High-temperature production of acerola vinegar using thermotolerant *Acetobacter senegalensis* A28

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Received 11 May 2018; accepted 4 September 2018

Abstract:

Acerola (*Malpighia emarginata*) vinegar is one kind of fruit vinegar produced using acetic acid fermented by acetic acid bacteria. In this study, *Acetobacter senegalensis* A28 was applied in acerola vinegar production at a high temperature. With 1.0% (v/v) of *A. senegalensis* A28 inoculum, the suitable conditions for production of acerola vinegar were determined to be pH 6.5, 20°Brix, 10^6 cells/ml, as well as 4.0% (v/v) of ethanol added to acerola juice. Moreover, a temperature of 35°C was found to be suitable for fermentation in both 100 ml and 2 litre working volumes. Total acid concentrations of 5.45% (w/v) and 4.90% (w/v), respectively, were achieved after 4 days of fermentation. Acid concentrations of 5.40% (w/v) and 4.70% (w/v) were obtained at 37°C after 5 days of fermentation in volumes of 100 ml and 2 litres, respectively. At pilot-scale fermentation of 20 litres at 37°C, acid concentration of 3.68% (w/v) was achieved after 5 days of fermentation.

Keywords: **acerola, acetic acid bacteria, *Acetobacter senegalensis,* fruit vinegar, thermotolerance.**

Classification number: **2.2**

Introduction

Fermentation products have been studied to improve their quality, productivity, scale, and diversity to meet consumers' increasing demands. Today, such research brings the possibility of the application of the results to industrial production [1]. One of these products is vinegar, a condiment that is indispensable in our daily diet; in addition, it plays an important role in supporting and maintaining human health. At present, many new types of vinegar are being studied and put into trial production at low cost. These are based on inexpensive and readily available raw materials, compared to traditional rice vinegar, in order to meet the current market's demands. Among the variety of methods of fermentation, sink methods are most commonly used. Much research has been conducted to explore the possibility of producing fruit vinegar from many sources, such as pineapple peel [2]. Acetic acid bacteria (AAB), one of the essential bacteria in vinegar fermentation, are being studied by scientists (e.g. oxidation mechanisms of ethanol to produce acetic acid, methods for identification, etc.), and achieve a variety of applications in practice. Thus, the process of vinegar fermentation is being optimised day by day. They are very diverse in nature (appearing more in fruits, grains, herbs, etc.) and are important in the food industry for oxidizing sugar and wine into acetic acid [3, 4]. Therefore, AAB play an important role in the industrial production of vinegar. In addition, they can be used in the production of cellulose and sorbose [5]. Temperature is a factor that plays an important role - it is the deciding factor in vinegar fermentation. However, climate change which is increasing global temperatures, is significant challenge for the fermentation ability of AAB. The optimal temperature for the fermentation of AAB is 28-30°C, and increased temperature has a strong influence on the production of vinegar [1, 6]. Therefore, today, the study of thermotolerant

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strains of AAB is an important consideration, not only for scientists but also for producers. In addition, the application of heat-resistant strains of AAB in large-scale industrial manufacturing is widely studied at present.

The goal of this paper is twofold: to determine the suitable proportion of ethanol to be added, and the favorable pH value, °Brix, and cell density; and to study high-temperature vinegar fermentation from acerola at working volumes of 2 litres and 20 litres.

Materials and methods

Culture and materials

Thermotolerant *A. senegalensis* A28 was isolated from a rice wine starter, identified, and stored at the Food Biotechnology Laboratory, Biotechnology Research and Development Institute, Can Tho University. Acerola which had ripened to a red colour was selected and purchased at An Nghiep market, Ninh Kieu district, Can Tho, Vietnam.

Examination of the characteristics of A. senegalensis A28

A. senegalensis A28 was cultured in yeast extract-peptone-glycerol-D_glucose (YPGD, yeast extract 5 g/l, peptone 5 g/l, glycerol 5 g/l, D-glucose 5 g/l) agar and broth for 24-48 hours at 35°C to observe the bacterial shape and cells under the microscope. Gram stain, catalase, and oxidase tests were conducted

Study of the suitable proportion of ethanol to be added to the acerola juice

The experiment was designed according to completely random design (CRD), with one factor, five levels (proportion of ethanol added (4, 5, 6, 7, 8% v/v)) and three replications. °Brix, pH, and cell density of the acerola juice were adjusted based on the suitable conditions selected from the previous section, and were supplied with a percentage of ethanol as experimental design. The amount of acid, pH value, and amount of total sugar were determined during 7 days of fermentation in aerobic conditions at 35°C.

