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Acetic Acid Production at High Temperature by Newly Isolated Thermotolerant *Acetobacter sicerae* A18

Huynh Xuan Phong*, Nguyen My Vi, Bui Thi Thao Anh, Nguyen Ngoc Thanh, Bui Hoang Dang Long, Ngo Thi Phuong Dung

Biotechnology Research and Development Institute, Can Tho University, Can Tho City, Vietnam

Email address

hxphong@ctu.edu.vn (H. X. Phong)

*Corresponding author

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Abstract

Acetic acid is an important organic acid used in many industries such as rubber processing and food processing. Thermotolerant microorganisms including acetic acid bacteria have been increasingly considered and developed in recent years, since they are able to be potentially and popularly utilized in many different issues of fermentation technology at high temperature, leading efficient production and reducing cooling cost. The objective of this study was to select the thermotolerant acetic acid bacteria for their application in acetic acid fermentation at high temperature. In this study, 9 isolates (A1, A3, A8, A18, A23, A47, A49, A50 and A52) were selected from 20 isolates with the acetic acid production ranged from 1.72% to 2.04% (w/v) at 37°C. The amplified 16S-rDNA sequences were employed for the identification of 9 selected strains of acetic acid bacteria. These strains were dominated by representatives from the *Acetobacter* genus with 4 species such as *A. pasteurianus*, *A. tropicalis*, *A. orientalis* and *A. sicerae*. *Acetobacter sicerae* A18 was identified as the most effective fermentative acetic acid bacterial strain at 39°C with acetic acid production was 2.56% (w/v) and productivity was 62.19%. Furthermore, the favorable conditions for acetic acid fermentation by the selected target strain *A. sicerae* A18 were found as follow: pH at 4.0, ethanol concentration at 5.0% (v/v) and the starter density at 10^5cells/mL; acid concentration and productivity were 2.82% (w/v) and 66.02%, respectively.

1. Introduction

Nowadays, the functional roles of thermotolerant microorganisms including acetic acid bacteria (AAB) as the inoculation resources have increasingly considered and developed, since they are able to be potentially and popularly utilized in many different issues of fermentation technology as well as manufacture of useful materials at high temperature, leading efficient production and reducing cooling cost [1-3]. The acetic acid bacteria have been used from ancient times in the domestic arts of vinegar and in the preparation of pickles, and the like, although the nature of the processes was hardly realized. Acetic acid bacteria are well known as Gram-negative or variable, ellipsoidal to rod-shaped cells. They have an obligate aerobic metabolism with oxygen as the terminal electron acceptor [4]. In nature, acetic acid bacteria are found in various kinds of fruits,

flowers, and other sources such as fermented foods in nature. Acetic acid bacteria have been classified into ten genera belonging to the family Acetobacteraceae: *Acetobacter, Gluconobacter, Acidomonas, Gluconacetobacter, Asaia, Kozakia, Zwaminathania, Saccharibacter, Neoasaia,* and *Granulibacter* [5-8]. Among of them, *Acetobacter* and *Gluconacetobacter* that relate to the thermotolerance, are the most popular and have been used for acetic acid fermentation, particularly for the vinegar production [9].

Acetic acid is well-known organic acid which is widely used in many fields and particularly in food industry. Recently, global warming and climate change influence to acetic acid production due to its effects to growth and development of AAB. Acetic acid bacteria that utilized in industries should not only produce acetic acid effectively, but also survive and ferment ethanol at high temperature condition. Recently, climate change and global warming have become major challenges for fermentation technology of AAB [1, 10]. The optimal temperature for fermentation is 30°C, and a temperature increase of only 2-3°C negatively influences AAB growth and development [11].

In Vietnam, acetic acid is usually produced by natural fermentation from fruit, alcohol, starch, etc. The fermentation process is not well conducted at smallholder scale so the quality of the product is not stable as well as low productivity. Temperature is one of the most important factors affecting to the acetic acid fermentation process, especially Vietnam is a tropical country and the temperature can increase to 37-40°C in summer [12]. The study of using thermotolerant AAB for acetic acid production could upgrade the product quality and minimize cost for precursors and for cooling. This study aimed to select thermotolerant AAB with their high acetic acid production as well as to determine the optimum conditions for acetic acid fermentation.

