Selection and Identification of Thermotolerant Acetic Acid Bacteria for Application in Acerola Vinegar Fermentation

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Abstract

Vinegar fermentation is essentially a two-step process comprising anaerobic conversion of sugars to ethanol and aerobic oxidation of ethanol to acetic acid. Vinegar can be produced from many juice extracts using yeast and acetic acid bacteria (AAB). This research identified the genetic relation between some thermotolerant AAB strains, and the factors that affected vinegar production from Acerola (*Malpighia emarginata*) juice at high temperatures. The interaction between yeast *Saccharomyces cerevisiae* and AAB was investigated. Yeast was fermented for one day before inoculating the AAB and fermenting the mash. Acid production was 2.67% (w/v). Eight thermotolerant AAB strains (A1, A3, A18, A19, A28, A31, A50, and A55) were selected which produced 3.68-4.24% (w/v) of total acid at 37°C. Strain A28 with acid levels of 2.84% (w/v) and 2.88% (w/v) was selected for fermentation at 38°C and 39°C, respectively. The 16S rDNA genes of the eight AAB strains were sequenced and compared with reference strains in GenBank. Results indicated that most of the strains were closely related to species in the genus *Acetobacter* including *Acetobacter tropicalis, A. pasteurianus, A. sicerae* and *A. senegalensis*. The highest acidity of Acerola vinegar fermentation was obtained for *A. senegalensis* A28 at 39°C, with 5.34% (w/v) and sugar concentration of 20.26°Brix, cell density of 10^6 cells/mL, and pH 6.5.

Keywords: Acerola, acetic acid bacteria, thermotolerant, vinegar fermentation

Introduction

Acetic acid bacteria (AAB) play an important role in food technology because of their ability to oxidize alcohol into acetic acid [1]. Besides vinegar production, AAB are utilized for manufacturing cellulose and sorbose. Natural production of vinegar depends on a mixed fermentation involving both yeast and AAB [2]. Vinegar fermentation is a two stage process; the first step is the anaerobic fermentation of conversion sugars to ethanol by yeasts, usually *Saccharomyces* species, and the second is the aerobic oxidation of ethanol to acetic acid by bacteria, usually *Acetobacter* species.

Vinegar is a traditional acidic condiment, widely produced from rice, malt, apples, wine, and various other agricultural materials [3]. It can be successfully produced from fruit juice. Acerola vinegar is a nutritious food with high contents of minerals and vitamins.

Recently, climate change and global warming have become major challenges for fermentation technology of AAB [4-6]. The optimal temperature for fermentation is 30°C, and a temperature increase of only 2-3°C negatively influences AAB growth and development [4, 7]. Using thermotolerant AAB for acetic acid production can upgrade the product quality and minimize the cost for precursors and cooling. This research evaluated and selected thermotolerant AAB strains to determine the optimal conditions for vinegar fermentation.

Materials and methods

Cultures

Twenty thermotolerant AAB strains and *Saccharomyces cerevisiae* were stored at the Food Microbiology Laboratory, Biotechnology Research and Development Institute, Can Tho University, Vietnam. *Acetobacter pasteurianus* was obtained from Yamaguchi University, Japan.

Investigation of simultaneous vinegar fermentative interaction between acetic acid bacteria and yeast

Yeast *S. cerevisiae* was cultured in YPD medium for 48 hours at 30°C. *Acetobacter pasteurianus* was cultured in YPGD medium with 10% potato extract for 48 hours at 30°C. Acerola juice extract was adjusted to 15°Brix by the addition of distilled water; the ratio of acerola juice and distilled water was 1:4. The pH of the acerola juice was adjusted to 5.0. Thirty milliliters of acerola juice extract was placed in each Falcon tube. The 1% (v/v) of *S. cerevisiae* cultivated in the medium (10^5 cells/mL) was primarily inoculated into the sterile fermenting medium of acerola juice. Next, the 1% (v/v) of *A. pasteurianus* inoculum (10^6 cells/mL) was also inoculated into the fermenting medium in the fermentation tubes and this was repeated after 1, 2, 3, and 4 days of fermentation. The acid content, pH value, and remained sugar were determined during 7 days of fermentation at 37°C.

