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Thermoresistant, Ethanol-Resistant and Acid-Resistant Properties of Acetic Acid Bacteria Isolated from Fermented Mango Alcohol

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Abstract

Vinegar production is seriously affected by the sensitivity of acetic acid bacteria (AAB) to high temperature, high ethanol concentrations, and high acetic acid concentrations. The aim of this study was to investigate the thermo-ethanol-acid tolerance characters of five AAB strains (VMA1, VMA5, VMA7, VMAM, VMAO) previously isolated from fermented mango alcohol and belonging to *Gluconoacetobacter* genera. As result, the five AAB strains exhibited good growth and acid production at temperatures up to 45˚C; they could tolerate and produce acetic acid at ethanol concentrations up to 20% (v/v). In addition, the studied strains showed growth at acetic acid concentrations up to 4.5% (w/v). Strains VMA7 and VMAO showed the highest resistance properties: they demonstrated acid production at 50˚C and VMAO could even grow at 60˚C; they tolerated and produced acetic acid at 25% (v/v) ethanol concentration; they showed resistance to acetic acid concentrations up to 6% (w/v). Considering all these properties, the use of these strains would seriously contribute to improving the quality of the vinegar produced and help to reduce the cooling water feeds in vinegar production especially in hot countries in the context of global warming.

Keywords

Vinegar, Acetic Acid Bacteria, Thermotolerant, Ethanol-Tolerant, Acid-Tolerant

1. Introduction

Acetic acid bacteria are a wide and well-distributed group that can be found in fruits, flowers, honey, soil, juices, and fermented beverages, among others [1]. Acetic acid bacteria (AAB) belong to the family *Acetobacteraceae* that are currently classified into nineteen genera but the main species in terms of fermentation belong to the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, and *Komagataeibacter* [2] [3]. AAB are characterized by their ability to oxidize carbohydrates, alcohols, and sugar alcohols (polyhydric alcohols or polyols) into their corresponding organic acids, aldehydes, or ketones, in a process termed "oxidative fermentation", from which they gain energy [4]. AAB are predominantly known for their use in the production of vinegar which is acetic acid production from ethanol by two membrane-bound enzymes pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (ALDH) [5] [6]. The greatest strength of AAB is their ability to use less biomass to produce large amounts of acetic acid compared to other bacteria that produce organic acids [7]. However, considering the industrial demand of high-performing bioprocesses, the production yield of metabolites obtained by acetic acid bacteria is still not satisfying [8]. Not only is their cultivation, isolation, and identification difficult, their cells are often present in a viable but not culturable state [5]. Moreover, most AAB are mesophilic and acetic acid fermentation is generally carried out at around 30˚C which requires a good cooling system to maintain optimum temperature under warm ambient conditions [9] [10]. With this type of AAB, even a 2˚C or 3˚C temperature elevation above 30˚C has a significant negative impact on the yield and acetification rate [11]. Recently, climate change and global warming have become major challenges for the fermentation technology of AAB [12]. Strains should then be temperature-resistant, particularly in tropical and sub-tropical regions [13]. In addition, to date, submerged fermentation (SF) is widely used for vinegar production but AAB must endure the high initial ethanol concentration and acetic acid accumulation causing acid stress that inhibits AAB cell growth and limits acid productivity [14]. Therefore, to overcome these problems, screening potential AAB strains that could be tolerant to different restrictive conditions is of capital importance.

The aim of this study was to investigate the thermo-ethanol-acid tolerance abilities of five *Gluconoacetobacter* strains isolated in previous studies from fermented mango alcohol. These strains could then be used as starters in order to improve the quality of the vinegar produced and to reduce the cooling water feeds during the acetification process, especially in hot countries in the context of global warming.

2. Materials and Methods

2.1. Bacterial Strains

Five (5) AAB strains VMA1, VMA5, VMA7, VMAM, and VMAO all belonging

to the genera *Gluconoacetobacter* previously isolated from fermented mango alcohol [15] were tested in this study. These strains were selected for their capacity to produce acetic acid.

