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Simultaneous production of acetic and gluconic acids by a thermotolerant *Acetobacter* strain during acetous fermentation in a bioreactor

Majid Mounir,[1,3,*] Rasoul Shafiei,[2] Raziyeh Zarmehrkhorshid,[3] Allal Hamouda,[1] Mustapha Ismaili Alaoui,[1] and Philippe Thonart[3]

Hassan II Institute of Agronomy and Veterinary Medicine (IAV), PO Box 6202, Rabat, Morocco,[1] Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran,[2] and Walloon Centre of Industrial Biology, University of Liege, B40, B-4000 Sart-Tilman, Belgium[3]

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The activity of bacterial strains significantly influences the quality and the taste of vinegar. Previous studies of acetic acid bacteria have primarily focused on the ability of bacterial strains to produce high amounts of acetic acid. However, few studies have examined the production of gluconic acid during acetous fermentation at high temperatures. The production of vinegar at high temperatures by two strains of acetic acid bacteria isolated from apple and cactus fruits, namely AF01 and CV01, respectively, was evaluated in this study. The simultaneous production of gluconic and acetic acids was also examined in this study. Biochemical and molecular identification based on a 16s rDNA sequence analysis confirmed that these strains can be classified as *Acetobacter pasteurianus*. To assess the ability of the isolated strains to grow and produce acetic acid and gluconic acid at high temperatures, a semi-continuous fermentation was performed in a 20-L bioreactor. The two strains abundantly grew at a high temperature (41°C). At the end of the fermentation, the AF01 and CV01 strains yielded acetic acid concentrations of 7.64% (w/v) and 10.08% (w/v), respectively. Interestingly, CV01 was able to simultaneously produce acetic and gluconic acids during acetic fermentation, whereas AF01 mainly produced acetic acid. In addition, CV01 was less sensitive to ethanol depletion during semi-continuous fermentation. Finally, the enzymatic study showed that the two strains exhibited high ADH and ALDH enzyme activity at 38°C compared with the mesophilic reference strain LMG 1632, which was significantly susceptible to thermal inactivation.

[**Key words:** *Acetobacter*; Thermoresistant; Bioreactor; Enzyme activity; Acetic acid; Gluconic acid]

Vinegar is considered an acidic product of special importance for the enrichment of our diet. It is the product of the oxidation of substrates that contain ethanol (1). Vinegar is derived from a two-stage fermentation process of agriculturally produced raw materials. The two-step process consists of the anaerobic conversion of sugars to ethanol followed by the aerobic oxidation of ethanol to acetic acid (2). This last step is facilitated by acetic acid bacteria (AAB).

The family *Acetobacteraceae* was first identified by Gillis and De Ley in 1980 (3). Since then, the development and application of new methodologies have significantly changed the taxonomy of AAB (4). At present, the family *Acetobacteraceae* (*Alphaproteobacteria*) consists of twelve genera: *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia* and *Ameyamaea* (5). Nevertheless, the genus *Frateuria* belongs to the *Xanthomonadaceae* family (*Gammaproteobacteria*) (6).

AAB are found in substrates that contain carbohydrates and/or ethanol, such as fruit juices, wine, cider, beer, and vinegar (7).

AAB need to be able to oxidize ethanol and tolerate increasing acetic acid concentrations to survive and serve as microbial cell factories for industrial vinegar production in a bioreactor (8,9). Strains should also be temperature-resistant, particularly in tropical and sub-tropical regions. In fact, due to associated economic profits, the production of vinegar by thermotolerant AAB has garnered significant interest (10). Specifically, global warming constitutes a significant challenge to the vinegar industry because large cooling systems are required to maintain the optimal temperatures for vinegar production (8,11). Ndoye et al. (8) isolated and studied thermoresistant AAB from over-producing crops, such as mangos and cereals, in Senegal and Burkina Faso, respectively (Sub-Saharan Africa). They isolated an *Acetobacter senegalensis* strain that was able to grow and oxidize ethanol at high temperatures (8).

The flavour of vinegar reportedly depends on the raw materials used in fermentation (12). Furthermore, selected starter cultures are observed to improve the quality of fermented foods, and aroma is one such quality (13). Therefore, gluconic acid has been proposed as a quality parameter because it contributes to the aromatic profile and viscosity of foods (14). Consequently, AAB strains that simultaneously produce gluconic acid and acetic acid during acetous fermentation are preferable when considering the sensory quality of the final product. In addition, cultivable and phenotypically stable thermotolerant AAB that can be exploited as biocatalysts are increasingly sought after for a number of biotechnological applications (15).

* Corresponding author. Hassan II Institute of Agronomy and Veterinary Medicine (IAV), PO Box 6202, Rabat, Morocco. Tel.: +212 663 736 975; fax: +212 537 774 889.
E-mail address: mounirmajid@gmail.com (M. Mounir).