Evaluation of the fermentative ability of thermotolerant acetic acid bacteria at different temperatures

Twenty strains of AAB and *A. pasteurianus* were cultured in YPGD medium with 10% potato extract for 48 hours at 30°C. Acerola juice extract was adjusted as previously explained. The 1% (v/v) of *S. cerevisiae* cultivated in the medium (10^5 cells/mL) was primarily inoculated into the sterile fermenting medium composed of acerola juice and incubated at 35°C. After 1 day of inoculation, the 1% (v/v) AAB inoculum (10^6 cells/mL) was inoculated into the fermenting medium in the fermentation tubes and incubated under aerobic condition at 37°C. The acid content, final pH value, and remained sugar were determined during 7 days of fermentation at 37°C. Strains of AAB were selected to test the fermentative capacity at 38°C and 39°C.

Identification of selected thermotolerant acetic acid bacteria

Eight selected AAB strains were cultured in PM medium at 37°C for 24 hours. 16S-rDNA sequences of 8 AAB strains were extracted using the protocol of the Molecular Biology Laboratory of the Biotechnology Research and Development Institute. The DNA extracted was used as a template in PCR with primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1525R (5’-AAAGGAGGTGATCCAGCC-3’) [8] and sequenced by the ABI system. The 16S rDNA sequences were then aligned with the data from GenBank and the NCBI Taxonomy Database to determine the scientific names.

Examination of the favorable conditions for vinegar fermentation

An experimental model was designed with three factors: sugars concentration (15, 20, and 25^{o}Brix), pH (3.5, 5.5, and 6.5), and cell density (10^3, 10^5, and 10^7 cells/mL). The acid content, pH value, and remained sugars were determined during 7 days of fermentation at 39°C. The validation experiment was carried out in a 5-L fermenter with 2 L of juice. The results were analyzed by Statgraphics Centurion XV (version 15.1.02) software. The least significant difference (LSD) with a confidence interval of 95% was determined.

Results and discussion

Simultaneous vinegar fermentative interaction between acetic acid bacteria and yeast

The ethanol content of the treatments fluctuated considerably during 7 days of fermentation. The ethanol contents of all treatments increased on the second and fifth days, and then fell on the sixth day of fermentation. The highest ethanol levels were obtained on the second day with 0.49 g L^{-1}, 0.55 g L^{-1}, 0.53 g L^{-1}, 0.55 g L^{-1}, and 0.55 g L^{-1} for treatments of AAB inoculum time at 0, 1, 2, 3, and 4 days, respectively. The result of ethanol content in this experiment was better than those obtained by Krusong and Vichitraka [9] who created ethanol concentrations after 24-48 hours ranging from 0.18 to 0.28 g L^{-1} at 30°C. Ethanol contents in this study ranged from 0.49-0.55 g L^{-1} at 37°C.

The highest acid production was obtained on the second day. It then declined gradually on the sixth day and rose slightly on the last day of fermentation (Figure 1). In particular, treatment B (inoculating AAB strains after 1 day fermentation of yeast) had the highest acid content with 2.76% (w/v) on the second day, with efficiency of sugar conversion at 26.2%. Treatment C (inoculating AAB strains after 2-day fermentation of yeast) had the highest sugar metabolism at 39%, but the highest acid content reached only 2.32% (w/v). Treatment A (yeast and AAB inoculated simultaneously) had the lowest acid content and fermentative performance at 2.12% (w/v) and 24.5%, respectively.

Acid contents of the treatments in our experiment were similar to the results of Krusong and Vichitraka [9]. When the yeast and AAB strain were inoculated at the same time, the effective fermentation was lower than in the other treatments. In contrast, the ethanol and acid content were higher for inoculating AAB after 1 day fermentation of yeast. Treatment B (inoculating AAB strains after 1 day fermentation of yeast) reached the highest acid level (2.76% w/v). Therefore, this treatment was chosen to conduct the following experiment.

