

Statistical and Biochemical Optimization of Acetic Fermentation for Vinegar Production

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How to cite this paper: Kourouma, M.C., Mbengue, M., Thioye, A. and Marone, A. (2025) Statistical and Biochemical Optimization of Acetic Fermentation for Vinegar Production. *Food and Nutrition Sciences*, 16, 1809-1827.
<http://doi.org/10.4236/fns.2025.1612106>

Received: September 3, 2025

Accepted: December 5, 2025

Published: December 8, 2025

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Abstract

This study aims to optimize the acetic fermentation process for vinegar production using three strains of acetic bacteria including KS1, KS2, KS3 by combining two approaches, statistical and biochemical. The results obtained with the BBD experiences led to the determination of the optimal values of the key fermentation parameters for each of the isolates. Maximum acidity was obtained at fermentation temperature, initial ethanol and acetic acid concentrations respectively of 37.5°C, 20% (v/v), 2% (w/v) for KS1; 45°C, 20% (v/v), 1 (w/v) for KS2 and 45°C, 5% (v/v), 1 (w/v) for KS3. The addition of CaCl₂ in optimal quantities has improved the acetic fermentation process, giving acetic acid bacteria better resistance to stress conditions, resulting in a considerable increase in biomass and acetic acid yield, with a maximum degree of 15.45 for KS2 and 15.30 for KS1. However, unlike previous isolates, the addition of CaCl₂ to the concentrations tested in this study have antagonistic effects on acetic acid production involving KS3, the degree of acetic acid therefore decreasing with increasing concentration of CaCl₂. The combination of the two optimizations, statistical and biochemical resulted in a considerable increase in the yield of acetic acid produced: with an increase of 525.77% for KS1; 504.90% for KS2 and 310.27% for KS3.

Keywords

Vinegar, Acetic Acid Bacteria, Box-Behnken Design, Calcium Chloride, Optimization

1. Introduction

Vinegar, is an acidic liquid, a fermented drink produced from various carbohydrate sources, including cereals, fruits, sugar, etc. [1] [2]. Vinegar is widely prized,

for its domestic use as a seasoning, preservative, cleaner; it is used in the food industry, where it is integrated into the production of ketchups, mayonnaise, and mustard; it is also used for its many health benefits due to their antimicrobial and antioxidant effects, hypoglycemic action, positive effect on the cardiovascular system, etc. [3]-[7].

Acetic acid is the main component of vinegar, it also gives it its characteristic smell and taste [8]. According to [9], the conversion of ethanol to acetic acid in vinegar production is believed to be the oldest microbial transformation process known. Acetic Acid can be obtained by two methods: chemical synthesis (mainly produced from petrochemical sources) or biological synthesis [10]. Among the chemical manufacturing processes, the most common are the carbonylation of methanol, the oxidation of aldehyde, and the oxidation of ethylene [11]. The biological pathway represents only about 10% of global production, but it remains important for vinegar production, as many global food purity laws stipulate that vinegar must be of organic origin [10] [12].

Acetic Acid Bacteria, responsible for the acetic fermentation leading to vinegar, are considered tedious due to their difficult growth on conventional culture media. Their cultivability is often weak and very irregular, thus affecting their productivity [13]. Strong from this observation, it is necessary to optimize the acetic acid fermentation process.

There are several factors that can affect the growth and survival of AAB, among which ethanol concentration, acetic acid concentration, temperature, oxygen and nutrient availability represent the most determining parameters [14]. Box-Behnken Design (BBD) is a statistical method used to screen and evaluate multiple variables efficiently. BBD is highly effective in identifying the key parameters influencing microbial growth and production. It serves as an initial step by assessing individual and interactive effects of variables [15]. Furthermore, many studies have suggested that the addition of Ca^{2+} could improve stress tolerance in living cells under non-ideal conditions [16].

The aim of this study is to optimize the production of biological acetic acid by combining two methods: statistical and biochemical. Specifically, it involves determining the values of key parameters of acetic fermentation using a BBD-type experimental plan, then testing the effect of adding gradual concentrations of calcium chloride (CaCl_2) on acetic acid production. The combination of these two methods would result in an increase in acetic acid yield for vinegar production.

2. Materials and Method

2.1. Bacterial Strains

Three (3) AAB strains namely KS1, KS2 and KS3 all belonging to the genera *Gluconoacetobacter* previously isolated from mango alcohol were used in this study. The species of the genus *Gluconoacetobacter* were chosen due to the stability of their key enzymes in acidic conditions, notably PQQ-ADH (considered as the key enzyme in vinegar production because of its essential role in the oxidation of eth-

anol to acetaldehyde) [17].

2.2. Evaluation of Acetic Acid Production Prior to Optimization

This is a test to evaluate the potential of acetic fermentation by strains KS1, KS2 and KS3 for vinegar production at laboratory scale.

2.2.1. Preparation of Microorganisms and Inoculum

First, the strains were cultured in 100 mL of YG broth (Yeast-extract, Glucose), containing respectively 3% (w/v) yeast, 3% (w/v) glucose. Then, the medium was incubated at 30°C until the optical density (OD600 nm) of the suspension reaches 0.4.

