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SHORT COMMUNICATION

Directional isolation of ethanol-tolerant acetic acid bacteria from industrial fermented vinegar

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Abstract The aim of this study was to isolate indigenous ethanol-tolerant acetic acid bacteria (AAB) from industrial fermented vinegar. As routine phenotypic methods for AAB isolation and identification appear to be very time-consuming and not so accurate, we adopted a two-step isolation strategy in the present study. In the preliminary screening step, GYEC agar plates with 3–10 % (v/v) ethanol as selective stress were utilized to recover potential AAB strains with ethanol tolerance from vinegar samples. In the rescreening process, acetic acid bacterial genus' specific *adhA* gene was amplified as an effective DNA target for directional detection of real AAB so that non-AAB isolates could be eliminated rapidly. In this way, an AAB pure isolate named ET-7-3 with 7 % (v/v) ethanol tolerance was successfully isolated and further identified to *Acetobacter pasteurianus* according to 16S rDNA sequencing and phylogenetic analysis. Summarizing, by the use of ethanol as selective stress combined with molecular identification method, ethanol-tolerant AAB could be isolated from industrial fermented vinegar with both efficiency and accuracy.

Keywords Vinegar · Acetic acid bacteria · Ethanol tolerance · Isolation · PCR

Abbreviations

AAB	Acetic acid bacteria
GYEC agar plate	Agar plates containing 1 % glucose, 1 % yeast extract, 3 % absolute ethanol, 2 % calcium carbonate, 1.5 % agar, w/v
PQQ	Pyrroloquinoline quinone
ADH	Alcohol dehydrogenase
adhA	Gene encoding subunit I of ADH

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Introduction

Vinegar is consumed worldwide as a food condiment and preservative. Although being different in production technology, the elemental fermentation process usually undergoes three steps, namely starch saccharification, alcohol fermentation and acetic acid fermentation [1, 2]. The oxidation of ethanol to acetic acid, which is the key step in vinegar production, is also the essential and distinctive characteristic of AAB, thus making them important microorganisms for vinegar industry [3]. Therefore, isolation of potential AAB has always been necessary keeping in view both the exploration of new and better acetic acid bacterial species for vinegar production and the improvement in their biotechnological performance.

Recently, research concerning tolerance and stress response to AAB related to industrial vinegar fermentation has been the renewed interest due to its potential not only in fundamental scientific research, but also of significant economic interest [4]. Several works on stressors such as thermic, acetic acid and sugar tolerance have been published [5–7]. However, to date, little is known about the tolerance and stress response of AAB to ethanol. It is well known that cost-efficient industrial vinegar production depends on, among other factors, rapid and high-yield conversion of ethanol to acetic acid, which in itself depends on the survival and performance of AAB under industrial conditions. High concentration of ethanol, as the alcohol fermentation product, can become a significant stress to inhibit acetic acid bacterial cell growth and then limit

acetification fermentation productivity [7]. Thus, in order to obtain the most suitable and best adapted AAB for vinegar production and to gain a better understanding of ethanol-resistant mechanism, indigenous ethanol-tolerant AAB must be recovered from industrial fermented vinegar first.

Unfortunately, traditional acetic acid bacterial isolation and identification methods based on an array of phenotypic tests were shown not to be completely reliable, and moreover, they are time-consuming [8]. In addition, one of the greatest hurdles when recovering potential AAB is due to the diversity and complexity of mixed microflora existing in vinegar fermentation process [9]. Although many efforts have been made to improve the methodology for AAB isolation and identification [8], novel efficient strategy for recovering indigenous ethanol-tolerant AAB from industrial fermented vinegar has yet to be developed.

