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METHODOLOGICAL ASPECTS OF REAL-TIME PCR USAGE IN ACETOBACTER DETECTION

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Abstract. Acetic acid bacteria occur in sugar and alcoholised, slightly acid niches such as flowers, fruits, beer, wine, cider, vinegar, souring fruit juices and honey. On these substrates they oxidize the sugars and alcohols, resulting an accumulation of organic acids as final products. These transformations are of considerable interest for the biotechnological industry. The best known industrial application of acetic acid bacteria is vinegar production. The study used a method Real-Time PCR allows the quantitative detection of the members of the acetic acid bacteria group including the well-known *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* species, and also other rare occurring but also acetic acid producing representatives as *Acidisphaera sp.*, *Acidocella sp.*, *Acidomonas sp.*, *Asaia sp.*, *Granulibacter*, *Kozakia sp.* and *Swaminathania sp.* The main objective of the present work is to test fast, sensitive and reliable technique such as Real-Time PCR and to detect acetic acid bacteria group without plating, isolated from white wine vinegar and directly from vinegar and the acetic acid bacteria grown in glucose medium (GYC) at 30°C, 48 h. The results obtained indicate that the amount of live acetic acid bacteria in vinegar during the fermentation process is not sufficient to detect them in this direct way.

Keywords: *Acetobacter aceti*, kit Acetics screening, white wine vinegar, DNA extraction, Real-Time PCR.

Introduction

Acetic acid bacteria (AAB) are important micro-organisms in the food and biotechnological industries because of their ability to oxidize many types of sugar and alcohols. The production of vinegar is one of the most important industrial processes in which these bacteria are involved [1]. Acetic acid bacteria are Gram negative, rod-shaped, peritrichously or polarly flagellated when motile, mesophilic and obligate aerobes. Most are

catalase positive and oxidase negative. These bacteria are capable of oxidizing sugars, sugar alcohols and alcohols to corresponding acids [2]. Over the past years, acetic acid bacteria have been the object of extensive research, resulting in a significant restructuration of their taxonomy and advances while understanding their physiology, metabolism and molecular biology and in methods for their isolation and identification [3].

In the food industry AAB are being used as main participants in the production of several foods and beverages, such as vinegar, cocoa, kombucha and other similar fermented beverages. However, their presence and activity can easily derive into spoilage of other foods or beverages such as wine, beer, sweet drinks and fruits [4].

As the AAB are specialized in rapid oxidation of sugars or alcohols, oxygen availability plays a pivotal role in their growth and activity. Their metabolic activity and growth is especially enhanced when oxygen is present or specifically added (for example in vinegar production). Their optimal pH is 5.5–6.3 [5]. The optimal growth temperature is 25–30°C yet some strains can grow very slowly at 10°C [6]. The most commonly species found in wine and vinegar are: *Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Gluconacetobacter hansenii*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter europaeus* [7]. *Gluconacetobacter europaeus* is one of the most prominent AAB species isolated from industrial submerged vinegar fermentors, with high resistance to acetic acid (more than 18%) [8]. *Acetobacter* species are commonly detected on grapes, in wine and in vinegar, with *A. aceti* and *A. pasteurianus* being the most abundant species [9].

Initially, the taxonomy of acetic acid bacteria was based on morphological and physiological criteria. However, phenotypic identification of strains of this group of bacteria, particularly on the species level, is not only inaccurate, but also laborious. The main reason for this difficulty is the instability of physiological traits, due to the presence of insertion elements in the genome of acetic acid bacteria. And one more thing, as the difficulty in managing these bacteria in routine laboratory techniques, due to their slow growth [10]. Gene detection methods based on molecular biology techniques are the common methods that are currently used for microbiological tests such as loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) techniques [11].

