Selection of Thermotolerant and non Overoxydative Acetic Acid Strains for Cost Effective Vinegar Production

ABSTRACT

The aim of this study was to select thermotolerant acetic acid bacteria with no overoxidation ability that would be useful for low-cost vinegar production. Acetic bacteria were isolated from palm wine and mango pulp after 3 to 5 days of fermentation. The isolates were first characterized by biochemical tests and were identified by sequencing of the ARNr 16 S gene. From the substrates collected, a total of 48 acetic acid bacteria were isolated. Among them, 8 strains showing inability to oxidate acetate to CO_2 and H_2O. Three strains of these 8 isolates belonging to *Gluconobacter oxydans* (Ski1) and to *Acetobacter ghanensis* (Fke 22 and Fk5) were able to grow and produce acetic acid at 37 °C and in culture medium containing 5 % of ethanol and until 10 % of acetic acid. This study allows to select three thermotolerant acetic acid bacteria strains with high amount of acetic acid production capacity but with no-overoxydation ability. These strains could then be useful as starters for low-cost vinegar production.

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Keywords: [Acetic acid bacteria, themotolerant strains, overoxydation, high acetic acid production capacity]

1. INTRODUCTION

Acetic acid (AA) is an organic acid of great global importance. The production of this acid has attracted more and more interest over the years [1]. A total of 75% of this acid is produced synthetically and only 10% of the world production is realized biologically [2]. AA production, based on microbial fermentation, is considered a clean and potential, alternative for the use of agricultural and biological wastes as carbon sources [3]. Acetic acid is the main component in vinegar and is also recognized as an effective antimicrobial compound that prevents the growth of pathogenic and spoilage organisms in fermented foods.

Acetic acid bacteria (AAB) are considered a large group of microorganisms that produce AA from ethanol. Indeed, acetic acid is the primary metabolite of AAB and is produced from the bioconversion of ethanol through two reactions catalyzed by the membrane-bound pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). ADH oxidizes the ethanol to acetaldehyde, which is then converted to acetic acid by ALDH and released into the surrounding environment [4].

These bacteria are widely distributed on the surface of flowers and fruits, in sweet substances or in alcoholic beverages [5]. They are strict aerobic, Gram-negative or variable and catalase-positive microorganisms [6]. The main species most used in vinegar production due to their significant abilities to oxidize ethanol to AA

belong to the genera Acetobacter, Gluconacetobacter, Gluconobacter and Komagataeibacter [7, 8] are the most frequently used species in vinegar production. Vinegar is produced by a two-stage fermentation process, in which fermentable sugars are first converted into ethanol by yeasts, and then, the ethanol is oxidized to acetic acid by acetic acid bacteria (AAB).

The optimal temperature of most AAB for AA production is 25 C to 30° C [9]. This mesophilic character of AAB, however, presents a considerable disadvantage for industrial applications because it requires a good cooling system to maintain optimum temperature under warm ambient conditions. With this type of AAB, even a 2˚C or 3˚C temperature elevation above 30˚C has a significant negative impact on the yield and acetification rate [10]. The high temperatures during the summer harvest period in some countries, in addition to the heat accumulated during fermentation, are a significant challenge for the vinegar industry [11]. In the vinegar production industry, temperature control leads to high energy consumption, resulting in increased production costs [12]. Recently, climate change and global warming have become major challenges for the fermentation technology of AAB [8]. It is thus recommended that strains be temperature- resistant, particularly in tropical and sub-tropical regions [13]. In this context, several authors have focused on the isolation and characterization of thermotolerant AAB from tropical products that are capable of producing AA at high temperatures, in order to minimize the loads due to the cooling system [14, 15, 16, 17].