So, in the present study, in order to isolate indigenous ethanol-tolerant AAB from industrial fermented vinegar, we adopted a two-step isolation strategy. In the preliminary screening step, GYEC agar plates with different ethanol concentrations (from 3 to 10 %, v/v) as selective stress were utilized to recover potential AAB isolates from vinegar samples based on their ethanol tolerance and hydrolysis ability [10]. During the rescreening process, acetic acid bacterial genus' specific *adhA* gene was amplified as an effective DNA target for directional detection of real AAB so that non-AAB isolates could be eliminated rapidly [11]. For further confirmation, 16S rDNA amplification and sequencing were carried out to identify isolates to genus level. Results showed that an AAB pure isolate named ET-7-3 with 7 % (v/v) ethanol tolerance was successfully isolated from industrial vinegar and identified as *Acetobacter pasteurianus* based on phylogenetic analysis of 16S rDNA sequences.

In conclusion, by the use of ethanol as selective stress combined with PCR-based molecular identification method, ethanol-tolerant AAB could be recovered from mixed microflora of industrial fermented vinegar in a rapid and accurate way.

Materials and methods

Vinegar sampling and AAB enrichment

Vinegar samples were collected from the fermentation workshop of Jiangsu Hengshun Vinegar Industry Co., Ltd. All the samples were taken aseptically and immediately stored at 4 °C before use. For indigenous AAB enrichment, vinegar samples were diluted with enrichment medium broth (1 % glucose, 1 % yeast extract, 3 % absolute ethanol) at the ratio of 1:10 (sample/medium) and incubated at 30 °C for 48 h with continuous and vigorous agitation in order to adapt acetic acid bacterial cells to rapid proliferation.

Preliminary screening of potential AAB strains with high ethanol tolerance

Traditional GYC culture medium (1 % glucose, 1 % yeast extract, 2 % calcium carbonate, 1.5 % agar, w/v) was slightly modified to gradient GYEC agar plates for screening of potential AAB strains with high ethanol tolerance. The GYC medium was first sterilized at 121 °C for 20 min in an autoclave. After cooling, a gradient volume of absolute ethanol (from 3 to 10 %, v/v) was added to each flask containing the same GYC medium to constitute an increasing percentage of ethanol, differing by 1 % from one flask to another. Each flask of GYEC solid medium with different ethanol concentrations (from 3 to 10 %, v/v) was poured to make GYEC agar plates.

The pre-enriched vinegar samples were then diluted serially, ranging from 10^{-1} to 10^{-7}, and 150 µl of 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread onto the GYEC agar plates with gradient ethanol concentrations (from 3 to 10 %, v/v) correspondingly and cultivated at 30 °C for 48–72 h.

Undergoing stress induced by different concentrations of ethanol, only acetic acid-producing microorganisms with comparatively high ethanol tolerance formed distinct clear zones around the colonies on GYEC agar plates. Isolates showing high ratios of clear zone diameter to colony diameter as well as high ethanol resistance were selected as potential AAB and purified by subsequent streaking for further examination.

Detection of AAB isolates by *adhA* gene amplification

DNA extraction was performed according to the method described by Ruiz et al. [12]. For each potential acetic acid bacterial strain studied, a single colony was picked from a fresh culture on GYC agar plates and resuspended in 50 µl sterile deionized water in a 1.5-ml Eppendorf tube. The mixture was vortexed for 15 s and then incubated at 95 °C for 15 min. After configuration (5,000 g, 4 °C, 5 min), the supernatant containing the released DNA was used as the template DNA for PCR amplification.

For AAB rapid molecular detection, the target *adhA* gene-specific primers (NuniADHfw: 5′-TGG(T/C)(A/T)CGG(C/T)AT(T/C)CC(G/C)GG-3′ and NuniADHrev: 5′-GT(G/C/A)GCGTC(A/G)GC(A/G)TGGAA-3′) designed by Trcek were used [11].

PCR amplification was carried out in an Eppendorf tube containing 1 µl bacteria extract and 1.0 mM dNTP, 2 µl 10× PCR buffer (TaKaRa, Japan), 20 pM of each primer and 1 Unit rTaq (TaKaRa) and supplemented by sterile water (Milli-Q) to a final volume of 20 µl. The cycling program started with initial denaturation of DNA at 94 °C for 5 min and continued with 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. At the end, a final

extension at 72 °C for 10 min was performed, followed by cooling down to 4 °C. Amplified DNA was electrophoresed in 1.0 % w/v agarose gels (Invitrogen, USA) with 1× TAE running buffer. The 250-bp DNA ladder marker (TaKaRa) was used as a length standard.