2. Materials and Methods

2.1. Cultures and Medium

Twenty isolates of thermotolerant AAB were maintained at Food Biotechnology Laboratory, Biotechnology Research and Development Institute, Can Tho University, Vietnam. The control strain *A. pasteurianus* from Yamaguchi University, Japan. YPGD medium included yeast extract 5.0 g/L, peptone 5.0 g/L, glycerol 5.0 g/L, D-glucose 5.0 g/L, and supplemented with 4% (v/v) ethanol.

2.2. Evaluation of the Fermentative Ability of Thermotolerant AAB at 37°C

Twenty-one isolates were cultured in YPGD medium with 10% extractive solution of potato for 48 hours at 30°C under aerobic condition (150 rpm). Until the cell density reached 10^7 cells/mL. YPGD medium containing 4% (v/v) ethanol was put 30 mL in falcon tube and the medium was sterilized at 121°C in 20 minutes. There were 63 tubes for triplicate. One percent (v/v) of starter was dispersed into YPGD medium contained in falcon tubes. The fermentation was conducted in 7 days at 37°C under aerobic condition (150 rpm). The data of acetic acid production was collected daily while final pH and remained glucose after fermentation were collected at the end of fermentation process.

2.3. Identification of the Selected Thermotolerant Acetic Acid Bacteria

The universal primers of alpha-protobacteria were used to identify selected thermotolerant AAB. Primers were used in this study included 27F (5'-AGAGTTTGATCCTGGCTC AG-3') and 1525R (5'-AAAGGAGGTGATCCAGCC-3') [6]. 16S rDNA extraction and PCR amplification were carried out at Biotechnology R&D Institute, Can Tho University, Vietnam. The amplified 16S rDNA products were sent to 1st BASE Company (Singapore) and Yamaguchi University (Japan) for sequencing. The 16S rDNA sequences were then aligned with the data from GenBank and the NCBI Taxonomy Database to determine the scientific names.

2.4. Evaluation of the Fermentative Ability of Thermotolerant AAB at 38°C and 39°C

Selected AAB isolates and *A. pasteurianus* were cultured in YPGD medium with 10% extractive solution of potato for 48 hours at 30°C under aerobic condition (150 rpm). Until the cell density reached 10^7 cells/mL. YPGD medium containing 4% (v/v) ethanol was put 30 mL in falcon tube and the medium was sterilized at 121°C in 20 minutes. There were 60 tubes for triplicate of two levels of temperature. One percent (v/v) of starter was dispersed into YPGD medium contained in falcon tubes. The fermentation was conducted in 7 days at 38°C and 39°C under aerobic condition (150 rpm). Acetic acid production was collected daily while final pH and remained glucose after fermentation were collected at the end of fermentation process.

2.5. Examination of the Favorable Conditions for Acetic Acid Fermentation

The favorable conditions for acetic acid fermentation at 39°C were conducted with 3 factors (starter density, initial pH and ethanol concentration). The selected AAB was cultured in YPGD medium with 10% extractive solution of potato for 48 hours at 30°C under aerobic condition (150 rpm). Until the cell density reached 10^7 cells/mL. YPGD medium was adjusted at 4, 5 and 6, then put 30 mL in falcon tube, and sterilized at 121°C in 20 minutes. There were 54 tubes for 2 replicates (Table 1). The ethanol concentrations were supplemented with 4, 5 and 6% (v/v) and cell suspension was dispersed into YPGD medium with the initial densities of 10^4, 10^5 and 10^6 cells/mL. The fermentation was conducted in 7 days at 39°C under aerobic condition (150 rpm). Acetic acid production of treatments was monitored daily using titration method whereas final pH and remained glucose after fermentation were collected at the end of fermentation process.