Apart from the thermotolerance of acetic acid bacteria challenge to be met, oxidation of acetic acid that had been accumulated in the culture medium, would occur due to some strains of AAB involved in the fermentation process [17, 18, 19]. Indeed, in addition to their ability to oxidise ethanol, some AA genera can further oxidise acetic acid to CO_2 and H_2O, generating the so-called acetate oxidation (overoxidation), that is carried out via the tricarboxylic acid cycle when there is a high level of dissolved oxygen and no ethanol in the medium [20]. Reasons for over-oxidation could be changes in the population or in the physiology of strains, stimulated by the lack of alcoholic substrate [21]. Currently, the acetic acid bacteria selected by vinegar manufacturers display low acetate oxidation, although prolonged incubation after ethanol depletion sometimes causes further acetate oxidation. Experiences accumulated for centuries have allowed some manufacturers to terminate vinegar fermentation when a small amount of ethanol still remains in the vinegar mash; this procedure has been thought to be the safest way to avoid acetate oxidation and prevent inferior vinegar fermentation [22]. Vinegar fermentation allowing acetic acid accumulation to be more than 4 % has also become an alternative way to overcome acetate oxidation. The aim of this study was to select thermotolerant acetic acid bacteria with no overoxidation ability that would be useful for low cost vinegar production.

2. MATERIAL AND METHODS

2.1 Isolation of Acetic Acid Bacteria

The biological material was rotten mango fruits collected from Sinématiali (9° 35′ North, 5° 23′ West) and Ferkessédougou (9° 35′ 37″ N, 5° 11′ 50 " West) and palm wine from Bingerville (5°21′20″ North, 3°53′07″ West. Samples were transferred to the laboratory of biotechnology University Felix Houphouet-Boigny Abidjan, Cote d'Ivoire. Approximately 10 g of mango fruit pulp were crushed and incubated at room temperature (28 to 30°C) for 3-5 days. Palm wine was left to ferment for 3 days [16].

The strains isolations were performed following Duthathai and Wasu [23] method which was modified by adding Nystatine (0.1%) to the medium after sterilization. The medium, GYC (Glucose Yeast Carbonate), had contained 0.5% glucose, 2% glycerol, 1% yeast extracts, 1% peptone, 1.5% potato extract, 1% $CaCO_3$, 4% ethanol, 1.5% agar and 0.0016% bromocresol green to monitor pH variation. After appropriate dilution in sterile

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saline solution, 0.1 mL of the fermenting samples (mango and palm wine) were plated on the GYC medium. Petri dishes were incubated at 30°C for 3-4 days under aerobic conditions.

Isolated colonies with yellow zones around were streaked on Hestrin-Schramm CaCO3-Agar medium to confirm acid production by formation of clear zones around colonies [24]. This medium was composed of 0.05% glucose, 0.3% peptone, 0.5% Yeast extract, 1.5% CaCO3, 1.2% agar and 4 % ethanol was added after sterilization. Culture was performed at 30ºC for 2-7 days. Confirmed acid forming colonies with the biggest clear zones were subjected to further biochemical tests. Acetic acid bacteria were identified based on following tests: Gram staining, catalase, oxidase. The strains isolated were kept at -80 °C in Luria Bertani medium supplemented with glycerol 20 % in Eppendorf tubes, for further studies.

Biochemical characterization regarding the oxidation of both acetic and lactic acids was performed. Overoxidation was investigated with HS broth containing bromocresol purple, 0.05 % glucose and 1 % acetic acid or lactic acid as carbon source. It was based on the capacity of strains to oxidate acetate to CO_2 and H_2O. Addition of acetic acid or lactic acid to the HS broth turns the purple medium to yellow. thus, one fresh colony of acetic acid bacteria was inoculated in 3 mL of HS broth and then incubated at 30 °C for 48 hours in aerobic atmosphere. Oxidation of acetic acid or lactic acid was assessed by the change of medium color back to purple due to pH rise [25].

2.2 Characterization of High Acetic Acid Producing and thermotolerant isolates

Effect of temperature on the growth of AAB strains was analyzed in liquid medium. Hestrin-Schramm broth modified by removal of glucose and citric acid, free of CaCO3 was used for that purpose. This basal medium was composed of 2.5% ethanol, 0.5% yeast extract, 0.5% peptone, 0.27% Na 2 HPO4 and 0.01% MgSO4) and adjusted to pH 6. Culture experiments were performed in 250 ml-Erlenmeyer flasks containing 150 ml of basal medium. The media were inoculated with 1.0 mL of a 24 hours pre-culture (OD600 = 0.5). Cultures were carried out in a shaking bath (150 rpm) at 30°C and 37°C.

