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Isolation, identification and characterization of thermo-tolerant acetic acid bacteria for semi-continuous acetous fermentation at high temperature

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Abstract

Nowadays, vinegar is industrially produced by mesophilic acetic acid bacteria (AAB). However, temperature fluctuation during acetous fermentation is inevitable and may cause process disturbances. This can be mostly avoided using thermo-tolerant AAB. The main purpose of the present study was to isolate thermo-tolerant AAB and then evaluate the fermentation performances. Twenty-eight different isolates were isolated from traditional Iranian vinegar. One of the isolates was able to grow and produce acetic acid in minimal culture media containing 5% (w/v) ethanol at 30-42 ºC. The 16s rRNA gene analysis showed that the selected thermo-tolerant isolate was *Acetobacter tropicalis* (L31). Acetous fermentation in a Lab-bioreactor showed that *Acetobacter tropicalis* (L31) grew in minimal culture medium, and produced 5% (w/v) acetic acid at 37°C in batch and semi-continuous fermentation mode. Fermentation time was significantly dependent on the dissolved oxygen (DO) concentration and acclimation of cells to low pH and acid stress. On average, acclimated cells produced 2.3-2.7 g $L^{-1}h^{-1}$ acetic acid during the production phase. The final yield was 87% at 37ºC in low and high DO concentrations. Low DO concentration (15%) during acetous fermentation caused longer fermentation time, but a large part of the cells (91%) grown under such condition were still viable even if oxygen flow was interrupted. In contrast, cells grown under high DO concentration were not able to tolerate oxygen deficiency. In conclusion, since *Acetobacter tropicalis* (L31) grew well in minimal culture medium and showed tolerance to high temperature and low DO, it is a potential isolate for vinegar starter production.

1. Introduction

Fermentation is one of the oldest food processing technologies in the world which has been passed down from generation to generation (Kebede et al., 2007). Vinegar as a well-known fermentative product that is defined as a solution which contains at least 4% acetic acid developed from ‘’acetous fermentation’’ of ethanol solutions (Hutkins, 2006).

Both of the well-defined traditional (slow) and submerged (fast) processes for vinegar production (Mamlouk et al., 2013) are used in Iran. Sprite vinegar is the main vinegar produced

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by the submerged process using distilled ethanol as the main carbon source. Due to the hot dry climate in the south of Iran, most vinegar producers are located in the north and central regions of Iran. However, vinegar production can be advantageous for southern regions because of the availability of certain agricultural products such as various kinds of date.

Generally, the fermentation process faces different challenges in hot and dry regions. One of the main challenges is temperature fluctuation, which may disturb the fermentation process and increase the cooling costs of the bioreactor (Ndoye, et al., 2007; Ndoye et al., 2006). Moreover, acetic acid bacteria (AAB), which are Gram negative bacteria, are routinely used in vinegar production. They are very susceptible to nutrient depletion, oxygen deficiency, temperature fluctuation, etc. (Hutkins, 2006; Qi et al., 2014; Shafiei, et al., 2013; Zheng et al., 2018). Therefore, using a thermo-tolerant acetic acid bacteria which can grow and produce acetic acid in a wide ranges of temperature (28-38°C) enables producers to produce a cost-effective vinegar by reducing the cooling costs (Ndoye, et al., 2006; Ndoye et al., 2007).

In spite of numerous reports about isolation, identification, and characterization of thermo-tolerant bacterial isolates, only a few bacterial isolates have been tested as a vinegar starter under the proper conditions (Mounir, Shafiei, Zarmehrkhorshid, Hamouda, Ismaili Alaoui, 2016; Mounir, Shafiei, Zarmehrkhorshid, Hamouda, Thonart, 2016; Ndoye et al., 2009).

There are some studies about vinegar starter production over a long period of time (Gullo et al., 2009; Lu et al., 1999; Mounir et al., 2015; Mounir, et al., 2016; Sokollek et al., 1997; Sokollek et al., 1998). Due to the diversity of traditional vinegars in Iran and the lack of comprehensive investigations on them, one of the main goals of the present study is to isolate, identify and characterize thermo-tolerant acetic acid bacteria from different kinds of traditional vinegar. This study also aims to evaluate the performance of selected thermo-tolerant isolate in submerged acetous fermentation at high temperature.

2. Materials and methods

2.1. Microorganisms

The mesophilic and thermo-tolerant acetic acid bacteria used in this study as reference bacteria are wild type strains coming from the Belgian Co-Ordinated Collections of Microorganisms (BCCM).

Acetobacter aceti (LMG 1535) and *Acetobacter pasturanius* (LMG 1625) were used as mesophilic reference strains (Ndoye et al., 2006). They consume ethanol and grow at 25-32°C. *Acetobacter senegalensis* (LMG 23690) was used as the thermo-tolerant strain. It can grow in liquid medium containing ethanol up to 42°C (Ndoye et al., 2006). In addition, this strain has been studied recently for vinegar starter production and gluconic acid production (Mounir, et al., 2016; Shafiei, et al., 2013; Shafiei et al., 2014; Shafiei et al., 2017).

