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ORIGINAL RESEARCH PAPER

DETERMINATION OF PRIMERS EFFICIENCY IN THE DETECTION OF *PEDIOCOCCUS* IN WINES

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Abstract: *Pediococcus* spp. are lactic acid bacteria which are considered spoilage in the wine making processes. Early detection can minimize the negative effects of *Pediococcus* spp on wine quality and prevent the development of advanced spoilage stages. The goal of this work was development and testing of a fast and efficient Real-Time Polymerase Chain Reaction (R-T PCR)-based method for easy detection of *Pediococcus* spp. strains in wine at the early stages of wine spoilage. As a result, primer sets for the Real time-PCR were designed to allow for correct and efficient detection of *Pediococcus* spp., and experimentally tested.

Keywords: *dilutions, DNA, lactic bacterium, microbiological, real-time PCR, wines spoilage*

INTRODUCTION

There are many factors influencing wine quality, the microorganism`s activity being one of the most important. A wide diversity of microorganisms is involved in the winemaking processes, determining the quality, evolution and organoleptic characteristics of wines. Wine spoilage could be caused by a lot of genera and species of yeasts and bacteria. Wine spoilage microbes are those microorganisms found at the wrong place and the wrong time, including microbes which are normally desirable and contribute to the quality of the end product [1]. That's why monitoring of the microbiological diversity present in grapes, musts and wines on different stages of the production process is crucial for the wine quality.

Pediococcus species is a group of microaerophilic, gram-positive, non-spore-forming and non-motile cocci [2] that largely act as a flavor-producing agent in wine and other fermented foods.

Pediococcus spp. are lactic acid bacteria present in grape musts and disappear almost completely during the winemaking process [3]. Together with bacterial species of the genera *Leuconostoc*, *Lactobacillus*, *Oenococcus oeni* [4 – 5], *Pediococcus* is a part of normal lactic acid bacteria microflora of musts and wines. These species can provoke the malolactic fermentation, which influences positively quality and organoleptic characteristics of wines. Though, uncontrolled multiplication of lactic acid bacteria, spontaneous malolactic fermentation can cause wine spoilage, loosing of the freshness, acidity, and stability of wines.

That's why, *Pediococcus* spp. are considered spoilage in the wine making processes. They are responsible for production of histamine, polysaccharides, acrolein formation from glycerol and increasing viscosity levels (wine ropiness) [1].

Pediococcus spp. can cause the formation diacetyl and glucan [6] causing undesirable olfactive changes in wines and instabilities. Early detection can minimize the negative effects of *Pediococcus* spp. on wine quality and prevent the development of advanced spoilage stages.

Traditional culture-based microbiological methods are time and labor consuming. These methods for contamination`s identification take 1 - 2 weeks and rely on growth on semiselective culture media or selective culture media, followed by final identification by biochemical and physiological analysis and morphology as determined by microscopic examination [7]. They don't give fast and conclusive results which can be used for technological decisions making. In case of spoilage bacterial multiplication, identification on the early stages is essential for keeping the high quality of the wines. Modern molecular biology methods, such as real-time PCR, demonstrate high efficiency in early detection of the potential spoilages in musts and wines and can be widely used in the winemaking process.

This study reports the results of primers efficiency determination for *Pediococcus* spp. identification in wines. Specific primers for *Pediococcus* spp. bacterium identification were designed based on the sequences publicly available in GeneBank database. One pair of primers proved to be the most efficient. All pairs of primers proved to be useful for an efficient, fast, sensitive and specific detection of spoilage *Pediococcus* spp. damnosus strains in wine by real-time PCR.

EXPERIMENTAL SETUP

Collection of sample

Total DNA was isolated directly from the red wine produced from endogenous grapevine variety Feteasca neagra at Technical University of Moldova (TUM). Wine was infected with *Pediococcus* spp., presenting the abnormal viscosity, non-typical sediment and lactic smell.