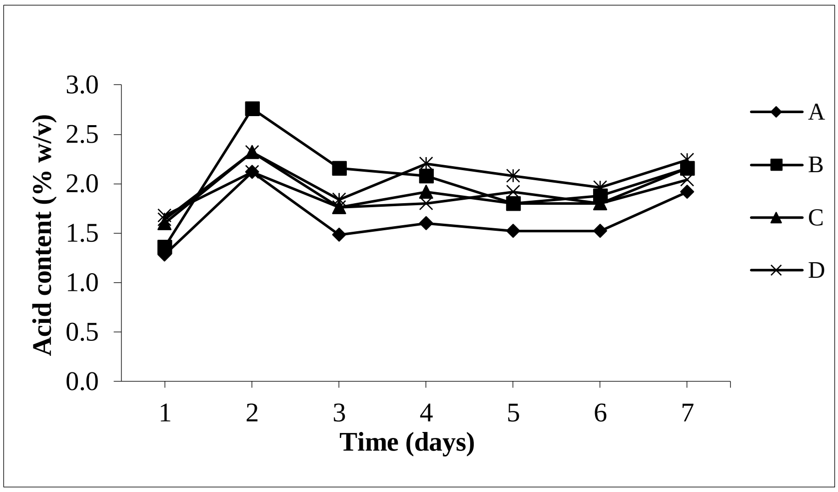

Figure 1. Acid production of five inoculation treatments at 37°C

*__Note:__ A: simultaneous inoculating yeast and AAB; B, C, D, and E: AAB inoculating after 1, 2, 3, and 4 days of yeast fermentation, respectively.

Fermentative ability of thermotolerant acetic acid bacteria

At 37°C, the fermentative ability of all the strains was relatively high and uniform. However, variations in acid content during the 7 days fermentation period changed significantly. From the first to the third day, acid content of the AAB strains increased continuously, then decreased on the fifth day, rose sharply on the sixth day and reduced slightly on the last day. On the sixth day of fermentation, strains A1, A49, A52, and A55 had high acid production with 4.24%, 4.16%, 4.12%, and 4.04% (w/v), respectively. Strain A1 had the highest acid content at 4.24% (w/v). The efficiency of sugar conversion of strain A50 was highest at 78.5% (data not shown).

These results were similar to Soha and Banerjee [10] who obtained acid content at 4.67% and final pH value at 4.0. Their research was conducted on banana juice with 7.77% alcohol level, and 15% *A. aceti* cell concentration at 37°C for 72 hours. Our study compared the fermentative capability among 20 strains at 37°C. Eight AAB strains, included A1, A3, A18, A19, A28, A31, A50, and A55 which produced 3.68-4.24% (w/v) of total acid were selected to study fermentation capacity at 38°C and 39°C.

At 38°C, the acid content of the treatments fluctuated considerably during the 7 days fermentation (Figure 2). Acid production of these strains stabilized from the start of fermentation until the fourth day, but then fell sharply on the fifth day. However, acid content of most of the AAB strains rose and reached maximum value on the last day. Strain A31 had the lowest acid production on the fifth day with 2.12% (w/v). However, on the last day of fermentation, acid content and sugar conversion of this strain reached maximum at 3.08% (w/v) and 23.4%, respectively. In contrast, strain A18 had the lowest acid content at 2.44% (w/v). However, the efficiency of sugar conversion of all eight strains was higher than *A. pasteurianus* at only 16.5%. At 38°C, the AAB strains required longer to adapt to the environmental conditions than at 37°C. Sossou *et al.* [11] demonstrated that the start of acetic acid synthesis was related to the increasing biomass of AAB to begin transforming ethanol into acetic acid.

