Optimising RAPD-PCR for Screening the Link of RAPD Markers to an Acid-resistant Gene in *Oenococcus oeni*

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RAPD-PCR conditions were optimised for screening RAPD markers linked to the acid-resistant gene in *Oenococcus oeni*. Two (S40, S333) out of 45 random primers were capable of producing stable polymorphism in *O. oeni* isolates. Thirty-three acid-resistant isolates and nine acid-sensitive isolates of *O. oeni* were used for screening RAPD markers linked to the acid-resistant gene. Specific bands of S40-1400 and S333-650 were amplified in 31 (94%) and 33 (100%) of 33 acid-resistant *O. oeni* isolates. The optimised RAPD-PCR method can potentially be used for the fast screening of acid-resistant *O. oeni* strains.

INTRODUCTION

Alcoholic fermentation by yeasts and the transformation of malic acid into lactic acid by lactic acid bacteria are two major steps in winemaking. Many winemakers favour malolactic fermentation because it improves the organoleptic properties of wines (Sancho *et al*., 1998). Among the many lactic acid bacteria species present in grapes, *Oenococcus oeni* is one of the species that triggers malolactic fermentation. Although many strains of *O. oeni* are well adapted to such a harsh ecological medium as wine, some strains do not grow in wine due to the presence of various compounds not favoured by the organisms (Augagneur *et al*., 2007). Environmental factors, such as low pH, high concentrations of alcohol and SO_2, and a lack of nutrients, also inhibit the growth of some strains of *O. oeni* (Nehme *et al*., 2008). In this context, selecting and using acid-resistant strains of *O. oeni* are increasingly being recognised as critical for successful malolactic fermentation (Li *et al*., 2006).

A traditional method for selecting acid-resistant *O. oeni* strains is to evaluate the ability of bacteria to adapt to various factors present in wine (pH, alcohol, SO_2, etc.) (Carreté *et al*., 2005). However, the wine industry needs various single-factor tests and multiple-factor tests to evaluate and select strains of interest. In addition, *O. oeni* usually grows slowly and requires a nutrient-rich medium and anaerobic environment. The traditional method does not meet the current needs of the industry because of its inefficiency and inaccuracy. Molecular-based methods have been approved as rapid and reliable tools for the screening of *O. oeni* strains (de Los Reyes-Gavilán *et al*., 1992). Very recently, a multiplex PCR method has been developed for identifying and typing *O. oeni* (Araque *et al*., 2009). With a broad aim of selecting acid-resistant strains of *O. oeni*, the objective of the present study was to optimise the RAPD-PCR, to characterise the acid-resistant strains and acid-sensitive strains of *O. oeni* at the DNA level, and to detect RAPD specific molecular markers linked to acid-resistant genes in *O. oeni*.

MATERIALS AND METHODS

Forty-two *O. oeni* isolates from the culture collection at the Microbiology Laboratory of the College of Enology, Northwest A & F University, China were used for this work. All these isolates were isolated during the natural fermentation process in wineries. In terms of the locations of the wineries, the identities of these isolates were initialised with 'NX', 'SD' and 'SX' respectively and followed by batch numbers. NX represents isolates from Guangxia Winery in NingXia Autonomous Region; SD represents isolates from Yantai Winery in ShanDong Province; and SX represent isolates from Yangling Winery in ShannXi Province, China. The susceptibilities of *O. oeni* isolates to acid (acid resistant or acid sensitive) were determined by the growth of bacteria in acidic culture. Briefly, each isolate was cultured in acidic tomato broth (ATB) (pH 4.8) and log phase bacteria were inoculated into the ATB (pH 3.2) at concentrations of 5×10^7 colony forming units (CFU)/mL. After incubation at 26°C for four days, the optical densities (OD) of the bacteria solutions were measured three times using a spectrophotometer at 600 nm. Isolates that yielded mean OD values ≥ 0.600 were considered as acid-resistant strains, while those with mean OD values ≤ 0.300 were considered to be acid-sensitive

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strains. We have determined that the method of using the OD value is closely correlated with the method of direct plating and counting CFU (R > 0.90) (data not shown) in determining the ability of *O. oeni* to grow at low pH. Among the 42 isolates, nine (NX-2g, NX-4f, NX-3c, NX-4a, NX-4d, NX-2h, NX-3e, NX-3d, and NX-3h) were determined as being acid-sensitive strains and the 33 other isolates as being acid-resistant strains. The ability of acid-resistant strains to grow at pH < 3.2 was assessed by culture bacteria under the same conditions as above, but at pH 3.0. After four days of growth, the OD values of all the strains were < 0.200, except for that of strain SD-2a, which was > 0.200 but < 0.300.