2.2.2. Fermentation Conditions

Acetic acid fermentations were carried out in YGEA fermentation medium containing 3% (w/v) Yeast, 1% (w/v) Glucose. Alcohol and acetic acid were added in a sterile manner at the respective concentrations of 5% (v/v) and 1% (w/v). The fermentation media were inoculated with 10% (v/v) of pre-culture of the microorganism concerned. The flasks were then incubated at 30°C with shaking at 150 rpm for a total of 16 days. Samples were taken every 48 hours to track and determine the amount of acetic acid produced

2.3. Statistical Optimization of Acetic Acid Production Using BBD

2.3.1. Statistical Experimental Plan

The experiments are performed by using a Box-Behnken-Design. The goal is to maximize the output variable, or response, which is affected by multiple independent variables, often known as input variables or factors [18]. This statistical approach provides information on the effects of experimental variables and total experimental error in the bare minimum of procedures required [19]. The advantage of BBD lies in reducing the number of experimental runs and allowing better estimation of quadratic relationships in systems with three or more variables [15].

The independent variables of acetic acid fermentation were X1, X2 and X3 respectively fermentation temperature, initial alcohol concentration and initial acetic acid concentration. Each variable was coded at 3 levels: -1, 0 and 1. **Table 1** below shows the variables, their symbols and code levels, it gives a range of these variables in the acetic fermentation process.

Table 1. Levels of factors and variables chosen for Box-Behnken experimental design.

Variable	Symbol	Code-variable level		
		-1	0	1
Temperature (°C)	X1	30	37.5	45
Alcohol (v/v)	X2	5	12.5	20
Acetic acid (w/v)	X3	0	1	2

2.3.2. Preparation of Microorganisms and Inoculum

First, the strains were cultured in 100 mL of YPG broth (Yeast-extract, Peptone, Glucose), containing respectively 3% (w/v) yeast, 1% (w/v) Peptone, 3% (w/v) glucose. Then, the medium was incubated at 37°C until the optical density (OD600 nm) of the suspension reaches 0.4.

2.3.3. Fermentation Conditions

The variables were combined in 15 experiments, repeated three times, which makes a total of 45 experiments for each isolate as described in **Table 2** below.

Table 2. Experimental design.

Run	Code level			Factors in real terms		
	X1	X2	X3	Temperature	Alcohol	Acetic Acid
1	-1	-1	0	30	5	1
2	-1	-1	0	30	5	1
3	-1	-1	0	30	5	1
4	0	-1	-1	37.5	5	0
5	0	-1	-1	37.5	5	0
6	0	-1	-1	37.5	5	0
7	0	-1	1	37.5	5	2
8	0	-1	1	37.5	5	2
9	0	-1	1	37.5	5	2
10	1	-1	0	45	5	1
11	1	-1	0	45	5	1
12	1	-1	0	45	5	1
13	-1	0	-1	30	12.5	0
14	-1	0	-1	30	12.5	0
15	-1	0	-1	30	12.5	0
16	-1	0	1	30	12.5	2
17	-1	0	1	30	12.5	2
18	-1	0	1	30	12.5	2
19	0	0	0	37.5	12.5	1
20	0	0	0	37.5	12.5	1
21	0	0	0	37.5	12.5	1
22	0	0	0	37.5	12.5	1
23	0	0	0	37.5	12.5	1
24	0	0	0	37.5	12.5	1
25	0	0	0	37.5	12.5	1
26	0	0	0	37.5	12.5	1
27	0	0	0	37.5	12.5	1

Continued

28	1	0	-1	45	12.5	0
29	1	0	-1	45	12.5	0
30	1	0	-1	45	12.5	0
31	1	0	1	45	12.5	2
32	1	0	1	45	12.5	2
33	1	0	1	45	12.5	2
34	-1	1	0	30	20	1
35	-1	1	0	30	20	1
36	-1	1	0	30	20	1
37	0	1	-1	37.5	20	0
38	0	1	-1	37.5	20	0
39	0	1	-1	37.5	20	0
40	0	1	1	37.5	20	2
41	0	1	1	37.5	20	2
42	0	1	1	37.5	20	2
43	1	1	0	45	20	1
44	1	1	0	45	20	1
45	1	1	0	45	20	1

Acetic fermentation batches were carried out with the YGKM fermentation medium of the following composition: yeast extract 5% (w/v); glucose 5% (w/v); K₂HPO₄ 3.3% (w/v); MgSO₄ 1.1% (w/v). The medium is first distributed in sterilized 250 ml Erlenmeyer flasks, before continuing with an addition of sterile ethanol (v/v) and acetic acid (w/v) at the specific concentrations listed in **Table 2**. The Erlenmeyer flasks were then inoculated with 10% (v/v) pre-culture of the concerned microorganism (KS1, KS2 and KS3) and incubated at the temperature described for each experiment (either 30°C, 37.5°C or 45°C) with agitation at 150 rpm for a total of 21 days. Samples were taken every 48 hours to determine the amount of acetic acid produced.

Experimental design and statistical analysis of the data were performed using STATISTICA 10 for Windows™.

2.4. Biochemical Optimization of Acetic Acid Production Using Calcium Chloride

The comparative effects of different concentrations of CaCl₂ on the rate of acetic acid production and on the biomass produced were determined.

Fermentation Conditions

The strains were cultured in 100 mL of YPG broth (Yeast-extract, Peptone, Glucose), containing respectively 3% (w/v) yeast, 1% (w/v) Peptone, 3% (w/v) glucose

and then, the medium was incubated at 37°C until the optical density (OD600 nm) of the suspension reaches 0.4.

