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COMPARATIVE ANALYSIS OF TWO METHODS FOR DETECTION THE DNA OF ACETIC ACID BACTERIA BY REAL-TIME PCR

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Abstract. Acetic acid bacteria (AAB) are very difficult to correctly identify at species levels based only on biochemical and physiological characteristics. For their proper identification, molecular analysis of the strains in comparison with reference species is recommended. In recent years, a variety of methods based on molecular techniques of DNA extraction and identification by polymerase chain reaction (PCR) have been used for identification of the genera, species and strains of AAB.

The study used a method Real-Time PCR allows the qualitative detection of the members of the acetic acid bacteria group including the well-known *Acetobacter, Gluconacetobacter* and *Gluconobacter* species.

The goal of this study was comparing two methods of DNA isolation from vinegar for further analysis by real-time PCR for detection of acetic acid bacteria (AAB). In this work, the Detection Kit B Acetics Screening from PIKA was used. The DNA was isolated using the same kit as recommended by manufacturer, and using DNAzol Reagent. For the study, acetic acid bacteria were isolated from unpasteurized white wine and apple vinegar in RAE medium at 30 ° C, 48-72 h.

As a result, we could identify the acetic acid bacteria from white wine and apple vinegar in the DNA samples isolated by two different methods. DNA was isolated from in equal quantities of the culture using DNAzole, amplification was successful and no inhibition of PCR was observed. The Ct values for this method were slightly lower for both samples compared to the values obtained for purified DNA using the kit buffer. The results of Ct obtained show that both methods of DNA extraction from acetic acid bacteria yielded amplifiable DNA, though in case of DNAzol the yield was slightly inferior compared to the genuine kit protocol.

Key words: Acetic acid bacteria, kit Acetics screening, DNAzol, Real-Time PCR, vinegar.

INTRODUCTION

Recently, the real-time PCR (r-t PCR) method has been increasingly used for the quantitative and qualitative determination of bacteria and has proven to be a fast, sensitive and accurate method

(Harms and al., 2003). It makes it possible to determine the initial template concentration and, therefore, to make an accurate estimation of cell numbers (Bleve and al., 2003). This method has been used to detect various bacteria including acetic acid bacteria (Luo and al., 2004).

In real-time PCR, DNA is measured during the exponential phase of PCR amplification. Accumulating product is detected as it is being amplified using fluorescent DNA probes. DNA extraction is usually affected by factors such as incomplete cell lysis, DNA sorption to a particular material, coextraction of enzymatic inhibitors and degradation or damage of DNA. Clearly, the application of a suitable DNA extraction protocol for a specific sample is essential for correct microbial diversity estimation (Fraga and al., 2014).

Theoretically, during PCR, each target sequence is amplified in proportion to the amount of target initially present in the sample (Figure 1.). Appreciation of this led to the development of method for quantifying gene expression called real-time PCR (Fraga and al., 2014).

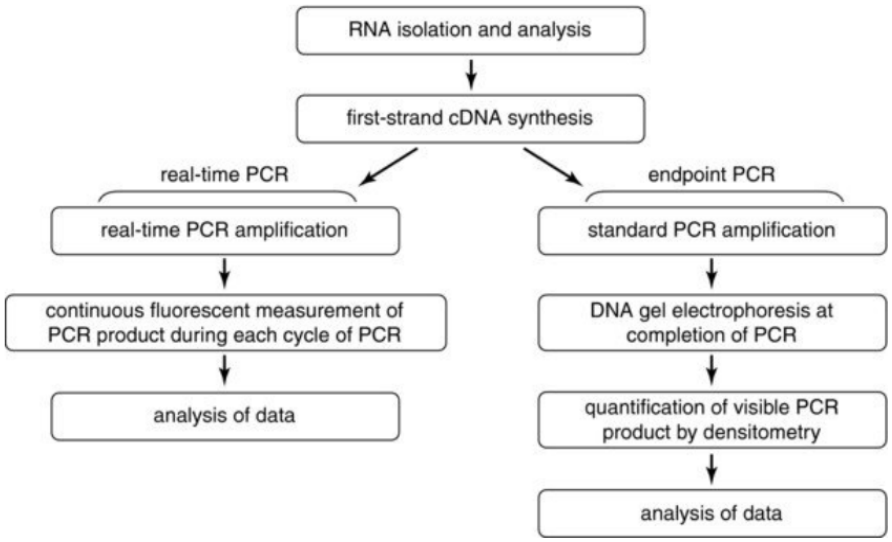


Fig. 1. Comparison of endpoint RT-PCR and real-time RT-PCR (Fraga and al., 2014)

Acetic acid bacteria play both negative and positive roles in the food industry. The intake of oxygen, involved in such usual enological practices as pumping over and racking may stimulate the growth of AAB, and this may lead to an undesirable production of acetic acid, acetaldehyde, ethyl acetate and dihydroxyacetone. Thus, to obtain good-quality wines, AAB populations must be kept as low as possible across the whole process (Drysdale and Fleet, 1989).