2.2. Analysis of Growth under Various Culture Conditions

2.2.1. Effects of Temperature on Growth Kinetics

The growth of the strains was assessed in liquid media. The bacterial suspension was prepared in YPG (Yeast-extract, Peptone, Glucose) medium and incubated at 30˚C until the optical density (OD600 nm) of the suspension reaches 0.4. 10% (v/v) of inoculum were then transferred into the fermentation medium YPG supplemented with 5% (v/v) ethanol and incubated at different temperatures (27˚C; 30˚C; 35˚C; 40˚C and 45˚C) with shaking at 150 rpm for a total of 15 days. Every two days, the bacterial growth was measured by spectrophotometry method (Thermo Scientific GENESIS 10S UV-VIS) at OD_{600}. The results obtained allowed us to draw the growth curve OD = f (t) in order to determine the maximum specific growth rate (μmax) for each temperature and draw the curve μmax = f (temperature).

2.2.2. High-Temperature Tolerance Ability

Samples were cultured on YPG medium plates supplemented with 5% (v/v) ethanol. The cultures were then incubated at different temperatures (35˚C; 40˚C; 45˚C; 50˚C; 55˚C; and 60˚C) for 5 days to examine the growth performance of the isolated strains at high temperatures.

2.2.3. High Ethanol Concentrations Tolerance Ability

All five bacterial isolates were screened for their ability to tolerate high ethanol concentrations. Samples were cultured on CARR solid medium supplemented with different ethanol concentrations ranging from 5% to 25% (v/v), using steak plate methods and incubated at 37˚C for five days.

2.2.4. High Acetic Acid Concentrations Tolerance Ability

The capacity of the isolated strains to resist acetic acid concentrations was evaluated. Samples were cultured on YGP medium broth in presence of 5% (v/v) ethanol concentration supplemented with acetic acid concentrations ranging from 0.5% to 6% (w/v) and incubated at 37˚C for five days.

2.3. Effects of Temperature and Ethanol on Acetic Acid Production

Effects of temperature on acetic acid production by the isolates were carried out on CARR agar medium (Yeast 3%, Ethanol 3%, Agar 2.5%). The plates were spot inoculated with pure pre-culture of bacterial strain and incubated at different temperatures 30˚C, 35˚C, 40˚C, 45˚C, and 50˚C.

Effects of ethanol on acid acetic production were also assessed on CARR medium supplemented with different ethanol concentrations ranging from 10% to 25% (v/v). The plates were spot inoculated and incubated at 37˚C.

In CARR medium, acid-forming strains produce a clear halo related to the

amount of the produced acid [16]. The capacity of the strains to produce acetic acid was determined by measuring the diameter of the halo every two days for 10 days.

2.4. Statistical Analysis

All experiments were performed in triplicate, and the mean and standard deviation were calculated. Honest Significative Difference (HSD) was detected using the test Tukey. The results were considered statistically significant if $p < 0.05$ using the SAS JMP Statistical Discovery Pro 16.0.0.

3. Results

3.1. Effect of Temperature on Growth Kinetics and Resistance to High Temperature

The effect of temperature on growth kinetics was assessed on YPG liquid media supplemented with 5% ethanol and incubated at different temperatures (27˚C; 30˚C; 35˚C; 40˚C and 45˚C). As it is given in Figure 1, Strains VMA1, VMA5,

