% for the TPC and TFC models, respectively, it can be said that the models are robust. This further confirms the TPC and TFC models for the two-stage fermentation of 'bignay.'

Physicochemical properties of bignay during alcohol fermentation

During the alcohol fermentation of 'bignay', the pH, alcohol content and total soluble solids were measured every two days as shown in Figure 2. The alcohol content of the fermenting bignay had an increasing trend while the total soluble solids decreased throughout the anaerobic alcohol fermentation process. The gradual decrease in pH of the fermenting bignay sample can be attributed to the production of organic acids by the yeast's utilization of sugars for its growth **(Zou et al., 2017**). Organic acids such as pyruvic acid, can be produced during glycolysis, and malic acid and citric acid can be produced during the tricarboxylic acid cycle **(Kumari, 2018)**.

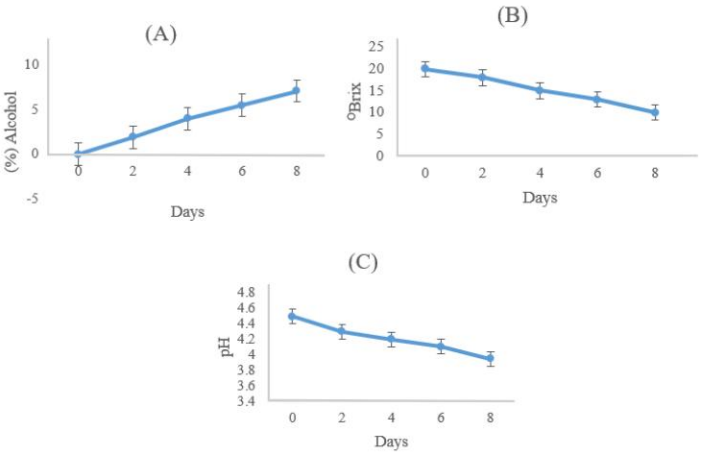

Figure 3 Changes in the physicochemical properties of the optimized product: (A) alcohol content, (B) pH, and (C) total soluble solids of 'bignay' juice during alcoholic fermentation (first stage of the fermentation process).

Physicochemical properties of bignay during acetic acid fermentation

The physicochemical properties of alcoholic 'bignay' during acetic acid fermentation were evaluated every five days until the suggested acetic acid fermentation time during the optimization process. The alcohol content, pH, TSS, and acetic acid content of 'bignay' during acetic acid fermentation are presented in Figure 3. The pH and the total soluble solids of the alcoholic bignay continued to decrease during the acetic acid fermentation as the acetic acid bacteria utilized the sugars and produced organic acids **(Zou et al., 2017)**.

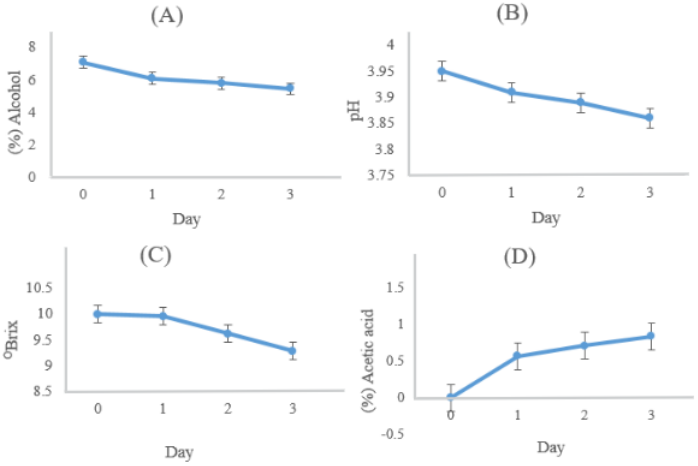

Figure 4 Changes in the physicochemical properties of the optimized product: (A) Alcohol content, (B) pH, (C) total soluble solids, and (D) acetic acid content of 'bignay' juice during acetic acid fermentation (second stage of the fermentation process).

Furthermore, the decrease in alcohol content, further decrease in the total soluble solid and subsequent increase in the total titratable acidity of 'bignay' juice during acetic acid fermentation supports the fact that there is an increase in the production of organic acids due to the oxidation of ethanol to acetic acid **(Deshmukh and Manyar, 2021; Chidi *et al.*, 2018).**

Comparison of fermented and unfermented 'bignay'

Unfermented 'bignay' was also subjected to the TPC and TFC analysis and the results were compared with the fermented 'bignay.' Results of the comparison, as displayed in Table 5, show that the fermented 'bignay' had a significant increase in both TPC and TFC compared to its unfermented counterpart. Increase in TPC and TFC of the fermented bignay is similar to other fermented berries **(Erskine *et al.*, 2023, Zhao et al., 2021).** The increase in TPC and TFC can be attributed to the

production and release of phenolic compound during fermentation **(Lin *et al,* 2014; Liu *et al,* 2023)**. The production of phenolic compounds is due to the possible biotransformation of precursor compounds such as other metabolites like tannins to gallic acid or free ellagic acid monomers due to the production of tannic acid hydrolase of *Saccharomyces cerevisiae* during the alcoholic fermentation. **(William *et al*., 2021; Chavez-Gonzalez *et al*., 2018).** Moreover, they may contain decarboxylases or reductases such as hydroxycinnamate decarboxylase, which transforms hydrocinnamic acids into caffeic, ellagic acid, and ferullic acid **(Antonio et al., 2016; William et al., 2021).** As for the release of phenolic compounds during fermentation, it is due to the hydrolytic nature of the organic acids produced during fermentation and production of cellulolytic enzymes during fermentation that destroy the cellular matrix in the fruit thereby releasing the stored phenolic compounds within the cellular matrix of the fruit that can be physically be extracted **(Sohail *et al*., 2022).**

Table 5 Effect of the optimized two-stage fermentation on the total phytochemical contents of 'bignay.'

PARAMETERS	BIGNAY SAMPLES		
	Unfermented	Fermented	Effect
TPC (GAE mg g^{-1})	218.656 ± 0.19^{b}	251.898 ± 0.30^{a}	Increase
TFC (QE mg g^{-1})	209.306 ± 0.13^{b}	249.013 ± 0.41^{a}	Increase

CONCLUSION

The resulting optimized process shows that using 75% fruit must and 3.5 days of acetic acid fermentation can help attain the maximum amount of total phenolic and total flavonoid content for the fermented bignay juice. Furthermore, comparing the TPC and TFC of the fermented and unfermented bignay, there was a notable increase in the TPC and TFC for the fermented bignay juice. The findings indicate that the optimized two-stage fermentation process was able to produce a phenolic-rich fermented bignay that can be used in the production of a functional beverage.

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