RESOLUTION OIV-OENO 414-2011

Enumerating yeasts of the species Brettanomyces bruxellensis using qPCR

THE GENERAL ASSEMBLY

IN VIEW of Article 2, paragraph 2, no. iv of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

CONSIDERING the work of the “Microbiology” Expert Group and the Methods of Analysis Sub-commission,

On the proposal of the Microbiology Expert Group and the Methods of Analysis Sub-commission,

DECIDES on the proposal of Commission II "Oenology" to include the following type IV method in section 4 of the “Compendium of international methods of analysis of wines and musts”:

Enumerating yeasts of the species Brettanomyces bruxellensis using qPCR

Type IV method

Warning to users

Phenol: All handling procedures involving phenol must be performed under a fume hood and gloves must be worn. All phenol-contaminated residues must be collected in suitable containers.

SYBR Green: This displays a non-zero mutagenicity, but one which is lower than that of ethidium bromide. The precautions for use must nevertheless be adhered to.

1. Scope of application

This protocol describes a method for enumerating yeasts of the species Brettanomyces bruxellensis in wine in bulk or bottled wines, using real-time qPCR (quantitative polymerase chain reaction) (qPCR). The analysis of wines during AF (alcoholic fermentation) and of musts has not yet been validated.

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2. Definition

The micro-organisms enumerated by this method are Brettanomyces bruxellensis yeasts which have a copy of the target gene

3. Principle

The PCR technique amplifies, by multiple repetition of an enzymatic reaction, a target DNA (deoxyribonucleic acid) region identified by two primers. The process involves repeating a three-step cycle:

- Denaturing the DNA by heating
- Hybridization of the primers
- Polymerization, carried out by the Taq (Thermophilus aquaticus) polymerase

However, unlike traditional PCR, qPCR can quantify the DNA amplified during the amplification process through the use of a fluorophore.

Until now two regions specific to the species have been used as targets. One region is the encoding gene for the 26S ribosomal RNA (ribonucleic acid) and the other the RAD4 gene [2, 3]. As with the FISH method, PCR is specific to Brettanomyces bruxellensis but has the advantage of being less expensive.

The distinctive feature of qPCR is that it is possible to read, after each amplification cycle, the fluorescence which increases exponentially as the DNA amplification proceeds. Many fluorescence techniques have been developed for this application. The SYBR® Green fluorophore has been found to be suitable for use with Brettanomyces.

- SYBR® Green fluorophore

This agent fluoresces strongly when it inserts itself non-specifically between the nucleotides in the double-stranded DNA. In contrast, it fluoresces only weakly when unbound. Using this technology, a merged curve can be generated at the end of the amplification that validates the specificity of the reaction.

- Internal standard

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In order to validate the DNA extraction and amplification stages, an internal standard has been integrated into the method (Lip4 Yarrowia lipolytica).

4. Reagents and products

All plastic consumables must be autoclaved beforehand to destroy any DNases (deoxyribonucleases), as must the Tris-HCl and TE (Tris EDTA, ethylene diamine tetra-acetic acid) buffer solutions, the ammonium acetate and the ultrapure water (18 M□). All the aqueous solutions are prepared using ultrapure water (18 M□). Some solutions are sterilized in an autoclave (indicated as "autoclaved"). Sterile ultrapure water (18 M□) is used, if possible, to prepare any solutions which are not autoclaved. It is not then necessary to work under sterile conditions.

- PVPP (eg: ISP Polyclar Super R or Sigma P6755-100G),
- Solutions at room temperature: Tris-HCl buffer, 10mM pH8, solution I (Tris-HCl 10mM pH8, EDTA 1mM, NaCl 100mM, SDS 1% (sodium dodecyl sulfate), Triton X-100 2%), TE (Tris-HCl 10 mM pH8, EDTA 1mM) autoclaved, 4M ammonium acetate, absolute ethanol,
- Provide one autoclaved, sterilized ultrapure (18 M□) water bottle (20mL) per qPCR plate,
- Solutions at 4°C: saturated phenol pH8: chloroform: IAA (isoamyl alcohol 24:25:1) and Rnase (ribonuclease) 1µg/µL
- Suspension at -20°C: internal standard, SYBR Green (e.g. iQ SBYR Green Supermix Bio-Rad 170-8884), primers 4µM Brett rad3, Brett rad4, YAL-F and YAL-R each one.
- Dry bath, set to 37°C.

All handling procedures involving phenol must be performed under a fume hood and gloves must be worn. All phenol-contaminated residues must be collected in suitable containers.