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This study aimed to isolate novel AAB strains whose features make them applicable for industrial use. Thus, we first isolated, identified and characterized novel AAB strains. We then examined the ability of these isolated strains to efficiently ferment ethanol into acetic acid in a lab-scale bioreactor at a high temperature. In addition, the ability of selected strains to simultaneously produce gluconic and acetic acids during acetous fermentation was assessed.

MATERIALS AND METHODS

Food samples Twenty-two food samples were used in this study. The samples were divided into three categories (traditional vinegars and wines, juices and honeys, and fruits). These samples were collected from different regions of Morocco based on different criteria, such as the climate (hot temperature), availability of local and natural products (non-use of pesticides), and the expertise of local residents. Immediately after collection, the samples were stored at 4°C.

Culture media and microorganisms GYEA [20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone of casein, 3% (w/v) ethanol and 1% (w/v) acetic acid] and GYEA/Mg^{2+} [20 g/L glucose, 5 g/L yeast extract, 3% (w/v) ethanol, 1% (w/v) acetic acid and 0.5 g/L $MgSO_4$] were used as enrichment media (16). Solid culture media (SGYEA and SGYEA/Mg^{2+}) consisted of the same components given above supplemented with 15 g/L of agar. GYEA/Mg^{2+} supplemented with 1 g/L K_2HPO_4 and 1 g/L $(NH_4)_2HPO_4$ was used as the fermentation medium (FM) for acetic fermentation in the bioreactor (17).

The isolated bacteria were compared with other mesophilic and thermotolerant AAB. The wild-type strains used in this study were obtained from the Laboratory of Microbiology of Gent (Belgium) (*A. senegalensis* LMG 23690T, *Acetobacter pasteurianus* LMG 1632, LMG 1607 and LMG 1701, *Acetobacter aceti* LMG 1531 and *Acetobacter cerevisiae* LMG 1625) and from the German Collection of Microorganisms and Cell Cultures (Germany) (*A. pasteurianus* DSM 2324 and *Acetobacter lovaniensis* DSM 4491). *A. senegalensis*, which can grow in liquid culture medium in the presence of ethanol and acetic acid at both mesophilic and thermophilic temperatures, was used as a thermotolerant reference strain (8,16).

Morphological, biochemical and metabolic characterization The morphology of bacteria, including their shape, size, arrangement, gram staining and motility, was characterized using cells grown on GYC at 30°C under aerobic conditions (18,19).

Conventional biochemical tests, such as catalase, oxidase, and growth in varying concentrations of ethanol and glucose, were employed according to literature (19). Overoxidation was tested in GYC medium (20 g/L glucose, 5 g/L yeast extract, 15 g/L agar, and 15 g/L $CaCO_3$) containing green bromocresol (0.022 g/L).

Carbohydrate assimilation tests were carried out using API 50CH strips (BioMerieux, France). The bacterial cells were first prepared in API 50CHL suspension medium, but that bromocresol purple was replaced with bromocresol green (20). The colour change was examined after 1, 2 and 3 days of incubation at 30°C.

Resistance to high temperatures Growth and acetification were tested at different temperatures: 30°C, 34°C, 35°C, 37°C, 40°C, and 41°C.

The thermoresistance basis of thermotolerant isolated AAB strains and *A. senegalensis* was compared. Pre-culture (100 mL) consisting of GYEA/Mg^{2+} medium containing 5% (v/v) of ethanol and 1% (v/v) of acetic acid was prepared in 500-mL flask for each strain. The flasks were inoculated with fresh colonies grown on plates after 48 h of incubation at 30°C and then incubated simultaneously and separately under agitation at two different temperatures, 30°C and 38°C.

Molecular tests The total genomic DNA of the selected strains was extracted from fresh cells grown on solid GYEA/Mg^{2+} medium using the Promega extraction kit (Promega, USA). The cells were resuspended in 600 μL of Nuclei Lysis Solution to which 3 μL of RNase and 200 μL of Protein Precipitation Solution were added. The DNA was precipitated by adding 600 μL of isopropanol, and it was then washed in 600 μL of 70% ethanol. The DNA was further resuspended in 100 μL of DNA Rehydration Solution (10 mM Tris and 1 mM EDTA) and preserved at 4°C. The quantity of extracted DNA was estimated by agarose gel electrophoresis.

The 16s rRNA gene was amplified using PCR and the following universal primers: 16SP0 5′-GAAGAGTTTGATCCTGGCTCAG-3′ for the coding segment and 16SP6 5′-CTACGGCTACCTTGTTACGA-3′ for the non-coding segment (8,21). The PCR reaction was performed in 200-μL Eppendorf tube containing 25 μL of Ready Mix (Promega), 2.5 μL of 16SP0 primer, 2.5 μL of 16SP6 primer, 2 μL of DNA template and 18 μL of sterile Milli-Q water.

The conditions of the PCR reaction, which was carried out in a thermocycler (Eppendorf, France) were as follows: an initial denaturation cycle at 95°C for 5 min, 25 denaturation cycles at 95°C for 30 s, primer annealing at 55°C for 30 s, primer elongation at 72°C for 2 min, and a final elongation cycle at 72°C for 10 min. The PCR products were then electrophoresed at 100 V for 20 min in a 1% agarose gel in 50× phosphate TAE buffer containing 1 μg/mL ethidium bromide.