Acetic fermentation tests were carried out in the YGKM fermentation medium. Sterile alcohol and acetic acid were added to the media respectively in optimal proportions, previously determined thanks to the results obtained with the experimental plan. For each of the isolates studied KS1, KS2 and KS3, six concentrations of CaCl₂ were evaluated: 0.00; 0.05; 0.10; 0.15; 0.20 and 0.25 (% w/v). The Erlenmeyer containing the fermentation medium were then each inoculated with 10% pre-culture, then incubated at the optimal temperature of each strain as determined by the experimental plan results with agitation at 150 rpm for a total of 21 days. Samples were taken every 48 hours to monitor and determine the amount of acetic acid produced, and the evolution of the biomass.

2.5. Estimate of Acetic Acid Production

1 ml of culture is taken from the fermentation medium then diluted to hundredth with distilled water. After homogenization, 20 mL of this solution are placed in an Erlenmeyer flask. 2 - 4 drops of phenolphthalein are added to assess the pH variation. The solution is then titrated against a 0.1 N NaOH solution. The amount of acetic acid produced in g per 100 mL was calculated using the following formula:

$$d^{\circ} = \frac{1000 \times C_b \times V_b \times P_m}{V_a \times m_v}$$

- C_b : Concentration of NaOH solution
- V_b : Average volume of NaOH needed to reach the end point
- P_m : Molar mass of acetic acid
- V_a : Volume of the sample
- m_v : Volumic mass of acetic acid

2.6. Statistical Analyses

All experiences were performed in triplicate, and the mean and standard deviation were calculated. An honest significant difference (DHS) was detected using the Tukey test. The results were considered statistically significant if $p < 0.05$ with SAS JMP Statistical Discovery Pro 16.0.0.

3. Results and Discussion

3.1. Acetic acid Production Tests

The selected isolates KS1, KS2 and KS3 were subjected to an acetic acid production test, in order to check their performance before starting the optimization processes. The results are shown in **Figure 1** below.

For the three isolates, fermentation starts weakly until the 2nd day, thus corresponding to their adaptation time. From the 2nd to the 5th day, isolate KS3 produces the most AA, then there is a greater production of AA by KS2 until the 16th day. KS1 shows the lowest production of AA throughout the experiment. The

maximum concentrations of AA, produced at the 14th day, are respectively 2.91 for KS1; 3.06 for KS2 and 2.93 for KS3.

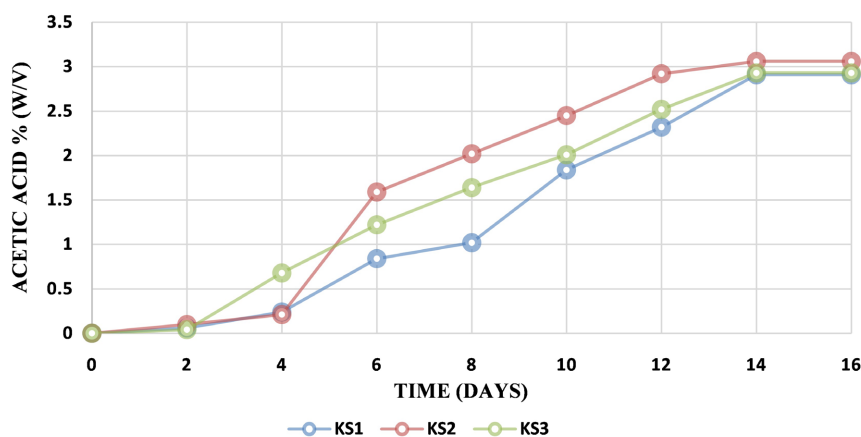


Figure 1. Acetic acid production test.

One of the most famous and important oxidative fermentation reactions of BA is acetic acid (vinegar) production from ethanol [20]. Acetic acid bacteria show differences in their ability to oxidize ethanol. The fermentative oxidation of ethanol into acetic acid depends on two successive sequential reactions, catalyzed respectively by pyrroloquinoline-alcohol dehydrogenase complex (PQQ-ADH) and aldehyde dehydrogenase (ALDH), both located on the periplasmic side of the inner membrane [21]. PQQ-ADH is considered the key enzyme in vinegar production due to its critical role in the oxidation of ethanol to acetaldehyde. With equal quantities of carbon source, nutrients, and under the same physiological conditions of temperature, pH, dissolved oxygen, etc., the production of acetic acid by potentially different isolates would therefore be governed by the activity of ADH and ALDH, and the mechanisms involved in resistance to alcohol and acetic acid. On the one hand, a difference in acetic acid concentration produced could therefore be explained by a difference in the expression of genes encoding their fundamental proteins. Indeed, [19] [20] reveal that a defect in ADH has been associated with reduced acetic acid resistance, and the elevation of ALDH activity by genetic amplification has been reported as an effective means for improving acid yields. It could then be assumed that the more the genes encoding these proteins are expressed, the faster the fermentation rate and the acetic acid yield increase. On the other hand, it has been revealed by several authors including [17] [21]-[23] that ADH in *Gluconoacetobacter* species was very stable under acidic conditions, which makes strains of the genus *Gluconoacetobacter* as well as those of the genus *Komagataeibacter* (genus derived from *Gluconoacetobacter*) are mainly involved in the manufacture of vinegars with a high acetic acid content.

