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

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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 acetic 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 gluconic 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.

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[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, Neosasaia, Granulibacter, Tantarchaeronia 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 temperature 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 acetic 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).
The purified PCR products were 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 (CTGGCTCACGAYGACCC), F2 (GAGCCAGCAGTCGGCGAAT), F3 (ACACAGTCGCAGGCCAACG), and F4 (CCAAACCGGCYGGGAGCAT) for the coding DNA segment and R1 (CTGGCTCACGATGATC), R2 (AAATTCTTCTGCTMCCCA), R3 (CCACATCTCACGACCAG) and R4 (TGTTGTCGAGGTTTCA) 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 other available in the GenBank/EBML/DDB database.

**Analytical methods**

The compositions of liquid culture media and fermentation broth were analysed by HPLC (Agilent 1100 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 (1%v/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 (Biofaffte, 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 operated using a continuous flow of sterile air at a rate of 1 VVM. The stirring rate was controlled to provide a minimum dissolved oxygen concentration. The fermentations were conducted at 38°C. The pre-culture was prepared in a 5-L embossed flask containing 1 L of GYE/Mg2+ 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 0.1 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 lyzed by sonication in a Bandelin Sonoliver (Germany) at 15 hertz 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 35°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.
Biochemical and molecular identification  Conventional biochemical and morphological tests performed following the guidelines of Bergey's Manual of Systematic Bacteriology (19) revealed that all the isolated bacteria were gram-negative, catalase-positive and oxidase-negative. Morphologically, they appeared as single or paired cocci on GC agar.

The analysis of liquid fermented media revealed that all strains were able to convert ethanol to acetic acid. They can also convert acetate to CO$_2$ and H$_2$O after a several days on brocormesol-containing GCY medium. Other biochemical tests, such as those for the growth in the presence of high glucose concentrations, utilization of ammonium, assimilation of different carbohydrates and utilization of ethanol, revealed that all strains belonged to the *Acetobacter* genus.

Molecular identification was performed according a phylogenetic analysis based on the sequencing of the gene coding for 16S ribosomal RNA, the component of the 30S small ribosomal subunit (29).

The studied species were identified based on reference strains by a digested PCR-amplified fragment analysis of the partial sequence of 16S rDNA. The alignments sequences were obtained from the GenBank databases (30).

AF01 and CV01, which were isolated from apple and cactus fruits, respectively, are closely related to *A. pasteurianus* [99% and 98% homology, respectively (query cover)]. However, TAV01, which was isolated from traditional apple vinegar, was assigned to *Glucosacetobacter xylinus* (more than 98% homology).

Phenotypic differentiation of selected thermoresistant strains  The thermoresistance features of the two studied strains, AF01 and CV01, required additional phenotypic differentiation. The assimilation of carbon sources by these strains was compared to that of wild-type strains using API 50CH strips (8).

The ability to assimilate carbon sources differed between the CV01 and AF01 strains and the wild-type *A. pasteurianus* LMG 1632 and *A. senegalensis* LMG 23690T strains. CV01 was the only strain that could assimilate L-xylene. AF01 was the only strain among the studied *A. pasteurianus* species that could assimilate both glyceral and D-mannose. In addition, the types of metabolized carbohydrates differed between CV01 and AF01. Therefore, the biochemical profiles of the two studied strains differed. These findings permit the genotypic and phenotypic differentiation of the two isolates.

Growth kinetics and fermentation of the isolated strains  In addition to the thermotolerance properties, this study aimed to identify novel features in selected strains intended for the production of fruit vinegar. To this end, the ability of selected thermotolerant strains to produce gluconic acid during acetic fermentation was assessed.

The first part of this study was performed on plates containing SGYEA medium, and the cells were incubated at four different temperatures: 30°C, 34°C, 37°C and 40°C. Bromocresol green (0.022 g/L) was added to the medium to reveal acidification and over-oxidation phenomena. The growth and bioconversion capacities of the two studied strains, AF01 and CV01, were compared with those of the thermoresistant reference strain *A. senegalensis* and other wild-type strains belonging to *A. pasteurianus*: LMG 1632, LMG 1607, LMG 1701, DSM 2324 and DSM 4491.

All of the strains grew well at 30°C. At temperatures greater than 34°C, almost all of the wild-type strains exhibited significant decreases in growth. AF01, CV01 and *A. senegalensis* grew well at 37°C, whereas the reference strains showed weak or no growth. Only AF01, CV01 and *A. senegalensis* continued to grow and produce acetic acid at 40°C. Of these strains, AF01 grew best at 40°C (Table 2).