According to *adhA* gene amplification result, the analyzed isolate obtaining a typical band of approximately 370 bp was most likely to be AAB and subjected to 16S rDNA sequencing for further identification, while non-AAB isolates were discriminated and eliminated at the same time.

Further Identification by 16S rDNA gene sequencing

Selected isolates were further identified by 16S rDNA gene amplification and sequencing. Genomic DNA was extracted as reported above. PCR amplification of 16S rDNA was performed using universal primers (27F: 5′-AGAGTTTGA TCMTGGCTCAG and 1492R: 5′-TACGGYTACCTTGT TACGACTT) [13, 14]. PCR amplification was carried out in 20 µl solution containing 1 µl bacteria extract and 1.0 mM dNTP, 2 µl 10 × PCR buffer (TaKaRa), 20 pM of each primer, 1 Unit LA Taq (TaKaRa) and sterile water (Milli-Q). The reaction was initiated at 94 °C for 5 min to denature the template DNA and then cycled 30 times at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 2 min. The samples were then incubated at 72 °C for 10 min for a final extension followed by cooling down to 4 °C. The amplified PCR product was purified with a gel extraction kit (OMEGA Bio-Tek) according to manufacturer's instructions. Directing sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd.

Phylogenetic analysis

Sequence contigs were assembled manually and subjected to BLAST program to find similarities with sequences deposited at GenBank (http://www.ncbi.nlm.nih.gov/blast) [15]. Multiple-sequence alignments and phylogenetic analysis were carried out using MEGA version 5.0 software [16]. The phylogenetic tree based on 16S rDNA was constructed according to the neighbor-joining method [17]. The homology of ethanol-tolerant isolate to other AAB species was compared according to their evolutionary divergence.

Results and discussion

Isolation of potential AAB strains with high ethanol tolerance

As AAB are fastidious microorganisms to isolate and cultivate despite the great number of growth media available [18], GYEC solid medium has been recommended as an ideal medium, for it enables most AAB to be recovered from traditional vinegars. Moreover, the halo formation caused by acid hydrolysis of $CaCO_3$ is one of the basic and dominant characteristics that associates an unknown colony with acetic acid bacterial group [3]. Therefore, in order to isolate potential AAB strains with high ethanol tolerance, GYEC agar plates with different concentrations of ethanol (from 3 to 10 %, v/v) were used as selective stress in preliminary screening step, and a total of 11 isolates showing 7 or 8 % ethanol tolerance (Table 1) as well as distinctive clear zones (Fig. 1) were picked up. They were named as ET-7-1, ET-7-2, ET-7-3, ET-7-4, ET-7-5, ET-7-6, ET-7-7, ET-7-8 and ET-7-9 with 7 % ethanol tolerance and ET-8-1 and ET-8-2 with 8 % ethanol tolerance, respectively. Table 1 also showed that there was no strain surviving on GYEC agar plates with ethanol concentration more than 8 % (from 9 to 10 %, v/v). This indicated that gradient ethanol concentration (from 3 to 10 %, v/v) could be utilized as an effective isolating stress for screening of ethanol-tolerant strains. However, it is important to note that these 11 ethanol-tolerant isolates should not be taken as real AAB arbitrarily, just because of the halo formation they have presented on GYEC agar plates. As halo formation is nothing but one of the essential phenotypes AAB strain possess—in other words, many other acid-producing microorganisms existing in the mixed microflora of industrial fermented vinegar may also present clear zone around colony on GYEC agar plates [10]—in the rescreening step, appropriate identification methodology should be utilized to make rapid and accurate detection of real AAB strains from all these 11 ethanol-tolerant isolates.