Genomic DNA was extracted from *O. oeni* isolates using a phenol/chloroform extraction method previously described by Doulis *et al*. (1999). Genomic DNA from the isolate SD-2a was used as template DNA and a random primer S333 was used as default primer for optimising the RAPD-PCR. Factors evaluated for RAPD-PCR optimisation included doses of Taq DNA polymerase, concentrations of dNTP, primer, Mg^{2+} and template DNA, and temperature for annealing. Single-factor tests were conducted to determine the best conditions for each factor. The RAPD-PCR reaction system was optimised through a combination of various factors as described by Schauder *et al*. (2001). The gradients of factors evaluated for RAPD-PCR optimisation were 0.5, 1.0, 1.5 and 2.0 units of Taq DNA polymerase; 80, 120, 160, 180 and 200 µmol/L of dNTP; 0.2, 0.4, 0.6 and 0.8 µmol/L of primer; 0.5, 1.5, 2.0, 2.5 and 3.0 mmol/L of Mg^{2+}; 5, 10, 20, 40 and 60 ng/L of DNA template; and 34, 36, 38 and 40°C of annealing temperature. PCR was conducted by annealing at 94°C for 5 min, followed by 45 cycles, each with l min at 94°C, l min at 36°C and l.5 min at 72°C, and a final extension at 72°C for 5 min. PCR products were examined using 2% agarose gel electrophoresis stained with ethidium bromide. The optimised conditions for each factor were achieved by examining the presence and specificity of bands in agarose gels.

Forty-five randomly selected primers (Table 1) previously used for screening *O. oeni* in China (Zhang, 2001) were synthesised by Generay Biotech Co. LTD (Shanghai, China). Primers were primarily screened with the optimised RAPD-PCR using DNA template from isolate SD-2a, and those that produced positive products were further selected in an allele pool, using the method described by Kilstrup *et al*. (1997). This two-step procedure for selecting primers was capable of characterising the genomic differences between bacteria isolates (Ingmer *et al*., 1999).

RESULTS AND DISCUSSION

According to the outcomes of repeated experiments for each factor examined, the optimised primary conditions were 1.0 U Taq polymerase, 160 µmol/L dNTP, 0.4 µmol/L random primer, 3.0 mmol/L Mg^{2+} and 10 ng DNA template in a 25 µL reaction system. Electrophoresis of comparisons of the gradients of each factor is shown in Fig. 1. Annealing temperature was optimised at 36°C and the PCR regimen was programmed as 94°C for 5 min, followed by 45 cycles of l min at 94°C, l min at 36°C and l.5 min at 72°C, and a final extension at 72°C for 5 min.

Among the 45 random primers tested, 40 (88.8%) yielded positive amplification of PCR products. The 40 primers were further tested on the allele pool and 36 primers produced stable and repeatable results. Of the 36 primers, six produced primer-specific bands in electrophoresis. The six primers were S25, S32, S33, S40, S178 and S333, which produced specific band sizes of 700, 600–700, >1500, 1400, 900 and 650 bp respectively. Using the six primers that yielded primer-specific amplifications, the DNA polymorphisms present in the gene pool were further verified in a single isolate of *O. oeni*. Results from repeated experiments revealed that only two of the six primers, S40 and S333, exhibited a stable polymorphism in single isolates. With the primer S40, genomic DNA of 31 (94%) of the 33 acid-resistant isolates amplified a clear and stable band of

TABLE 1
The random primers screened for RAPD-PCR.