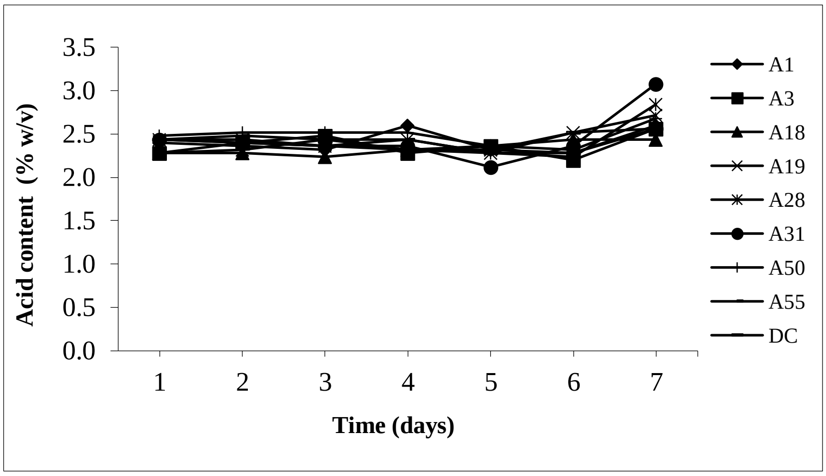

Figure 2. Acid production of eight selected AAB strains at 38°C

(DC: <u>Acetobacter</u> <u>pasteurianus</u>)

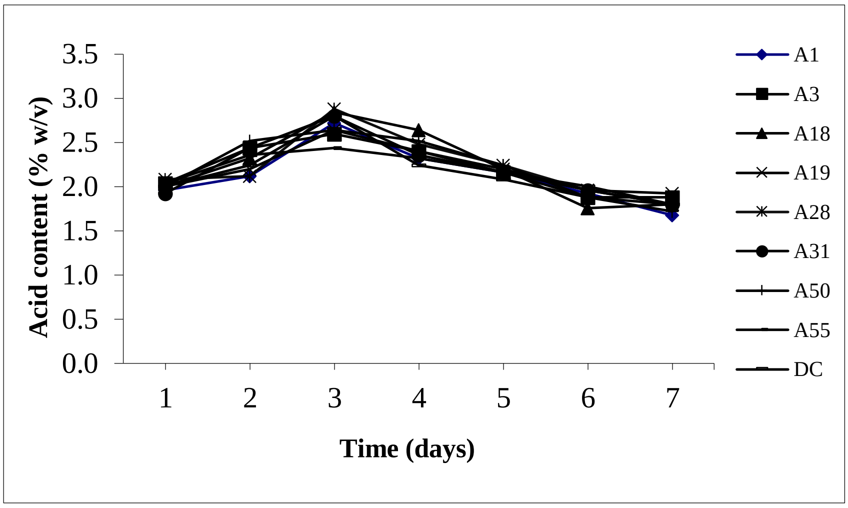

Figure 3. Acid production of eight selected AAB strains at 39°C
(DC: Acetobacter pasteurianus)

At 39°C, acid contents of most strains increased from the first to the third day of fermentation and then decreased gradually (Figure 3). The acid content of strain A28 peaked at 2.88% (w/v), whereas the acid level of strain A55 hit a low point of 2.44% (w/v). The sugar conversion efficiency of strain A28 was the highest with 28.0%, and the lowest was 19.2% for strain A50. With fermentation conducted at 38°C and 39°C the maximum acid contents of these strains were lower than for fermentation at 37°C. Based on fermentation capacity at 38°C and 39°C, strain A28 was selected for study of the optimal condition of acerola vinegar production at 39°C.

Identification of selected thermotolerant acetic acid bacteria

Eight selected AAB strains (A1, A3, A18, A19, A28, A31, A50, and A55) were identified at the species level by sequencing techniques. Results showed that all identified strains belonged to the *Acetobacter* genus with high identities from 97-100%. In 8 AAB identified strains, three (A3, A19, and A31) were *A. tropicalis* species, 2 (A1 and A55) were *A. pasteurianus* species, A18 and A50 belonged to *A. sicerae* species, and strain A28 was *A. senegalensis* species. These bacteria are commonly found in acetic acid fermentation. The bacteria have been studied regarding heat resistance, substrate fermentation, and enzyme activity. Ndoye *et al.* [6] isolated two strains of thermotolerant *A. tropicalis* CWBI-B418-B419 and *A. pasteurianus* CWBI from fruits which produced high acetic acid concentration at 38°C. Furthermore, Kanchanarach *et al.* [8]

successfully isolated two thermotolerant strains *A. tropicalis* SKU1100 and *A. pasteurianus* SKU1108 with high fermentative ability at 42°C.