Study of the suitable levels of °Brix, pH, and cell concentration for vinegar fermentation

The experiment was designed according to CRD, with 3 factors, 3 levels, and 3 replications: °Brix (15, 20, 25); pH (3.5, 5, 6.5); cell concentration (10^3, 10^5, 10^7 cell/ml). *A. senegalensis* A28 was cultured in a YPGD medium for 48 hours at 30°C, and the acerola juice was diluted with distillated water at a ratio of 1:4. °Brix, pH, and cell density were adjusted according to the experimental design, sterilised with $NaHSO_3$ (140 mg/l), and supplied with 4% (v/v) ethanol. The amount of acid amount, pH value, and amount of total sugar were determined during 7 days of fermentation in aerobic conditions at 35°C.

Study of vinegar fermentation with a working volume of 100 ml

The experiment was designed according to CRD with 3 levels of temperature: 35°C, 37°C and 39°C, and 3 replications. °Brix, pH, cell density, and the percentage of ethanol in the acerola juice were adjusted based on the suitable conditions selected from the previous sections. The prepared acerola juice with a volume of 100 ml was incubated at 35°C, 37°C, and 39°C. The amount of acid, pH value, and amount of total sugar were determined during 7 days of fermentation in aerobic conditions.

Study of vinegar fermentation with a working volume of 2 litres

The experiment was designed according to CRD with 3 levels of temperature: 35°C, 37°C and 39°C, and 3 replications. °Brix, pH, cell density, and the percentage of ethanol in the acerola juice were adjusted based on the suitable conditions selected from the previous sections. The prepared acerola juice with a volume of 2 litres was incubated at 35°C, 37°C, and 39°C. The amount of acid, pH value, and amount of total sugar were determined during 7 days of fermentation in aerobic conditions.

Study of vinegar fermentation with working volume of 20 litres

The experiment was designed according to CRD with 2 levels of temperature (37°C and 39°C) and 3 replications. °Brix, pH, cell density, and the percentage of ethanol in the acerola juice were adjusted based on the suitable conditions selected from the previous sections. The prepared acerola juice with a volume of 20 litres was incubated at 37°C and 39°C. The amount of acid, pH value, and amount of total sugar were determined during 7 days of fermentation in aerobic conditions.

Acerola vinegar products with the working volumes of 2 litres and 20 litres underwent preliminary analysis using quality indicators following Vietnam's standard No. 3215:79. This included: sensory evaluation (colour, odour, and clarity) by 10 people, as well as pH, amount of retentive ethanol, and amount of total acid amount.

Data analysis

The results of these experiments were calculated using Microsoft Excel 2013 (Microsoft Inc., USA) software and statistically analyzed using Statgraphic Centurion XV (Statgraphics Technologies Inc., USA).

Results and discussion

Morphological and biochemical characteristics of A. senegalensis A28

The colonies of bacteria were circular, convex, opaque, and beige, and the cells were coccoid when observed under the microscope. The results of the Gram stain, oxidase, and catalase tests showed that *A. senegalensis* A28 is Gram negative, oxidase negative, and catalase positive. In summary, the morphological and some of the biochemical characteristics of *A. senegalensis* A28 are completely suitable with previous studies of such colonies: circular, convex, opaque, beige, coccoid cells; Gram negative, oxidase negative, and catalase positive [7].

The suitable proportion of ethanol to be added for vinegar fermentation

The initial acerola juice was acidic so it had a lower amount of total acid (3.6% (w/v) and 0.3% (w/v), in turn) and a higher pH value after we adjusted to pH 6.5. Moreover, the amount of total sugar in the juice after we adjusted 20°Brix doubled in comparison to what it had been initially (62.1 g/l and 116.5 g/l). The results of the experiments with 4, 5, 6, 7, and 8% (v/v) ethanol added to the prepared acerola juice are presented in Fig. 1.

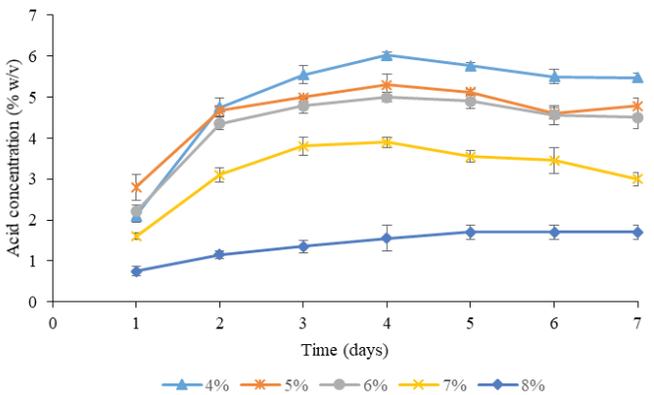

Fig. 1. Total acid production by *A. senegalensis* A28 at different ethanol concentrations.