2.2. Vinegar samples and culture media

Thirty-one vinegar samples from different regions of Iran were collected. The regions were chosen on the basis of various features such as availability of different fruits, climate and living traditions.

Acetic acid bacteria were isolated by several different enrichment and isolation culture media. Enrichment media (100 mL) were prepared in 500 mL baffled flasks. The AE medium contained 5 gL^{-1} glucose, 3 gL^{-1} yeast extract, 4 gL^{-1} casein peptone, 5% (w/v) ethanol and 1% (w/v) glacial acetic acid. The YGM/Mg^{2+} contained 10 gL^{-1} yeast extract, 20 gL^{-1} glucose, 20 gL^{-1}, Manitol and 0.5 gL^{-1} $MgSO_4$. These two culture media were used as the enrichment culture media (Ndoye et al., 2006; Sokollek et al., 1997; Sokollek et al., 1998).

GYE agar (20 gL^{-1} glucose, 5 gL^{-1} Yeast extract, 5 gL^{-1} casein peptone, 50 gL^{-1} ethanol, 15 gL^{-1} $CaCO_3$, 0.022 gL^{-1} green bromocresol and 15 gL^{-1}

agar) was used as an isolation culture medium. In addition, it was used to determine over-oxidation or sub-oxidation of ethanol in the isolates. Broth GYE supplemented with different ethanol and acetic acid concentrations and without calcium carbonate and green bromocresol was also used to test the growth and acetic acid production under different conditions (section 2.5 and 2.6).

2.3. Isolation procedures and preservation of bacteria

Five mL of each vinegar sample was inoculated into each flask containing enrichment culture media. They were then incubated at 30 and 38ºC for 2-10 days on a shaker (120 rpm). Growth was monitored by checking the turbidity (OD_{540nm}). If OD_{540nm} was higher than one, 100 µL of the culture media suspension was transferred to the solid culture media (GYE agar) and inocubated at 30ºC and 38°C.

Isolates were selected on the basis of colony and/or cellular morphologies; consequently, the probability of isolating different species from the samples increases irrespectively to their relative presence (Gullo et al., 2006).

The purified isolates were stored in Microbank^{Tm} vials at -80ºC for long term storage according to the procedures presented by the PRO-LAB Company.

2.4. Molecular and biochemical identification of bacterial isolates

Morphological, biochemical and molecular tests were done to confirm that the isolated bacteria belong to acetic acid bacteria (AAB).

Morphological characteristics including shape, size, arrangement, Gram staining and motility were determined from cells grown at 30ºC on GYE agar under aerobic condition (Cleenwerck et al., 2002). Overoxidation capacity was tested by using the chalk-ethanol test of Carr and Passmore (Brenner et al., 2005). Carbohydrate assimilation tests were determined using API 50 CH Stripes (BioMerieux) (Ndoye et al., 2006).

Molecular identification was done based on 16s rRNA gene sequencing. Total genomic DNA of the selected isolates was extracted from fresh growing cultures on YGM/Mg^{+2} by using an extraction kit from the NucleoSpin® Company.

PCR amplification of the 16s rRNA gene was performed based on the procedures previously described by Ndoye, et al. (2006). Analysis of sequence was done by using the Blast program from GenBank and was compared with others available in the GenBank/EMBL/DDBJ database via BLASTN from NCBI and then aligned with reference sequences included in those databases.

The phylogenetic relationships among species were analyzed based on 16S rRNA gene sequences of 1,384 bases using the neighbor-joining (NJ) approach listed in the MEGA (Molecular Evolutionary Genetic Analysis) version X software. For NJ analysis, the distance between sequences was calculated using Kimura's two parameter model. Bootstrap values were obtained for 1,000 randomly generated trees.

2.5. Nutritional requirements of isolated bacteria

To obtain isolates capable of growing and producing acetic acid in minimal culture media, two minimal culture media (GYE_5 and GYE_2) were used. GYE_5 contained: $(NH_4)_2HPO_4$ 0.7 gL^{-1}, $MgSO_4$ 0.2 gL^{-1}, glucose monohydrate 2 gL^{-1}, yeast extract 5 gL^{-1} and ethanol 50 gL^{-1}. GYE_2 has the same composition except that it contained 2 gL^{-1} yeast extract. All the components were sterilized at 121 ºC for 20 min. except the glucose which was sterilized separately.

2.6. Determination of growth and concentrations of acetic acid, ethanol and glucose

Bacterial growth was monitored by measuring absorbance at 540nm against a non-inoculated medium as a blank (Ndoye et al., 2006).

Acetic acid concentration, glucose and ethanol

concentration in vinegar samples and culture media in the end of fermentation were determined by high performance liquid chromatography. Culture samples were collected and centrifuged (3000 ×g, 10 min). The supernatants were then filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The HPLC analyses were performed using an Agilent 1110 series HPLC equipped with a Supelcogel C 610H column preceded by a Supelguard H pre-column (oven temperature 40 °C) and a differential refraction index detector (RID, detection cell maintained at 35°C). An isocratic mobile phase consisting of 0.1 % H_3PO_4 (in MilliQ water) was used at a flow rate of 0.5 mL min^{-1}. The method lasted for 35 min at a maximum pressure of 60 bars (Shafiei, et al. 2017; Shafiei, et al. 2013).