3.2. Optimization of Acetic Acid Production by Statistical Tools Using BBD

Each isolate was involved in a series of 15 acetic fermentation experiments, re-

peated three times. Each experiment involved is a combination of different values of the key fermentation parameters, namely the fermentation temperature and the initial concentrations of ethanol and acetic acid. The averages of the responses obtained for each combination are presented in the following **Tables 3-5**.

Table 3. Average responses obtained for KS1 based on combinations.

Combination	Temperature	Alcohol	Ac. Acid	Mean response
C1	30	5	1	1.64
C2	30	12.5	0	1.25
C3	30	12.5	2	1.83
C4	30	20	1	2.69
C5	37.5	5	0	2.00
C6	37.5	5	2	2.77
C7	37.5	12.5	1	3.18
C8	37.5	12.5	1	3.2
C9	37.5	12.5	1	3.28
C10	37.5	20	0	3.00
C11	37.5	20	2	4.70
C12	45	5	1	2.82
C13	45	12.5	0	3.00
C14	45	12.5	2	3.80
C15	45	20	1	4.00

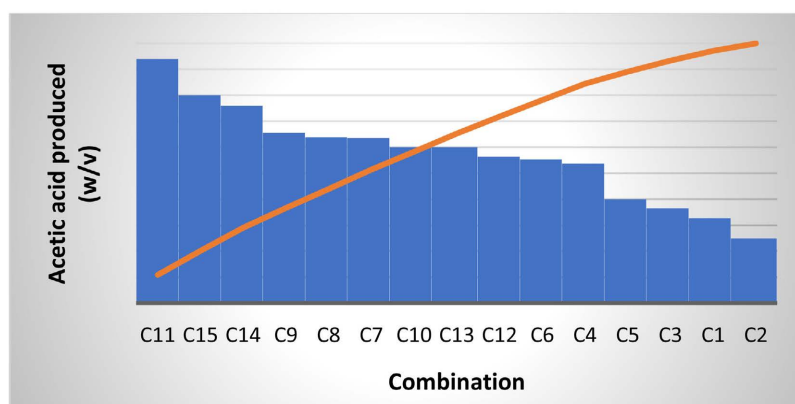
Table 4. Average responses obtained for KS2 based on combinations.

Combination	Temperature	Alcohol	Ac. Acid	Mean response
C1	30	5	1	1.68
C2	30	12.5	0	1.25
C3	30	12.5	2	3.11
C4	30	20	1	3.52
C5	37.5	5	0	2.51
C6	37.5	5	2	3.01
C7	37.5	12.5	1	4.00
C8	37.5	12.5	1	4.01
C9	37.5	12.5	1	3.99
C10	37.5	20	0	1.97
C11	37.5	20	2	4.78
C12	45	5	1	3.00
C13	45	12.5	0	3.01
C14	45	12.5	2	5.01
C15	45	20	1	5.48

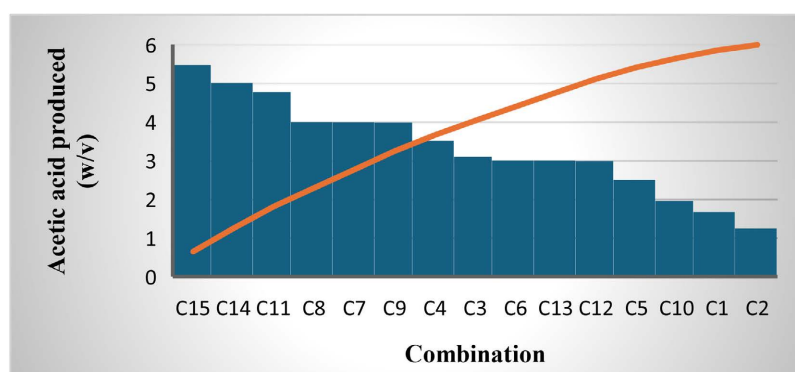
Table 5. Average responses obtained for KS3 based on combinations.

Combinaison	Temperature	Alcohol	Ac. Acid	Mean response
C1	30	5	1	1.77
C2	30	12.5	0	2.66
C3	30	12.5	2	2.85
C4	30	20	1	3.43
C5	37.5	5	0	2.19
C6	37.5	5	2	3.00
C7	37.5	12.5	1	3.6
C8	37.5	12.5	1	3.6
C9	37.5	12.5	1	3.6
C10	37.5	20	0	3.1
C11	37.5	20	2	3.9
C12	45	5	1	5.6
C13	45	12.5	0	5.00
C14	45	12.5	2	3.00
C15	45	20	1	3.2

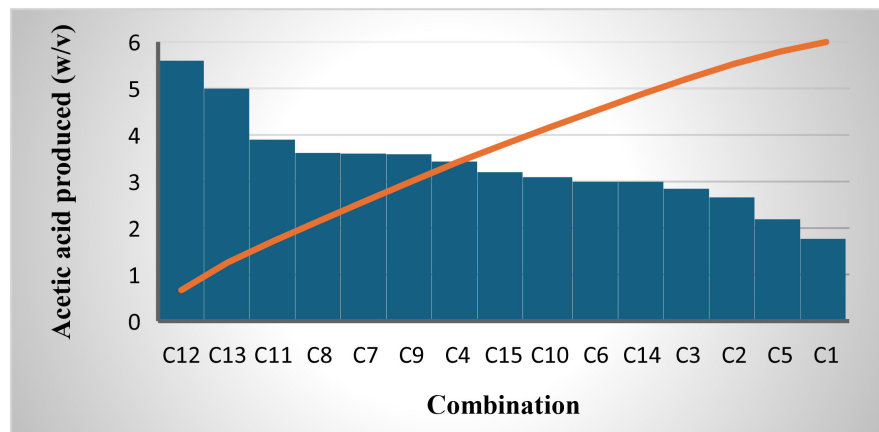
For each isolate, the responses were ranked in descending order of importance from highest to lowest, as illustrated by **Figure 2**.