PCR substances	Specifications	CAS Number
4.1 ammonium acetate	>98%	631-61-8
4.2 phenol:chloroform:IAA (24:25:1)	Ultra	136112-00-0

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4.3 proteinase K	1215 U/mg proteins (16.6 ng/ml)	39450-01-6
4.4 SDS	>99% Ultra	151-21-3
4.5 Tris base	>99.8% Ultra	77-86-1
4.6 BSA	Molecular biology grade	9048-46-8
4.7 saturated phenol pH 8		108-95-2
4.8 PVPP 360kDa		9003-39-8
4.9 RNase A	70 U/mg in solution	9001-99-4
4.10 TE pH8	Ultra	Tris : 77-86-1 EDTA : 60-00-4
4.11 Primers 25nmol		-

5. Apparatus

Plastic consumables: 2mL screw-capped microtubes, 1.5 and 1.7mL microtubes, white (10 µL), yellow (200 µL) and blue (1000 µL) pipette tips for micropipettes P20, P200, P1000, P5000, 96-well PCR microplates and optical film, non-powdered gloves

Glass beads (Ø 500 µm)

Bottle (20mL) autoclaved (for ultrapure [18 Mo] sterilized water, one per qPCR plate), 15 and 50 mL Centrifuge tubes

Equipment:

- automatic pipettes (P20, P200, P1000, P5000)
- microtube centrifuge
- automatic stirrer to split cells (eg. GenieDisruptor)
- Thermocycler coupled to a spectrofluorimeter (optical system to detect the fluorescence generated during the real-time PCR reactions)
- Magnetic stirrer
- Stop watch

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- dry bath set to 37°C
- autoclave
- 100mL volumetric flasks
- 50mL volumetric flasks
- 10mL volumetric flasks
- 100mL beakers
- 50mL beakers
- 10mL beakers
- Magnetic stirring bars

6. Sampling (sample preparation)

6.1. Enumerating the samples:

The samples are removed either directly into bottles for analysis or into pre-sterilized sample flasks.

No interference with the method has been observed from the yeasts tested (including K1 and L2056) when the yeast populations are not greater than 5.10^6 CFU/mL (colony forming units). There is no data for populations larger than this figure; consequently, avoid measuring wines during AF.

NB: When enumerating yeasts using standard microbiology methods of analysis (growth in agar growth medium , optical density), the results are expressed in CFU/mL (colony forming unit). Conversely, enumeration resulting from the analysis by qPCR is expressed in GU/mL (genetic unit).

6.2. Preparing the internal standard

Grow *Yarrowia* in liquid YPD (yeast peptone dextrose) at 28°C up to an OD_{600} (optical density at 600 nm) of 1 (approximately 48 hrs).

After estimating the $OD_{600\ nm}$ dilute to 1.0×10^6 CFU/mL in isotonic saline solution (1 OD = 1.0×10^7 CFU/mL).

Transfer a 110µL sample of the 1.0×10^6 CFU/mL culture into a 1.7mL microtube and

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add 110µL of 40 % glycerol to obtain a population of 5.0 x 10^5 CFU/mL. Mix and store at -80°C. One tube can be used to process 5 wine samples.

Perform an enumeration simultaneously to check the titer of the suspension

6.3. Preparing the solutions

100mL of Tris-HCl pH8 10 mM: weigh 0.121 g of tris base (eg.Trizma base) and dissolve in 80mL of ultrapure [18 Mo] water. Adjust the pH using HCl. Make up to 100mL. Autoclave.

100mL TE: weigh 0.121 g of tris base and dissolve in 80mL of water. Adjust the pH using HCl. Add 37.2 mg of EDTA. Adjust the pH to 8 (to assist the dissolution of the EDTA) then make up to 100mL. Autoclave.

100mL solution I: prepare 50mL of TE 2x and add 10mL of 1M NaCl, 10mL of SDS 10% (dissolved by heating gently) and 2 g of Triton X100, then make up to volume.

4M ammonium acetate: dissolve 15.4 g of ammonium acetate in 50 mL ultrapure [18 Mo] water qs to 50mL.

100mL phenol:chloroform:IAA (25:24:1): add 48mL of chloroform and 2mL of isoamyl alcohol to 50mL of phenol saturated with TE buffer pH8. Store at 4°C.

RNase A 1µg/µL: dilute a 70U/mg solution of RNase A (e.g. Sigma, R4642-50MG, stored at –20°C) with ultrapure [18 Mo] water. The specified concentration of the RNase stock is indicated on the tube and in the specification sheet for the batch. The diluted solution should be kept at not more than 4°C for up to 3 weeks.