Rapid and sensitive detection and enumeration of microorganisms has always been a challenge for the food industry [12]. Real-time PCR (rt-PCR) has evolved into a fast, sensitive and accurate tool for quantifying bacteria in environmental samples [13]. It makes it possible to determine the initial template concentration and, therefore, to make an accurate estimation of cell numbers [14]. This method has been used to detect various bacteria [15]. The real-time polymerase chain reaction (real-time PCR) was first introduced in 1992 by Higuchi and coworkers and allows precise quantification of specific nucleic acids in a complex mixture by fluorescent detection of labeled PCR products. Fluorophore-coupled nucleic acid probes interact with the PCR products in a sequence-specific manner and provide information about a specific PCR product as it accumulates. Detecting the PCR product in real-time involves the use of specific fluorescent probe (e.g., Taqman) or nonspecific dye (such as SYBR Green I) [16]. Real-time PCR (rt-PCR) has evolved into a fast, sensitive and accurate tool for detection bacteria in environmental samples [17]. This method has been used to detect various species bacteria but, so far, it was little used to AAB analyses [18].

The present study was undertaken to develop a real-time PCR-based assay for the rapid screening of low concentrations of *Acetobacter* species. The main objective of the

present work is to test fast, sensitive and reliable technique such as real-time PCR (rt-PCR) and screening the presence for the whole group of acetic acid bacteria, *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* in the vinegar in fermentation processes.

Materials and methods

In our research molecular detection of *Acetobacter* has been done using kit “**For everyone Detection Kit B Acetics Screening**” which contains all the materials necessary for this determination. The **qPCR** reaction was performed according to the manufacturer's protocol (#2401-15 *4eTM for everyone Detection Kit B Acetics Screening User Guide*) [19]. Unless the reaction PCR tubes supplied by the manufacturer have been replaced with the tubes **BIORAD 96-well plates**, these being considered suitable for the detection of *Acetobacter* bacteria. Determined parameters of FAM and HEX are subsequently needed to evaluate the results table 1.

Table 1

Evaluation of PCR results		
Detection of target (FAM dye)	Control reaction (HEX dye)	Result
+	+	DNA of acetic acid bacteria is present
+	-	DNA of acetic acid bacteria is present
-	+	DNA of acetic acid bacteria is not detected
-	-	Result is not evaluable: <ul style="list-style-type: none"> • Either: Repeat the DNA extraction with a smaller amount of sample • or: Dilute extracted sample with Rehydration buffer (1:100 to 1:1000) and repeat PCR

In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The C_t (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). C_t levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the C_t level the greater the amount of target nucleic acid in the sample). WVDL real time assays undergo 40 cycles of amplification.

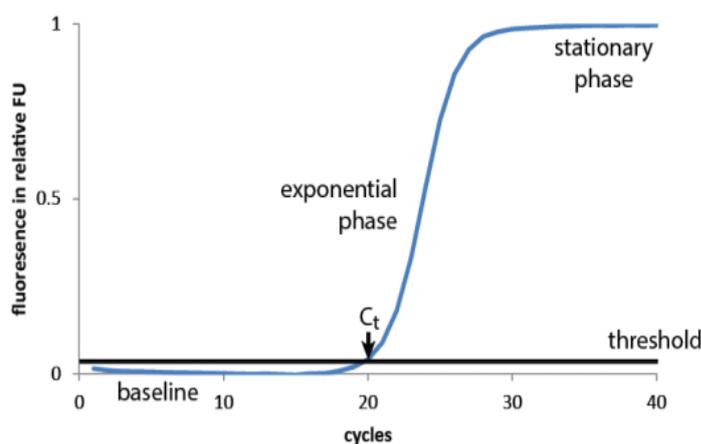


Figure 1. Example of Real Time chart –PCR.

The interpretation is as follows:

- ✓ FAM Channel detects target organisms:
 - a. Ct \leq 38: Reaction is positive
 - b. Ct 38 – 40: Reaction is critically low, repeat the sample preparation and/or the PCR
 - c. Ct $>$ 40: Reaction is negative
- ✓ VIC/HEX Channel detects internal positive control:
 - a. For the internal positive reaction a Ct value \leq 35 is expected
 - b. If the Ct value is between 38-40, the control reaction has to be assessed as inhibited/negative
 - c. In case of a positive sample with Ct values \approx 20 – 25, the internal positive control may show higher Ct values or fail completely

The following samples were used for detection: vinegar obtained in TUM laboratory from white Nova grapes; m/o isolated from untreated vinegar on GYC medium; positive control - DNA provided; negative control. Used vinegar was in the fermentation process, namely in the stationary phase of growth of acetic acid bacteria, when the number of live and active bacterial was maximum.