Isolation of the wine DNA

Total DNA was extracted from wine using polyvinyl-pyrrolidone (PVP)-based DNA extraction methods [8]. The procedure was carried at the room temperature. Briefly, pellet formed after centrifugation of 10 mL of wine was re-suspended in 0.6 mL of the Extraction buffer (Tris-HCl 0.2M *p*H 8.0, NaCl 0.25M, Na2EDTA 0.025M, SDS 5 % w/v) and heated at 65 ^{o}C for 1 hour. All reagents were molecular biology grade (Sigma-Aldrich). Then 60 mg of PVP powder and with 0.5 volume of ammonium acetate solution (7.5 M) were added to the sample and incubated on ice for 30 min. After 10-minute centrifugation at 10000 g the supernatant was transferred to a fresh tube, mixed with an equal volume of isopropanol and incubated at -20 for 30 minutes. The samples were centrifuged, the pellet washed with 70 % ethanol, air dried and dissolved in 50 µL of water; 2 µL of the resulting DNA solution was used per PCR reaction. The qualitative and quantitative verification of the isolated DNA was made by spectrophotometric analyses at Genova Nano Micro-volume spectrophotometer.

Polymerase chain reaction amplifications

The Real-Time PCR allows precise quantification of specific nucleic acids in a complex solution by fluorescent detection of labeled PCR products.
In a real time PCR, a positive reaction is detected by accumulation of a fluorescent signal. For the experiments, we used SYBR Green I nonspecific dye as the fluorescent agent.
The Ct (cycle threshold) represents the number of cycles required for the fluorescent signal to cross the threshold (i.e., to pass the background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample. The lower the Ct level the higher is the concentration of target nucleic acid in the sample. Around 40 cycles of amplification are usually needed [9].
In our research molecular detection of *Pediococcus* spp. has been done at real-time PCR Detection Systems CFX96 TouchTM BIORAD. PCR conditions were as recommended by SybrGreen producer (Applied Biosystems) - initial incubation at 50 ^{o}C for 2 minutes, initial denaturation at 95 ^{o}C for 2 minutes, and alternation of 95 ^{o}C for 15 sec. and 60 ^{o}C for 1 minute for 40 cycles. For melting curve construction, samples were heated to 95 ^{o}C for 15 seconds, then incubated at 60 ^{o}C for 1 minute (1.6 ^{o}C /sec ramp rate), then heated to 95 ^{o}C for 15 seconds (0.15 ^{o}C /sec ramp rate). Cycling conditions are shown in Figure 1. The detection of *Pediococcus* spp. bacteria was made at SYBR channel.

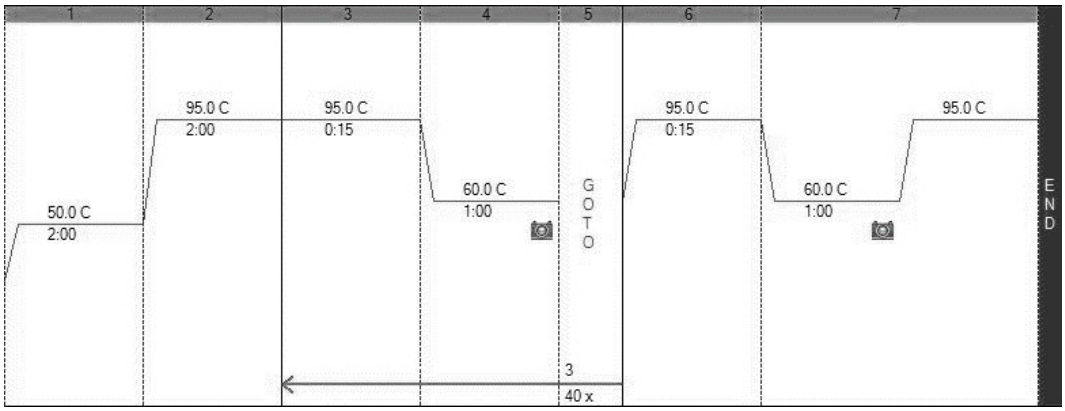

***Figure 1**. Temperature cycling conditions*

RESULTS AND DISCUSSION

We developed three pairs of primers for detection of *Pediococcus* species, basing on the sequence of AM899859.1:1-704 *Pediococcus* damnosus partial rpoA gene for DNA-directed RNA polymerase subunit alpha, strain LMG 16740. Primer names, sequences and characteristics are shown in Table 1.