Brett 4µM primers: using 100 µM stock solutions of primers (in the supplier's tubes), mix 4 µM Brett rad3 (GTTCACACAATCCCCTCGATCAAC) and 4 µM Brett rad4 (TGCCAACTGCCGAATGTTCTC) qs to 1mL with ultrapure [18 Mo] water). Store for up to 1 year at –20°C.

YAL 4µM primers: using 100 µM stock solutions of primers (in the supplier's tubes), mix 4 µM YAL-F (ACGCATCTGATCCCTACCAAGG) and 4 µM YAL-R (CATCCTGTCGCTCTTCCAGGTT) qs to 1mL with ultrapure [18 Mo] water). Store for up to 1 year at –20°C.

7. Procedure

Sample to be analyzed: shake the bottle to homogenize its contents.

For a corked bottle: disinfect the neck of the bottle with 70% alcohol and uncork over a naked flame, using a corkscrew disinfected with 70% alcohol.

Transfer a 15-20mL sample of the wine into a 30-mL, sterile, plastic, single-use bottle.

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The steps at which the protocol may be paused are identified by a * (max. interruption time, T°).

7.1. Separating the cells

This step must be duplicated.

The handling procedures must be carried out under a confined microbiological safety cabinet dedicated to this purpose.

- take a 1mL sample of wine and transfer to a 2mL screw-capped microtube
- add 20µL of internal standard, at a concentration of 5.0 x 105 CFU/mL
- centrifuge for 30 sec. at 9,300g
- eliminate the supernatant by gently inverting the microtube
- suspend the pellet in 1mL of Tris-HCl 10 mM pH 8
- centrifuge for 30 sec. at 9,300g and eliminate the supernatant.
- vortex briefly to suspend the pellet in the residual fluid * (3 months, -20°C).

One tube will be used for extracting the DNA and the other will be stored at –20°C until validated results have been obtained.

7.2. Extracting the DNA

From a fresh or frozen pellet. Do not process more than 24 samples at the same time.

- add **PVPP** (1% of final mass/volume) by weighing add **0.3 g** of **200-500µm glass beads**
- **add 200µL of solution I**
- **add 200µL of phenol:chloroform:IAA (**24**:25:1**)
- disrupt the cells with the automatic stirrer (for example a **GenieDisruptor) 4x80 sec.** with cold intervals (-20°C refrigerated unit) lasting for about 80sec between each disruption phase
- add **200µL of TE**
- centrifuge for **5min at 15700*g***.
- **carefully** collect 400µL of the upper aqueous phase in a 1.7mL microtube. **If the**

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two phases mix, repeat the centrifugation step.

- add **1mL of absolute ethanol** and mix the tube by inversion 4-5 times * **(a few hours, room T°)**
- centrifuge for **5 minutes at 15700*g*** and eliminate the supernatant by inverting the microtube
- suspend the pellet in **400µL of TE and 30µL of RNase at a concentration of 1 µg/µL**
- incubate the solution at **37°C for 5 minutes (then readjust to 48°C)**
-
- add **10µL of 4M ammonium acetate + 1mL of absolute ethanol**; mix by inversion
- centrifuge for **5 minutes at 15700*g***
- eliminate the supernatant by inversion; use filterpaper to absorb the final drops
- dry the pellet (leave the open tube in the dry bath at 48°C, for approximately 1 hour)
- add 25µL of TE to the pellet, vortex and leave at 4°C for between 1 and 18 hrs (to assist the solubilisation of the DNA). Mix using the automatic stirrer * **(a few weeks, -20°C)**

7.3. qPCR

For each sample of wine, provide 2 wells with Brett rad3/4 primers and 2 internal standard wells with YAL primers. For each plate, provide a negative control with TE for each pair of primers to be carried out as the final operation. Also perform a positive control on the *Brettanomyces bruxellensis* DNA available at -20°C. To prepare the positive control, add 5µL stock solution (4.5 UG / ml) in a final reaction volume of 25 µL.