(a)



(b)



(c)

Figure 2. Decreasing ranking of the average responses obtained (a) KS1; (b) KS2; (c) KS3.

KS1

The best response, *i.e.* 4.70% (w/v) is obtained with the C11 combination; the values of fermentation temperature and initial ethanol and acetic acid concentrations involved being respectively 37.5°C, 20% (v/v), 2% (w/v). The lowest response, *i.e.* 1.25% (w/v) is obtained with the C2 combination; the values of fermentation temperature and initial ethanol and acetic acid concentrations involved being, respectively 30°C, 12.5% (v/v), 0% (w/v).

KS2

The best response, *i.e.* 5.48% (w/v) is obtained with the C15 combination; the values of fermentation temperature and initial ethanol and acetic acid concentrations involved being respectively 45°C, 20% (v/v), 1% (w/v).

Just like KS1, the lowest response of 1.25% (w/v) is obtained with the C2 combination; the values of fermentation temperature and initial ethanol and acetic acid concentrations involved being respectively 30°C, 12.5% (v/v), 0% (w/v).

KS3

The best response, *i.e.* 5.6% (w/v) is obtained with the C12 combination; the values of fermentation temperature and initial ethanol and acetic acid concentrations involved being 45°C, 5% (v/v), 1% (w/v), respectively.

The lowest response, 1.77% (w/v) is obtained with the C1 combination; the values of fermentation temperature and initial ethanol and acetic acid concentrations involved being respectively 30°C, 5% (v/v), 1% (w/v).

3.3. Optimization of Acetic Acid Production by Biochemical Tools Using Calcium Chlorure

The effect of six calcium chloride concentrations (0.00%; 0.05%; 0.10%; 0.15%; 0.20% and 0.25%) on acetic acid fermentation process was tested using isolates KS1, KS2 and KS3.

The results obtained are illustrated by the following **Figures 3-5**.

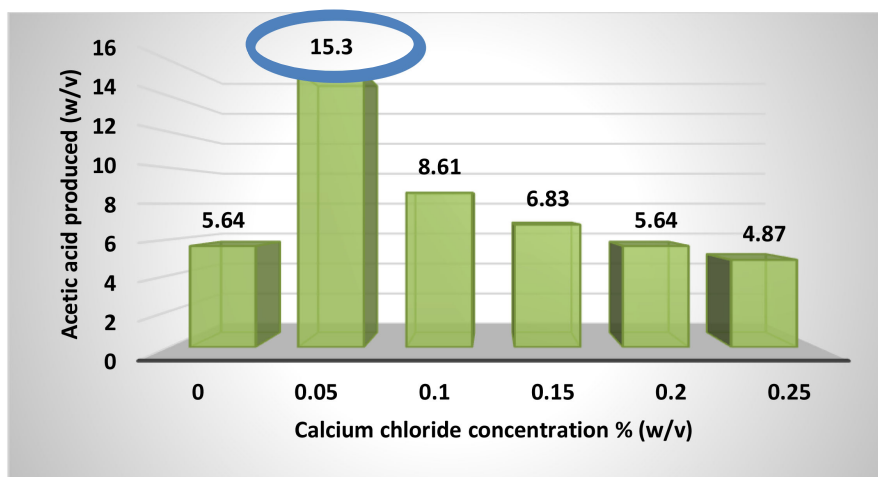


Figure 3. Evolution of the quantity of acetic acid produced by KS1 depending on the CaCl_2 concentrations.

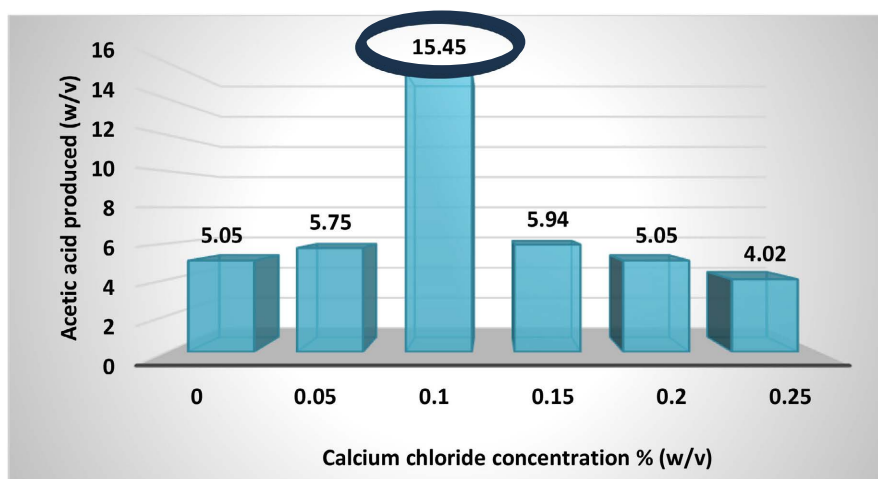


Figure 4. Evolution of the quantity of acetic acid produced by KS2 depending on the CaCl_2 concentrations.