***Table 1**. Primer sequences and characteristics*

Name	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC %	Self 5` compl	Self 3' compl
p45	TTGAAGGC GTAGTTGA AGATGTCA	Plus	24	144	167	60.50	41.67	3.00	3.00
p46	GACACGCT CGATTGGG GTATAA	Minus	22	475	454	60.22	50.00	4.00	2.00
p47	GCGTAGTT GAAGATGT CACACAAA	Plus	24	150	173	60.03	41.67	5.00	0.00
p48	GCATATCA TCCTTGCG AGCCTTA	Minus	23	425	403	60.87	47.83	4.00	2.00
p49	GGTGCAGC GGTTACAA GCA	Plus	19	89	107	61.26	57.89	4.00	0.00
p50	AGATTTTT CGTCGTCA CTTTCAAT CTTT	Minus	28	229	202	60.68	32.14	5.00	1.00

We performed BLAST analysis of these primers in GeneBank database to check their specificity and inclusivity. Table 2 shows the number of hits of these primers to DNA sequences of different members of *Pediococcus*.

Table 2. *Pediococcus species recognized by primers*

	Number of hits in Gene Bank database					
Primer name	***Pediococcus damnosus***	***P. inopinatus***	***P. cellicola***	***P. parvulus***	***P. siamensis***	***P. ethanolidurns***
p45	9	7		5	1	1
p46	9	7	1	5	1	1
p47	9	7		5	1	1
p48	9	7	1	5	1	1
p49	9		1	5	1	1
p50	9			5		

As it can be seen from the Table 2, primer pairs P45-46 and P47-48, originally designed for nested PCR analysis, can recognize five *Pediococcus* species (P. damnosus, P. inopinatus, P. parvulus, P. siamensis, P. ethanolidurans), while the primer pair P49-P50, originally designed for real-time PCR analysis, can recognize two species of *Pediococcus* (P. damnosus and P. parvulus). Primer pairs P45-P46 and P47-48 were designed to meet the same requirements as real time PCR primers, except for the length of the amplified fragment. However, during experimental testing of these primers in real-time PCR, all three primer pairs showed satisfactory results, with the pair p49-p50 being the best performing.

Pairs of primers P45-46, P47-48, P49-50 were checked by running the real-time PCR amplification using DNA purified from wine as a template. Each pair of primers showed positive results in the *Pediococcus* spp. bacterium identification. Figure 2 and 3 demonstrate the results of the analyses for the P45-46 pair of designed primers. The reaction is positive, i.e., pair 45-46 is sensitive for *Pediococcus* spp. identification.

Figure 2 shows the growth of the amplification curve, Ct value= 26.33. Figure 3 demonstrates single well pronounced peak with the melt temperature= 80.50 ^{o}C.

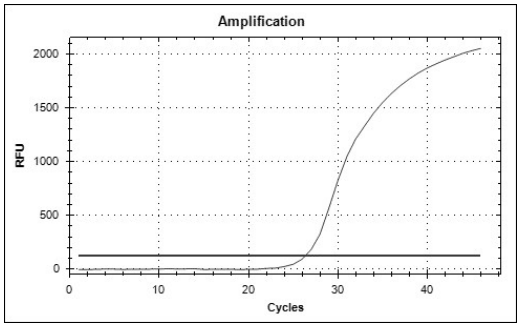

Figure 2*. Amplification curve obtained by P45-46*

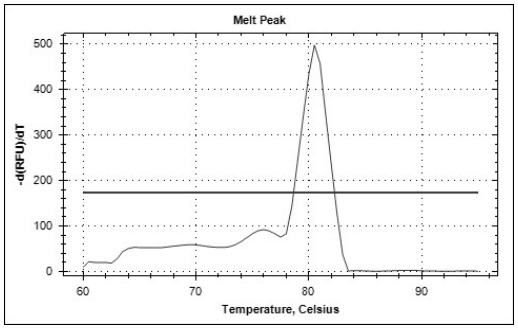

Figure 3*. Melt curve of the fragment obtained by P45-46*

Figure 4 and 5 demonstrate the results of the analyses for the P47-48 pair of designed primers. The results are similar to the previous ones, obtained by P45-46. The reaction is positive, i.e., pair 47-48 is sensitive for *Pediococcus* spp. identification.