PCR amplification programme:

Cycle number	Time (seconds)	Temperature (°C)

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1	**180**	**95**
40	**30**	**95**
	10	**64.6**
The merged curve is established after 90°C by reducing the heat by 0.5°C every 10 seconds		

Num. of Brett wells = Num. of YAL wells = 2 x num. of samples + 2

The table below indicates, as a function of the number of samples, the number of wells and the quantity of each constituent of the mixture.

number of samples	number of wells	water at18 M□ (µL)	iQ SYBR Green Supermix (µL)	Mixture of 4 µM primers (µL)
1	4	26.3	65.6	13.1
2	6	36.8	91.9	18.4
3	8	47.3	118.1	23.6
4	10	57.8	144.4	28.9
5	12	68.3	170.6	34.1
6	14	78.8	196.9	39.4
7	16	89.3	223.1	44.6
8	18	99.8	249.4	49.9
9	20	110.3	275.6	55.1
10	22	120.8	301.9	60.4
11	24	131.3	328.1	65.6
12	26	141.8	354.4	70.9

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13	28	152.3	380.6	76.1
14	30	162.8	406.9	81.4
15	32	173.3	433.1	86.6
16	34	183.8	459.4	91.9
17	36	194.3	485.6	97.1
18	38	204.8	511.9	102.4
19	40	215.3	538.1	107.6
20	42	225.8	564.4	112.9
21	44	236.3	590.6	118.1
22	46	246.8	616.9	123.4
23	48	257.3	643.1	128.6

- remove the Brett 4 µM and the YAL 4 µM primers from the freezer
- remove the SYBR Green (4°C if tube in current use, otherwise –20°C)
- prepare a Brett mixture and a YAL mixture using the quantities shown in the table above as a function of the number of samples.
- apply 20µL of mixture to the bottom of each well
- add 5µL of homogenized DNA solution to the automatic stirrer (or 5µL of water for the negative controls)
- adjust the optical film and load the plate

7.4. Reading the results

- remove the plate and place it directly in the bag for disposal **(do not open it)**
- set the baseline to 100.

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- analyze (in the order indicated below):
    - the negative controls, which should not produce a signal. If a Ct of less than 37 is observed, repeat the process, changing all the solutions,
    - the positive control on Brett: its Ct must be approximately 25, with a melting point of 82.5°C (± 0.5°C),
    - YAL internal standards: if a Ct is obtained, check the melting point of the product (84°C ± 0.5°C). If the product does not conform, the absence of a Brett signal cannot be interpreted,
    - samples: check the Tm of the *Brettanomyces bruxellensis* product (82°C ± 0.5°C). If and only if the Tm is acceptable, check the exponential profile of the amplification. Then record the Ct values and plot them onto the standard curve.

NB: the Ct represents the time needed for the fluorescence of the target sequence to reach a threshold value. Consequently, it is the minimum number of PCR cycles required for the fluorescent signal to emerge from the background noise.

8. Calculations (Results)

Five *Brettanomyces bruxellensis* strains were inoculated at different concentrations, from 3,1 x 10^5 to 3 UFC/mL, on 14 wines (3 white wines, 2 rosé wines, 9 red wines whose phenolic compound content varied widely). The DNA was then extracted in the presence of 1% PVPP.

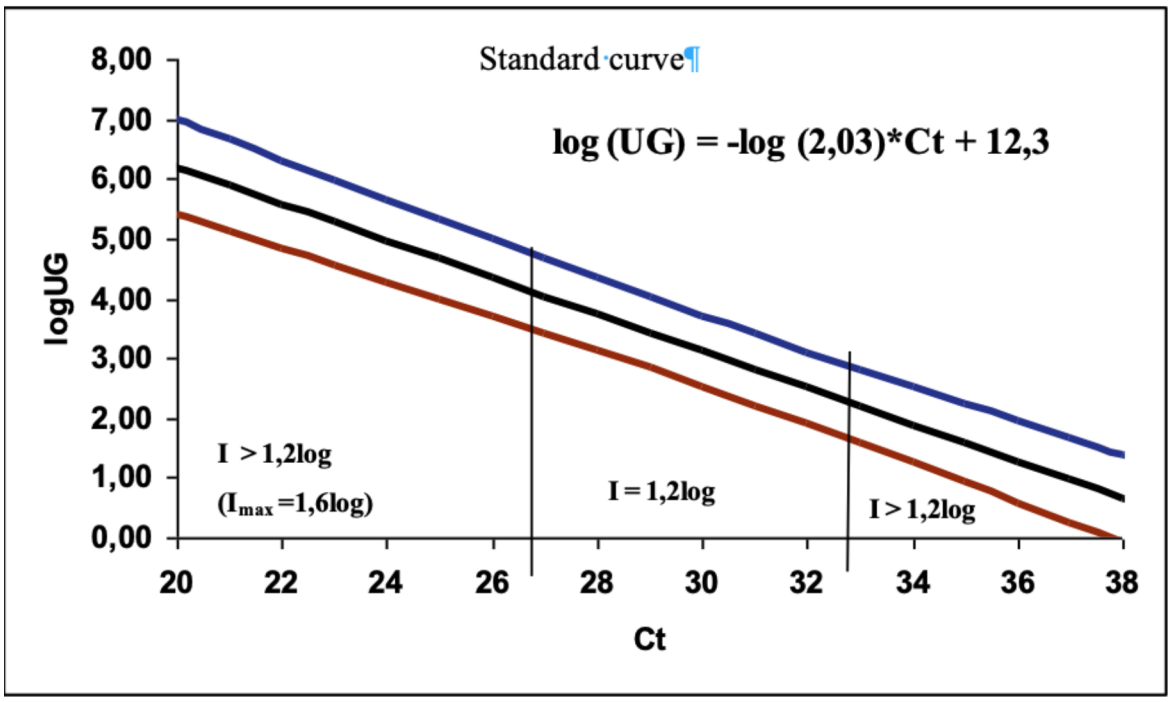
A standard curve was established from the set of results obtained on the different combinations of wines and strains.