In addition, the growth kinetics and fermentation behaviour of AF01 and *A. senegalensis* on liquid medium were compared. Fig. 1 shows that AF01 produced more biomass than *A. senegalensis* at both 30°C and 38°C. The average values of the final turbidity measured at 540 nm were 1.29 and 1.42 for the AF01 strain at 30°C and 38°C, respectively; these values were 0.94 and 0.76 for *A. senegalensis* at 30°C and 38°C, respectively.

Moreover, AF01 produced more biomass during fermentation at 38°C than at 30°C (Fig. 1). This finding suggests that AF01 is thermotolerant.

Furthermore, AF01 produced more acetic acid than *A. senegalensis*. At 30°C, AF01 and *A. senegalensis* produced an average of 43.67 g/L and 43.56 g/L acetic acid, respectively, whereas these levels were 45.1 g/L and 31.16 g/L, respectively, at 38°C. Notably, AF01 produced more acetic acid at 38°C than at 30°C.

Furthermore, AF01 strain was able to oxidize all available ethanol at both temperatures, 30°C and 38°C. This strain also oxidized almost all available ethanol after only 26 h at 38°C, whereas the same result was obtained after 48 h at 30°C. Therefore, this bacterium is thermostolerant.

<table>
<thead>
<tr>
<th>Source of isolation</th>
<th>0A, 3E</th>
<th>0.2A, 3E</th>
<th>1A, 3E</th>
<th>1A, 5E</th>
<th>2A, 5E</th>
<th>3A, 5E</th>
<th>4A, 5E</th>
</tr>
</thead>
<tbody>
<tr>
<td>DV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
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<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

* a Origin of selected strains: DV, date vinegar; RAV, rosemary apple vinegar; TAV, traditional apple vinegar; CV, cactus vinegar; AJ, apple juice; AF, apple fruit.

b Cultivation of selected strains on GYEA solid culture media at various concentrations of acetic acid (v/v) and ethanol (v/v), e.g., 0.2A, 3E: GYEA solid culture medium containing 0.2% (v/v) acetic acid and 3% (v/v) ethanol.

c Level of growth: ++, very good growth; +, good growth; +, weak growth; -, very weak growth; --, no growth.

### TABLE 2. Comparison of growth, acidification and over-oxidation capacities between selected strains (AF01 and CV01) and other AAB

<table>
<thead>
<tr>
<th>Strain</th>
<th>30°C</th>
<th>34°C</th>
<th>37°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. pasteurianus</em> (LMG 1632)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AF01</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CV01</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>A. senegalensis</em> (LMG 23690T)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. pasteurianus (DSM 2324)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. pasteurianus (LMG 1607)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. pasteurianus (LMG 1701)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A.Lovaniensis (DSM 4491)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Growth: --, no growth; +, weak growth; +, good growth; ++, very good growth.

Acidification: +, positive test; -, negative test.

Over-oxidation: +, positive test; -, negative test.
Acetic acid fermentation by AF01 and CV01 strains in a bioreactor

Semi-continuous fermentation was performed in a 20-L bioreactor for both selected strains, AF01 and CV01. AF01 was tested first. This strain was thermotolerant on solid and liquid media. Fig. 2A shows that AF01 exhibited a 23-h lag phase. Subsequently, the cells grew exponentially, and their growth slightly slowed after the addition of the second batch, ultimately reaching an OD of 1.54 before the third batch was added. In parallel, acetic acid accumulated in the culture during this phase at an average rate of 0.83 g/L/h. Immediately after the addition of the third batch, the cell growth and acetic acidification rates considerably decreased. The final acetic acid concentration reached 7.64% at the end of the fermentation.

The CV01 strain was also tested in a parallel bioreactor. As shown in Fig. 2B, the lag phase of this bacterium was significantly shorter (5 h) than that of AF01. Furthermore, the production of acetic acid began later in the mid-log phase. The biomass and acetic acidification rates slightly decreased and then recovered after the addition of each batch due to the dilution that results from the addition of fresh medium. In total, 10% (w/v) of acetic acid was produced at an average rate of 1.08 g/L/h. Furthermore, the residual amount of ethanol after fermentation with this strain is lower than that obtained after fermentation with AF01 (7.97 g/L with CV01 compared with 19.02 g/L with AF01).

