Analysis of alcohol dehydrogenase activity and fermentation characteristics of Acetobacter pasteurianus JST-S strain

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Abstract: Alcohol dehydrogenase activity (ADH), acetic acid yield, and tolerance to temperature and acid-induced stress are important bacterial strain parameters for the industrial production of acetic acid or vinegar. In this study, we evaluated and compared multiple features between A. pasteurianus JST-S strain, screened in the laboratory, and A. pasteurianus CICC 20001, a commonly used industrial strain. The ADH enzyme activity peaked at 8.22 U mg⁻¹ for JST-S compared with the 7.62 U mg⁻¹ for CICC 20001. Further, the ADH protein level was higher in JST-S than in the CICC 20001 strain. Comparative analysis of growth and cell morphology of the two strains indicated that the acetic acid tolerance of JST-S is superior to that of CICC 20001. Further, when the two strains were used for semi-continuous fermentation in 4 batches, the total acid production in fermentation broth with the JST-S peaked at 62.96 ± 1.42 g L⁻¹ compared with 56.83 ± 1.12 g L⁻¹ for the CICC 20001 strain. Thus, the JST-S strain seems to have better fermentation characteristics than the commonly used industrial strain. Based on all our observations, we propose that A. pasteurianus JST-S may be applied for cost-effective industrial production to obtain a high concentration of acetic acid.

1. Introduction

Acetic acid bacteria, also known as Acetobacter, are widely used in the brewing of vinegar¹-². Current industrial-scale production of acetic acid utilizes two broad categories of bacteria, namely Acetobacter and Gluconobacter³⁴. CICC 20001 and AS 1.41, which can oxidize ethanol to acetic acid, are the main A. pasteurianus strains used in China for the industrial brewing of vinegar⁵-⁶.

Previous studies have shown that acetic acid bacteria can convert ethanol into acetic acid, and is associated with membrane-bound pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) in the ethanol oxidizing respiratory chain⁷-⁹. PQQ-ADH is localized on the periplasmic side of the A. pasteurianus inner membrane and is a complex consisting of 3 subunits, a dehydrogenase subunit (subunit I), a cytochrome c subunit (subunit II), the smallest subunit, subunit III⁷-¹⁰. Previous studies have suggested that PQQ-ADH may affect the fermentation characteristics of A. pasteurianus in response to stress resulting from elevated temperatures or high concentrations of acid or alcohol¹¹-¹³. PQQ-ADH is a unique member of the alcohol dehydrogenase family and is...
essential for acetic acid bacteria oxidation of ethanol into acetic acid[14].

An increasing number of studies have been undertaken to establish the relationship between the key enzymes of the ethanol oxidizing respiratory chain and acetic acid yield [5,14,15]. However, there is very little data that elucidates the relationship between the enzyme dynamics in the ethanol oxidizing respiratory chain, the enzymatic protein expression and the tolerance of acetic acid bacteria under high temperature, acid, and alcohol stress.

In this study, the ADH activity and the tolerance of the *A. pasteurianus* JST-S strain during the fermentation process were compared to that of *A. pasteurianus* CICC 20001. The acid resistance mechanism of *A. pasteurianus* JST-S was evaluated by growth assay, scanning electron microscopy and by examining ADH enzyme activity and expression by SDS-PAGE gel electrophoresis. Additionally, we carried out semi-continuous fermentation to assess the suitability of each strain for application in industrial vinegar fermentation.

2. Materials and methods

2.1. Bacterial strains and media

*A. pasteurianus* CICC 20001 (also known as Huniang 1.01) was obtained from the China Center of Industrial Culture Collection. *A. pasteurianus* JST-S was isolated from solid fermentation substrate of vinegar (Yantai Di Boshi brewing machine Co., Ltd., Yantai, China) stored in the laboratory at the College of Food Science and Engineering, the Key Laboratory for Agricultural Products Processing of Anhui Province, Hefei University of Technology. All *Acetobacter* strains were maintained on seed medium consisting of 10 g L⁻¹ glucose, 5 g L⁻¹ yeast extract, 1.1 g L⁻¹ MgSO₄, 3.3 g L⁻¹ K₂HPO₄. YPGD medium (5 g L⁻¹ yeast extract, 5 g L⁻¹ peptone, 5 g L⁻¹ glucose, 5 g L⁻¹ glycerol, 17 g L⁻¹ agar) supplemented with different concentrations of acetic acid was used in growth-monitoring experiments.