2.7 Acetous fermentation

To test the ability of selected isolate to oxidized ethanol to acetic acid, the fermentation process was performed in a Lab-scale bioreactor (Bio Lafitte, France), with a total volume of 20 L and a working volume of 15 L. The bioreactor was equipped with three probes used for monitoring temperature, pH and dissolved oxygen. A cross-shaped sparger and two marine-blade impellers (left-handed orientation) were used to aerate the culture medium. The air inlet was set at 1vvm. Dissolved oxygen (DO) concentration was regulated automatically by changing the impeller rotation speed based on the oxygen demand during the growth of bacteria. Since acetic acid bacteria are strict aerobes, two critical thresholds were set for the dissolved oxygen (DO) concentration: 45% and 15% for cultivation under high DO and low DO concentrations, respectively, and the pH was monitored but not regulated. Temperature was set at 37 ±0.5°C.

A minimal culture media was used for acetous fermentation. It contained 2 gL^{-1} glucose, 2 gL^{-1} yeast extract, 1 gL^{-1} $(NH4)_2$ HPO_4, 5% (w/v) ethanol, 0.5% (w/v) acetic acid.

The bioreactor was inoculated by pre-culture (fresh cells, OD_{540nm} about 1.5) prepared in a 5L baffled flask containing 800 mL minimal culture medium under aerobic condition (130 rpm) at 37°C.

After inoculation, the pH, ethanol concentration, acetic acid concentration and biomass were determined by the methods previously explained.

2.9. Flow cytometric analysis

Cells withdrawn from the bioreactor were harvested (5000 ×*g,* 10 min, 30°C) and washed with Phosphate buffer solution (50 mM, pH 7.4). The washed cells were suspended in fresh GYE broth containing 5% (w/v) ethanol as the main carbon source. Cell viability was determined using Thiazole orange (TO) and 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) as fluorescent dyes according to the protocol previously explained and used by Shafiei et al. (2013).

2.8. Statistical analysis

The data presented in the "acetous fermentation" section are the means of at least two independent fermentation runs.

3. Results

3.1. Analysis of vinegar samples

Various types of Iranian vinegar produced from different fruits were collected from different regions and analyzed. Generally, residual glucose concentration was low meaning that ethanol fermentation was done properly. Most of the vinegars had a low concentration of residual ethanol; however, some types of vinegar (such as A1, G1, G2, G4, R1, etc.) contained higher concentrations of residual ethanol (Supplementary files: Table 1S).

3.2. Isolation of bacteria and growth in different culture media

A total of 28 AAB were isolated from

different fruits were collected from different regions and analyzed. Generally, residual glucose concentration was low meaning that ethanol fermentation was done properly. Most of the vinegars had a low concentration of residual ethanol; however, some types of vinegar (such as A1, G1, G2, G4, R1, etc.) contained higher concentrations of residual ethanol (Supplementary files: Table 1S).

3.2. Isolation of bacteria and growth in different culture media

A total of 28 AAB were isolated from traditional vinegars using different enrichment and isolation media at 30 and 38°C. Thirteen isolates originated from grape vinegar and 15 isolates were isolated from other types of vinegar. We could not isolate acetic acid bacteria from some samples even after enrichment in different culture media (Supplementary files: Table 1S). In addition, it seems that some vinegar samples with low concentrations of ethanol (<0.25% w/v) and also old vinegars had lower bacterial population; therefore, a long period of enrichment (about 10 days) was needed to observe detectable growth. Isolation of bacteria needs different kinds of culture media to ensure that all the present bacteria have the ability to grow. GYE medium can support the growth of all acetic acid bacteria (Gullo et al., 2006; Gullo et al., 2008; Gullo et al., 2012). However, even after a long incubation under optimum conditions, we could not detect the growth of acetic acid bacteria in some types of vinegar samples, especially in older ones (age>3 years). This can be explained on the basis that AAB are fastidious microorganisms and their cultivation on synthetic culture media is difficult. In addition, the suggested culture media are not able to support the growth of all AAB (Gullo et al., 2006). Moreover, it has been proven that storage conditions affect the culturability of AAB, for example AAB proliferated in bottled wine are more difficult to isolate and cultivate (Bartowsky et al., 2008). The phenomenon of viable but non-culturable (VBNC) cells has been ascribed to AAB where their population is very often under estimated (Bartowsky et al., 2008; Shafiei, et al., 2013; Shafiei, Delvigne, & Thonart, 2013). Some methods, such as epifluorescence staining techniques, Real time PCR and DGGE electrophoresis, have been described for detection and enumeration of AAB in foods (Bartowsky et al., 2008; De Vero et al., 2008) whereas double layer-agar and AE-Medium has partially reduced the problem of isolation and cultivation of AAB (Sokollek et al., 1998). Thus, it seems that in the cases where we could not isolate bacteria from the vinegar sample, bacteria were either in a VBNC state or other cultivation techniques were necessary.