Acetic acid production was monitored every 24 h for 10 days and measured as total acidity using titration with 0.1N NaOH against phenolphthalein as pH indicator [26]. The experimental results were expressed as mean ± standard deviation (SD) of triplicate.

Isolates displaying non overoxidation properties with acid production at 37°C were evaluated for resistance to different ethanol and acetic acid concentrations at 37°C and further identified by molecular characterization.

2.3 Ethanol and acetic acid tolerance ability of the AAB isolates

AA Bacterial tolerance against acetic acid and alcohol were analyzed in HS broth. In the case of acid stress, the broth was supplemented with acetic acid concentrations ranging from 1 to 10%; for alcohol tolerance, ethanol concentrations in the broth were varied from 3 to 10%. Ten (10) ml of each acid or alcohol solution were seeded with 1 ml of a 0.5 OD_{600} preculture. Bacterial growth was monitored for turbidity using spectrophotometer (PIOWAY Medicallab UV 752, China) after incubation at 37°C for 48 h by measuring optical density at 600 nm absorbance [27]. Experiments were carried out in triplicate.

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2.4 Molecular characterization of selected strains

The genomic DNA preparation was performed by the heat shock methods. Bacteria were grown in YGM/Mg2+ agar medium for 24 h at 30°C. Cell suspensions were made using some colonies in 400 µl of milli-Q water into Eppendorf tubes. The tubes were then frozen at -20°C for 20 min before heating them in a heat block at 100°C for 20 min. Afterwards, tubes were centrifuged at +4°C for 10 min at 14000 rpm. The supernatants containing total genomic DNA were poured in new sterile tubes and conserved at -20°C. Then, the 16S rDNA gene was amplifed by using an universal primer consisting of a forward primer 16F27: 5′-AGAGTTTGATCCTGGCTCAG-3′ and a reverse primer 16R1522: 5'- AAGGAGGTGATCCAGCCGCA-3', localized respectively on positions 8-27 and 1541-1522. The PCR kit, Hot Master Mix 5 PRIME (Dominique Deutcher, France) was used. Each amplification reaction was performed in a total volume of 50 µl containing 25 µl of Master mix; 2 µl of each primer; 17 µl of RNAse free water and 4 µl of extracted DNA.

PCR amplification was carried out in a thermocycler (Mastercycler Personal, Eppendorf, Pecq, France) with the following procedure: a first denaturation step of the DNA at 95°C for 2 min, then denaturation at 95°C for 1 min, primers annealing at 58°C for 30 sec, primers extension at 65°C for 2min and a final extension cycle at 65°C for 7 min. A total of 35 cycles were done. Isolates were then identified by sequencing the PCR products in a sequencer ABI 3730xl 96-capillary DNA Analyzers (Eurofins genomics, Paris, FRANCE). The analyses were performed by SANGER methods. Sequences analyses were performed with National Center for Biotechnology Information (NCBI) software using nucleotide Basic Local Alignment Search Tool (BLAST) method (http://www.blast.ncbi.nlm.nih.gov).

3. RESULTS AND DISCUSSION

3.1 Macroscopic, microscopic and biochemical properties of isolated strains

The aim of this study was to look for bacterial strains with no-overoxidation capacity as well as ability to grow well at high temperatures and produce high level of acetic acid. Physiological, biochemical and molecular studies were performed to characterize selected strains.

From the substrates collected, palm wine and fermented mango pulp, a total 48 of acetic acid bacteria were obtained. All isolates showed yellow zone on GYC medium and clear zones on HS $CaCO_3$-Agar plates. These strains belonged to gram negative, morphologically long, rod and coccobacillus shape motile by means of peritrichous flagella. They were all fermenting alcohol into acetic acid, negative for oxidase, and positive for catalase. Positive result for acetate oxidation occurred in 40 of the 48 acetic acid bacteria isolates (Figure 1).