Fermentation medium contained 10 g L⁻¹ glucose, 5 g L⁻¹ yeast extract, 1.1 g L⁻¹ MgSO₄ꞏ7H₂O, 3.3 g L⁻¹ K₂HPO₄, and different concentrations of ethanol. Fermentation medium were divided into 50 mL aliquots in 250 mL Erlenmeyer flasks and then sterilized at 121 °C for 20 min prior to the addition of ethanol and acetic acid. To prepare seed culture, cells were cultured on a Zhp Serias thermostat incubator with shaker (ZHP-Y2102L; Shanghai Sanfa Scientific Instruments Co., Ltd., Shanghai, China) at 30 °C for 24 h at 150 rpm. Fermentation medium was inoculated with 10% seed culture and incubated at 32 °C at 150 rpm. The chemicals used in the study were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd., Beijing, China.

2.2. Determination of ADH enzyme activity

To determine ADH enzyme activity in response to varying concentrations of ethanol and acetic acid, strains were inoculated and cultivated in fermentation medium containing 2%, 4%, 6%, or 8% (v/v) ethanol or 0.3%, 0.6%, 0.9%, or 1.2% (v/v) acetic acid at 32 °C for 60 h at 150 rpm. In addition, strains were also inoculated in fermentation media and cultivated at 28 °C, 32 °C, 36 °C, or 40 °C for 60 h at 150 rpm in order to evaluate ADH enzyme activity under different temperatures. Following incubation, samples were centrifuged at 8000 × g, at 4 °C for 10 min and washed 3 times using 0.01 mol L⁻¹ PBS (pH 7.4) buffer. The crude enzyme was extracted using methods described previously [15-16]. ADH activity was determined with potassium ferricyanide as an electron acceptor coupled with catalytic dehydrogenation of substrate by the enzyme[17]. All ADH activity assays were performed at 25 °C. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of substrate per minute. The protein concentration was measured by the modified Lowry method and bovine serum albumin as a standard protein[18].

2.3. Analysis of ADH protein expression by SDS-PAGE gel electrophoresis

Strains were cultivated at 32 °C for 24 h in fermentation media with 6% (V/V) ethanol and 0.3% (V/V) acetic acid. The ADH enzyme extracts were obtained from 20 mL fermentation broth and analyzed by SDS-PAGE gel electrophoresis. Briefly, the fermentation broth was centrifuged at 8000 × g, at 4 °C
for 10 min and cells were collected. The cells were washed three times with 0.01 mol/L PBS (pH 7.4) buffer and then suspended in suspension buffer (10 mmol L⁻¹ Tris-HCl pH 8.0, 5 mmol L⁻¹ EDTA, 1 mmol L⁻¹ PMSF) for 10 min in a 20:1 proportion (20 mL fermentation liquid mL⁻¹ suspension buffer). The suspended cells were lysed by ultrasonic cell disruption (JY92-II, Xinzhi Biotechnology Co. Ltd., Ningbo, China) using pulses (200 W) lasting 5-10 s, 60 times on ice followed by centrifugation at 10000 × g at 4 °C for 12 min and storing the supernatant containing ADH enzyme at –80 °C.

Sample aliquots (30.0 µL) were mixed with 10 µL protein sample loading buffer and boiled alongside protein ladder aliquots (20 µL) in a boiling water bath for 10 min and loaded on SDS-PAGE gels for electrophoresis, which was run initially at 60 V for 30 min followed by 80 V for 4 h. Following protein separation, the gels were Coomassie-stained for 30 min followed by overnight de-staining. The samples were then photographed on a gel-imager (Bio-Rad, USA).