Sound winemaking practices were considered to be sufficient to inhibit the growth of these organisms. These practices include the maintenance of anaerobic conditions by blanketing the wine with an inert gas or filling containers completely, as well as the correct use of sulphur dioxide (SO_2). However, it has become increasingly evident that, in some cases, these organisms can survive and even multiply under the anaerobic or semi-anaerobic conditions found in winemaking (Wessel du Toit and Pretorius Isak, 2002).

In recent years, there has been renewed interest in the acetic acid bacteria associated with wine. Since the last comprehensive review of acetic acid bacteria associated with wine was published more than a decade ago, recent research is linked to the development of modern methods for the detection of acetic acid bacteria (Amerine and Kunker, 1968; Poblet and al., 2000).

As already mentioned, it is important to detect the presence or absence of a specific microorganism, in our case acetic acid bacteria, by a simple and accurate method. Although real-time PCR is accurate, it is also expensive and complex. However, PCR is also one technique that detects relatively low concentrations of acetic acid bacteria DNA in food (Ángel González and al., 2006).

In the present study, real-time PCR was used and compared two types of reagent for the isolation of genomic DNA of acetic acid bacteria. The aim of the study was to analyze the use of DNAzole for isolating DNA from acetic acid bacteria for real-time PCR analysis. The present study

provides detection studies for *A. aceti* and other AABs by two methods.

METHOD AND MATERIALS

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) (Mitina and al., 2019).

In research molecular detection of Acetobacter has been done using manufacturing kit ”For everyone Detection Kit B Acetics Screening” (PIKA Weihenstephan, Germany) which contains all the materials necessary for this determination. Template DNA for PCR was prepared by two methods: (1) lysis buffer from kit; (2) by extraction with the DNA isolation reagent DNAzol (Gibco BRL, Gaithersburg, MD, USA). All steps were performed at room temperature; DNA preparations were stored at −20°C until needed.

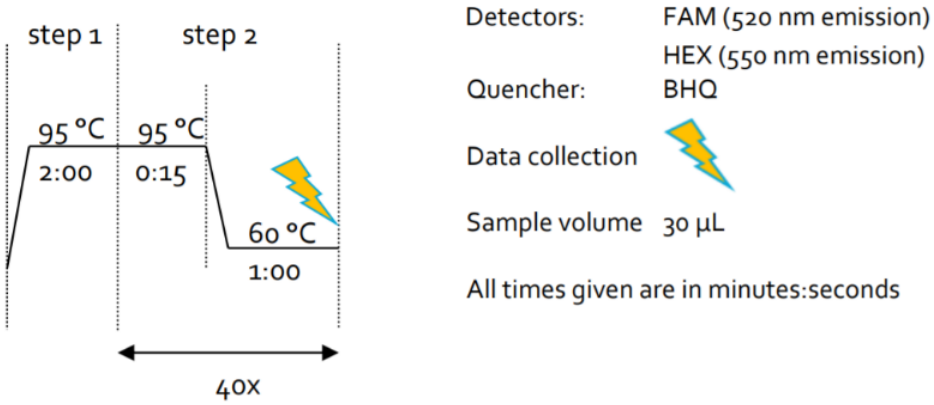


Fig. 2. Temperature scheme of thermocycler (User guide, 2003).

The source of acetic acid bacteria was two types of bacteria Acetobacter previously isolated from two different natural vinegars: apple cider and white wine (Boistean and al., 2020 a). Culture isolates were identified on the basis of colony characters, growth characters and morphological characteristics, which later on were confirmed by biochemical tests were also performed for the identification of pure culture. Acetic bacteria were isolated and plated on selective medium RAE and the petri dishes, incubated at temperature 30°C for 48-72h until the maximum growth of colonies (Boistean and al., 2020 b).

The qPCR reaction was performed according to the manufacturer's protocol 2401-15 4eTM for everyone Detection Kit B Acetics Screening User Guide (User guide, 2003). Cycling conditions are shown in Figure 2.