Figure 4 shows the growth of the amplification curve, Ct value=26.06. Figure 5 demonstrates single well pronounced peak with the melt temperature = 80.50 ^{o}C.

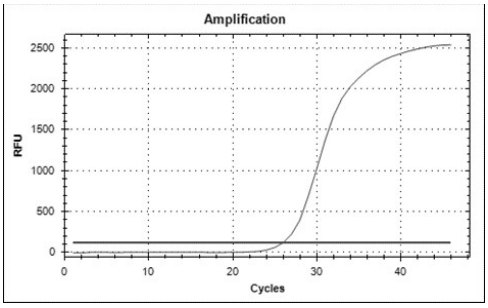

Figure 4. *Amplification curve obtained by P47-48*

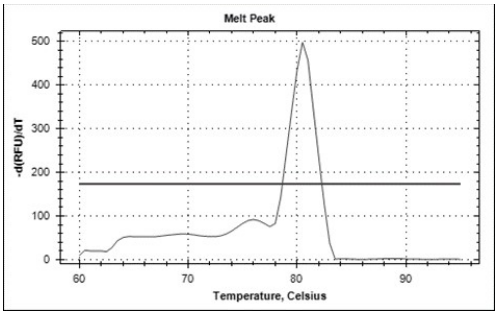

Figure 5. *Melt curve of the fragment obtained by P47-48*

Figure 6 and 7 demonstrate the results of the analyses for P49-50 pair of designed primers. That pair of primers shows better results comparing to previous two pairs of primers. The reaction is positive, i.e., pair 49-50 is sensitive for *Pediococcus* spp. identification.

Figure 6 shows the growth of the amplification curve, starting from the earlier cycles of amplification. Ct value = 22.51.

Figure 7 demonstrates single well pronounced peak with melt temperature = 77.0 ^{o}C. Thus, the last primer pair was capable of detecting the target sequence at an earlier cycle.

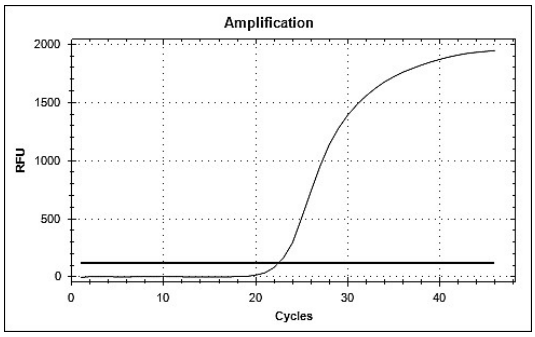

Figure 6. *Amplification curve obtained by P49-50*

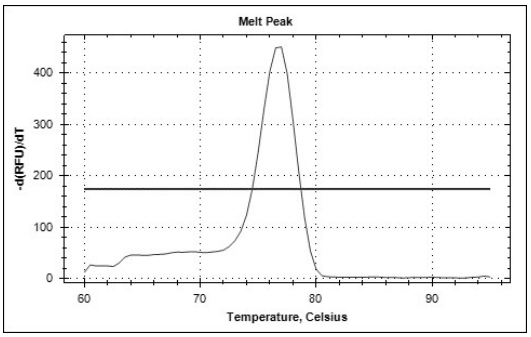

Figure 7. *Melt curve of the fragment obtained by P49-50*

As a result of the first part of the experiments, we saw that all three pairs of primers were suitable for *Pediococcus* spp. identification in wines using real-time PCR. Pair P49-50 showed the best results of the earlier identification and higher sensibility.