2.4. Growth analysis of the strains
To observe and compare bacterial growth, strains were cultivated at 30 °C for 24 h. They were then transferred to the same Erlenmeyer flasks sterilized at 121 °C for 20 min and diluted 10⁸ times with stroke-physiological saline solution (0.9 g NaCl per 100 mL distilled water). The samples (0.1 mL) were then inoculated in YPGD medium containing 1.5%, 1.8% or 2.1% (v/v) acetic acid at 32 °C for 3 days.

2.5. Morphological analysis of strains under scanning electron microscope
Morphological features of strains were analyzed using the scanning electron microscope (SEM), following previously reported procedures with some modifications[19-21]. Briefly, liquid culture was centrifuged at 8000 × g for 10 min at 4 °C followed by suspension into larger 50 mL fermentation medium containing 6% (v/v) ethanol and 2% (v/v) acetic acid and incubation at 32 °C for 14 h. Bacterial suspensions were pelleted by centrifugation and cell pellets were washed three times with 0.01 mol L⁻¹ PBS (pH 7.4) buffer followed by fixation at 4 °C by 25% (v/v) glutaraldehyde for 4 h and subsequent centrifugation. The samples were dehydrated using a stepwise increment of ethanol concentration (35%, 60%, 85%, 95%, 100% (v/v)) and vacuum freeze-dried for 6 h. Finally, bacterial cells were coated with gold for 90 s and observed under a scanning electron microscope (JEM-2100F; JEOL, Japan).

2.6. Analysis of repeated batch fermentation
In order to study the fermentation characteristics of *A. pasteurianus* CICC 20001 and JST-S, the strains were inoculated in fermentation media containing 8% (V/V) ethanol and cultivated at 32 °C for 96 h. Subsequently, the fermented broth was collected and rapidly centrifuged at 8000 × g for 10 min at 4 °C to obtain cells. Cells were collected under aseptic conditions and again inoculated in fermentation media containing 8% (v/v) ethanol and cultivated at 32 °C for 60 h. To perform semi-continuous fermentation, the process was repeated four times. The acidity of fermentation broth was measured by 0.1 mol L⁻¹ NaOH with phenolphthalein as an indicator of pH[12,22-25]. Samples were collected every 12 h and total acetic acid content was determined using the above titration method. The bacterial biomass was measured as described previously[26].

3. Results and discussion

3.1. ADH enzyme activity of JST-S and CICC 20001 strains under varying initial ethanol contents
We first set out to determine the strain specific dynamics of ADH enzymatic activity under varying ethanol concentration in the culture broth. Our results are shown in Fig. 1. The ADH enzyme activity of both strains showed dynamic trends with an initial proportional increase when ethanol concentration was within a range of 2–4%. The enzyme activity of *A. pasteurianus* CICC 20001 and JST-S peaked with values 5.91 U mg⁻¹ and 6.48 U mg⁻¹, respectively, when ethanol concentration was 4% and after 36 h of fermentation. Higher concentrations of ethanol (6–8%) resulted in reduced peak
activity of ADH enzymes in both strains. At 8% ethanol concentration, the peak ADH enzyme activity, which occurred at 42 h of fermentation, was the lowest for both CICC 20001 and JST-S strains at 3.31 U mg\(^{-1}\) and 4.15 U mg\(^{-1}\), respectively. These results implied that strain growth may be inhibited under high alcohol conditions, which led to the prolonged fermentation time for ADH enzyme activity to peak. The peak ADH enzyme activity of \textit{A. pasteurianus} JST-S at alcohol concentrations 2 and 4% was higher than that of the CICC 20001 strain, which indicated that the ability of \textit{A. pasteurianus} JST-S to oxidize ethanol might be better than that of the CICC 20001 strain.

![Figure 1](image_url)

**Figure 1.** ADH enzyme activity under different ethanol concentrations in culture media. (A), \textit{A. pasteurianus} CICC 20001; (B), \textit{A. pasteurianus} JST-S. The different ethanol concentrations are as indicated by the key. Data are represented as the average of three replicate measurements. Error bars show standard deviations.