The results are obtained in GU/mL (genetic unit/mL) from the standard curve

- $\log GU = -\log(2.03) \times Ct + 12.34$

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9. Method characteristics: intra-laboratory validation parameters

9.1. Linearity, repeatability and reproducibility [4]

The six-point calibration curve was prepared in the range of 0 to $2x10^5$ CFU/mL of the L02I1 strain in a wine with four replicates. This population range was selected according to the usual levels of *Brettanomyces bruxellensis* in wines. The measured log GU vs. theoretical log GU relationship was described by simple regression analysis. Regression parameters, slope and intercept were determined as shown in the Table below. The regression model was accepted with a risk □=1% and the chosen linearity domain validated since no model error was detected.

Fidelity of the method was compared to that obtained with the classical culture method. Three operators prepared DNA extracted from a wine inoculated with the L02I1 strain at two levels: $1.9x10^4$ (high) or $1.9x10^2$ (low) CFU/mL. Four repeats of PCR were performed for each DNA extract. The standard deviation for repeatability and reproducibility, respectively S_r and S_R, were calculated from log GU values for both

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levels (table below). For the qPCR method, both S_r and S_R were similar for the low population level, but S_R was greater than S_r at high population levels. Both standard deviations were twice as high as those obtained with the classical microbiology method. This effect was attributed to the increased number of steps during the qPCR method.

Table

Parameter	Values
Regression equation	
Range (CFU/mL)	0 to $2x10^5$
Slope (±SD)	0.957 (0.044)
Intercept (±SD)	-0.049 (0.142)
Regression model	F_{obs}>F(1.18) : Linear model accepted
Model error	F_{obs}<F(4.18) : No model error
Fidelity	
S_r qPCR (low/high)	0.26/0.25
S_r microbio (low/high)	0.17/0.04
S_R qPCR (low/high)	0.29/0.41
S_R microbio (low/high)	0.17/0.04
Accuracy	
Mean 43 samples (D)	2.39 (qPCR)/2.25 (microbio)
S_R D	1.18

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Equality test $W=D/S_R$ D	0.11<3 accuracy acceptable

9.2. Limit of detection (LoD) and limit of quantification (LoQ) [4]

LoD and LoQ indicate the sensitivity of the method. LoD is the lowest population detected by the method; LoQ is the minimum of the population that can be quantified accurately. In food product analysis, these parameters are calculated from the background. However in qPCR there is no background. We thus used two other approaches to evaluate LoD and LoQ. The first method uses slope, intercept and standard error on intercept obtained from linearity validation experiments. With this method, LoD and LoQ values of 3 and 31 GU/mL respectively were obtained. In the second approach, the LD was obtained from the population level resulting in one negative result from 10 independent measurements. Analysis of our data obtained from 14 wines inoculated with five strains revealed that 96% of samples (48/50) containing 101 to 250 CFU/mL resulted in positive signals, while 83% (49/59) were positive if they contained 26 to 100 CFU/mL and 65% (44/68) if 5 to 25 CFU/mL. Thus the limit of detection evaluated from this method would be in the range of 26-100 CFU/mL. By the systematic repetition of each PCR assay, an LoD of 5 CFU/mL was certified thanks to probability calculations $(1-p)^2$. Indeed for 5 CFU/mL, 88% of samples were positive. This increased to 97% for 25 CFU/mL.

10. References

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129: 237-243.

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