Table 1. *Treatments of the three factors for one replicate.*

[Ethanol] (% v/v)	pH	Starter density (cells/mL)	pH	Starter density (cells/mL)	pH	Starter density (cells/mL)
		10^4		10^4		10^4
4	4	10^5	5	10^5	6	10^5
		10^6		10^6		10^6
		10^4		10^4		10^4
5	4	10^5	5	10^5	6	10^5
		10^6		10^6		10^6
		10^4		10^4		10^4
6	4	10^5	5	10^5	6	10^5
		10^6		10^6		10^6

2.6. Data Analysis

The data were analyzed by Statgraphics Centurion XV (version 15.1.02). Statgraphics Centurion XV was used to test for the least significant difference (LSD) with the confidence interval of 95% and Microsoft Excel was used to process tables and graphics.

3. Results and Discussion

3.1. Fermentative Ability of Thermotolerant AAB at 37°C

The acetic acid production of 20 selected isolates and the control strain *A. pasteurianus* were summarized in Table 2. The results show that isolates A3, A47, A8, A50, A49, A1, A52, A18, and A23 produced the high values of acetic acid after 7 days of fermentation at 37°C. The acetic acid concentration of *A. pasteurianus* was 1.32% (w/v), lower than all tested AAB isolates (1.36-2.04% w/v). Markedly, the acid concentrations of 9 isolates were 2.04, 2.04, 1.84, 1.80, 1.80, 1.76, 1.76, 1.76, and 1.72% (w/v), respectively. These 9 isolates were selected for identification and fermentation at 38 and 39℃. These results were appropriate with the study of Kanchanara et al. [3] with *A. pasteurianus* MSU10, IFO3191, and SKU1108 in YGPD medium containing 4-6% (v/v) ethanol at 37°C with the acetic acid production were ranged from 2% to 3% (w/v).

Table 2. *Acid production and productivity of 21 AAB at fermentative temperature 37°C.*

AAB	Acid concentration (% w/v)	Productivity (%)
A1	1.76 ^{abc}	42.75
A3	2.04 ^{a}	49.56
A8	1.84 ^{ab}	44.70
A11	1.48 ^{cde}	35.95
A13	1.36 ^{de}	33.04
A16	1.56 ^{bcde}	37.90
A18	1.76 ^{abc}	42.75
A19	1.48 ^{cde}	35.95
A22	1.60 ^{bcde}	38.87
A23	1.72 ^{abc}	41.78
A24	1.60 ^{bcde}	38.87
A28	1.60 ^{bcde}	38.87
A29	1.52 ^{bcde}	36.92
A31	1.56 ^{bcde}	37.90
A46	1.56 ^{bcde}	37.90
A47	2.04 ^{a}	49.56
A49	1.80 ^{abc}	43.73
A50	1.80 ^{abc}	43.73
A52	1.76 ^{abc}	42.75
A55	1.68 ^{bcd}	40.81
Control	1.32 ^{e}	32.07
CV	15.29%	

*Note: The values were arithmetic means of triplicates.

3.2. Identification of the Selected Thermotolerant AAB

Nine AAB isolates (A1, A3, A8, A18, A23, A47, A49, A50, and A52) were selected based on the fermentation results at 37°C and their purified PCR products were sent to the 1st Base company (Singapore) for identifying at the species level. The identification results were summarized in Table 3. The nucleotide sequences of selected isolates were compared with Gene Bank of NCBI by BLAST tool. It was clear that almost identified strains belonged to *Acetobacer* genus with high identities, from 96 to 100%. There were 4 isolates (A3, A23, A47 and A49) of *A. tropicalis,* 2 isolates (A1 and A52) of *A. pasteurianus*, 2 isolates (A18 and A50) of *A. sicerae,* and 1 isolate A8 of *A. orientalis*. These bacteria were found commonly in the acetic acid fermentation, especially in high temperatures acetic acid fermentation processes. *A. tropicalis* SKU1100 and *A. pasteurianus* SKU1108 were selected with fermentable ability at 42°C [13]. Similarly, 2 strains *A. tropicalis* CWBI-B418-B419 and *A. pasteurianus* CWBI were isolated from fruits with high fermentation at 38°C [10].