Primer	Sequence	Primer	Sequence	Primer	Sequence
S1	GTTTCGCTCC	S16	TTTGCCCGGA	S31	CAATCGCCGT
S2	TGATCCCTGG	S17	AGGGAACGAG	S32	TCGGCGATAG
S3	CATCCCCCTG	S18	CCACAGCAGT	S33	CAGCACCCAC
S4	GGACTGGAGT	S19	ACCCCCGAAG	S34	TCTGTGCTGG
S5	TGCGCCCTTC	S20	GGACCCTTAC	S35	TTCCGAACCC
S6	TGCTCTGCCC	S21	CAGGCCCTTC	S36	AGCCAGCGAA
S7	GGTGACGCAG	S22	TGCCGAGCTG	S37	GACCGCTTGT
S8	GTCCACACGG	S23	AGTCAGCCAC	S38	AGGTGACCGT
S9	TGGGGGACTC	S24	AATCGGGCTG	S39	CAAACGTCGG
S10	CTGCTGGGAC	S25	AGGGGTCTTG	S40	GTTGCGATCC
S11	GTAGACCCGT	S26	GGTCCCTGAC	S90	AGGGCCGTCT
S12	CCTTGACGCA	S27	GAAACGGGTG	S178	TGCCCAGCCT
S13	TTCCCCCGCT	S28	GTGACGTAGG	S288	AGGCAGAGCA
S14	TCCGCTCTGG	S29	GGGTAACGCC	S333	GACTAAGCCC
S15	GGAGGGTGTT	S30	GTGATCGCAG	S412	GGGACGTTGG

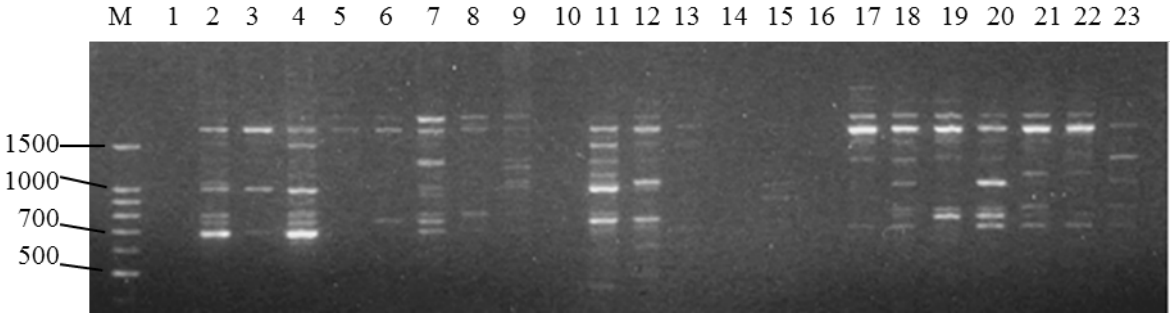

FIGURE 1

RAPD profiles obtained from *O. oeni* using different concentrations of five primary factors. Lanes 1 - 4 represent 0.5, 1.0, 1.5, 2.0 UTaq DNA polymerase; 5 - 9 represent 80, 120, 160, 180, 200 µmol/L dNTP; 10 - 13 represent 0.2, 0.4, 0.6, 0.8 µmol/L of primer; 14 - 18 represent 0.5, 1.5, 2.0, 2.5, 3.0 mmol/L of Mg^{2+}; 19 - 23 represent 5, 10, 20, 40, 60 ng of genomic DNA, respectively. M: Molecular marker.