In almost all the treatments, the amount of acid produced increased from the 1st day to the 4th day and decreased in the last 3 days, especially, the treatment that had 4% ethanol added. Following 7 days of fermentation, *A. senegalensis* A28 in the acerola juice containing 4% (v/v) ethanol produced a larger amount of acid than the other treatments; the highest amount of acid produced was 6.02% (w/v) on the 4th day. The pH value dropped dramatically compared to the initial value (ranging from 4.18 to 3.59 on the final day). The total amount of sugar used in all these treatments was not particularly high (from 13.09 g/l to 11.03 g/l). These results were also suitable with studies of the supplemented the YPGD medium with 4% ethanol [3]. However, the treatment that added 2% and 3% ethanol (v/v) will need to be studied in order to understand more clearly the suitable percentage of ethanol for this strain to grow in acerola juice. In summary, the research supports the finding that the treatment that adds 4% ethanol (v/v) is the suitable concentration (with the acid produced amounting to 6.02% w/v).

The suitable conditions for vinegar fermentation

The results of testing different conditions over 7 days of fermentation are shown in Fig. 2. The surface plotting was constructed using Statgraphics software.

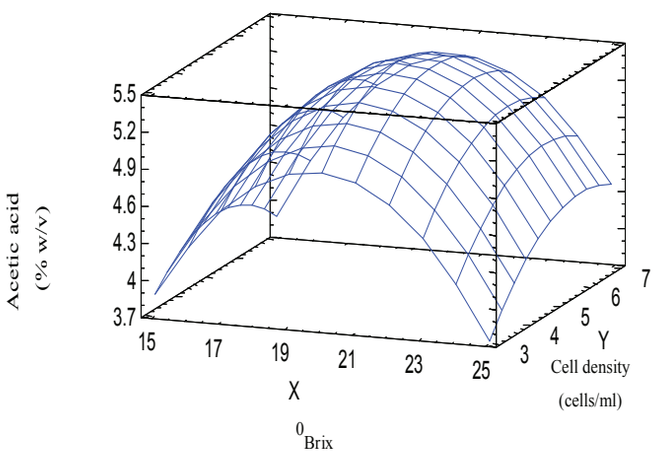

Fig. 2. Surface plotting of acetic acid production.

The final equation in terms of coded factors: Acid content = -19.4669+1.997×X+ 0.798333×Y+0.449444×6.5-0.0468×X×X+0.019×X×Y-0.0288333×X×6.5-0.0825×Y×Y-0.0316667×Y×6.5+0.0488889×6.5×6.5-0.0005×X×Y×6.5

Here, X is the concentration of sugars and Y is cell density. The results were obtained after solving the equation X=20.26°Brix, Y=10^6 cells/ml. As a result, the optimal condition for fermentation was determined as 20.26°Brix (concentration of sugars), initial pH 6.5, and 10^6 cells/ml (cell density). The highest amount of acid produced was 5.88% (w/v). After fermentation, the pH values were reduced. However, thermotolerant bacteria can grow in pH conditions ranging from 3.0 to 7.7. In addition, the

optimal fermentation conditions for acerola vinegar in this experiment were similar to the conditions for the fermentation of banana vinegar: the highest acetic acid level obtained was 4% (v/v) after 6 weeks of fermentation at 37-38°C in a medium containing 5% ethanol (v/v), 20.62 g/l used sugars, and 10^5 cells/ml [8].

Vinegar fermentation at 35°C, 37°C, and 39°C with a working volume of 100 ml

The results of fermentation with *A. senegalensis* A28 in 100 ml of prepared acerola juice are presented in Fig. 3. The amount of total acid of treatments incubated at 35°C and 37°C was much higher than others. Indeed, *A. senegalensis* A28 incubated at 35°C produced the largest amount of acid on the 4th day of fermentation (5.45% w/v); while for the incubation of bacteria at 37°C, the highest amount of acid produced 5.4% (w/v) on the 5th day because when incubating at 37°C, bacteria need more time for their development and growth. In order to save both time for fermentation costs of production for treatment at 35°C, day 4 was selected, as the amount of acid produced then was greater than on the other days. Moreover, the pH value after 7 days of fermentation decreased considerably compared to the initial values (3.19 for the 37°C treatment, and 3.68 for the 35°C treatment). The amount of sugar used for treatments incubated at 35°C and 39°C was very low (11.37 g/l and 14.31 g/l, respectively). In comparison, 2.52% (w/v) of acid was achieved from *A. tropicalis* DK4 after 7 days of fermentation in YPGD containing 4% (v/v) ethanol at 39°C. That is, the amount of acid produced in this study was much higher [9]. This research shows that the fermentation capacity of *A. senegalensis* at three levels of temperatures, 35°C, 37°C, and 39°C. 35°C was the most suitable temperature for the fermentation of acerola juice.