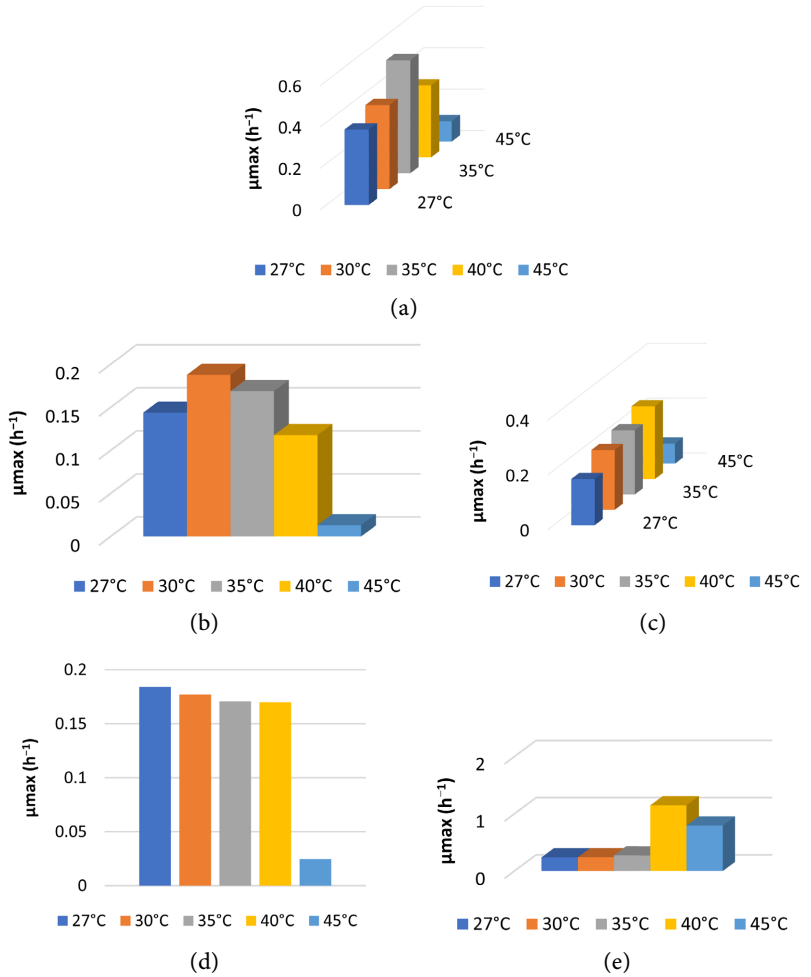

Figure 1. Effect of temperature on growth. (a): VMA1; (b): VMA5; (c): VMA7; (d): VMAM; (e): VMAO.

and VMAM presented their maximum specific growth rate respectively at 35˚C, 30˚C, and 27˚C (μmax = 0.546 h^{-1}; 0.1877 h^{-1}; 0.1842 h^{-1}) but showed good growth at 40˚C. Strains VMAM exhibited good growth at 30˚C, 35˚C and 40˚C with relatively close values of μmax (respectively μmax = 0.177 h^{-1}; 0.1706 h^{-1}; 0.1699 h^{-1}) but the statistical analysis showed significant differences between the values. As for strains VMA7 and VMAO, they demonstrated their maximum specific growth rate at 40˚C (respectively μmax = 0.2651 h^{-1}; 1.147 h^{-1}) but still exhibited a growth at 45˚C.

In addition, the thermo-resistance properties of the five strains were evaluated on YPG agar plates in presence of 5% (v/v) ethanol concentration and incubated at different temperatures ranging from 35˚C to 60˚C. The results are presented in Table 1. All five strains showed a great growth up to 45˚C. At 50˚C, all strains exhibited poor growth except VMAO which showed medium growth. No growth was observed above 50˚C except for VMAO who demonstrated thermo-resistance until 60˚C.

Table 1. Temperature resistance properties of the bacterial isolates.

Strains N˚	35˚C	40˚C	45˚C	50˚C	55˚C	60˚C
VMA1	+++	+++	+++	+	−	−
VMA5	+++	+++	+++	+	−	−
VMA7	+++	+++	+++	+	−	−
VMAM	+++	+++	+++	+	−	−
VMAO	+++	+++	+++	++	++	+

+++: good growth; ++: medium growth; +: poor growth; −: negative growth.

3.2. Resistance to High Ethanol Concentrations

The ethanol resistance properties of the isolates were investigated on CARR medium agar plates supplemented with ethanol ranging from 5% - 25% (v/v) and incubated at 37˚C. According to the results in Table 2, all the isolates showed a good growth of up to 15% (v/v) alcohol. The ethanol resistance decreased when the concentration of alcohol exceeded 15%, but remains relatively important for strains VMA1, VMA5, VMA7, and VMAO which showed medium growth at 20% (v/v) ethanol. These later exhibited high tolerance to ethanol and were able to grow in presence of 25% (v/v) ethanol. These strains were regarded as alcohol-tolerant.

Table 2. Alcohol tolerance ability of the bacterial isolates.

Strains N˚	5% alcohol	10% alcohol	15% alcohol	20% alcohol	25% alcohol
VMA1	+++	+++	+++	++	+
VMA5	+++	+++	+++	++	+
VMA7	+++	+++	+++	++	+
VMAM	+++	+++	+++	+	−
VMAO	+++	+++	+++	++	+

+++: good growth; ++: medium growth; +: poor growth; −: negative growth.