Table 3. *The identification results of 9 selected AAB strains.*

Strains	Species	Identity (%)
A1	*Acetobacter pasteurianus*	100%
A3	*Acetobacter tropicalis*	97%
A8	*Acetobacter orientalis*	99%
A18	*Acetobacter sicerae*	99%
A23	*Acetobacter tropicalis*	96%
A47	*Acetobacter tropicalis*	98%
A49	*Acetobacter tropicalis*	96%
A50	*Acetobacter sicerae*	97%
A52	*Acetobacter pasteurianus*	98%

3.3. Fermentative Ability of Thermotolerant AAB at 38°C and 39°C

Nine selected isolates (A3, A47, A8, A50, A49, A1, A52,

A18, and A23) were chosen for acetic acid fermentation at 38 and 39°C in 7 days using YPGD medium containing 4% (v/v) of ethanol. The strain *A. pasteurianus* was also used for comparison in this experiment. Experimental data in Figure 1 shows that *A. sicerae* A18 had the highest acetic acid concentration of 1.80% (w/v) with the productivity of 43.73%.

Table 4. *Acid concentration and productivity of 10 AAB at fermentative temperature 38°C.*

AAB	Acid concentration (% w/v)	Productivity (%)
A1	1.84 ^{abc}	44.70
A3	1.64 ^{c}	39.84
A8	1.80 ^{bc}	43.73
A18	1.80 ^{bc}	43.73
A23	2.04 ^{ab}	49.56
A47	1.80 ^{bc}	43.73
A49	2.08 ^{ab}	50.53
A50	1.88 ^{abc}	45.67
A52	2.12 ^{a}	51.50
Control	1.24 ^{d}	30.12
CV	15.78%	

*Note: The values were arithmetic means of triplicates.

At 38°C, acetic acid bacterial strains needed more time for adaption to environmental temperature so all isolates slowly reached the maximum acetic acid concentrations (Figure 1). The maximum acid concentrations and productivities were 1.64-2.12% and 39.84-51.50%, respectively. *A. pasteurianus* produced only 1.24% of acid concentration and productivity of 30.12%. The beginning of acetic acid synthesis was related to the increasing biomass of acetic acid bacterial for transforming ethanol into acetic acid [14]. Microorganisms needed time to adapt (the log phase) because at that time microorganism activity was not enough strong, so generated acid content was not high. In the lag phase, the bacteria had enough energy that was used for the synthesis of acetic acid [4].

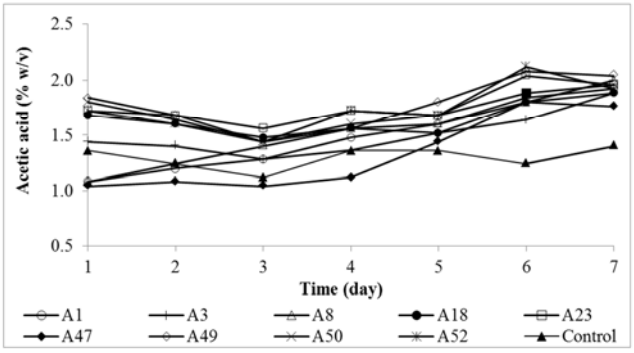

Figure 1. *Acid production of 9 thermotolerant acetic acid bacterial strains at 38°C. Fermentation was carried out triplicate in 30 mL YPGD containing 4% (v/v) of ethanol in 7 days; A1, A52: A. pasteurianus; A3, A23, A47, A49: A. tropicalis; A8: A. orientalis; A18, A50: A. sicerae; control: A. pasteurianus.*

In contrast, at 39°C, 10 isolates adapted rapidly with this temperature (Figure 2). In the third day of fermentation, acetic acid concentartions reached the maximum values and stabilized until the last day of fermentation. The maximum acid concentration and productivity peaked at 2.56% (w/v) and 62.19%, respectively, by *A. sicerae* A18. Acid concentration was not significantly different in comparison with *A. pasteurianus* A1 (acid concentration of 2.52% w/v). Compared to the study of Phong [12], the acetic acid concentration of 2.52% (w/v) was achieved from *A. tropicalis* DK4 after 7 days of fermentation in YPGD containing 4% (v/v) ethanol at 39°C, the acetic acid content of *A. sicerae* A18 in this study is greater.