Molecular detection of real AAB by *adhA* gene amplification

However, traditional acetic acid bacterial identification methods based on an array of morphological, physiological and biochemical tests are generally laborious, time-consuming and often not straightforward to interpret [8]. From industrial and technological point of view, routine analyses of a mass of samples require rapid and reliable techniques for taxonomical identification of AAB [19]. Therefore, the application of molecular methods, based on identification of specific DNA segments, could be a proper solution for quick and accurate identification of these microorganisms [20]. For example, Trcek et al. have analyzed the suitability of *adhA* gene encoding subunit I of AAB PQQ-dependent alcohol dehydrogenase for construction of DNA probes and designed two pairs of specific primers (NuniADHfw with nacetirev) and (NuniADHfw with NuniADHrev) based on variable and conserved regions of AAB *adhA* sequences. The former primer set was tested

Table 1 Ethanol tolerance statistics of acetic acid-producing microorganisms growing on GYEC agar plates with different concentrations of ethanol and numbers of ethanol-tolerant isolates selected in the preliminary screening step

Ethanol concentrations of GYEC agar plates (v/v, %)	Dilution of AAB-enriched vinegar samples			Numbers of ethanol-tolerant isolates
	10^{-5}	10^{-6}	10^{-7}	
3	+++	+++	++	None
4	+++	+++	++	None
5	+++	+++	++	None
6	+++	++	+	None
7	++	++	+	9
8	+	+	−	2
9	−	−	−	None
10	−	−	−	None

+++, very good growth with distinctive clear zone; ++, good growth with legible clear zone; +, slight growth; −, no growth

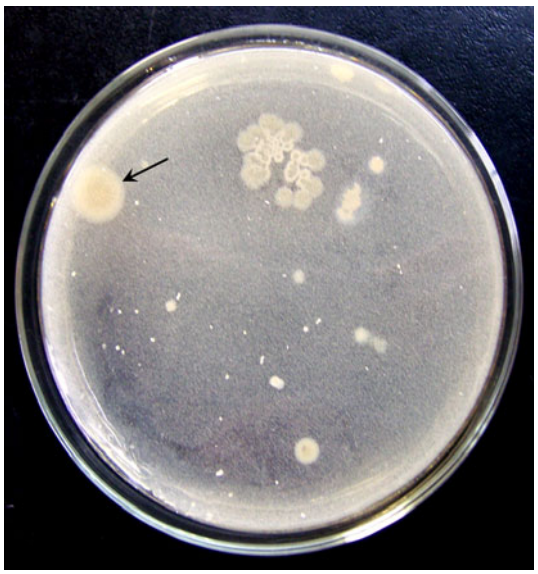

Fig. 1 Clear zone produced by potential AAB growing on GYEC agar plate. *Arrow* indicates a potential acetic acid bacterial candidate isolate with distinctive clear zone around its colony

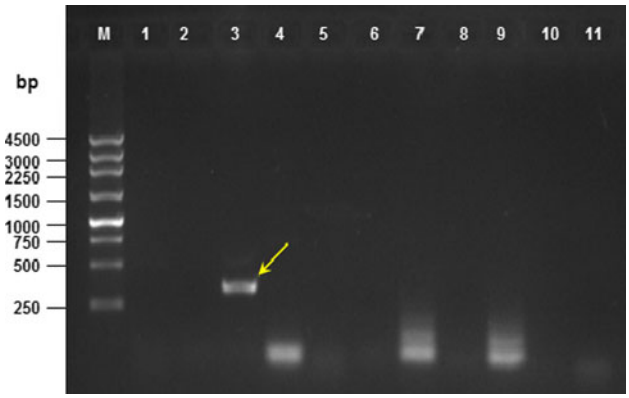

Fig. 2 Amplification of *adhA* gene of 11 potential AAB isolates with high ethanol tolerance. *Lanes*: M, 250-bp DNA ladder marker (TaKaRa); 1, isolate ET-7-1; 2, isolate ET-7-2; 3, isolate ET-7-3; 4, isolate ET-7-4; 5, isolate ET-7-5; 6, isolate ET-7-6; 7, isolate ET-7-7; 8, isolate ET-7-8; 9, isolate ET-7-9; 10, isolate ET-8-1; 11, isolate ET-8-2. *Arrow* indicates an approximately 370-bp amplicon obtained from isolate ET-7-3