The reaction PCR tubes supplied by the manufacturer have been replaced with the tubes BIORAD 96-well plates, which have shown themselves to be more suitable for this unit Bio-Rad CFX-96 Connect R-T System (Bio-Rad, USA). The determined results were evaluating 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: - Either: Repeat the DNA extraction with a smaller

Detection of target (FAM dye)	*Control reaction (HEX dye)*	*Result*
		amount of sample - Or: Dilute extracted sample with Rehydration buffer (1:100 to 1:1000) and repeat PCR

As all kits include an internal inhibition control reaction besides the detection of the target microorganism, a 2-channel real time PCR thermocycler is needed (FAM and HEX/VIC).

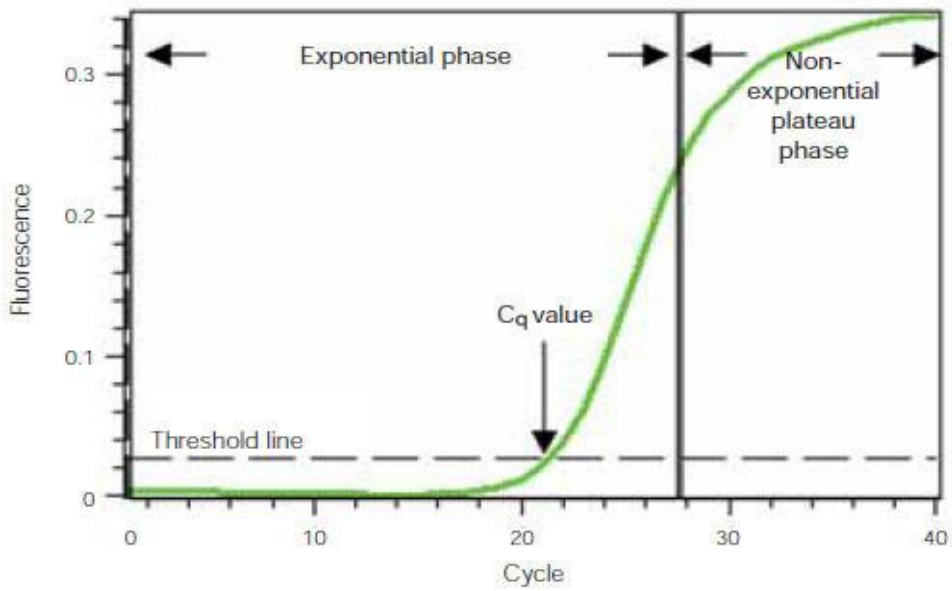


Fig. 3. Baseline-subtracted fluorescence versus number of PCR cycles (Mitin and Mitina, 2020)

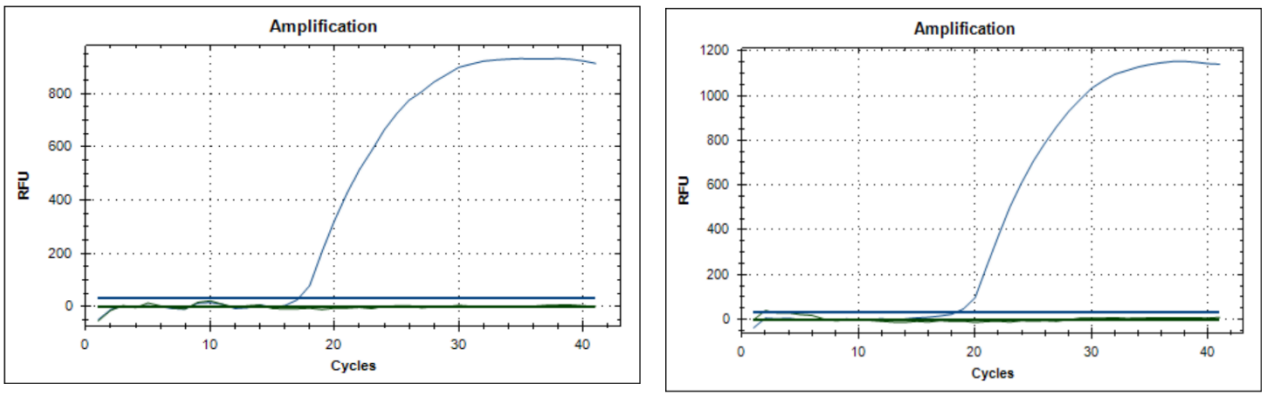
In real-time PCR, the fluorescent signal increases in proportion to the accumulation of the product. A standard curve can then be constructed that displays fluorescence versus the number of cycles (Fig. 3). The quantification cycle (Cq), also called the threshold cycle (Ct), is the number of cycles required for the fluorescent response signal to cross the threshold (Mitin and Mitina, 2020).