Table 5. *Acid production and productivity of 10 AAB at fermentative temperature 39°C.*

AAB	Acid concentration (% w/v)	Productivity (%)
A1	2.52 ^{a}	61.22
A3	2.08 ^{abcd}	50.53
A8	2.40 ^{ab}	58.30
A18	2.56 ^{a}	62.19
A23	2.12 ^{abcd}	51.50
A47	1.52 ^{d}	36.92
A49	1.76 ^{bcd}	42.75
A50	1.68 ^{cd}	40.81
A52	1.76 ^{bcd}	42.75
Control	2.28 ^{abc}	55.39
CV	13.04%	

*Note: The values were arithmetic means of triplicates

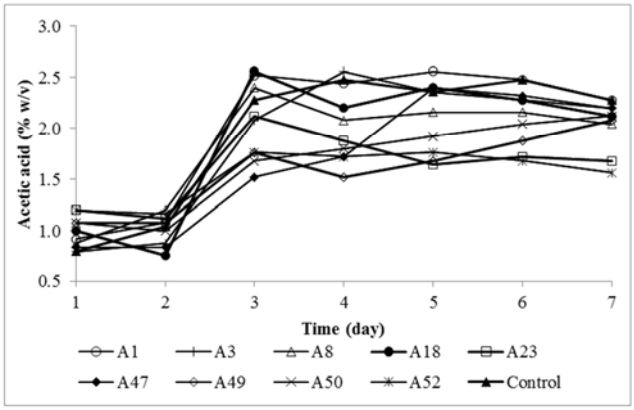

Figure 2. *Acid production of 9 thermotolerant acetic acid bacterial strains at 39°C. Fermentation was carried out triplicate in 30 mL YPGD containing 4% (v/v) of ethanol in 7 days; A1, A52: A. pasteurianus; A3, A23, A47, A49: A. tropicalis; A8: A. orientalis; A18, A50: A. sicerae; control: A. pasteurianus.*

During fermentation at 38°C and 39°C, *A. sicerae* A18 was selected for study the favorable conditions for acetic acid fermentation at 39°C. *Acetobacter sicerae* A18 was short rod shape, Gram negative (Figure 3) and isolated from fermented pickle in Binh Duong province, Vietnam.

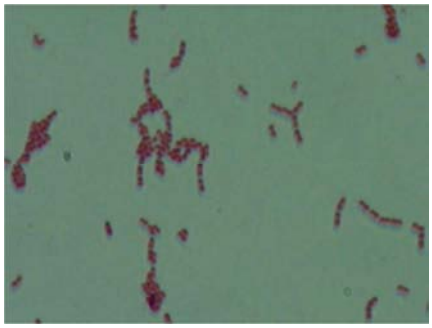

Figure 3. *Acetobacter sicerae A18.*

3.4. Examination of the Favorable Conditions for Acetic Acid Fermentation

To determine the favorable conditions for acetic acid fermentation, *A. sicerae* A18 was used in experimental design with three independent factors at 39°C including ethanol concentration (4, 5 and 6% v/v), initial pH (4, 5 and 6) and starter density (10^4, 10^5 and 10^6 cells/mL). Effects of ethanol concentration, pH and cell density on acid production by *A. sicerae* A18 at 39°C after 6 days of fermentation were summarized in Table 6.

Table 6. *Effect of ethanol concentration, pH and cell density on acid production (% w/v) by A. sicerae A18 at 39°C.*

