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SHORT COMMUNICATION

# Identification of a second *PAD1* in *Brettanomyces bruxellensis* LAMAP2480

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**Abstract** Volatile phenols are aromatic compounds produced by some yeasts of the genus *Brettanomyces* as defense against the toxicity of hydroxycinnamic acids (*p*-coumaric acid, ferulic acid and caffeic acid). The origin of these compounds in winemaking involves the sequential action of two enzymes: coumarate decarboxylase and vinylphenol reductase. The first one converts hydroxycinnamic acids into hydroxystyrenes, which are then reduced to ethyl derivatives by vinylphenol reductase. Volatile phenols derived from *p*-coumaric acid (4-vinylphenol and 4-ethylphenol) have been described as the major contributors to self-defeating aromas associated with stable, gouache, wet mouse, etc., which generates large economic losses in the wine industry. The gene responsible for the production of 4-vinylphenol from *p*-coumaric acid has been identified as *PAD1*, which encodes a phenylacrylic acid decarboxylase. *PAD1* has been described for many species, among them *Candida albicans, Candida dubliniensis, Debaryomyces hansenii* and *Pichia anomala*. In *Brettanomyces bruxellensis* LAMAP2480, a 666 bp reading frame (*DbPAD*) encodes a coumarate decarboxylase. Recent studies have reported the existence of a new reading frame belonging to *DbPAD* called *DbPAD2* of 531 bp, which could encode a protein with similar enzymatic activity to *PAD1*. The present study confirmed that the transformation of *Saccharomyces cerevisiae* strain BY4722 with reading frame *DbPAD2* under the control of the *B. bruxellensis ACT1* promoter, encodes an enzyme with coumarate decarboxylase activity. This work has provided deeper insight into the origin of aroma defects in wine due to contamination by *Brettanomyces* spp.

**Keywords** *Brettanomyces bruxellensis* · *PAD1* gene · *p*-Coumaric acid · Volatile phenols

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## Introduction

Species belonging to the genus *Brettanomyces* found in varied wine-producing areas have been described as the main contaminants of wine (Chatonnet et al. 1992; Suárez et al. 2007). The presence of these yeasts in wine has been associated with the appearance of phenolic aromas described as "medicinal", "horsy'', "paint'', etc. which decrease the sensorial parameters of this product (Garijo et al. 2014; Di Toro et al. 2015).

Production of these *off-flavors* are the result of hydroxycinnamic acids (HCA) metabolization found in must (Vaquero et al. 2007; Campolongo et al. 2014), as

*p*-coumaric acid, caffeic acid and ferulic acid, which are the main phenolic acids present in plants (Puupponen-Pimiä et al. 2001). Although HCAs contain antimicrobial properties, microorganisms that ferment grape juice are able to metabolize phenolic acids into less toxic products such as vinyl or ethyl derivatives (Suárez et al. 2007). These latter products are responsible for the negative sensorial properties attributed to wines (Cavin et al. 1998; Dias et al. 2003). The wine yeast *Saccharomyces cerevisiae* is able to metabolize *p*-coumaric acid to produce 4-vinylphenol. The *PAD1* gene described in *S. cerevisiae* codes for a phenylacrylic acid decarboxylase protein, which is responsible for the metabolism of the HCA to vinyl derivatives. A similar activity has been identified in other yeast species, including *Candida albicans, Candida dubliniensis, Candida guillermondii,* and *Pichia anomala* (Stratford et al. 2007; Huang et al. 2012). This activity has also been observed in bacteria with the identification of the gene *pdc* in *Lactobacillus plantarum* (Divie 1997) and *padC* in *Bacillus subtilis* (Cavin et al. 1998), coding for a phenolic acid decarboxylase.

Harris et al. (2009) reported a gene sequence coding for an enzyme with possible decarboxylase activity from *Dekkera anomala*. This study provides a partial sequence of a potential coumarate decarboxylase (CD) protein, that is not homologous with other phenylacrylic acid decarboxylases described in yeast and bacteria. On the other hand, Godoy et al. (2014) described the gene coding for a phenylacrylic acid decarboxylase enzyme from *Brettanomyces bruxellensis* (gene *DbPAD*), which has 43 % identity with the *PAD* nucleotide sequence from different ascomycetes, and 34 % identity with similar gene sequences in bacteria.