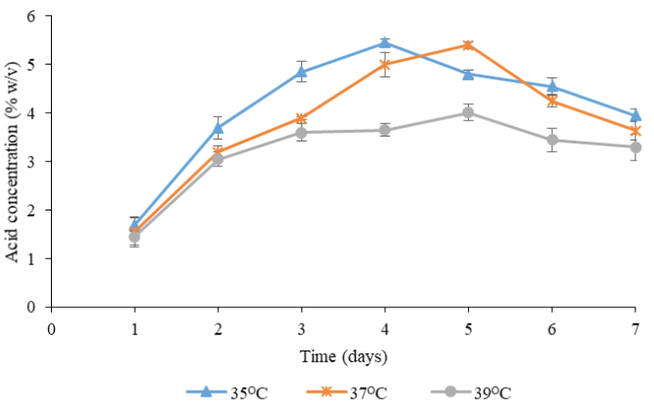

Fig. 3. Total acid production by *A. senegalensis* A28 at a working volume of 100 ml.

Vinegar fermentation at 35°C, 37°C, and 39°C at a working volume of 2 litres

The fermentation results of *A. senegalensis* A28 in 2 litres at 35°C, 37°C, and 39°C over 7 days are presented in Fig. 4. The amount of acid for treatment at 35°C was always higher than two other treatments during fermentation, and, at 35°C, the highest amount of acid was produced on the 4th day (4.9% w/v). However, the amount acid produced in the two treatments at 37°C and 39°C increased from day 1 to day 5 and decreased over the last two days because the bacteria needed time for development and growth. Thus, as with the above experiment, the amount of acid on the 4th day was selected. Moreover, on the 5th day of fermentation, the amount of acid produced was 4.7% (w/v). Subsequently, the range of pH values at the three levels of temperature was 3.58 to 4.01 over 7 days of fermentation. With treatment at 35°C, the amount of sugar used by the bacteria was the lowest (9.9 g/l). In addition, the most suitable temperature for *A. senegalensis* A28 was 28°C [7]. Previous works show that when the temperature increases, the amount of acid produced decreases [1, 10]. Thus, we see that at 35°C, the amount of acid produced was the largest; it decreased when temperature was increased to 37°C and 39°C. In conclusion, experiment shows the fermentation capacity of bacteria at temperatures of 35°C, 37°C, and 39°C at the working volume of two litres.

According to the experiments at the working volume of 100 ml and 2 litres, 35°C was the suitable temperature level for fermentating with acerola juice and *A. senegalensis* A28. However, at 37°C and 39°C, this strain also demonstrated good fermentation capacity and good ability for growth. The purpose of these experiments was to test the fermentation ability of *A. senegalensis* A28 at high temperatures; hence, these two temperatures were selected for fermentation at a larger scale.

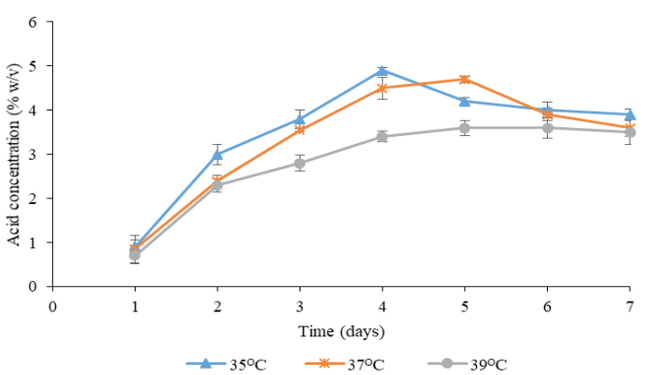

Fig. 4. Total acid production by *A. senegalensis* A28 at a working volume of 2 litres.

Vinegar fermentation at 37°C and 39°C at a working volume of 20 litres

A. senegalensis A28 was fermented with 20 litres of prepared acerola juice, and incubated at 37°C and 39°C in aerobic conditions for 7 days. The results are presented in Fig. 5. Over 7 days of fermentation, *A. senegalensis* A28 produced a higher amount of acid when incubated at a working volume of 20 litres at 37°C (3.68% (w/v) on day 5) in comparison to incubation at 39°C. The amount of acid increased from the 1st day to the 5th day, and decreased over the last 2 days; thus, on days 6 and 7, the amounts of ethanol and sugar were not enough for the bacteria to use for growth and to produce acid.