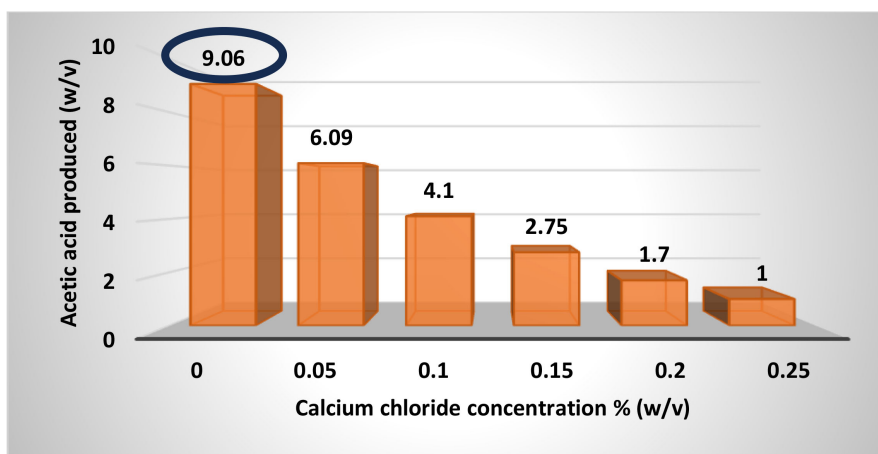


Figure 5. Evolution of the quantity of acetic acid produced by KS3 depending on the CaCl_2 concentrations.

✓ **KS1**

The maximum concentration of AA is produced at 0.05% CaCl₂ and the minimum at 0.25% CaCl₂. At CaCl₂ concentrations greater than or equal to 0.10% (w/v), a decrease in acetic acid production is observed. However, for CaCl₂ concentrations of 0.10% and 0.15%, respectively, the quantities of AA produced remain higher than those obtained without addition of CaCl₂ (0% (w/v)). It was also noted an equivalence between the quantities of acetic acid produced at 0% CaCl₂ and those produced at 0.20% CaCl₂ ($p > 0.05$, indicating that there are no statistically significant differences).

✓ **KS2**

A significant increase ($p < 0.05$) in acetic acid production is noted as the concentration of CaCl₂ increases, with a production optimum at 0.10% CaCl₂. Beyond this concentration, a decrease in the quantity of AA produced is observed with a minimum production at 0.25% CaCl₂. However, the amount of AA produced at 0.15% CaCl₂ remains higher than that produced without adding CaCl₂. It should also be noted that the quantity produced at 0.20% CaCl₂ is substantially equal to that produced at 0% CaCl₂, nevertheless statistical analyses show a significant difference between the two values ($p < 0.05$).

✓ **KS3**

A decrease in the acetification yield is observed with KS3. Unlike previous isolates, it would seem that calcium chloride had a negative effect on the productivity of KS3, with a drastic decrease in the amount of acetic acid produced as the concentration of CaCl₂ increased in the reaction medium.

In the pursuit of optimization, a comparison of the results obtained with the different isolates according to the concentration of CaCl₂ involved was carried out. The following **Figure 6** illustrates a comparison report.

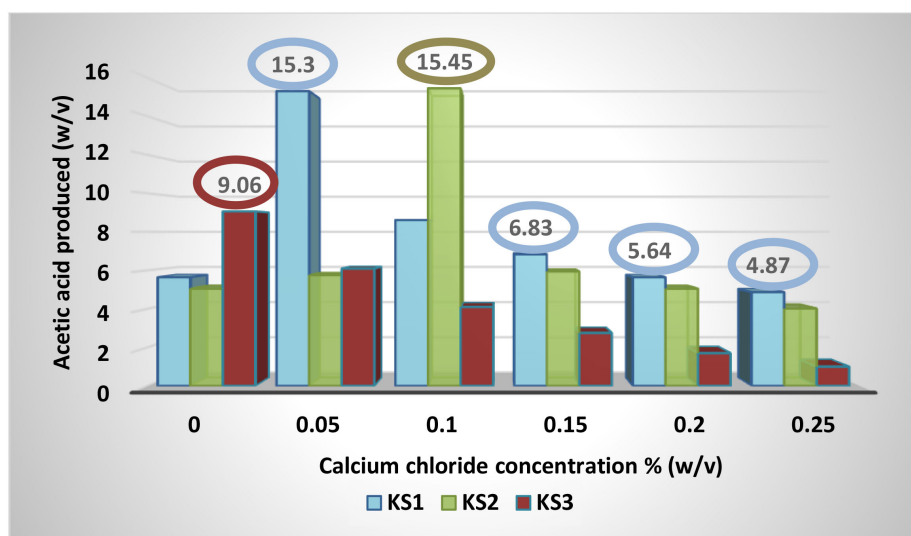


Figure 6. Comparison of the productivity of isolates KS1, KS2 and KS3 according to the studied CaCl₂ concentration.

Figure 6 shows the maximum acetic degrees for each isolate based on the amount of calcium chloride added in the reaction medium.

- ✓ At a zero concentration of CaCl_2 , KS3 has the highest productivity, which is 9.06° (w/v) acetic acid.
- ✓ At CaCl_2 values of 0.05; 0.20 and 0.25% (w/v), KS1 displays a higher productivity than isolates KS2 and KS3, with a maximum of 15.30° at 0.05% CaCl_2 (w/v), and an acetic degree of 6.83° (w/v) at 0.20% CaCl_2 , which remains interesting, since most commercial vinegars mention an acetic degree of around 6% (w/v).
- ✓ KS2 produces the highest amount of acetic acid, at 15.45% following the addition of 0.10% CaCl_2 .