Favorable conditions for acerola vinegar fermentation

Strain A28 was identified as the optimal condition for fermentation for three factors at 39°C including sugar concentration (15, 20, and 25°Brix), initial pH (3.5, 5.0, and 6.5), and cell density (10^3, 10^5, and 10^7 cells/mL). Surface plotting (Figure 4) and contours (Figure 5) were constructed using the Statgraphics software. The highest acid content was achieved at 5.40% (w/v).

The final equation in terms of coded factors was represented as 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. Where X was sugar concentration and Y was cell density. The results obtained after solving this equation were X = 20.26°Brix, Y = 10^6 cells/mL.

Thus, the optimal condition for fermentation was determined at 20.26°Brix, initial pH 6.5, and 10^6 cells/mL. With sugar concentration of 20.26° Brix, the additional sugar was not too low or too high for fermentation. The efficiency of fermentation was low with a low sugar source, while with a high sugar content the sugar metabolized into gluconic acid. After fermentation the pH value reduced, however, thermotolerant bacteria can grow in pH conditions from 3.0 to 7.7. Yamada *et al.* [12] isolated 64 strains of AAB from fruits, berries, and fermented products on nutrient medium at pH 3.5. The optimal fermentation conditions of acerola vinegar in their experiment were similar to conditions in the fermentation of banana vinegar. Banana vinegar achieved the highest acetic acid concentration at 4.0% (w/v) after 6 weeks of fermentation at 37-38°C in medium containing 5.0% (v/v) ethanol, 20.62 g L^{-1} sugar, and 10^5 cells/mL [13].

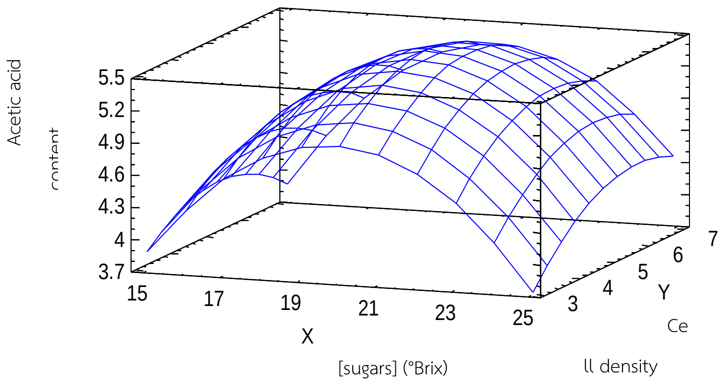

Figure 4. Surface plotting of acetic acid production

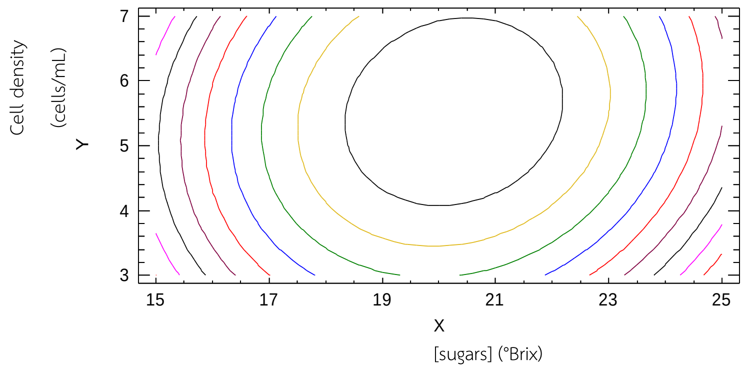

Figure 5. Contours of acetic acid production

The acerola vinegar product had a bright yellow color, no cloudiness, mild aromatic smell and a typical sweet-sour taste. In the validation experiment, the acid content of acerola vinegar reached 5.34% (w/v) after 5 days fermentation at 39°C. The acid content in the validation experiment was very similar to the results calculated according to the three experimental factors. After fermentation, the efficiency of sugar conversion was relatively high (62.3%).

