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EVALUATION OF THERMOTOLERANT *ACETOBACTER PASTEURIANUS* STRAINS ISOLATED FROM MOROCCAN FRUITS CATALYZING OXIDATIVE FERMENTATION AT HIGH TEMPERATURE

**M. MOUNIR^{1,3}, R. SHAFIEI², R. ZARMEHRKHORSHID³, A. HAMOUDA¹,
M. ISMAILI ALAOUI¹ and P. THONART³**

1 : Hassan II Institute of Agronomy and Veterinary Medicine (IAV), PB 6202 Rabat. Morocco

2 : Department of Biology, Faculty of Science, University of Isfahan, Isfahan. Iran

3 : Wallon Centre of Industrial Biology. University of Liege, B40. B-4000 Sart-Tilman. Belgium

ABSTRACT

Six strains of acetic acid bacteria were isolated from Moroccan local products and their potential as industrial strains was evaluated in lab-bioreactor. Three of them, namely TAV01, AF01 and CV01, isolated from traditional apple vinegar, apple and cactus fruit, respectively were selected and their responses to high temperature were assessed. Morphological and biochemical identification confirmed that these strains belong to *Acetobacter* species. Their growth and acetic acid production were compared with the thermoresistant reference strain, *Acetobacter senegalensis* and mesophilic strains of *Acetobacter pasteurianus*. The two strains AF01 and CV01 showed abundant growth and noticeable acetic acid production ability at high temperatures (38 to 41°C). A thermophilic character was observed for AF01 strain. Indeed, this bacterium grew better at 38 than 30°C.

Key words: vinegar, *Acetobacter*, thermoresistance, bioreactor, enzyme activity, semi-continuous fermentation, gluconic acid, acetic acid.

INTRODUCTION

In Morocco, the agricultural and agro-industrial activities generate large quantities of grade-outs and substandard fruits which constitute a substantial economic loss and a real nuisance to the environment. These raw materials, rich in organic substances, can be recycled and processed by biotechnological processes which are the best solution to avoid human and animal health risks (Benkerroum 2013).

The valorization of these low commercial quality fruits through biotechnological processes is therefore essential. Such valorization brings on the domestic market a new generation of highly prized and often imported products like vinegar (Ndoy et al. 2006).

The objective of this study is the isolation of novel acetic acid bacteria strains from Moroccan habitats for industrial use. In the present study, we were first interested in isolation, identification and characterization of novel AAB strains. Then, in the second part, the ability of the isolated strains to perform efficient acetic acid fermentation at high temperature was evaluated.

MATERIALS AND METHODS

Samples, culture media and microorganisms

A total number of twenty two food samples (traditional vinegars and wines, juices and honeys, and fruits) were collected from different regions of Morocco on the basis of different criteria (temperature, availability of local and natural products and living knowhow).

GYEA medium, composed of glucose, yeast extract, peptone of caseine, ethanol and acetic acid and GYEA/Mg²⁺ medium, composed of glucose, yeast

extract, ethanol, acetic acid and MgSO₄ were used as enrichment media (Shafiei et al. 2013). Solid culture media (SGYEA and SGYEA/Mg²⁺) consisted of the same components given above supplemented by 15g/L of agar was used for screening.

Isolated bacteria were compared with other mesophilic and thermotolerant acetic acid bacteria as reference bacteria. The wild type strains used were obtained from Laboratory of Microbiology of Gent (*Acetobacter senegalensis* LMG 23690T, *Acetobacter pasteurianus* LMG 1632, LMG 1607 and LMG 1701, *Acetobacter aceti* LMG 1531 and *Acetobacter cerevisea* LMG 1625) and from German Collection of Microorganisms and Cell Cultures (*Acetobacter pasteurianus* DSM 2324 and *Acetobacter loveniensis* DSM 4491). *Acetobacter senegalensis* was used as a thermotolerant reference strain.

Morphological and biochemical characterization of the isolated strains

Firstly, a morphological characterization under microscope was done on the isolated bacteria which could grow on the solid or liquid media. This characterization including shape, size, arrangement, Gram staining and motility was carried out from cells grown on GYC at 30°C under aerobic condition (Cleenwerck et al. 2002 and De Ley et al. 1984).

Conventional biochemical tests such as catalase, oxidase, growth in different concentration of ethanol and glucose were done according to Bergy's manual of systematic bacteriology (De Ley et al. 1984).