For the determination of primers efficiency, we analyzed the serial dilutions of the template DNA by real-time PCR. For this experiment, serial dilutions of the control DNA isolated from wine infected with *Pediococcus* spp 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, 10-8 were made. The experiment was made for all three pairs of designed primers. Each curve represents different dilutions of DNA sample high enough for identification by designed primers. The result of the serial dilutions experiments for *Pediococcus* spp. identification in wines using real-time PCR can be seen on the Figure 8.

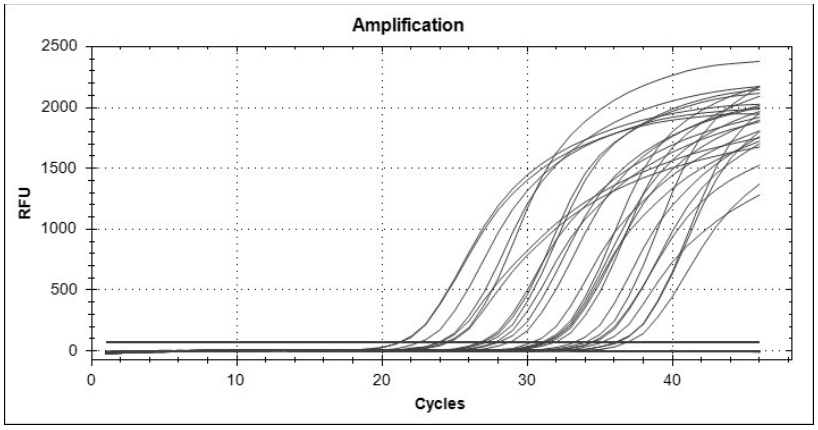

Figure 8. *Serial dilution chart for all primers*

Based on the obtained results, necessary calculations for the primers efficiency determination were made and correlation curves constructed.
Based on the average Ct values and quantity log correlation, the curves for each pair of primers were constructed. They can be seen on the Figures 9, 10 and 11.
The PCR efficiency for each individual sample was derived from the slope of the regression line fitted to a subset of baseline-corrected data points in the log-linear phase. Calculations were made using the Primer Efficiency Calculator [10].
Figure 9 shows the correlation between the Ct values obtained by the pair of primers 45-46 and log of the DNA template dilution factor. Pair of primers 45-46 demonstrated high efficiency of *Pediococcus* spp. identification. All the primers with the PCR efficiency higher than 90 % are considered efficient and can be used for the amplification [11].

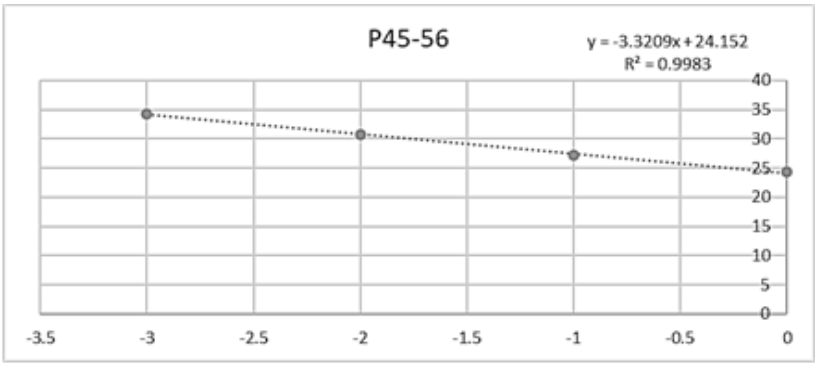

Figure 9. *Correlation curve obtained by P45-46*

Figure 10 shows the correlation between the Ct values obtained by the pair of primers 47-48 and log of the DNA template dilution factor. Pair of primers 47-48 demonstrated only 84.88 % efficiency of *Pediococcus* spp. identification by PCR. This value is below the acceptable limits. However, this primer pair can still be used for the higher DNA concentration in the samples and can't be used for the low genetic material quantities because of the possible inconclusive results.