The influence of calcium chloride on the biomass of the isolates was also studied through the determination of the Optical Density, which evolution is proportional to the biomass formed. The following **Figures 7-9** show respectively the evolution of OD for KS1, KS2 and KS3 according to the concentration of CaCl_2 involved in the acetification process.

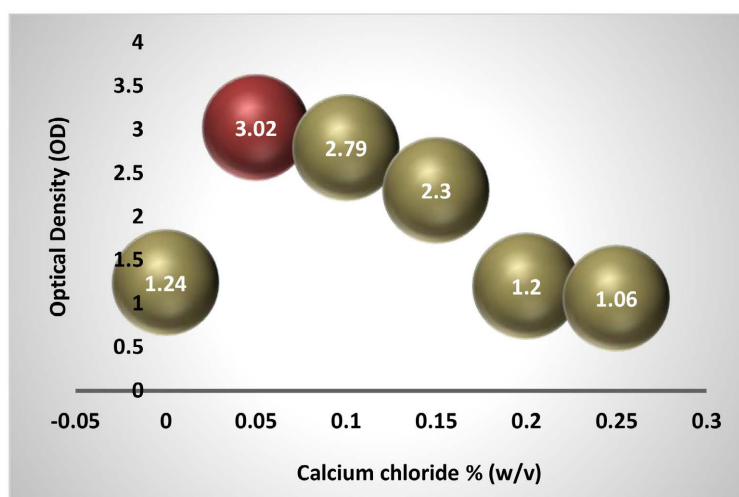


Figure 7. Evolution of the OD of KS1 based on CaCl_2 concentrations.

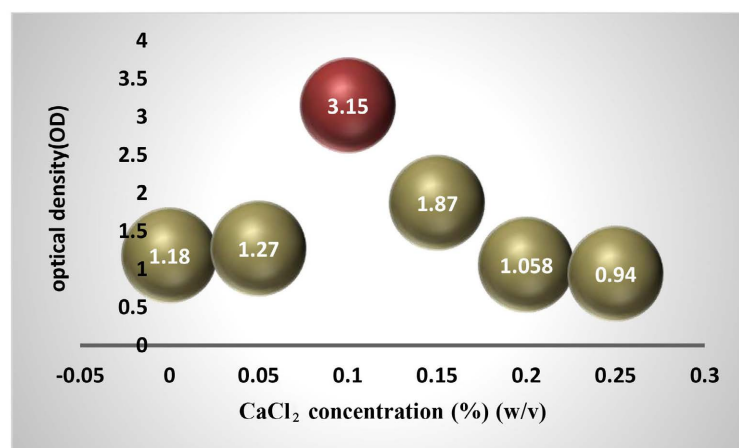


Figure 8. Evolution of the OD of KS2 based on CaCl_2 concentrations.

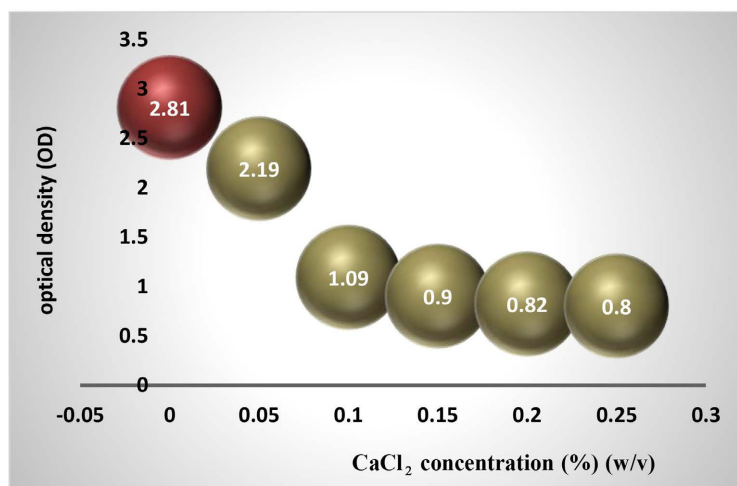


Figure 9. Evolution of the OD of KS3 based on CaCl₂ concentrations.

It is observed for KS1 a maximum of biomass at 0.05% (w/v) of CaCl₂, for KS2 at 0.10% (w/v) and for KS3 at 0% (w/v). The evolution of the biomasses of KS1, KS2 and KS3 seems to follow the same trend as that of the evolution of the acetic acid produced. Indeed, for each isolate, the maximum productivity is correlated with the maximum biomass production; similarly, the smallest values for acetic acid were obtained with the lowest values for biomass.

Numerous studies have suggested that adding Ca²⁺ could improve the stress tolerance of living cells under non-ideal conditions [16]. In this study, the effect of calcium chloride on vinegar production by three isolates of acetic bacteria, namely KS1, KS2 and KS3 was tested. The gradual addition of CaCl₂ at different concentrations seems to have a very positive effect on isolates KS1, and KS2 with an optimum production of acetic acid at 0.05% and 0.10% CaCl₂ respectively. Above these values, a decrease in acetic acid yield was observed. Compared to the control (CaCl₂ concentration = 0%), the addition of 0.05% of CaCl₂ results in an increase of 271% of the yield of acetic acid with KS1 and the addition of 0.10% of CaCl₂ results in an increase of 306% of the yield of acetic acid with KS2. However, unlike previous isolates, the addition of CaCl₂ to the concentrations tested in this study would have antagonistic effects on acetic acid production involving KS3, the degree of acetic acid therefore decreasing with increasing concentration of CaCl₂.