Acid and ethanol resistance test

After selection of AAB strains on the basis of morphological and metabolic tests, a series of screening test was performed using streaked plates. During screening, the concentrations of acetic acid (AA) and Ethanol (ETOH) increased gradually. The following concentrations were used for acetic acid and ethanol successively; 0.2%, 1%, 2% to 8% and 3%, 4% to 8% (w/v).

Resistance to high temperatures

Growth and acetification were tested under different temperatures: 30°, 34°, 35°, 37°, 38°, 39°, 40°, 42° and 43°C.

A comparison on a thermotolerance basis between AF01 strain isolated from apple and *Acetobacter senegalensis* strain was performed. 100ml of preculture using GYEA/Mg²⁺ medium was prepared for each strain in 500 ml Erlenmeyer flasks. These Erlenmeyer flasks were inoculated with fresh colonies grown on plates after 48 h of incubation at 30°C.

Flasks containing fresh GYEA/Mg²⁺ were subsequently inoculated with 5ml of the same preculture and incubated simultaneously and separately under agitation (120rpm) at two different temperatures, 30°C and 38°C. The optical density, ethanol and acidity were measured each two-hours.

Growth and fermentation kinetics of the selected strains

Three strains, among the six isolated strains from the Moroccan local products, were selected, studied and compared with other mesophilic and thermoresistant strains. TAV01, AF01 and CV01 are the names proposed for the three selected strains isolated from traditional apple vinegar, apple and cactus fruits, successively. Growth kinetics of these isolates was monitored and compared with *A. senegalensis* both in flask and plates.

RESULTS

Isolation of adapted thermotolerant acetic acid bacteria

The Monitoring of fruit preparations cultures in GYEA liquid medium revealed variability in terms of acidity changes. A gradual increase in acidity was noticed particularly for dried fig, apple and cactus samples. The final values of acidity with these three kinds of fruits were: 3.75, 4.5 and 3.9, respectively.

Microscopic observation of inoculated culture media showed a great diversity of microorganisms (size and number) for all kinds of fruits. However, a significant number of cocci and small size bacteria were observed in the case of cactus and apple fruits. Yeasts were also observed under microscope for date samples which was probably due to their high sugar content.

Furthermore, a similar isolation procedure of AAB from liquid samples (traditional vinegars, juices, honey and traditional alcohols) in GYEA/Mg²⁺ liquid medium was performed. Successful results were obtained from date, apple and cactus vinegars. It is noticeable that these samples exhibited a significant growth at 30°C.

A strain isolated from the traditional apple vinegar noted TAV01, showed a capacity of growth and acetate production on plates with high concentrations of ethanol and acetic acid. These strain could grow normally in the medium containing a concentration of 6%(v/v) of ethanol and 6%(v/v) of acetic acid. The growth of this bacterium was also tested at various temperatures: 30, 35, 37 and 39°C on flasks. TAV01 could grow very well at 35°C (figure 2).

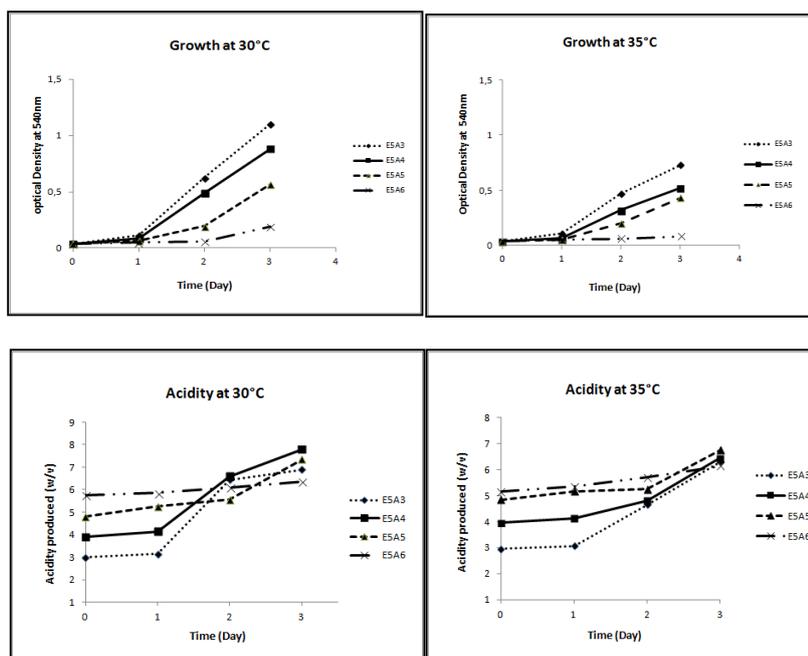
Table 1. Thermoresistance test of the selected strains on GYEA solid medium