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Some genera of AAB can further oxidise the produced acetic acid to CO_2 and H_2O, resulting in so-called acetate oxidation (ethanol overoxidation). This ability is useful for distinction from the genus *Gluconobacter*, which does not have the same capability [28]. Overoxidation was investigated with HS broth containing bromocresol purple; it was based on the capacity of strains to oxidate acetate to CO_2 and H_2O (Table 1). The

positive results of acetate overoxidation are shown in the color change of medium from the initial color, purple to yellowish and back to purple [25].

The 8 acetic acid bacteria showing negative results of acetate overoxidation were selected for further test namely the evaluation of their thermotolerance capacity. In this research, isolates from mango were Fki5, Fke22, Ski1, Ski 2 and Ski 12 while palm wine isolates were Pal1, Pal 9 and Pal 11.

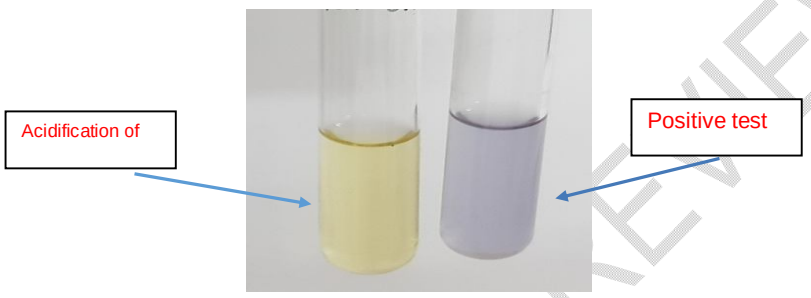

Fig. 1. Acetate oxidation test. Overoxidation of the isolates on HS broth containing bromocresol purple.

The positive results of ethanol overoxidation are shown in the color change of medium from the initial color, purple to yellowish and back to purple

Table 1. Biochemical and microscopic tests of the selected non overoxydative AAB isolates after 3-4 days incubation at 30°C

Isolates	Biochemical			Microscopic	
	Catalase	Oxydase	Overoxidation	Shape	Gram
Pal 1	+	-	negative	coccobacille	gram -
Pal 9	+	-	negative	coccobacille	gram -
Pal 11	+	-	negative	coccobacille	gram -
Fki 5	+	-	negative	cocci	gram -
Ski 2	+	-	negative	coccobacille	gram -
Fke 22	+	-	negative	coccobacille	gram -
Ski 1	+	-	negative	coccobacille	gram -
Ski 12	+	-	negative	coccobacille	gram -

Most of the tested isolates were able to grow at 30°C and to produce maximal acetate ranging from 20.42± 0.16 to 25.005± 0.60 g/L starting from the 11th day to the 15th day of cultivation (Figure 2). Although the optimal growth temperature of AAB is between 25 and 30°C, three isolates Fki 5, Fke 22 and Ski 1 were able to develop growth without any appreciable lag phase at 37°C, and present similar amount of acid production (21.87±0. 0.60 to 24.82±0.70 g/L) to that at 30°C on the thirteenth day. This result is in accordance with the

findings of El-Askri et al. [29] who asserted that the fermentation efficiency of thermotolerant strains for vinegar production at 38–40 °C was similar to that of mesophilic strains at 30 °C. Thermotolerant strains may have acquired their growth phenotype by adapting to habitats characterized by higher temperatures, such as tropical regions [30]. Thermal adapted strains are very useful in the submerged fermentation process as temperatures rise more than the optimal value for AAB growth because of the rapid heat accumulation. And the use of these strains can decrease the electricity consumption required for cooling by up to 8.5%.