Principally, higher temperature (38°C) inhibited the growth of AAB. Just two vinegar samples contained AAB bacteria which could grow in GYE broth at 38°C after 10 days.

All isolated bacteria formed colonies and produced acetic acid in GYE agar. Accordingly, they dissolved $CaCO_3$ and formed halo zones around the colonies after 24-48 h at 30 ºC. They could also convert the indicator color from blue to yellow. The yellow color disappeared after a few days and was converted to blue after a long incubation at 30 ºC indicating acetate over-oxidation (the utilization of acetate by bacteria).

Nine of the isolated bacteria grew slowly (longer than 120 h) on GYE agar and formed small yellow halo around their colonies due to low concentration of acetic acid at 30 °C; their growth on AE agar were also restricted at 30 ºC and even after long incubation small colonies appeared on AE agar. Moreover, they exhibited slow growth and acetic acid production on YGM/Mg^{2+}. Therefore, due to their slower growth rate, they were ignored for further studies. The rest of the isolates (19 isolates) (Supplementary files: Table 2S) were able to grow and produce acetic acid at different temperatures.

3.3. Nutritional requirement of isolated bacteria

Referring to the previous points above, most of the isolates showed considerable growth in enriched media, such as AE, GYE agar and YGM/Mg^{2+}, at 30 ºC. In some preliminary experiments, glucose, peptone and yeast extract content of the GYE agar medium were decreased step by step. The results showed that the growth of all isolates was influenced moderately by decreasing the glucose concentration. It was also observed that the isolated bacteria grew and produced acetic acid very slowly in the absence of glucose. As indicated by Gullo et al. (2008), the *Gluconobacter* species, *Acetobacter* species, and *Gluconoacetobacter* species are the most isolated bacteria that are responsible for vinegar production as well as wine and beer spoilage. In the present study, the isolated and selected bacteria belong to the *Acetobacter* genera, and all of them have been previously detected in various fermented products (Gullo et al., 2008). In addition, it was proven that all the selected isolates are over-oxidizers meaning that they can consume acetic acid as a carbon source in the absence or low concentration of ethanol. Although, over-oxidation is not a desirable feature for strains used in vinegar production, it can be inhibited by changing the environment condition. Thus, it seems that this feature does not cause a problem for vinegar production.

The best carbon sources for *Acetobacter* sp., in descending order are ethanol, glycerol, and lactate (Brenner et al., 2005; Tesfaye et al., 2002). In this study, it was observed that glucose depletion in rich culture medium (GYE agar) had moderate effect on growth and acetic acid production.

Peptone depletion adversely affected the growth and activity of some isolates whereas yeast extract depletion (in the presence of peptone) did not influence growth and acetic acid production. In the present study, isolated bacteria grew well in culture media supplemented with peptone and yeast extract, but most of them showed slow growth in the culture media with low concentration of organic nitrogen sources. The nitrogen source is essential for acetic acid bacteria during fermentation, and it has great influence on the course of fermentation. The nitrogen source also considerably affects stress acclimation of acetic acid bacteria during vinegar fermentation (Álvarez-Cáliz et al., 2012). From an economic point of view, peptone cannot be used in vinegar production on an industrial scale. On the other hand, it causes turbidity in the final product (Ndoye, et al., 2007; Ndoye, et al., 2007), and therefore strains which are capable of growth in minimal media are desired. Yeast extract and ammonia can be used together to supply nitrogen sources; however, in this study it was shown that most of the isolates, except for L31, failed to adapt to this condition.

Among nineteen isolates presented in Table 2S, L31 and *Acetobacter senegalensis* LMG 23690 (the thermo-tolerant reference strain) were able to grow moderately and convert ethanol to acetic acid effectively in GYE_2 agar (GYE containing 2 gl^{-1} yeast extract).

3.4. Growth in different concentrations of ethanol

All the isolates were tested in solid culture media (GYE agar) to screen those which were able to grow at high temperature. Results showed that growth at high temperature was dependent on ethanol and acetic acid concentrations. All the isolates grew in GYE agar containing less than 4% (w/v) ethanol and 0% (w/v) acetic acid at 30-40°C. It was shown that 5% (w/v) ethanol is the maximum ethanol concentration in broth medium that can be tolerated by some isolated bacteria. Higher ethanol concentrations (6-10 % (w/v)) caused a dramatic fall in growth and the rate of acetic acid production. Just nine isolates, particularly L31, were able to grow and produce acetic acid on GYE agar containing 5% (w/v) ethanol at 30-40°C (Table 2S).

3.5. Biochemical and molecular identification

Biochemical and molecular identification were performed on the nine isolates explained in the previous sections. All of these were Gram negative, with single or paired cocci on GYE agar. Elongated and filamentous shapes were observed, especially during cultivation of some isolates in the broth culture media. All the isolated bacteria formed pale to light cream colonies.

HPLC analysis revealed that all the isolates were able to convert ethanol to acetic acid. They used lactate and acetate as a carbon source and converted acetate to CO_2 and H_2O after a few days. Other biochemical tests, such as oxidase, catalase, growth in the presence of high glucose concentrations, ammonium utilization, different carbohydrates assimilation and ethanol utilization, revealed that all isolates belong to AAB (Supplementary file: Table 3S).