Conclusions

Eight thermotolerant AAB strains (A1, A3, A18, A19, A28, A31, A50, and A55) were selected and produced 3.68-4.24% (w/v) of total acid at 37°C. Identification indicated that the majority of the strains were closely related to species in the genus *Acetobacter* including *A. tropicalis*, *A. pasteurianus*, *A. sicerae*, and *A. senegalensis*. In acerola vinegar fermentation at 39°C with *A. senegalensis* A28, the highest acidity was obtained at 5.34% (w/v) with sugar concentration of 20.26°Brix, cell density of 10^6 cells/mL, and pH 6.5. This result indicated the potential of using thermotolerant acetic acid bacteria for vinegar production at high temperatures.

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References

[1] K. Matsushita. Thermotolerant acetic acid bacteria: Its physiological features and application for high temperature oxidative fermentation. *Proceeding of Satellite Seminar in Asian Core Program,* (2009) The National University of Laos, Laos.

[2] M.R. Adams. Vinegar. In: Wood, Brian, J.B. (Ed.), Microbiology of Fermented Foods. Blackie Academic and Professional, London, (1998) 1-44.

[3] J.I. Horiuchi, .T Kanno, and M.I. Kobayashi. New vinegar production from onions. *J Biosci Bioeng.* 88 (1999) 107-109.

[4] A. Saeki, G. Theeragool, K. Matsushita, H. Toyama, N. Lotong, and O. Adachi. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at high temperatures. *Biosci. Biotechnol. Biochem*. 61 (1997) 138-145.

[5] D. Moonmangmee, O. Adachi, Y. Ano, E. Shinagawa, H. Toyama, G. Theeragool, N. Lotong, and K. Matsushita. Isolation and characterization of thermotolerant *Gluconobacter* strains catalyzing oxidative fermentation at higher temperatures. *Biosci. Biotechnol. Biochem*. 64 (2000) 2306-2315.

[6] B. Ndoye, S. Lebecque, R. Dubois-Dauphin, L. Tounkara, A.T. Guiro, C. Kere, B. Diarawa, and P. Thonart. Thermoresistant properties of acetic acid bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. *Enzyme Microb. Technol.* 39 (2006) 916-923.

[7] S. Ohmori, H. Masai, K. Arima and T. Beppu. Isolation and identification of acetic acid bacteria for submerged acetic acid fermentation at high Temperature. *Agric Biol Chem*. 44 (1980) 2901-2906.

[8] W. Kanchanarach, G. Theeragool, T. Yakushi, H. Toyama, O. Adachi, and K. Matsushita. Characterization of thermotolerant *Acetobacter pasteurianus* strains and quinoprotein alcohol dehydrogenases. *Appl Microbiol Biotechnol.* 85 (2009) 741-751.

[9] W. Krusong and A. Vichitraka. An investigation of simultaneous pineapple vinegar fermentation interaction between acetic acid bacteria and yeast. *Asian Journal of Food and Agro-Industry* 3 (2010) 192-203.

[10] P. Soha and S. Banerjee. Optimization of process parameters for vinegar production using banana fermentation. *International Journal of Research in Engineering and Technology* 9 (2013) 501-514.

[11] S.K. Sossou, Y. Ameyapoh, S.D. Karou, C. de Souza. Study of pineapple peelings processing into vinegar by biotechnology. *Pak. J. Biol. Sci.* 12 (2009) 859-865.

[12] Y. Yamada, R. Hosono, P. Lisdyanti, Y. Widyastuti, S. Saono, T. Uchimura and K. Komagata. Identification of acetic acid bacteria isolated from Indonesian sources, especially of isolates classified in the genus *Gluconobacter*. *J Gen Appl Microbiol.* 45 (1999) 23-28.

[13] N.T.M. Hien and N.M. Thuy. The relationship between acetic acid content and ethanol, sugar, bacteria in the production of banana vinegar. *Can Tho Univetsity Journal of Sciences* (2014) Special issue: *Argiculture,* 76-83.