Cultures Strain	Culture media													
	0A, 3E		0.2A, 3E		1A, 3E		1A, 5E		2A, 5E		3A, 5E		4A, 5E	
	35 °C	41 °C	35 °C	41 °C	35 °C	41 °C	35 °C	41 °C	35 °C	41 °C	35 °C	41 °C	35 °C	41 °C
<i>DV</i>	++	+	++	+	+	+	+	±	±	-	±	-	-	-
<i>RAV</i>	++	++ +	++	+	++	++ +	±	±	++	+	+	±	±	-
<i>TAV01</i>	++	+	++ +	-	++	-								
<i>CV01</i>	+	+	++	++	++ +	++	++	++	++	++	++	++	+	±
<i>AJ</i>	+	++	+	+	++	+	++	++	+	±	±	+	±	-
<i>AF01</i>	++	++	++	++ +	++	++	++	++ +	++	++	++	+	+	± ±

A : acetic acid ; E : ethanol ; exp : 0.2A,3E : GYEA/Mg²⁺ culture medium containing 0.2% (w/v) of acetic acid and 3%(v/v) of ethanol.

DV : Date Vinegar ; RAV : Rosemary Apple Vinegar ; TAV : Traditional Apple Vinegar ; CV : Cactus Vinegar ; AJ : Apple Juice ; AF : Apple Fruit.

+++ : very good growth; ++ : good growth; + : weak growth; ± : very weak growth ; - : no growth.



ESA3 : GYEA/Mg²⁺ culture medium containing 5% (v/v) of ethanol (96,2%) and 3% (v/v) of acetic acide.
 ESA6 : GYEA/Mg²⁺ culture medium containing 5% (v/v) of ethanol (96,2%) and 6% (v/v) of acetic acide

Figure 2. Growth and acetate production of TAV01 strain at 30 and 35°C.

The graphs show that TAV01 strain is able to produce acetate in a medium containing high starting concentrations of acetic acid and ethanol (E5A3, E5A4, E5A5 and E5A6) as well as at 30°C and at 35°C.

Identification of selected strains

All the isolated bacteria were Gram negative, catalase positive and oxydase negative. Morphologically, they were single or paired cocci on GYC agar. Elongated shapes were observed especially for DV strains in liquid media. All the isolated bacteria formed pale to light cream colonies. Overoxidation of acetate to CO₂ and H₂O was also noted for all isolates on bromocresol containing GYC medium.

The analysis of liquid fermented media revealed that all of them were able to convert ethanol to acetic acid. They could also convert acetate to CO₂ and H₂O after a few days on solid medium. Other biochemical tests such as growth in the presence of high glucose concentrations, ammonium utilization, different carbohydrates assimilation and ethanol utilization, revealed that all strains belong to *Acetobacter* genus.

Growth kinetics and fermentation of the isolated strains

All selected strains showed good growth at 30 °C. From 34 °C, almost all the wild type strains showed a significant decrease in growth. At 37 °C AF01, CV01 and *Acetobacter senegalensis* strains showed good growth, while the other reference strains showed weak or no growth. At 40°C, AF01 and CV01 strains remain beside *Acetobacter senegalensis* the only strains that had appropriate growth and could produce acetic acid in the medium. The AF01 strain remains the one that had the best growth at high temperature (40°C) (Table 2).

Table 2. Comparison of growth, acidification and overoxidation capacities between selected strains (AF01 and CV01) and other AAB

Strain	30°C			34°C			37°C			40°C		
	G	A	O	G	A	O	G	A	O	G	A	O
LMG 1632	++	+	+	+	+	-	±	+	-	-	-	-
AF01	++	+	+	++	+	+	++	+	+	++	+	+
CV01	++	+	+	++	+	+	+	+	+	+	+	+
<i>A. senegalensis</i>	++	+	+	++	+	+	+	+	+	+	+	+
DSM 2324	+	+	-	±	-	-	-	-	-	-	-	-
LMG1607	++	+	+	++	+	+	+	+	+	±	-	-
LMG 1701	++	+	+	++	+	+	±	-	-	-	-	-
DSM 4491	++	+	+	++	+	-	±	-	-	±	-	-

(G) Growth : (-) no growth, (±) weak growth, (+) good growth, (++) very good growth. (A) Acidification : (+) positive test; (-) negative test. (O) over-oxidation: (+) positive test; (-) negative test.

In addition, a comparison of the thermotolerance properties between AF01 strain and *Acetobacter senegalensis* on liquid medium was performed. The results of this comparison are given in Figures. 3.