Molecular identification done for L31 showed that it belongs to the *Acetobacter* genius. A nearly complete 16S rRNA gene sequence of L31 (1384 nucleotides) was obtained. Based on the constructed phylogenetic tree (Figure 1), *Acetobacter tropicalis* (L31) completely resembles *Acetobacter tropicalis* Ni-6B. The GenBank/EMBL/DDBJ accession numbers for 16S rRNA, of *Acetobacter tropicalis* (L31) is MK100347. *Acetobacter tropicalis* (L31) was deposited in the microbial culture collection at the University of Isfahan (Deposit number ISF2016121).

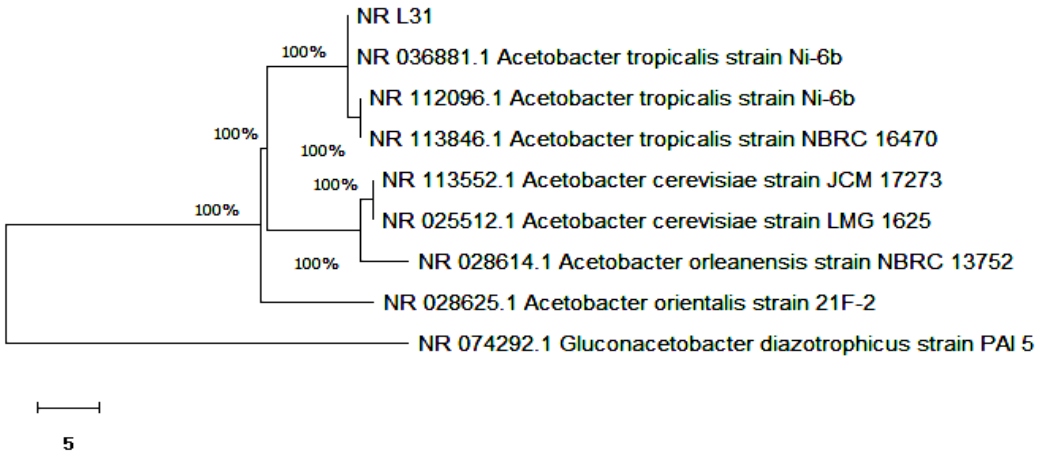

Figure 1. Phylogenetic relationships of *Acetobacter* strain isolated from apple vinegar. The phylogenetic tree based on 16S rRNA gene sequences was constructed by MEGA X software and the neighbor-joining (NJ) method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications. *Gluconacetobacter diazotrophicus* PAI5 was used as an outgroup.

3.6. Growth and acetic acid production at high temperature

All the isolates were tested in the solid culture media (GYE agar) to screen those which were able to grow at high temperature. Results showed that the selected isolates and the thermo-tolerant reference strain, *Acetobaceter senegalensis* (LMG 23690) exhibited substantial growth and acetic acid production at 30 °C in GYE broth (Table 2S). At 37ºC, there was a dramatic decline in growth and acetic acid production for all the isolates except for *Acetobacter tropicalis* (L31).

At temperatures above 37ºC, growth and acetic acid production of *A. tropicalis* (L31) and *A. senegalensis* were significantly dependent on the initial ethanol and acetic acid concentration (Table 1). However, *Acetobacter tropicalis* (L31) and *A. senegalensis* (LMG 2369) grew and produce acetic acid at 37.5 ºC in GYE broth supplemented with 5 % (w/v) ethanol.

Growth on the solid culture media at 37 ºC (Supplementary file: Table 2S) showed that most of isolated bacteria could tolerate ethanol up to 5 % (w/v), but in the broth culture media they did not exhibit such growth and acetic acid production (comparison between Table 2S and Table 1). By comparing the growth of isolated bacteria in the presence of ethanol on the solid culture media and broth culture media, it seems that the solid culture media can only be used for a preliminary screening and selection whereas tolerance to ethanol and high temperature must be tested in the broth culture media. In fact, the evaporation of ethanol during preparation and incubation of the solid culture media may cause a dramatic decline in ethanol concentration; hence, the results are not comparable in the two different media (solid and broth media).

It was also appears that increasing the temperature up to 34ºC has a moderate effect on the growth and acetic acid production of all selected isolates and reference strain (*A. senegalensis*) (Table 2s and 1), but at temperature above 35ºC growth of non-thermo-tolerant acetic acid bacteria (AAB) decreased sharply. In other words, it seems that limited temperature fluctuations (Table 1) can influence the concentration of ethanol which can be tolerated by a certain strain. This fact must be considered by researchers in explanation of the potential of isolates because in large scale acetous fermentation temperature fluctuation is inevitable. Therefore, on one hand, isolates which are going to be used in industrial vinegar production must be able to tolerate higher concentrations of ethanol and acetic acid, but on the other hand, their performance must not be effected by a few temperature fluctuations.

Table 1. Comparison of growth and acetic acid production of Acetobacter tropicalis (L31) in GYE broth with two reference strains (A. senegalensis and A. aceti) at different temperatures and ethanol concentrations.