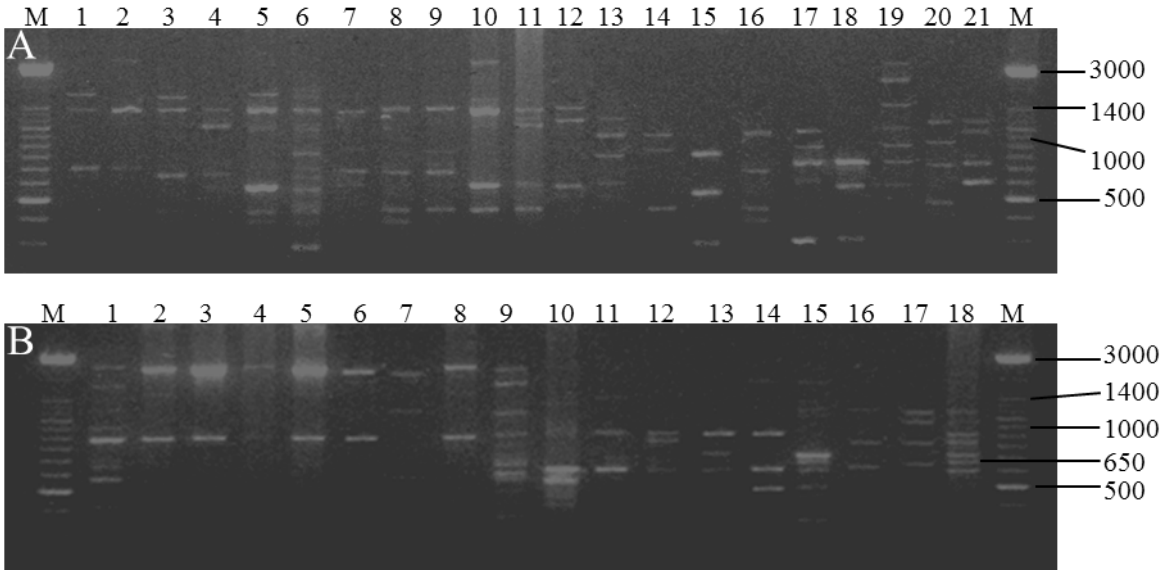

FIGURE 2

A. Genomic DNA amplification in single *O. oeni* isolates using primer S40. Lanes 1 - 12 represent acid-resistant isolates: SX-1a, SD-1f, SD-2hg, SD-2ji, SD-2a, NX-3g, NX-2f, NX-3f, NX-1g, NX-1h, NX-2b, SD-1b; Lanes 13 - 21 represent acid-sensitive isolates: NX-2g, NX-4f, NX-3c, NX-4a, NX-4d, NX-2h, NX-3e, NX-3d, NX-3h. M: Molecular marker. A band of 1400 bp was amplified in the acid-resistant isolates but not in the acid-sensitive isolates, except for NX-3e. **B.** Genomic DNA amplification in single *O. oeni* isolates using primer S333. Lanes 1 - 9 represent acid-sensitive isolates: NX-2g, NX-4f, NX-3c, NX-4a, NX-4d, NX-2h, NX-3e, NX-3d, NX-3h; Lanes 10 - 18 represent acid-resistance isolates: SX-1a, SD-1f, SD-2hg, SD-2ji, SD-1b, SD-2a, NX-3g, NX-2f, NX-3f; M: Molecular marker. A band of 650 bp was amplified in the acid-resistant isolates, but not in the acid-sensitive isolates, except for isolate NX-3h.

1400 bp. In contrast, of the nine acid-sensitive isolates, eight (88.9%) did not exhibit the specific band of 1400 bp. Figure 2A shows the comparison of representative acid-resistant isolates and acid-sensitive isolates amplification with primer S40, from which we can see that all acid-resistant isolates exhibit the band 1400 bp, but that all acid-sensitive isolates except for NX-3e did not exhibit the band 1400 bp. With the primer S333, a band of 650 bp was present in 33 (100%) of the 33 acid-resistant *O. oeni* isolates, but not in eight (88.9%) of the nine acid-sensitive isolates (except for isolate NX-3h) (Fig. 2B).

The presence and specificity of bands derived from the two primers indicate that the specific bands of S40-1400 and S333-650 are molecular markers linked to the gene of acid resistance in the 42 isolates of *O. oeni* screened. The markers of the two specific bands linked to the gene for acid resistance in *O. oeni* isolates were achieved through selection from 45 random primers and confirmation in a single *O. oeni* isolate. No reports could be found on established acid-resistant *O. oeni* strains and the relevant technological properties of such strains. However, the linkage between tolerance to stress and technological properties has been observed

in *O. oeni* strains. For example, in one study, two types of *O. oeni* strains with different physiological characteristics relevant to environmental stress were distinguished using PCR and electrophoresis (Renouf *et al*., 2009). In another study, positive correlations between malolactic activity and the ability to develop and tolerate stress conditions in selected *O. oeni* strains were observed using RAPD-PCR (Capozzi *et al*., 2010). Nevertheless, the linkage between physiological characteristics and tolerance to stress in *O. oeni* has not been confirmed by researchers nor widely accepted by the wine industry. One objective of the present trial was to establish a method for fast screening acid-resistant *O. oeni* strains. Although preliminary data from this work was promising, future studies are definitively needed to further characterise the *O. oeni* strains and to assess the methodology by determining the genotypes of *O. oeni* strains and sequencing the PCR products derived from each primer used.

CONCLUSIONS

In summary, the findings of this study demonstrate the presence of DNA polymorphism in genomic DNA between acid-resistant and acid-sensitive *O. oeni* strains. The PCR conditions were optimised and the RAPD-PCR reaction system was established and found to be a suitable application in our laboratories. We have been using this technique in our laboratories for characterising *O. oeni* isolates and there is the potential of its application in the winemaking industry for the fast screening of acid-resistant *O. oeni* strains.

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