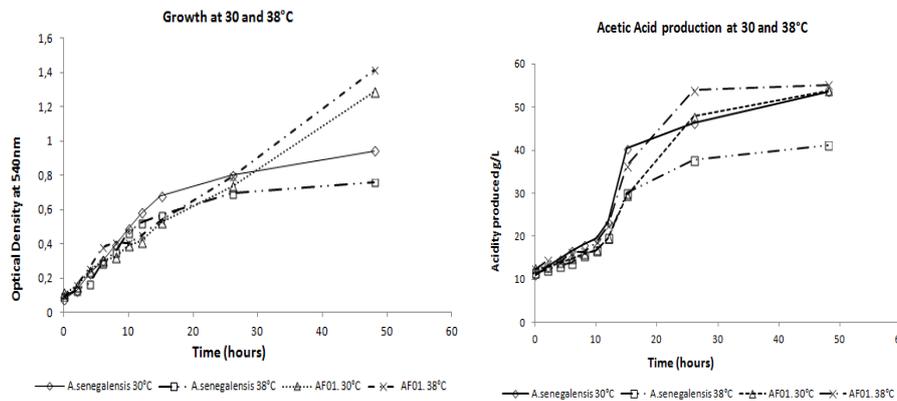


Figure 3. Growth and acetic acid production of AF01 (Δ and \times) and *Acetobacter senegalensis* (\diamond and \square) strains at 30 and 38 °C on GYEA liquid medium supplemented with 0.5g/L of $MgSO_4$.

Results in Figure 3 show that AF01 strain produced more biomass compared to the *Acetobacter senegalensis* strain both at 30 °C to 38 °C. The average values of the final turbidity measured at 540nm are: 1.286 and 1.415 for the AF01 strain respectively at 30 and 38 °C; 0.943 and 0.761 for the *Acetobacter senegalensis* strain respectively at 30 and 38°C.

It is also noticeable that the AF01 strain produced more biomass during fermentation at 38°C than at 30°C (Fig. 1). This finding suggests that the AF01 strain has a thermophilic character.

Fig. 2 shows that AF01 strain produced also more acetic acid compared with *Acetobacter senegalensis*. Average quantities produced at 30°C are 43.67 g/L and 43.56 g/L respectively for AF01 and *Acetobacter senegalensis* strains, while those produced at 38°C are 45.1 g/L and 31.16 g/L respectively for AF01 and *Acetobacter senegalensis* strains. It is considerable that for the same strain, AF01, the amount of acetic acid produced at 38°C is higher than that produced at 30°C.

DISCUSSION

The present study aimed at isolating AAB strains which tolerate high temperature from Moroccan local products. It has been already reported that some strains of AAB were isolated from date and strawberry fruits (Sharafi et al. 2010; Kregiel et al. 2012).

As it can be seen in Table 1, except for date vinegar and traditional apple vinegar isolates, all strains showed abundant growth at either 35 or 41°C in different culture media. For all strains, resistance to high temperature decreased by increasing concentrations of acetic acid and ethanol. Similar effect of increasing ethanol concentrations on growth have been reported by Maal and Shafiei (2010). It should be noted that some of the isolated strains especially AF01 exhibited thermoresistant and thermophilic characters. Indeed, it showed a better growth at 41°C compared to that observed at 35°C. Besides that, traditional apple vinegar isolate showed a weak growth at 41°C compared with the others. However, this strain exhibited more resistance to acetate and ethanol at 35°C.

The cultures of TAV01 strain exhibited a formation of non-soluble substances which gather in the form of filaments. After revelation with Lugol's solution (1/10) and the red of Congo, this substance was recognized as cellulose. Later studies aiming the optimization of the production of this substance could be considered. Therefore, this phenomenon disadvantages the use of this bacterium for industrial production of vinegar.

The comparison of the five thermotolerant strains revealed that AF01 and CV01 strains isolated from apple and cactus fruit, respectively were the most thermoresistant strains (good growth at 35 and 41°C).

CONCLUSION

In the present study, we isolated and characterized acetic acid bacteria from twenty two Moroccan foods and fruits. Then, growth kinetics of fermentation of the selected ones was investigated.

TAV01 strain was able to produce acetate in a medium containing high starting concentrations of acetic acid and ethanol. In addition, this strain could produce cellulose in the culture medium.

The study of the fermentation of the AF01 strain in liquid medium revealed that this strain has more resistance to temperature compared to *Acetobacter senegalensis* strain. In addition, this bacterium showed thermophilic properties.

According to these results, the three strains were selected for further investigations.

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