Culture medium	**GYE broth**																					
Ethanol concentration (% w/v)	2.5	3.5	4.5	5	2.5	3.5	4.0	4.5	5	2.5	3.5	4.0	4.5	5	2.5	3.5	4.5	5	2.5	3.5	4.5	5
Temperature	**30 ºC**				**35.5 ºC**					**36.5 ºC**					**39 ºC**				**42 ºC**			
A. tropicalis (L31)	++	++	++	++	++	++	++	+	+	++	++	+	+	+	++	++	+/-	+/-	+	+/-	+/-	+/-
A. senegalensis (LMG 23690)	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	++	+/-	+/-	+	+/-	+/-	+/-
A. aceti (LMG 1535)	++	++	++	++	++	+	+/-	+/-	+/-	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-

(++): Good growth and acetic acid production. (+): Slow growth and acetic acid production. (+/-): Weak or no growth

(++): Good growth and acetic acid production. (+): Slow growth and acetic acid production. (+/-): Weak or no growth

3.7. Acetous fermentation

To evaluate the ability of the selected isolate, *Acetobacter tropicalis* (L31), to oxidize ethanol to acetic acid, acetous fermentation was performed in a Lab-bioreactor using minimal culture medium at 37°C. In the first step, we studied vinegar production in batch fermentation mode. A semi-continuous fermentation was then used to evaluate the ability of cells to oxidize ethanol continuously. In addition, the vitality (performance) of *Acetobacter tropicalis* (L31) to low dissolved oxygen was also determined. In most modern industrial vinegar production units, submerge semi-continuous fermentation or submerge continuous fermentation are the main methods used. In this mode of fermentation vinegar production continues in sequential cycles (Gullo et al., 2014). Our results showed that cells were progressively acclimated to the harsh conditions (low pH and high acetic acid concentration) of fermentation, and consequently, the number of cells increased in the second fermentation cycle. Different criteria, such as acclimation to specific raw materials, rapid acidification, and phage resistance, are considered in the selection of industrial starter cultures (Gullo et al., 2014).

Based on the obtained results, *Acetobacter tropicalis* (L31) started to grow in minimal medium with a short lag phase (about 4 h), if the inoculum had been cultivated in culture medium containing the same composition, *Acetobacter tropicalis* (L31) oxidized 5% (w/v) ethanol to acetic acid in the presence of initial 0.5% (w/v) acetic acid at 37°C (Figure 2). Although, acetic acid was produced mainly during stationary phases, it was also produced during the exponential phase. As Figure 2 shows, a great part of glucose was not consumed by the bacterial cells when ethanol was available as a carbon source. In addition, gluconic acid was not produced during acetous fermentation by *Acetobacter tropicalis* (L31). At the end of fermentation, the final concentration of acetic acid was 48 gl^{-1} (4.8% w/v). Thus, with regard to the initial concentration of acetic acid (5 gl^{-1}), the yield was 87%.

As it is shown in Figure 2, each acetous fermentation cycle lasted about 34 h under high dissolved oxygen (DO) concentration (45%) (Figure 2). It was revealed that the length of the lag phase and production time was completely dependent on DO concentration and also acclimation of cells to fermentation condition.

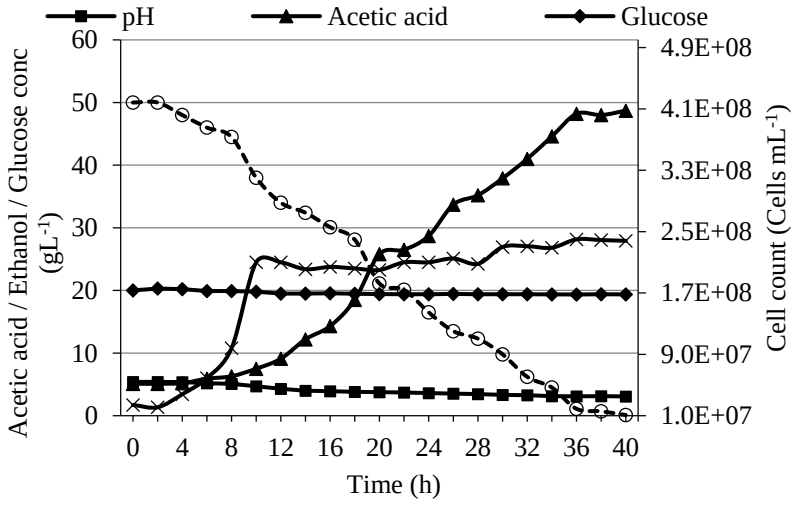

Figure 2. Batch acetous fermentation by Acetobacter tropicalis (L31) in minimal medium at 37°C.

Glucose is not consumed by cells in the presence of ethanol. In addition, cells produced the major part of acetic acid during the stationary phase.

We used the semi-continuous fermentation mode to produce vinegar by *Acetobacter tropicalis* (L31). Oxidation of 50 gl^{-1} ethanol lasted about 40 h during the first cycle (Figure 3). On average, 1.3 $gl^{-1}h^{-1}$ acetic acid was produced during the production phase (10^{th}-36^{th} h) of the first cycle.