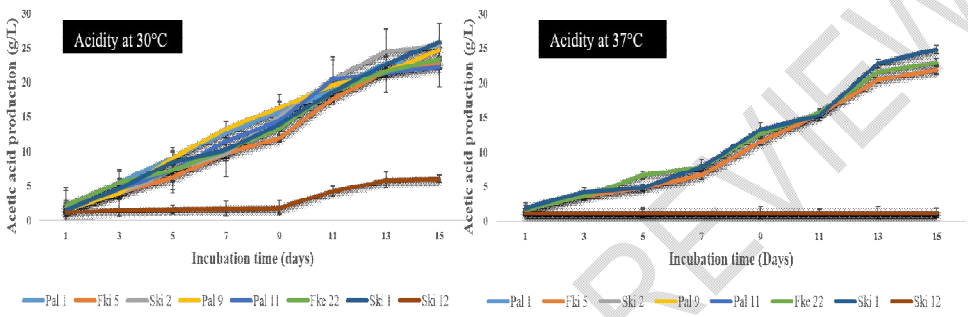

Figure 2: Effect of temperature on acetic acid yield by the 8 selected AAB with no overoxidation capacity.

Cultures were incubated at 30°C and 37°C in modified HS broth supplemented with ethanol 2.5% as sole carbon source for 15 days.

3.2 Acetic acid bacteria species and ethanol and acetic acid tolerance ability of selected strains

The 16S rDNA from the selected isolates was sequenced. According to a Blast search done on the GenBank database, only three of the selected strains were belonging to acetic acid bacteria. Indeed, strain Ski1 have 100 % identity to several *Gluconobacter oxydans* (e.g: strains with accession numbers MT974237.1; MT974235.1; GU205101.1 with 100 % identity) while Fke 22 and Fk5 strains have greater than 99% identity to various *Acetobacter ghanensis* (mainly the strains with accession numbers MT544655.1 with 99 % identity; MT544653.1 with 99 % identity; HE979554.1 with 100 % identity). Thus, the strains Ski1, Fke 22 and Fk5 were confirmed to belong to *Gluconobacter oxydans* (Ski1) and to *Acetobacter ghanensis* (Fke 22 and Fk5).

Generally, Acetic acid bacteria have been classified into two genera, *Acetobacter* and *Gluconobacter*. This classification is based on their ability to oxidize ethanol and glucose. Acetobacter bacteria oxidize ethanol more strongly than glucose, while *Gluconobacter* bacteria oxidize glucose more strongly than ethanol, which is one of the reasons why Acetobacter are used for vinegar making and *Gluconobacter* for gluconate and ketogluconate production. Despite its inability to further oxidize acetic acid, it is slower in ethanol oxidation when compared to Acetobacter [31]. Acetate is the most predominant organic acid in vinegar, and gluconate is the second one. It is reported that gluconate add flavor to vinegar therefore the production of gluconic acid by *Gluconobacter* would be beneficial to the industrial process [32].

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Concerning, the *Acetobacter ghanensis* is an acetic acid bacteria belongs to genus *Acetobacter. A. ghanensis* was firstly isolated from Ghanaian cocoa beans in Ghana [33]. The novel strains had phenotypic characteristics that enabled them to be differentiated from phylogenetically related Acetobacter species, i.e. they were motile, did not produce ketogluconate from glucose, were catalase-positive and oxidase-negative, grew on yeast extract with 30 % glucose, grew weakly on glycerol but not on maltose or methanol as carbon sources, and did not grow with ammonium as sole nitrogen source and ethanol as carbon source.

The response of the three isolates to stress conditions, in terms of growth capability has been investigated using different parameters, in order to provide useful information on their ability to initiate and carry out the fermentation (Figure 3). For this purpose, 48 h cultivation at 37°C under different ethanol and acid concentrations was conducted with the thermotolerant acetic acid bacteria isolates.

In the case of 48 h at 37°C cultivation under different ethanol concentrations, Ski1 (*Gluconobacter oxydans*) and *Acetobacter ghanensis* strains Fke 22 and Fk 5 showed significant growth in the media with 3 to 5 % ethanol at the early stage. As the ethanol concentration increased, 6 to 10%, growth of all AAB strains sharply decreased (Figure 3 a).