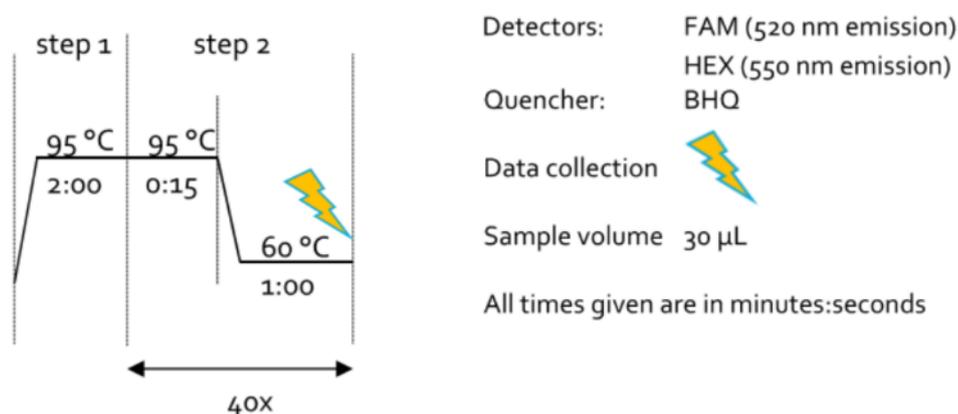


Figure 2. Temperature scheme of thermocycler.

For the reaction mixture suitability test, we used Platinum PCR Supermix (Invitrogen). A sample containing TaqMan Universal Master Mix II was used as a positive control. The detection of acetic acid bacteria was done at FAM™ (520 nm emission) channel, of Internal Positive Control at HEX® (550 nm emission) channel. Cycling conditions are shown in “Figure 2”. All DNA extractions were performed in duplicate.

Results and discussion

In order to evaluate the suitability of the kit for the analysis of vinegar, we first tested its functionality. We performed PCR analysis of the positive control DNA (purified DNA of acetic bacteria) included in the kit, and used to polymerase-containing mixes – one included in the kit, the other TaqMan Universal Master Mix II. Figure 3a and 3b shows the results of this analysis. As one can see, both enzyme mixes successfully carried out the

amplification, and the fluorescent signal could be detected through both FAM and HEX channels. Thus, the test gives valid results. However, the enzyme mix included in the kit gave a better performance, amplifying the DNA more efficiently and giving a lower Ct value.

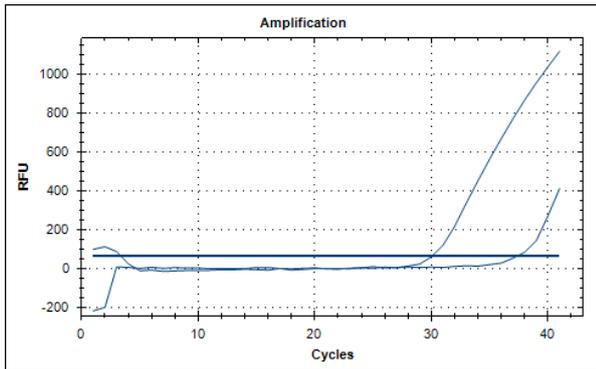


Figure 3a. Positive control FAM chart of DNA amplification.

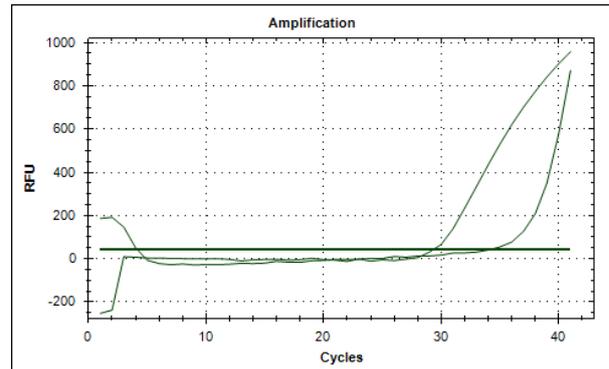


Figure 3b. Positive control HEX chart of DNA amplification.

Next, we analyzed the following samples: DNA purified directly from vinegar and DNA purified from the colonies inoculated from this vinegar on the media (2 samples of each), using the enzyme mix included in the kit. The results of this analysis are shown on figure 4 *a* and *b*.