### 3.2. ADH enzyme activity of JST-S and CICC 20001 strains at different temperatures

Temperature is an important factor affecting enzyme activity [27]. Accordingly, we observed significant differences in the enzyme activity in either of the \textit{A. pasteurianus} strains with alterations in incubation temperature (Fig. 2). The peak ADH enzyme activities of both strains were relatively small (CICC 20001: 5.16 U mg\(^{-1}\) and JST-S: 5.64 U mg\(^{-1}\)) when the fermentation temperature was at 28 °C. When fermentation temperature was 32 °C, the peak value of ADH enzyme activities of \textit{A. pasteurianus} was at the highest (CICC 20001: 7.62 U mg\(^{-1}\) and JST-S: 8.22 U mg\(^{-1}\)). With increase in incubation temperatures, we observed a dramatic attenuation of enzyme activity with lower peak values (CICC 20001: 3.22 U mg\(^{-1}\) and JST-S: 6.32 U mg\(^{-1}\) at 36 °C; CICC 20001: 1.32 U mg\(^{-1}\) and JST-S: 2.09 U mg\(^{-1}\) at 40 °C). Thus, at lower fermentation temperatures the two strains display high enzyme activity with negligible difference but beyond a certain temperature range, the peak activity values are dramatically reduced, showing significant differences between the strains. Comparing the values between the two strains, we concluded that the maximum enzyme activity of the \textit{A. pasteurianus} JST-S was higher than that of the CICC 20001 strain, implying that the JST-S strain is more thermo-tolerant. Therefore, the JST-S strain may be more suitable for industrial vinegar fermentation at higher temperatures than CICC 20001 strain, as it may reduce cooling costs.
3.3. ADH activity in JST-S and CICC 20001 strains under different acid stress conditions

Previous studies have shown that vinegar yield was directly proportional to the stability of ADH enzyme activity under high acidity \cite{11}. A comparative analysis of ADH activity from both strains revealed that with initial acetic acid concentration in the range of 0.3\%–0.6\%, the ADH activity in either strain increased gradually and peaked after 30 h of fermentation (Fig. 3). When initial acetic acid content was 0.6\%, the ADH activity peaks of both CICC 20001 and JST-S strains were at the highest, 7.05 U mg\(^{-1}\) and 7.89 U mg\(^{-1}\), respectively. With further increase in initial acetic acid concentration (0.9\%–1.2\%), the activity of ADH enzyme decreased in both strains. The peak value of ADH enzyme activity was not only the most attenuated (CICC 20001: 2.41 U mg\(^{-1}\) and JST-S: 3.72 U mg\(^{-1}\)) at 1.2\% initial acetic acid concentration, but was also delayed by 6 h compared to the lower initial acetic acid concentrations (Fig. 3). Thus, an initial acetic acid concentration within the range of 0.3–0.6\% resulted in higher enzyme activity, achievable after shorter fermentation durations in either strain. However, further increase in the acid concentration may result in the repression of enzyme activity or protein expression, based on the highly attenuated peak activity values attained after longer durations of fermentation. A comparative analysis of the enzyme activity suggests that the \textit{A. pasteurianus} JST-S strain might present superior acid resistance than the CICC 20001 strain.

Figure 3. ADH enzyme activity under different initial acetic acid contents (A), \textit{A. pasteurianus} CICC 20001; (B), \textit{A. pasteurianus} JST-S. The different acetic acid concentrations are as indicated by the key. Data are represented as the average of three replicate measurements. Error bars show standard deviations.
3.4. JST-S strain shows better growth at higher acetic acid concentrations
We cultivated *A. pasteurianus* CICC 20001 and JST-S strains in YPGD media supplemented with acetic acid at different concentrations and compared the growth of the two strains. As shown in Fig. 4, the number of *A. pasteurianus* CICC 20001 and JST-S colonies decreased with an increase in acetic acid concentration. While the number of colonies showed no obvious visual differences at 1.5% acetic acid concentration, at 1.8%, the number of colonies in either strain decreased significantly, indicating that 1.8% acetic acid had a slight inhibitory effect on the growth rate of the strains. At 2.1% concentration, the number of colonies decreased dramatically, with the number of *A. pasteurianus* JST-S strain colonies significantly higher than that of the CICC 20001 strain (Fig. 4). Thus, high concentrations of acetic acid negatively affect the growth of either bacterial strain, with *A. pasteurianus* JST-S strain displaying superior acetic acid tolerance, compared with the CICC 20001 strain.