RESULTS AND DISCUSSION

Ideally, DNA extraction methods should be simple, fast, and efficient. The choice of method often lies between the cost of materials, labor costs, optimal DNA yield, and the removal of substances that can interfere with the PCR reaction. Thus, it is necessary to check the applicability of the method for specific types of products. The main criteria for this check are based on quantitative and qualitative analysis.

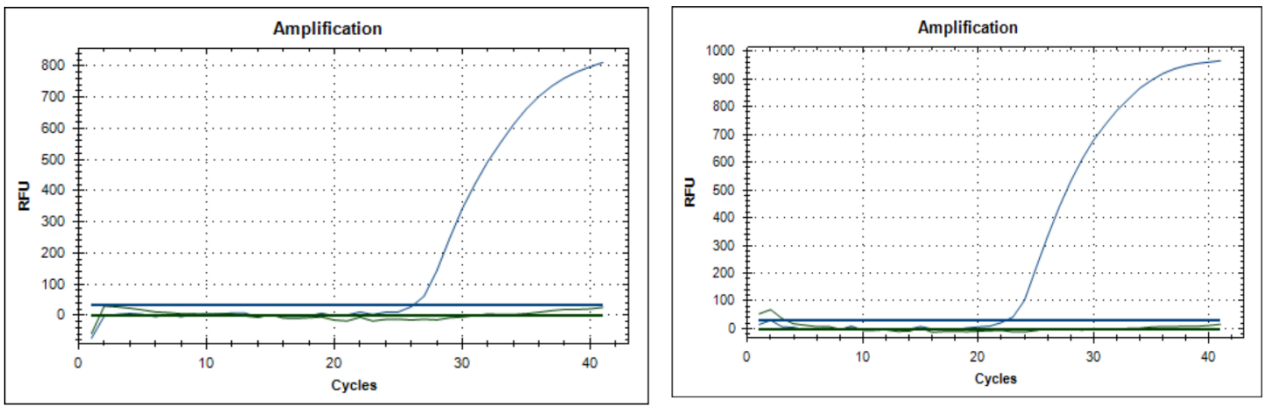
Figure 4 a and b, shows the amplification curve when the DNA isolated from bacteria from apple and wine vinegar was used as a template. One can see, that the Ct value for the DNA isolated from the bacteria from apple vinegar is 17.14, and for the DNA isolated from the bacteria from white wine vinegar is 18.39.

When DNA was isolated from the same culture volume by DNAzol, the amplification was successful, and no PCR inhibition was observed. The Ct values were slightly lower for both samples, compared to those obtained for the DNA purified by kit lysis buffer. the Ct values obtained were 26.15 for the DNA from apple and 22.51 for the DNA from white wine vinegar. (Figure 5, a and b).



a) **b)**

Fig. 4. The amplification curve for DNA of bacteria from **a)** apple and **b)** white wine vinegar, isolated by lysis buffer from kit.



a) **b)**

Fig. 5. The amplification curve for DNA of bacteria from a) apple and b) white wine vinegar, isolated by DNAzol.

Thus, we could obtain PCR-quality DNA from the acetic acid bacteria with both methods.

Analyzing the data presented in Table 2, we see that DNA isolated from apple cider vinegar using a kit crosses the threshold line along the FAM channel at Ct = 17.14, while DNA isolated using ADNzol crosses the threshold line along the FAM channel at Ct = 26.15 ... The data for white wine vinegar also shows that crossing the threshold for DNA isolated by the kit at Ct = 18.39, and DNA isolated by ADNzol at Ct = 22.51.

Table 2. Ct values for the DNA of bacteria from a) apple and b) white wine vinegar isolated by two different methods

Apple vinegar		***White wine vinegar***	
Kit	DNAzol	kit	DNAzol
17.14	*26.15*	*18.39*	*22.51*

Thus, the ADNzol DNA isolation method is less effective and cannot be a good analogue to the manufacturer's kit.

CONCLUSION

The r-t PCR method is currently one of the most accurate methods for detecting various microorganisms at the DNA level. The analysis of scientific articles proves that this method is poorly studied for the detection of acetic acid bacteria. It is possible that one of the reasons may be not only the cost of the process, but also the inaccessibility of kits for the detection of AAB. Therefore, in our

study, we compared two methods for isolating the DNA of acetic acid bacteria, which can be reliably determined using the example of vinegar. Previously carried out methods for determining DNA in different types and quantities proved that for detection it is necessary to isolate bacteria or accumulate in a larger quantity than in which they are in wine. The methods used in these studies have led to the conclusion that the use of ADNzole is not an alternative or better substitute for a production kit.

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