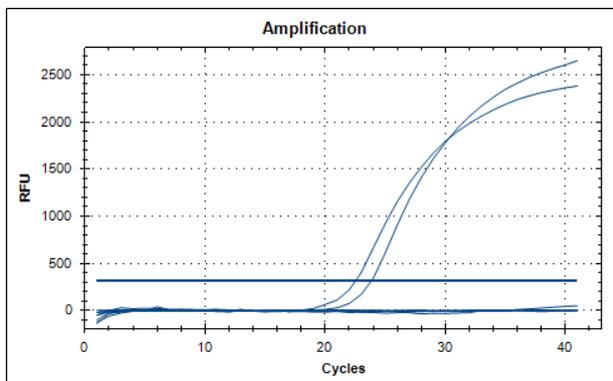


Figure 4a FAM chart of DNA amplification.

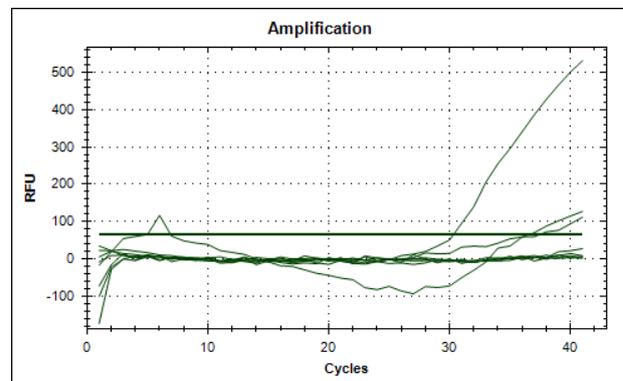


Figure 4b HEX chart of DNA amplification.

The only two samples resulting in amplification in Figure 4a are the samples containing the DNA purified from the two bacterial colonies. In Figure 4b, the three curves crossing the baseline are the amplification graphs of the negative control sample and the same DNA isolated from the two bacterial colonies which gave the signal on FAM channel. Thus, the test is valid for those three samples (negative control and two bacterial colonies). We could not detect any signal on either FAM or HEX channel for the samples containing DNA isolated directly from vinegar. This could be due to the low amount of the cells containing in the vinegar, but rather, taking into account no signal from HEX channel for these samples, to the bad DNA quality and PCR inhibition. Below is the table summarizing the Ct value data from HEX and FAM channels.

Analyzing the data presented in table 2 we can see than the FAM channel crosses the threshold line at Ct = 23.84 and the HEX channel at Ct = 37.56, and therefore we get $\Delta Ct = 29 < 37$, so we can confidently assert that the microorganisms isolated from untreated vinegar on GYC medium belong to the genus *Acetobacter*.

Tabel 2

Sample	Analyze of date RT-PCR		FAM*	HEX**
	Cycle quantification, C _q			
	C _q FAM	C _q HEX		
M/o colonies isolated from untreated vinegar on GYC medium	23,84	37,56	+	+
White wine vinegar (untreated)	Didn't intersect	Didn't intersect	-	-

* - 520nm; ** -550nm

From these results we conclude that this method is not effective for the detection of acetic bacteria in vinegar, probable they are in a small number, and the solution containing DNA proved to be diluted. Then, it is recommended another AND method extraction, filtering the vinegar in order to be able to accumulate and grow a larger amount of bacteria on the filter.

Conclusions

The detection and identification of AAB are important to better understand AAB population dynamics, as well as to maintain the quality of the final product in vinegar production. The isolation of strains and their maintenance in pure cultures is useful for industrial purposes, but many methods are time consuming and expensive. New techniques bypass this culture step and enable the identification of microorganisms that are difficult for traditional methods.

In this study of these 2 samples that were simultaneously amplified, only bacteria isolated showed a positive reaction and the presence of acetic bacteria of the genus *Acetobacter*. A sample taken from vinegar during the fermentation period did not give a positive result, despite the fact that the phase was selected with the greatest accumulation of acid bacteria.

At the end of this work we can draw the following conclusions, firstly, that isolated bacteria from live vinegar belong to the genus *Acetobacter*, secondly, that even using vinegar in the phase of maximum accumulation of acetic acid active bacteria, this is not enough to detect them using PCR real-time.

Acknowledgments

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