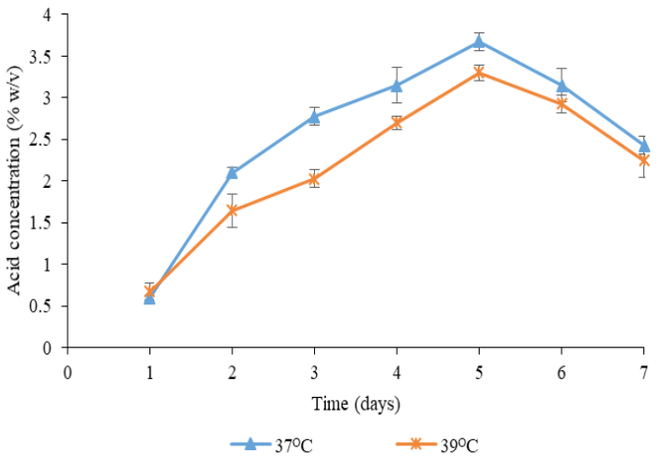

Fig. 5. Total acid production by *A. senegalensis* A28 at a working volume of 20 litres.

At the two levels of temperature, the highest amount of acid was produced on the 5th day of fermentation, so day 5 was selected. The pH value of acerola juice declined dramatically compared to what it had been initially (both 3.52 for the two treatments on the final day). as the amount of sugar used by the bacteria with treatment at 37°C was lower (7.79 g/l). This experiment illustrates that at 37°C, *A. senegalensis* A28 has better fermentation capacity. In comparison with the working volume of 100 ml and 2 litres, the amount of acid produced with the working volume of 20 liters was continuously lower because of the aerobic condition for the fermentation of acetic acid bacteria [11]. Indeed, when bacteria were incubated at larger scale, the surface that was in contact with the atmosphere was decreased, leading to a decline in the amount of acid.

In addition, for acerola vinegar products at a working volume of 2 litres and 20 litres, sensory evaluation by 10 people was undertaken, as was analysis of pH, the amount of acid, and the amount of retentive ethanol. The results of physical and chemical indicators show that the pH and the amount of acid produced correlate with each other. The highest amount of acid produced in five treatments was 5.9% (w/v). The amount of acid in the vinegar was equivalent to that of some vinegar products in supermarkets, such as apple vinegar, rice fermented vinegar, and the like (approximately 4-7% acid, depending on the products). In addition, the amount of retentive ethanol in the acerola vinegar products was very low (the largest was 0.032%); thus, it would not affect consumers' health. The results of the sensory evaluation following TCVN 3215:79 in almost all treatments showed a general score that was higher than 15.2. This means that acerola vinegar products were evaluated as being of 'moderately good' quality. However, treatment at 39°C at a working volume of 2 litres obtained a score of 13.0, that is, a 'medium' standard of quality. Moreover, acerola vinegar products that were incubated at 37°C at working volumes of 2 litres and 20 litres attained higher scores than the others.

Conclusions

The addition of 4% (v/v) ethanol to acerola juice (pH 6.5, 20°Brix), initial bacterial cell density of 10^6 cells/ml, and a fermentation temperature of 35°C are suitable conditions for acerola vinegar fermentation using *A. senegalensis* A28. The amounts of acid at 5.45% (w/v) and 4.90% (w/v) were achieved at working volumes of 100 ml and 2 litres, respectively, after 4 days of fermentation. The amount of acid at a working volume of 100 ml was 5.4% (w/v) and at a working volume of 2 litres was 4.7% (w/v). The acerola juice was fermented for 5 days at 37°C. At a working volume of 20 litres, the highest amount of acid was 3.68% (w/v) when incubated for 5 days at 37°C. In addition, evaluation of organoleptic, physical, and chemical indicators illustrate that treatment at 37°C produced a delicious product that is suitable for the tastes of consumers.

ACKNOWLEDGEMENTS

This research was jointly supported by the Ministry of Science and Technology of Vietnam (contract Nr. 09/2014/HĐ-NĐT); the Advanced Programme in Biotechnology, Can Tho University; and the New Core-to-Core Programme (2014-2019). We would like to thank Prof. Dr Kazunobu Matsushita and Assoc. Prof. Dr Toshiharu Yakushi (Department of Biological Chemistry, Yamaguchi University) for their support and guidance with acetic acid bacteria identification under the JSPS and SSSV exchange research at Yamaguchi University, Japan.

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