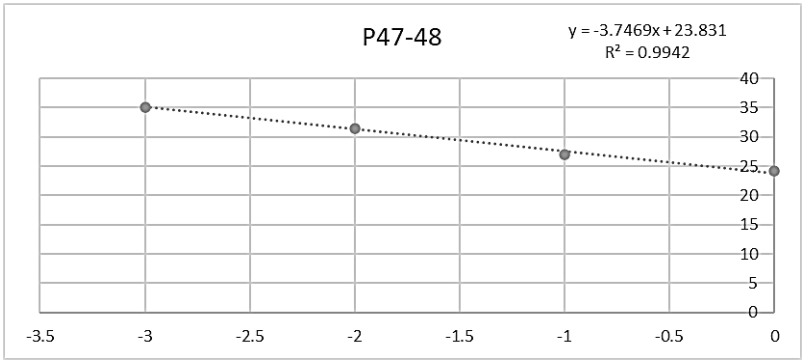

***Figure 10**. Correlation curve obtained by P47-48*

Figure 11 shows the correlation between the Ct values obtained by the pair of primers 49-50 and log of the DNA template dilution factor. It is different from the previous plots in the way it had five dilution points. One dilution point was excluded because of high standard deviation between the replicas. In case of using this primer pair, a Ct value for additional dilution factor (10-4) was obtained. It gives the evidence of a better performance of this primer pair for qPCR analysis compared to other tested primer pairs. The primer pair 49-50 gave the efficiency close to 100 %, and demonstrated the lowest Ct value of the primers tested.

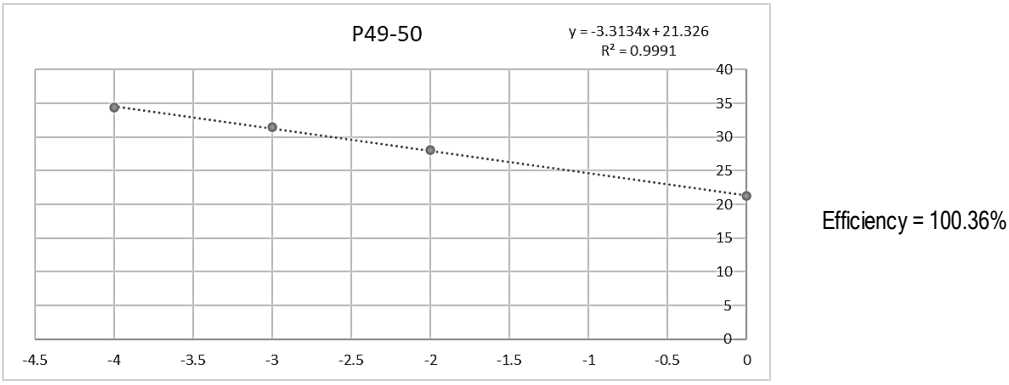

***Figure 11**. Correlation curve obtained by P49-50*

In conclusion, from these results we may say that all three pairs of primers are efficient for PCR identification of *Pediococcus* spp. in wines, with the 49-50 pair of primers demonstrating the optimal performance. The designed pairs of primers can be used for molecular analyses and *Pediococcus* spp. identification in wines on the early stages because of the high specific sensibility to the pathogen and experimentally proved efficiency.

CONCLUSIONS

The present work showed that rpoA gene for DNA-directed RNA polymerase subunit alpha is a good candidate gene for primer design, and specific and efficient primers for *Pediococcus* spp. identification can be constructed basing on its sequence. GeneBank Blast analysis is a good tool for selecting the degree of specificity and inclusivity of the primer pair, and, depending on the need, more inclusive or more specific primer pairs can be designed.

Unexpectedly, a primer pair 45-46, resulting in the synthesis of a fragment of 332 bp, which is longer then optimal for real time PCR (normally up to 150 bp), still gave a good efficiency of about 100 %. However, the primer pair 49-50, synthesizing a fragment of an optimal length (141 bp) outperformed this primer pair, amplifying the fragment at a higher template dilution (10^{-4}).

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