[22] recommended calcium chloride (CaCl₂) as a source of calcium ions (Ca²⁺) to support cellular function and facilitate cell recovery and protection after stress. The latter reports that the addition of an optimal concentration of CaCl₂ would increase the number of viable cells by inhibiting their senescence by maintaining the membrane structure, resulting in an increase in biomass, which is correlated with an increase in the formation of the product, acetic acid in this study. In the same vein, [23] argued that Ca²⁺ ions act as essential electrolytes for maintaining microbial cell wall permeability and stability. [24] pointed out that CaCl₂ appears to increase the robustness of the cell wall in the process of acetylation, which is important for the development of bacterial cell walls, in order to limit their per-

meability and give greater stability under stress conditions, by changing the electrolyte conformation under high temperature conditions. This would be due to the increase in phosphatidylglycerol, which is a major anionic phospholipid involved in the integrity of the cell wall. In addition, [25]-[28] report that divalent metal cations such as Ca^{2+} function as prosthetic groups for AAB enzymatic activities, promoting growth and oxidation, but also improving the production of acetic acid at high temperatures. The addition of calcium chloride could therefore improve the bacteria's thermo-tolerance mechanisms, thus reducing the negative effects of high temperatures.

On the other hand, [29] [30] showed that adding CaCl_2 during acetic acid production could lead to a modification of key membrane-bound enzymes, which would positively affect the stability of these enzymes, and, therefore, the fermentation process. Studies by [31]-[33] show that these characteristics would be related to the activity of PQQ-ADH, one of the main process enzymes responsible for converting ethanol into aldehyde, first step in the production of acetic acid.

Given all of the above about the positive effects of calcium chloride, it might be suggested that the antagonistic effect of calcium chloride observed with KS3 isolate would be related to the concentrations of CaCl_2 used in this study. For our upcoming studies, it is therefore imperative to test lower concentrations of calcium chloride (*i.e.* below 0.05%).

The addition of CaCl_2 is therefore effective for improving the acetic fermentation process. The use of CaCl_2 leading to better resistance to stress conditions, results in an increase in the yield of biological acetic acid and a decrease in the reduction of cooling costs.

3.4. Combined Effect of the Impacts of Statistical and Biochemical Optimization

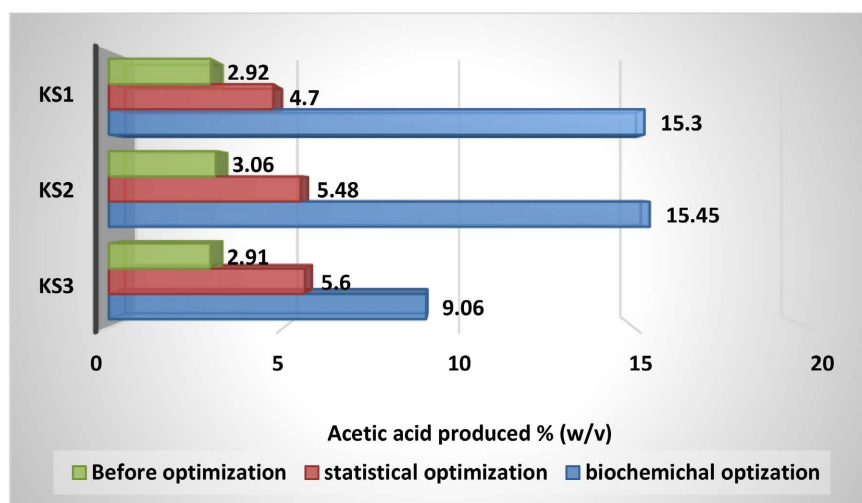


Figure 10. Evolution of the acetic degree before and after optimizations.

Such a comparative study is necessary in order to show the importance and the

inescapable impact of the optimization of the acetification process on the acetic acid yield. It should be noted that the quantity of acetic acid produced by the isolates follows a very positive evolution after optimizations. Indeed, we note an evolution from 2.92 to 15.3, which is an increase of 525.77%; from 3.06 to 15.45 for KS2, an increase of 504.90% and from 2.91 to 9.06 for KS3, an increase of 310.27% (**Figure 10**).

These very promising results were conducted at the laboratory scale, pilot-scale production trials will be carried out in our upcoming work to validate our results on a larger scale.

4. Conclusion

This study allowed us to optimize the acetic fermentation process of three AAB strains namely KS1, KS2 and KS3, thanks to the use of statistical tools, and also by biochemical approach. The responses obtained with the Box-Behnken Design led to the determination of the optimal values of the key fermentation parameters for each of the isolates. The addition of CaCl₂ in optimal quantities has improved the acetic fermentation process, giving acetic acid bacteria better resistance to stress conditions, resulting in a considerable increase in biomass and acetic acid yield for strains KS1 and KS2. However, the addition of CaCl₂ to the concentrations tested in this study have antagonistic effects on acetic acid production involving KS3. The combination of the two optimizations, statistical and biochemical resulted in a considerable increase in the yield of acetic acid produced: with an increase of 525.77% for KS1; 504.90% for KS2 and 310.27% for KS3.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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