No	Factors			Responses			
	Ethanol (% v/v)	pH	Cell density (cells/mL)	Sugar (g/L)	pH	Acid concentration (% w/v)	Productivity (%)
1	4	4	10^4	7.24	3.84	2.46^{bc}	59.71
2	4	4	10^5	7.04	3.87	2.46^{bc}	59.71
3	4	4	10^6	6.97	3.88	2.64^{b}	64.08
4	4	5	10^4	8.51	4.65	1.62^{ghi}	39.32
5	4	5	10^5	8.38	4.68	1.74^{fgh}	42.23
6	4	5	10^6	8.51	4.69	1.68^{gh}	40.78
7	4	6	10^4	9.60	3.87	1.74^{fgh}	42.23
8	4	6	10^5	7.10	3.84	1.80^{fg}	43.69
9	4	6	10^6	8.90	3.95	1.80^{fg}	43.69
10	5	4	10^4	8.50	3.97	2.60^{b}	63.11
11	5	4	10^5	8.81	3.97	2.82^{a}	66.02
12	5	4	10^6	8.08	3.97	2.84^{a}	66.50
13	5	5	10^4	9.06	4.88	1.50^{hij}	36.41
14	5	5	10^5	9.20	4.87	1.62^{ghi}	39.32
15	5	5	10^6	9.19	4.98	1.50^{hij}	36.41
16	5	6	10^4	9.28	4.44	1.68^{gh}	40.78
17	5	6	10^5	8.37	4.44	2.10^{de}	50.97
18	5	6	10^6	9.20	4.38	1.86^{efg}	45.15
19	6	4	10^4	6.35	3.91	1.98^{def}	48.06
20	6	4	10^5	6.32	3.90	2.22^{cd}	53.88
21	6	4	10^6	6.05	3.91	2.22^{cd}	53.88
22	6	5	10^4	7.20	3.86	1.32^{j}	32.04
23	6	5	10^5	7.17	3.94	1.38^{ij}	33.50
24	6	5	10^6	6.93	3.92	1.38^{ij}	33.50
25	6	6	10^4	8.06	5.37	1.74^{fgh}	42.23
26	6	6	10^5	7.93	5.36	1.74^{fgh}	42.23
27	6	6	10^6	8.52	5.31	1.68^{gh}	40.78

The results show that the highest acid concentrations were achieved at 2.84 and 2.82% (w/v) from the treatment 12 (5% v/v ethanol, pH 4.0, and initial cell density of 10^6 cells/mL), and treatment 11 (5% v/v ethanol, pH 4.0, and initial cell density of 10^5 cells/mL), respectively. These two treatments were also had the highest productivities of 66.50 and 66.02%, respectively. At the same conditions of ethanol concentrations and pHs, the acid concentrations were significantly lower when the cell density was at 10^4 cells/mL (treatment 10, acid concentration of 2.60% w/v). Treatment 3 (4% v/v ethanol, pH 4.0, and initial cell density of 10^6 cells/mL) had acid concentration of 2.64% (w/v), as high as the acid concentration of treatment 10 (5% v/v ethanol, pH 4.0, and initial cell density of 10^4 cells/mL). It revealed that the effect of initial cell density on the acid production and productivity because this treatment had lower ethanol concentration. Kanchanarach et al. [3] showed that the thermotolerant *A. pasteuriaus* SKU1108 strain produced only 1.6% acetic acid at 37°C in the YPGD containing 6% (v/v) ethanol. Because of the lower initial cell density, comparison between treatment 11 and 12, the favorable conditions for acid production by *A. sicerae* A18 at 39°C was determined as follows: 5% (v/v) of ethanol, pH 4.0, and initial cell density of 10^5 cells/mL.

4. Conclusions

Nine thermotolerant acetic acid bacterial isolates (A1, A3, A8, A18, A23, A47, A49, A50 and A52) were selected based on their high acid production at 37°C, which acetic acid concentrations were in range of 1.72- 2.04% (w/v) after 7 days of fermentation. The acid concentrations of these selected strains were 1.64-2.12% (w/), corresponding to the productivities of 39.84-51.50% at 38°C. *A. sicerae* A18 had the highest acetic acid concentration at 2.56% (w/v) with the productivity of 62.19% at 39°C. Nine selected isolates were identified as 4 species including *A. pasteurianus*, *A. tropicalis*, *A. sicerae,* and *A. orientalis*. Three factors were identified that initial pH 4.0, ethanol concentration of 5.0% (v/v), and 10^5 cells/mL of starter density were the most suitable for acetic acid fermentation of strain *A. sicerae* A18 in YPGD medium at 39°C; the acid concentration and productivity were 2.82% (w/v) and 66.02%, respectively.

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