Acetic acid tolerance is a crucial ability allowing AAB to stably produce large amounts of acetic acid. In this experiment, the growth of Fke 22 identified as *Acetobacter ghanensis* was almost unaffected to increasing acid content, whereas the Ski1(*Gluconobacter oxydans*) and Fki5 (*A ghanensis*) displayed sharp decrease after 6% acetic acid concentration (Figure 3b). The two A. ghanennsis strains were differentiated by their ability to sustain increasing acid concentrations. Several molecular machineries responsible for acetic acid tolerance in AAB have been reported, including (1) prevention of acetic acid influx into the cell, (2) acetic acid assimilation, (3) acetic acid efflux by transporter or pump, and (4) protection of cytoplasmic proteins against denaturing by general stress proteins [34].

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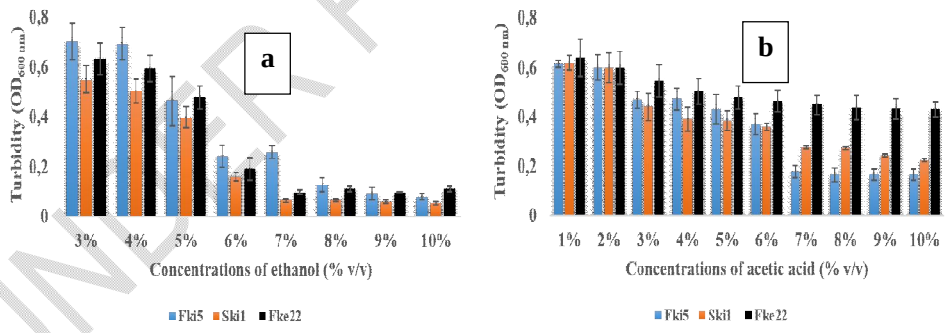

Fig. 3. Growth of 3 thermotolerant selected acetic acid bacteria isolates in 48h cultivation at 37°C

under ethanol (a) and acid stress (b). The selected acetic acid bacteria strains were cultivated in HS broth at 37°C for 48 h

From a technological point of view, there are two well defined methods to produce vinegar: traditional (slow) and submerged (fast) processes [35]. Traditional methods are commonly referred to as “surface culture

fermentations" and AAB grow abundantly on the media. To date, submerged fermentation (SF) is widely used for vinegar production [36].

This new strain, *Acetober ghanensis*, also has been found in many fruits jujube, kaffir lime, pineapple, rose apple in Thailand [37]. Artnarong et al. [38] isolated this strain from palmyra palm fruit; the authors found that it could produce high acetic acid 5.64% in ethanol 6% after 55 days and showed negative overoxidation. Obviously, this bacterium could produce acetic acid faster than spontaneous fermentation thus it was possible to use it as the starter culture for vinegar fermentation. Several studies have performed acetic acid bacteria isolated from fruits such as, cherry, mango etc and characterized their properties. For examples, the Acetobacter strain grew well in modified Caar media with 4% and 5% ethanol, but the growth decreased at higher 6% (v/v) ethanol concentration [39]. Kommanee et al. [37] found that the growth of *Acetobacter pasteurianus* increased maximally at 4% (v/v) ethanol and the cells decreased at 6% and 8% ethanol concentration.

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4. CONCLUSION

This study allowed to select three thermotolerant acetic acid bacteria strains including Ski1, Fke 22 and Fk5 with high amount of acetic acid production capacity but without acetic acid overoxydation ability. Moreover, the selected strains are able to oxidize ethanol to acetate under stress conditions occur during acetic acid production process. Overcoming the acetification constraints is a main goal in order to facilitate innovation in submerged fermentation and to create new industry-challenging perspectives. These strains belonging to *G. oxydans* and *A. ghanensis* species could then be used as starters. Their utilization would help reduce the cooling water feeds during the acetification process especially in hot countries in the context of global warming. Moreover, the constraints of rigorous monitoring of alcohol levels, necessary to overcome acetate oxidation that increment the outlay of production, will be alleviated.

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