AF01 was sensitive to increased acetic acid concentrations, likely because acetic acid affects cell growth (31). This result corroborated the evolution of the biomass.

The ability of the isolated strains to produce gluconic acid during acetous fermentation was also investigated. CV01 was the only strain that transformed some of the glucose present in the fermentation medium to gluconic acid while oxidizing ethanol (Fig. 2B). The final amount of gluconic acid produced at the end of cultivation in the bioreactor was 11.03 g/L. This production represents an additional advantage for this strain because gluconic acid and its derivatives are widely applicable in the food and pharmaceutical industries (32). Specifically, gluconic acid is considered a sensory quality indicator in the production of vinegar.

The microbial production of Na-gluconate in batch processes at high temperatures (38–40°C) was also investigated by Zarmehrkhorshid et al. (33).

Gluconic acid increases the biotechnological value of produced vinegar. Gluconic acid has been proposed as quality parameter of traditional balsamic vinegar because it contributes to the aromatic profile and viscosity of the vinegar (34,35). Acetic acid fermentation contributes to the vinegar aroma profile by converting some compounds present in fruit juices to aromatic compounds, such as gluconic acid and acetoin, which are obtained from glucose and DL-lactate, respectively (36).

Some AAB have been reported to produce gluconic acid from glucose. This reaction is catalysed by glucose dehydrogenase (GDH) (32,33). However, few studies reported the simultaneous ability of
AAB strains to produce acetic and gluconic acids in the same fermentation cycle (25,37).

With regard to the acclimation and heritable adaptation of AAB strains in growth culture conditions, Azuma et al. (38) reported that the genetic instability and hyper-mutability of A. pasteurianus strains are responsible for the gradual acquisition of resistance against high temperatures. Furthermore, the cultivability and phenotypic stability of AAB species were studied by Gullo et al. (15). The oxidation of ethanol to acetic acid by A. pasteurianus AB0220 remained stable during long-time preservation (LTP), which confirmed the suitability of the LTP technique for AAB belonging to A. pasteurianus.

Several fermentation cycles of AF01 were monitored in the bioreactor. AF01 was very sensitive to changes in the fermentation conditions of the cultivation medium during later cycles, despite its thermoresistance. This behaviour was considered a negative characteristic of this strain regarding its use in industrial vinegar production.

**Enzymatic study** The activity of enzymes responsible for the acetic fermentation process was analysed in the selected thermoresistant strains, AF01 and CV01, to confirm the thermoresistance of the enzymes. The activities of ADH and ALDH in AF01 and CV01 were compared to those of the thermoresistant reference strain A. senegalensis and the mesophilic strain A. pasteurianus (LMG1632). The enzymes were incubated for 10, 20, 30 and 40 min at 30°C, 34°C and 38°C, and the residual enzyme activity was then measured.

Fig. 3 shows that the three strains, CV01, AF01 and A. senegalensis, exhibited high ADH and ALDH enzyme activity at 30°C and 38°C compared with the mesophilic reference strain LMG1632. However, ADH and ALDH were more resistant to deactivation at 38°C in the mesophilic reference strain than in the other strains. In addition, the thermal inactivation rates of ADH and ALDH were higher at 38°C than at 30°C for all of the strains, and ADH was more thermosensitive than ALDH. Thus, ADH was less thermostable than ALDH. These results agree with the thermotolerant characteristic of this bacterium observed.

A comparative study of acetic acid productivity of wild-type AAB used for ethanol oxidation was reported by Gullo et al. (39). Overall, mesophilic strains exhibited a significant acetic acid yield with respect to thermotolerant species (39). However, thermo-adapted strains (which stably perform efficient acetic fermentation at high temperatures) were obtained after a number attempts to isolate thermotolerant mutants (39,40). Thus, some authors have elucidated the role of a number of genes involved in AAB thermostolerance (39,41).

Two practical conclusions can be deduced from this study: (i) Because AF01 and CV01 could grow and produce acetic acid at high temperatures under semi-continuous conditions, they may be useful as industrial thermostolerant strains, especially in sub-tropical regions. Therefore, future studies should test these strains in a pilot plant bioreactor. In addition, their ability to cost-effectively produce starter should be studied. (ii) Unlike most AAB strains, CV01 was able to simultaneously produce gluconic acid and acetic acid during acetous fermentation. This feature is very important because it is rarely observed in AAB and helps improve the taste of vinegar.

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