3.3. Acetic Acid Concentrations Tolerance Ability

The acetic acid resistance properties of the isolates were studied on YPG medium broth in presence of 5% (v/v) ethanol concentration supplemented with acetic acid concentrations ranging from 0.5% - 6% (w/v) and incubated at 37˚C for 5 days. As it is shown in Table 3, all the strains could tolerate up to 4.5% acetic acid concentration. Strains showed really good growth up to 4% (w/v) acetic acid concentration; strains VMA1 showed poor growth at 4.5% (w/v) acetic acid concentration and no growth was observed at 5% (w/v). Strains VMA5 and VMAM showed poor growth at 5% (w/v) acetic acid and no growth was observed above for these isolates. As for strains VMA7 and VMAO, they demonstrated medium growth at 5% and 5.5% (w/v) acetic acid and showed a tolerance at 6% (w/v) acetic acid.

Table 3. Acetic acid resistance ability of the bacterial isolates.

Strains N˚	0.5%	1%	1.5%	2%	2.5%	3%	3.5%	4%	4.5%	5%	5.5%	6%
VMA1	+++	+++	+++	+++	+++	+++	+++	+++	+	−	−	−
VMA5	+++	+++	+++	+++	+++	+++	+++	+++	++	+	−	−
VMA7	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
VMAM	+++	+++	+++	+++	+++	+++	+++	+++	++	+	−	−
VMAO	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+

+++: good growth; ++: medium growth; +: poor growth; −: negative growth.

3.4. Effects of Temperature on Acetic Acid Production

The effect of temperature variation on the acetic acid production capacity of the five strains was investigated at different temperatures ranging from 35˚C to 50˚C in a CARR medium containing green bromocresol as a pH indicator by measuring the diameter of the halo formed by AAB and related to the amount of the produced acid. After 10 days of incubation, results are presented in Figure 2. Maximum acid production capacity was obtained at 40˚C for VMA1, VMA5, and VMAM. VMA1 produced the same amount of acid at 40˚C as well as at 35˚C. Strains VMA5 and VMAM showed a bit of more acid amount production at 45˚C than at 35˚C. As for strains VMA7 and VMAO, they showed their maximum acid production at 45˚C. Our results also showed that no acetic acid production was observed above 45˚C except for VMA7 and VMAO. Yet, results of temperature tolerance showed all tested strains could grow up to 50˚C. At 50˚C of temperature, a long lag time of 6 days was observed for VMA7 and VMAO.

3.5. Effects of Ethanol on Acetic Acid Production

The influence of ethanol concentration on produced acetic acid was also assessed on CARR medium plates supplemented with ethanol ranging from 10% to 25% (v/v) and incubated at 37˚C for 10 days. As it is shown in Figure 3, maximum