3.5. Cell morphology is better conserved in the JST-S strain under acid stress
SEM is a conventional method to study the surface changes of microbiological cells [28-30]. We examined the cell morphology and membrane response to high acetic acid concentration (2%) in culture media for the *A. pasteurianus* JST-S and CICC 20001 strains (Fig. 5). Acetic acid, a metabolic byproduct of acetic acid bacteria, exhibits significant toxicity to the cells on accumulation in the cell-culture media [30]. Resistance to acetic acid has been shown to be dependent on the cell membrane characteristics [31-32]. As shown in Fig. 5, *A. pasteurianus* JST-S and CICC 20001 strains showed irregular rod-shaped cells having rough surface, along with the appearance of some irregularities on the cell surface of some cells displaying ruptured folds as indicated by the white arrows. These results suggest that high acetic acid concentration in the media environment can lead to detrimental changes in osmotic pressure within some *A. pasteurianus* JST-S and CICC 20001 cells, resulting in their rupture. We hypothesize that the surface structure of acetic acid bacteria was altered to adapt to the new acetic acid environment in order to maintain ion transport or transmembrane transport and combat acetic acid stress. Further, *A. pasteurianus* JST-S cells displayed higher degree of surface smoothness and less disrupted cells compared with *A. pasteurianus* CICC 20001, implying a lower susceptibility of the JST-S strain to the toxic effects of acetic acid.

Figure 4. Comparative growth of the two strains under different acetic acid concentrations. Growth was examined under acetic acid concentrations of 1.5%, 1.8% and 2.1%. Growth was evaluated based on the number of colony forming units. B1–3: growth of *A. pasteurianus* JST-S and H1–3, growth of *A. pasteurianus* CICC 20001.

3.6. Comparative analysis of ADH protein expression using SDS-PAGE
The molecular weight of the ADH protein as observed on SDS-PAGE appeared slightly different between the two strains (Fig. 6). The molecular weight range of subunit I and subunit II is reported to be 71–85 kDa and 44–55 kDa, respectively [39]. As seen in Fig. 6, one clear band appeared between the
66.2 and 97.4 kDa molecular weight markers, which we estimated to be approximately 79 kDa. Another clear band appeared above the 43 kDa molecular weight marker band and was estimated to be approximately 44 kDa. The two ADH bands obtained from the JST-S strain were stronger compared with those from the *A. pasteurianus* CICC 20001, suggesting that ADH protein expression could be higher in the *A. pasteurianus* JST-S than in the CICC 20001 strain.

3.7. Performance in repeated batch fermentation

As shown in Fig. 7, the total acid content, the ethanol content and the biomass of *A. pasteurianus* JST-S and CICC 20001 showed similar trends with fermentation time. Within the first 24 h of fermentation, the strains displayed slow growth kinetics while adapting to the fermentation conditions. During this time, both ethanol utilization and acetic acid yield were low. After 24 h of fermentation, both strains entered a stage of rapid growth with elevated acetic acid production. After fermentation for 96 h, the total acid content in the system reached the highest values for both *A. pasteurianus* strains (CICC 20001: 54.14 g L\(^{-1}\), JST-S: 63.54 g L\(^{-1}\)); the ethanol residue was at the minimum (CICC 20001: 7.13 g L\(^{-1}\), JST-S: 3.92 g L\(^{-1}\)); and the biomass was at maximum (CICC 20001: 0.281 g L\(^{-1}\), JST-S: 0.328 g L\(^{-1}\)). A comparison between the two strains suggested that *A. pasteurianus* JST-S showed higher ethanol utilization rate and better growth during the entire fermentation process in the first batch fermentation than the *A. pasteurianus* CICC 20001 strain.
Figure 7. Comparison of the first batch fermentation characteristics of *A. pasteurianus* JST-S and CICC 20001. The total acid content is indicated by squares, the biomass by circles, and the ethanol content by triangles. In each case, the filled shape refers to *A. pasteurianus* JST-S strain, while the open shape refers to *A. pasteurianus* CICC 20001 strain. Data are represented as the average of three replicate measurements and error bars show standard deviations.