Interestingly, using some of the cells from the first cycle (10% of working volume) as a starter for the second cycle decreased the ethanol oxidation time to 29 h during the second cycle (41^{st} to 62^{nd} h). On average, 2.36 $gl^{-1}h^{-1}$ acetic acid was produced during the production phase (46^{th}-62^{nd} h) of the second cycle. In the same way, the third cycle (63^{rd}-82^{nd} h) was completed in 28 h, and on average 2.73 $gl^{-1}h^{-1}$ acetic acid was produced during the production phase (68^{th}-82^{nd} h). This means that acclimation to acidic conditions may increase the capacity of cells to consume ethanol fast.

The total number of cells in each fermentation cycle was also dependent on acclimation of cells to the condition of acetous fermentation. As Figure 3 shows, the total number of cells by microscopic count was 2.02 $\times 10^8$ mL^{-1} at the end of the exponential phase of the first cycle. However, it increased to 3.5 $\times$ 10^8 mL^{-1} at the end of exponential phase of the second and third cycles.

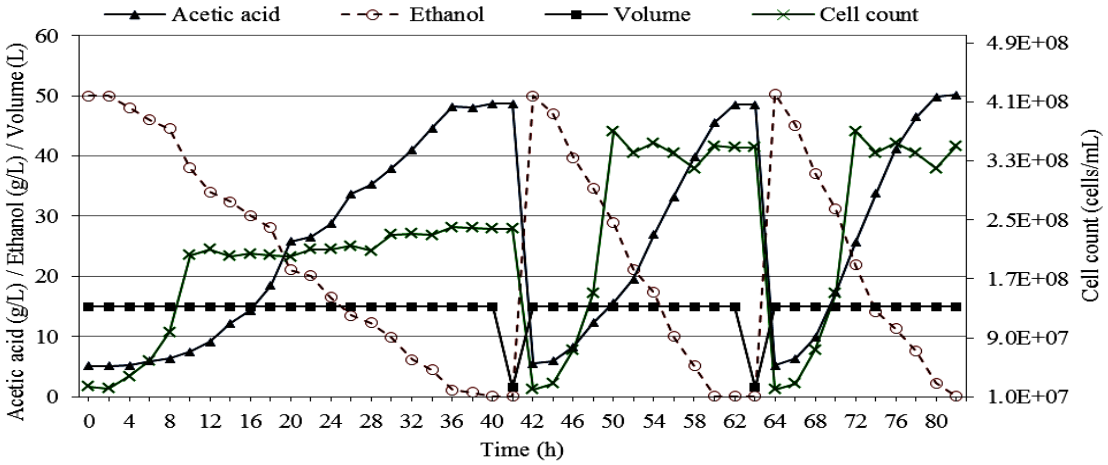

Figure 3. Semi-continuous acetous fermentation by *Acetobacter tropicalis* (L31) in minimal culture medium at 37°C under high dissolved oxygen concentration. Fermentation was done in Lab-scale bioreactor (working volume 15 L) in three sequential cycles (0-41^{st} h, 41^{st}-61^{st} h and 61^{st}-82^{nd} h). In the end of each cycle, 13.5 L of culture medium was withdrawn, and replaced by fresh culture medium. Cells grew and produced acetic acid faster during the second and third cycles.

Sensitivity of cells to oxygen deficiency was also determined using CTC as a marker for cell viability (total dehydrogenases activity). As it is seen in Figure 4a, the number of viable cells decreased sharply after interruption of oxygen, and just 28% of cells were capable of CTC reduction after harvesting from the bioreactor. This means that cells grown in high dissolved oxygen (Figure 3) are very sensitive to oxygen deficiency.

It was also shown that *Acetobacter tropicalis* (L31) was able to produce acetic acid in minimal medium in batch and semi-continuous mode in low dissolved oxygen. If the dissolved oxygen concentration decreased to 15%, the lag phase increased considerably (7 h). In addition, low concentration of oxygen influenced the length of the production phase, and increased it to 52 h during the first cycle. Interestingly, in low DO concentration the total number of cells increased and reached 2.74×10^8 cells ml^{-1}. However, compared to the growth of *Acetobacter tropicalis* (L31) in the presence of high DO, (Figure 3), the acetification rate was significantly lower (1.24 gL^{-}

$^{1}h^{-1}$) in the production phase (stationary phase). Conversely, as Figure 4b shows, when cultivating cells under low DO concentration almost all the cells (91%) remained viable if oxygen flow was interrupted, indicating that cells grown in low oxygen concentration can efficiently tolerate oxygen depletion. There have been many studies about the susceptibility of AAB to DO deficiency done in the past years (Qi et al., 2014; Zheng et al., 2018). Generally, oxygen deficiency can enhance the effect of stress during acetous fermentation (Gullo et al., 2014). In contrast, proper oxygen supply can improve cell tolerance (Zheng et al., 2018). In this study, we evaluated the effect of oxygen flow interruption on total cellular dehydrogenases as a viability indicator. It was also observed that tolerance to O_2 deficiency was dependent on the rate of oxygen supply during acetous fermentation. In other words, cells were very vulnerable to oxygen deficiency if they were grown under high oxygen concentration. In AAB, O_2 acts as a final electron acceptor. O_2 consumption results in the formation of ATP production. Acetic acid readily traverses the cell membranes, and enter cytoplasmic space.