for its availability with most acetic acid bacterial strains, and the latter one was demonstrated to be useful in the detection of *A.aceti* directly from cider vinegar [11, 21]. Here in our study, for rapid and accurate selection of real AAB from all these 11 ethanol-tolerant isolates, acetic acid bacterial genus' specific *adhA* gene (370 bp) was used as a DNA target for molecular detection. With the help of primers NuniADHfw and NuniADHrev, PCR amplification was successfully carried out. A specific amplicon of approximately 370 bp was obtained from isolate ET-7-3 (Fig. 2), while negative PCR results were observed in the rest of the 10 isolates. So a tentative identification was achieved based on this band position, which indicated that isolate ET-7-3 could be classified to acetic acid bacterial group.

Further identification of AAB isolates by 16S rDNA sequencing and phylogenetic analysis

Identification of AAB isolate ET-7-3 was further confirmed by 16S rDNA sequencing and phylogenetic analysis. Approximately 1.5-kb fragment of the 16S rDNA gene of isolate ET-7-3 was amplified and sequenced. Hagstrom found that 16S rDNA sequence presenting more than 97 % similarity was a reasonable level of grouping bacteria into species [22]. With universal primers 27F and 1492R, 1452 bp of isolate ET-7-3 16S rDNA gene was amplified, sequenced and submitted to GenBank for BLAST. Nucleotide–nucleotide BLAST result showed 99 % sequence similarity with the first hit of *Acetobacter pasteurianus*. To understand the taxonomic position of isolate ET-7-3, a phylogenetic neighbor-joining tree was constructed, and it showed high phylogenetic affiliation with other members

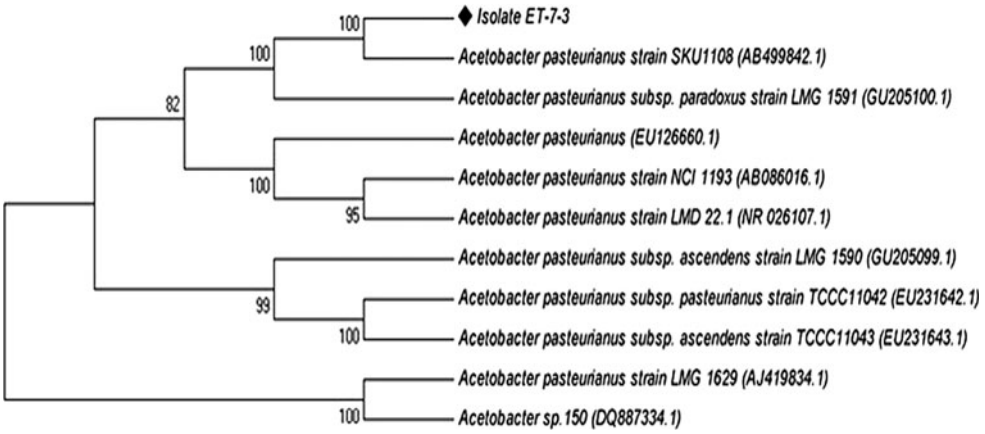

Fig. 3 A phylogenetic tree based on 16S rDNA sequences constructed by neighbor-joining method. The sequences are compared with 16S rDNA sequences obtained from NCBI database. Accession numbers are shown in parentheses. Phylogenetic analysis results show the homology of isolate ET-7-3 to other *Acetobacter pasteurianus* strains

of *Acetobacter pasteurianus* species (Fig. 3). Therefore, isolate ET-7-3 could be identified as a strain of *A. pasteurianus*.

Conclusions

In conclusion, we have successfully recovered an indigenous acetic acid bacterial isolate ET-7-3 with high ethanol tolerance from industrial fermented vinegar. Results demonstrated that the directional isolation and molecular identification strategy we adopted was both efficient and accurate.

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