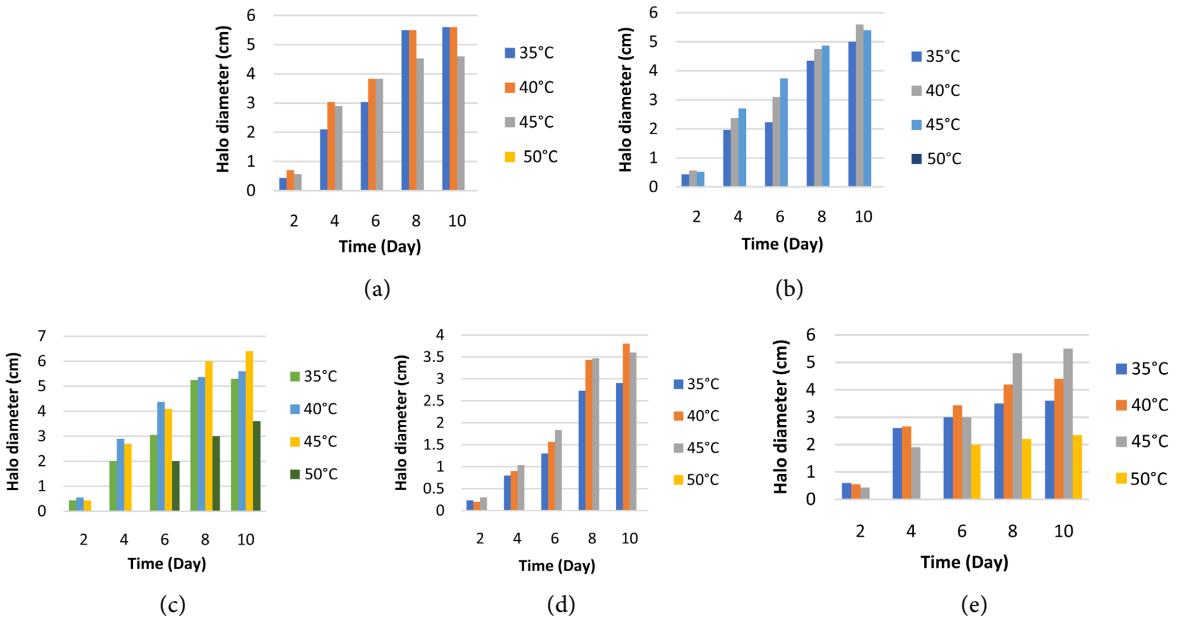

Figure 2. Effect of temperature on acetic acid production. (a): VMA1; (b): VMA5; (c): VMA7; (d): VMAM; (e): VMAO.

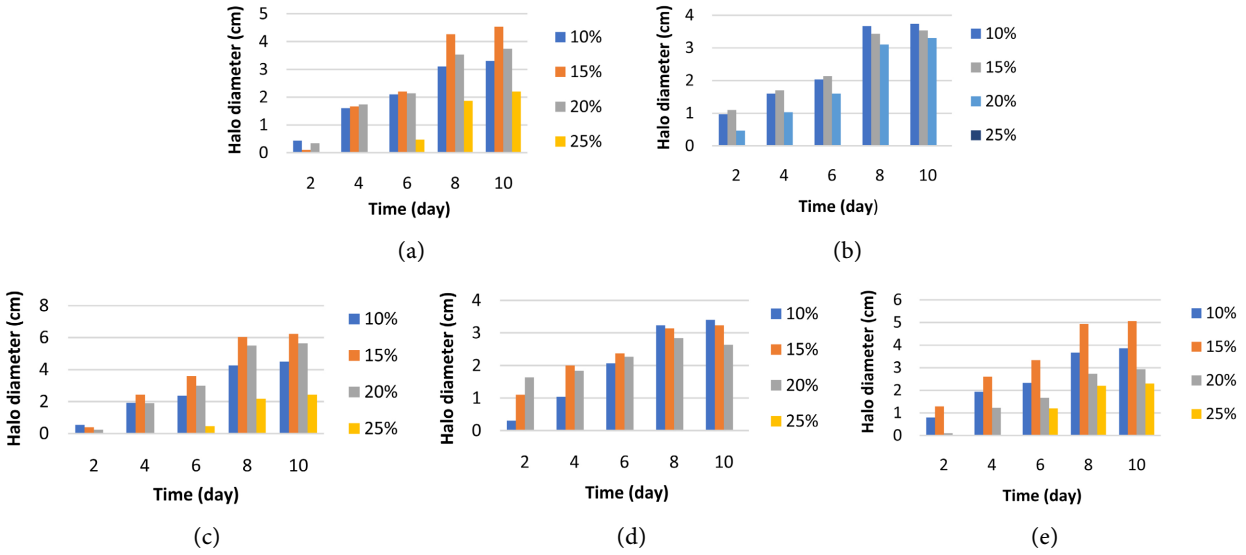

Figure 3. Effect of ethanol concentrations on acetic acid production. (a): VMA1; (b): VMA5; (c): VMA7; (d): VMAM; (e): VMAO.

acid production was obtained at 15% (v/v) ethanol for strains VMA1, VMA7, VMAO, and 10% (v/v) ethanol for strains VMA5, VMAM. All strains produced considerable amounts of acid up to 20% ethanol. Strains VMA1 and VMA7 showed a higher amount of acetic acid at 20% than at 10% (v/v) ethanol. Only strains VMA1, VMA7 and VMAO demonstrated acid production at 25% (v/v) ethanol. However, at this ethanol concentration, a long lag phase of 6 days was observed.