We observed that *A. tropicalis* (L31) acclimated readily to a minimal culture medium meaning that it is possible to use it for sprite vinegar production. In addition, in vinegar fermentation, the acetification rate is directly associated with bacterial alcohol oxidation ability (Qi et al., 2014). *A. tropicalis* (L31) produced 2.3-2.7 $gL^{-1}h^{-1}$ acetic acid by ethanol oxidation at 37°C. With regard to the high temperature (37°C), this acetification rate is in the normal range (Lee et al., 2016; Qi et al., 2014).

Intracellular ATP has adverse effects on cell viability (Hutkins, 2006). ATP is necessary to get rid of adverse effects of intracellular acetic acid. Sufficient oxygen supply enhanced ethanol oxidation and acetic acid production (Zheng et al., 2018). Since the acetification rate was much lower in low DO concentration, it seems that the tolerance of cells to oxygen flow interruption is a function of acetic acid concentration. In other words, low DO concentration caused lower acetic acid production. Then, by interruption of oxygen flow, cells were able to tolerate lower concentration of acetic acid.

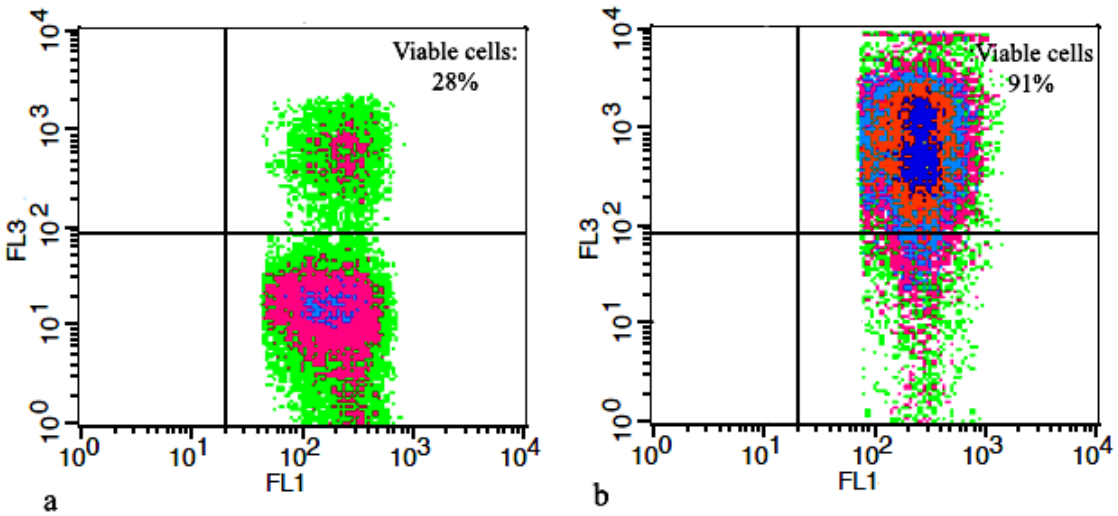

Figure 4. Viability of *Acetobacter tropicalis* (L31) during semi-continuous fermentation. Cells were harvested from bioreactor, washed and incubated in the presence of CTC. a) Shows the viability of cells grown under high dissolved oxygen concentration. b) Shows the viability of cells grown under low dissolved oxygen concentration. Cell viability was measured by determination of total cellular dehydrogenase activity using CTC as fluorescent dye. The horizontal axis (FL1) shows the fluorescent intensity of TO while the vertical axis (FL3) shows the fluorescent intensity of CTC.

4. Conclusions

In the present study, we isolated AAB from different kinds of vinegar. Then, a targeted screening approach was performed on the isolates considering some important phenotypic features such as growth in different concentrations of ethanol and acetic acid at high temperature, growth in minimal culture media, and susceptibility to low dissolved oxygen. Based on the obtained results, one of the isolates, *Acetobacter tropicalis* (L31) was able to tolerates, grow, and produce acetic acid at a higher temperature in both enriched and minimal culture media. Three practical consequences can be deduced from these results and must be considered when isolated AAB are planned to be used in the vinegar industry. i) Abundant growth on selective minimal culture media is essential for an isolate to be used in the vinegar industry. In addition, it must remain viable for a long time under lab conditions. ii) Normally, the amount of dissolved oxygen (DO) fluctuates during acetous fermentation, thus an isolate should be checked to see if it can tolerate DO fluctuations. iii) Temperature may also fluctuate during acetous fermentation; therefore, an ideal isolate for vinegar industry should tolerate a wide range of temperatures (30-42°C). Thus, since *Acetobacter tropicalis* (L31) grew well in minimal culture medium in both low and high DO and showed tolerance to high temperatures and DO fluctulations, it seems that it is a potential isolate for vinegar starter production.

Conflict of interest

All authors declare that they have no competing interests.

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