The PCR reaction products were purified using a PCR Preps Wizard kit (Promega) and quantified on an agarose gel.

The purified PCR product was sequenced according to the Sanger method using a Big Dye Kit and a 3730 DNA analyser (Applied Biosystems) (8). The following primers were used for sequencing: F1 (CTGGCTCAGGAYGAACG), F2 (GAGGCAGCAGTRGGGAAT), F3 (ACACCARTGGCGAAGGC), and F4 (GCACAAGCGGYGGAGCAT) for the coding DNA segment and R1 (CTGCTGGCACGTAGTTAG), R2 (AATCCTGTTYGCTMCCCA), R3 (CCAACATCTCACGACACG) and R4 (TGTGTAGCCCWGGTCRTAAG) for the non-coding DNA segment.

The products of the sequencing reaction were assembled using the CodonCode Aligner program. The sequence was then analysed using the BLAST algorithm from GenBank of National Center for Biotechnology Information (NCBI) and was compared with others available in the GenBank/EMBL/DDBJ database.

Analytical methods The compositions of liquid culture media and fermentation broth were analysed by HPLC (Agilent 1110 series; Agilent Technologies, CA, USA) using the method described by Shafiei et al. (16,22).

The total amount of produced biomass was determined by measuring the absorbance of the culture on a spectrophotometer at 540 nm (O.D. measured by spectrophotometer). For all samples, the cultivated broth was diluted to an O.D. of less than 0.8.

The total acidity (%w/v) of the samples was measured via titration with 0.5 N NaOH using phenolphthalein as a pH indicator.

Growth and fermentation kinetics of the selected strains Two strains were selected, studied and compared with other mesophilic and thermoresistant strains. The growth kinetics of these isolates was monitored in flasks and bioreactor and compared with those of *A. senegalensis*.

A 20-L stirred tank bioreactor (Biolafitte, France) was used for the fermentations. The bioreactor was divided into a headspace volume and a working volume of 15 L. A computer connected to the fermenter allowed for the control of fermentation parameters: temperature, agitation, partial pressure of dissolved oxygen and pH. The reactor was aerated using a continuous flow of filtered sterile air at a rate of 1 VVM. The stirring rate was controlled to provide a minimum dissolved oxygen concentration 60%. The fermentations were conducted at 38°C.

The pre-culture was prepared in a 5-L embossed flask containing 1 L of GYEA/Mg^{2+} culture medium. The flask was inoculated with fresh cells that were grown for 48 h on plates. The flask was then incubated on a shaker (120 rpm) at 38°C.

After 24–36 h of incubation (O.D. between 0.2 and 0.4), the pre-culture was used to inoculate the bioreactor. The bioreactor was filled with fermentation medium (FM) with initial ethanol and acetic acid concentrations of 5% (v/v) and 1% (v/v), respectively.

The fermentations were semi-continuous: when the concentration of the substrate (ethanol) approached 0, 3 L of the cultivation medium was replaced by the same volume of fresh FM.

Enzymatic study The activities of the enzymes responsible for the production of acid, namely alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), were studied at the tested temperature (38°C). The cells were centrifuged, washed and used to determine the enzymatic activity according to the procedure used by Wood et al. (23) and Blandino et al. (24) and adjusted by Ndoye et al. (8), which utilizes ferricyanide as an electron acceptor.

A protease inhibitor cocktail (P8849; Sigma Aldrich, Germany) was added to the washed cells before lysis. The cell suspension was lysed by sonication in a Bandelin Sonifier (Germany) (15 bursts of 30 s with intermittent cooling) (25). The cells were maintained on ice during sonication.

The specific enzyme activity is expressed as units per milligram of proteins, and the protein content was determined through a Bradford assay using bovine serum albumin (BSA) as a standard (8,23–27).

RESULTS AND DISCUSSION

Isolation of adapted thermotolerant AAB This study aimed to isolate thermotolerant AAB strains from Moroccan products. The screening of liquid samples (Table 1) showed that all but the date vinegar and traditional apple vinegar strains abundantly grew at either 35°C or 41°C in different culture media. The thermoresistance of all of the strains inversely correlated with the concentrations of acetic acid and ethanol. A similar relationship between thermoresistance and ethanol concentration has been reported by Maal et al. (28). Notably, the AF01 strain, which was isolated from apple fruits, grew better at 41°C than at 35°C. Moreover, the traditional apple vinegar isolate weakly grew at 41°C compared with the other strains. However, this strain was more resistant to acetate and ethanol at 35°C than the other strains.

The comparison of the five thermotolerant strains revealed that AF01 and CV01, which were isolated from apple and cactus fruits, respectively, were the most thermoresistant strains (good growth at 35°C and 41°C). Therefore, AF01 and CV01 were selected for subsequent investigations.