4. Discussion

The use and application of AAB have a long history in fermentation processes

and the most known exploitation of AAB is the oxidation of ethanol into acetic acid commonly known as vinegar production. During the acetification process, the activity of AAB is affected by many variables such as temperature, ethanol concentration, acidity accumulation, and dissolved oxygen [17]. Screening potential AAB which could be tolerant to different drastic conditions is of great importance. In this study, five strains tolerant to temperatures, ethanol, and acetic acid respectively greater than or equal to 50˚C, 10% (v/v), and 4.5% (w/v) were isolated from fermented mango alcohol. These strains were regarded as *Gluconoacetobacter* from the previous study [15].

Temperature is one of the most important environmental factors that affect the growth and survival of microbes [18]. Most of the acetic acid bacteria are mesophilic with an optimum growth temperature of 30˚C, but strains that can grow at a temperature of 42˚C have been also reported and known as thermotolerant strains [19]. However, since vinegar fermentation in submerged culture is an exothermal process, where temperature increases up to about 35˚C or higher, the performance of traditional mesophilic strains would be badly affected resulting in a severe reduction in fermentation rate and efficiency [20]. Generally, the fermentation process faces different challenges in hot areas. In addition, recently, climate change and global warming have become major challenges for the fermentation technology of AAB due to their effects on the growth and development of AAB [12]. Cooling equipment must be used to overcome high temperatures, which would require additional costs [21]. Therefore, an ideal isolate for the vinegar industry should tolerate a wide range of temperatures (30˚C - 42˚C) [22]. Currently, the use of thermotolerant and thermophilic AAB is unavoidable in vinegar production in order to reduce in a considerable way the cooling water expenses during production in bioreactor and to prevent great mortality during storage after processes such as atomization, freeze-drying [23]. In addition, cultivable and phenotypically stable thermotolerant AAB that can be exploited as biocatalysts are increasingly sought after for a number of biotechnological applications [24] [25]. Thermotolerant AAB is AAB that belongs to the same genera or same species as mesophilic strains but can grow at a temperature of 5 to 10˚C higher than generic mesophilic strains do, and these strains differ from thermophilic strains that could grow above 60˚C [20]. Currently, studies on the selection of AAB are focused on the screening of heat-resistant bacteria from products of tropical and sub-tropical regions such as those isolated from products of Sub-Saharan Africa [21] [26]. These strains may have acquired resistance to high temperatures and have obtained phenotypic proliferation under stress conditions by adapting to their habitats; indeed, AAB has been reported to exhibit phenotype instability, which can occur by either, temporal acclimation or heritable adaptation [27] [28]. In this study, all five isolates AAB showed a great growth up to 40˚C and tolerated up to 50˚C. Strains VMA7 and VMAO showed their highest specific growth rate at 40˚C and VMAO even resisted at 60˚C. Moreover, all five isolates presented their highest acid acetic production capacity at temperatures ≥ 40˚C. Strains VMA7 and VMAO even presented the ability to

produce a significant amount of acid acetic at 50˚C with a lag phase of 6 days. These strains were then regarded as thermotolerant AAB. These findings are consistent with those reported by [27] who isolated AAB from cocoa beans in Côte d'Ivoire capable to grow at 50˚C. Also, these results are greater than many of those already reported by researchers. In fact, those reported by [17] [19] [23] [28] [29], presented isolated AAB strains with temperature tolerance that does not exceed 45˚C.

AAB can produce acetic acid from ethanol through oxidative fermentation; Ethanol is first oxidized to acetaldehyde by a membrane-bound pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH); subsequently, acetaldehyde is oxidized to acetic acid by aldehyde dehydrogenase (ALDH) [30]. Ethanol is then the main carbon source for AAB and it is noteworthy that acetic acid fermentation using a high concentration of ethanol can improve the content of organic acid and the quality of vinegar [31]. However, this alcohol can itself become an extreme stressing factor when it is above 7% (v/v) [32]. In addition, for certain vinegar production such as orange vinegar, where orange wine can have an alcoholic content of 13% - 14%, a dilution has to be made in order to facilitate the action of acetic acid bacteria; the later causes a variation in the concentration of nutrients, and a solution with minerals and a source of nitrogen had to be added [33]. Moreover, it has been proven that over-oxidation can be avoided through high initial ethanol concentration [34]. Thus, for the optimum growth of AAB and the high-quality vinegar, ethanol must be precisely controlled and ethanol-tolerant AAB needs to be screened out [17]. In this study, all strains showed a growth of up to 20% (v/v) alcohol. Strains VMA1, VMA5, VMA7, and VMAO showed an extreme resistance to high ethanol concentrations (25% (v/v)). In addition, all five isolates presented their highest acid production capacity at ethanol concentrations greater or equal to 10% (v/v). Moreover, strains VMA1 and VMA7 produced a higher amount of acetic acid at 20% than at 10% (v/v) ethanol and these last two-plus VMAO could even produce acetic acid at 25% (v/v) ethanol. These strains were regarded as extremely tolerant to ethanol. Yet, in previous studies, [15] demonstrated that strains VMAM and VMAO showed ethanol tolerance only up to 10% (v/v) and VMA7 only up to 15% (v/v) ethanol. This increase in the ethanol resistance of these 3 strains might be explained by the fact that in this present study, strains were incubated at 37˚C instead of 30˚C. Indeed, some authors have reported that the ADH of thermotolerant strains exhibited higher resistance to ethanol at elevated temperatures [31] [35]. The findings in this study are consistent with those reported by [36] but are greater than those reported by [17] [20] [37] [38].