Repeated batch fermentation is a common practice in industrial deep acetic acid fermentation owing to the higher acetic acid production rate and yield along with reduced running costs\(^1\). The results of repeated batch fermentation of *A. pasteurianus* CICC 20001 and JST-S are shown in Fig. 8 and Table 1. Both *A. pasteurianus* CICC 20001 and JST-S strains showed similar trends in total acetic acid production with respect to time with repeated batch fermentation (Fig. 8). In all 4 fermentation batches (A–D in Fig. 8), the JST-S strain showed a higher average acetic acid production capacity of 62.96 ± 1.42 g L\(^{-1}\), while the average acetic acid content in *A. pasteurianus* CICC 20001 was significantly lower at 56.83 ± 1.12 g L\(^{-1}\) (Table 1). These results imply that *A. pasteurianus* JST-S has better fermentation characteristics in terms of acetic acid production (As shown in Table 1) and could be used for cost-effective industrial acetic acid production.

Figure 8. Repeated batch fermentation of *A. pasteurianus* CICC 20001 and JST-S. A, B, C and D represent the first, second, third and fourth batches of fermentation broth, respectively. The total acid produced is indicated by the closed circles for *A. pasteurianus* CICC JST-S, and closed squares for *A. pasteurianus* CICC 20001 strain. The data are represented as the average form three replicate measurements and the error bars show standard deviations.

4. Conclusion
In our study, we found that ADH enzyme activity of *A. pasteurianus* JST-S strain was decisively superior to that of the CICC 20001 strain under varying fermentation conditions. Furthermore, higher ADH enzymatic protein expression in the JST-S strain, as analyzed by SDS-PAGE, in addition to
superior growth characteristics and conservation of cell morphology under high acetic acid concentrations implied greater tolerance of the JST-S strain to extreme acidic conditions. The results of semi continuous fermentation suggested that *A. pasteurianus* JST-S strain could better withstand the additional stress induced by repetitive batch culturing in the industrial production setting. Future studies to elucidate the metabolic mechanisms resulting in higher tolerance of JST-S strain and adaptability to industrial production should be attempted with an aim to further improve acid yield by genetic engineering and proteomic manipulations. This research must also be undertaken to the study production of the different flavors of vinegar.

Table 1. Determination of different parameters in the repeated batch fermentation process with *A. pasteurianus* CICC 20001 and JST-S strains

| Strain   | Biomass g L⁻¹ | Total acetic acid content g L⁻¹ | Qmax g L⁻¹ h⁻¹ | ADHmax U mg⁻¹ |
|----------|---------------|-------------------------------|-----------------|---------------|
| First batch |               |                               |                 |               |
| A        | 0.28±0.02     | 54.68±1.36                    | 0.873±0.04      | 7.62±0.06     |
| B        | 0.32±0.02     | 62.96±1.42                    | 1.046±0.03      | 8.22±0.04     |
| Second batch |            |                               |                 |               |
| A        | 0.33±0.01     | 56.72±1.32                    | 1.155±0.04      | 7.74±0.03     |
| B        | 0.36±0.03     | 62.68±1.48                    | 1.191±0.07      | 8.86±0.05     |
| Third batch |           |                               |                 |               |
| A        | 0.34±0.02     | 56.26±1.68                    | 1.139±0.05      | 7.76±0.04     |
| B        | 0.38±0.01     | 62.76±1.54                    | 1.099±0.06      | 8.92±0.06     |
| Forth batch |             |                               |                 |               |
| A        | 0.35±0.03     | 56.83±1.12                    | 1.10±0.04       | 7.63±0.07     |
| B        | 0.39±0.02     | 62.13±1.44                    | 1.297±0.05      | 8.96±0.06     |

Notes: A, *A. pasteurianus* CICC 20001; B, *A. pasteurianus* JST-S; Qmax, Maximum fermentation intensity; ADHmax, Maximum ADH activity

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