Acetic acid is very important in the industrial sector; the demand for acetic acid has been steadily increased because of its use in a variety of industries [39]. Today, its biological production accounts for only about 10 percent of world production but it remains important for vinegar production, because many of the world food purity laws stipulate that vinegar used in foods must be of bio-

logical origin [40] [41]. Nevertheless, the increasing acetic acid concentration during the fermentation process is not favorable to the cell. Acetic acid accumulation causes acid stress that inhibits AAB growth [42]. Thus, how AAB survives and subsists under an extreme acidic environment is always being an interesting question in the industrial and fundamental research fields [43]. The main reason acetic acid is toxic to microorganisms is its ability to cross the cell membrane and enter cells. This increases intracellular acetic acid concentrations and disrupts some physiological functions of the cell membrane [44]. Considering that acetic acid is the main organic acid in vinegar fermentation, high acid tolerance ability is important for industrial strains. In this study, it has been shown that all the isolated strains can tolerate up to 4.5% (w/v) of acetic acid. The highest acid resistance was observed with VMA7 and VMAO who demonstrated resistance at 6% (w/v) of acetic acid at 37˚C in presence of 5% ethanol and were then regarded as acid-resistant. The adaptation to these extreme conditions must be the consequence of genome mutations and rearrangements [45]. The mechanism of acid resistance of AAB is still being studied; [46] reported that *Gluconacetobacter europaeus* V3 facing increased concentration of acetic acid changed the total fatty acid composition of cells; [42] reported that *Komagataeibacter europaeus* V3, a highly acetic acid-tolerant strain within acetic acid bacteria, has developed a strategy to resist high concentrations of acetic acid, which consists of increasing the cell size; [47] showed that *K. europaeus* CGMCC 20445 regulates the gene expression levels of cell envelope proteins and stress-responsive proteins to adapt to the gradual increase in acidity during acetic acid fermentation; [48] demonstrated that under the condition of 0.5%, 1.0% and 1.5% acetic acid, the intracellular ammonium concentration increased by 1.49, 1.78 and 2.07 times; [49] related that Pyrroloquinoline quinine (PQQ)-dependent alcohol dehydrogenase (ADH) has a resistance to acetic acid and some major enzymes in the TCA cycle, citrate synthase and aconitase have been promoted the TCA cycle associated with acetic acid assimilation. Due to outstanding tolerance towards acetic acid, AAB Acid Resistance (AR) mechanisms will add valuable knowledge to the encyclopedia of bacterial AR strategies [50].

5. Conclusion

The oxidation of ethanol to acetic acid in order to produce vinegar is one of the best well-known use of AAB in industries. However, during the acetification process, AAB are affected by many variables such as temperature, ethanol concentration, acidity accumulation. This study allowed us to test thermotolerance, ethanol-tolerance, and acid-tolerance properties of five AAB strains isolated from previous studies. The tested strains have the ability to grow and produce acetic acid at temperatures ≥ 45˚C; they tolerate high concentrations of ethanol ≥ 20% (v/v). In addition, they show resistance to acetic acid concentrations ranging 4.5% - 6% (w/v). These strains were regarded as thermo-ethanol-acetic acid-tolerant and could be used as a starter in vinegar production in order to

reduce the cooling cost of bioreactor and enhance the quality of the produced vinegar.

Conflicts of Interest

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

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