Bioinformatic analysis of the *DbPAD* gene identified two open reading frames of 666 and 531 bp, called *DbPAD* (Godoy et al. 2014) and *DbPAD2,* respectively. Therefore, this study describes the sequence of *DbPAD2* and show that it codes for an enzyme with phenylacrylic acid decarboxylase activity (Pad).

## Materials and methods

### Strains and growth conditions

*B. bruxellensis* LAMAP2480, *S. cerevisiae* BY4722 and *E. coli* DH5α were obtained from the strain collection of the Applied Microbiology and Biotechnology Laboratory, University of Santiago, Chile. *B. bruxellensis* LAMAP2480 was grown in YPD media (Difco Laboratories, Detroit, USA) at 28 °C for 96 h. Strain *E. coli* DH5α (Promega, USA) was inoculated in LB media (0.05 g yeast extract $l^{-1}$, 0.1 g tryptone $l^{-1}$ and 0.1 g NaCl $l^{-1}$) and grown at 37 °C for 24 h. *S. cerevisiae* BY4722 was grown in SD media (2 % (w/v) glucose and 0.67 % (w/v) YNB without amino acids) supplemented with 0.01 g leucine $l^{-1}$ and 0.02 g uracil $l^{-1}$ at 28 °C for 24 h. The culture media were supplemented with 100 mg *p*-coumaric acid $l^{-1}$ to induce enzymatic activity.

### PCR–SOEing PCR

In order to obtain constant levels of gene expression at all times the *ACT1* promoter (Hernandez-Garcia and Finer 2014) from *B. bruxellensis* was used. Thus the *DbPAD* and *DbPAD2* reading frames, and *ACT1* promoter were amplified from the *B. bruxellensis* LAMAP2480 genome (Wizard®, Genomic DNA purification (Promega, USA)). *ACT1/DbPAD* and *ACT1/DbPAD2* constructs were generated using SOEing PCR (Horton et al. 1989; Thornton 2016), with the primers described in Table 1.

### *ACT1/PAD1* cloning and transformation of *S. cerevisiae*

The constructs *ACT1/DbPAD* and *ACT1/DbPAD2* were ligated in the pGEM-T Easy vector (Promega, WI, USA) and expressed in strain *Escherichia coli* DH5α (Godoy et al. 2014). Both were sequenced at Macrogen®, Seoul, Korea.

The constructs were digested with *Eco*RI and *Sac*I, purified with Zymoclean gel DNA recovery kit (Zymo research) and cloned in the modified yEPACT4 vector (Sánchez-Torres et al. 1998). With the aim of evaluating the promoter sequence association of *B. bruxellensis* in *S. cerevisiae,* a vector named yEPACT4B was created by replacing the *ACT1* promoter of *S. cerevisiae* by the *B. bruxellensis* LAMAP2480 promoter. Finally, the strain *S. cerevisiae* BY4722 was transformed by electrotransformation (Becker and Guarente 1991). The colonies obtained were selected on SD media supplemented with 0.02 g uracil $l^{-1}$.

**Table 1** Oligonucleotides used to amplify the *DbPAD*, *DbPAD2* reading frames and *ACT1* promoter

| Gene | Primer | Sequence (5′–3′) |
|---|---|---|
| *ACT1* | | |
| | *ACT1F* | AAGAATTCGTCGATGTGCATAAATATCA |
| | *ACT1*R | TGATAGCAAGGGAGTTTCATACCAGAACCGTTATCAATAAC |
| *DbPAD* | | |
| | *DbPADF* | AAGAGCTCGTCGATGTGCATAAATATC |
| | *DbPADR* | AACCATGGCTAAAAGGTAATTGCATCAG |
| *DbPAD2* | | |
| | *DbPAD2F* | GTTATTGATAACGGTTCTGGTATGAAACTCCCTTGCTATCA |
| | *DbPAD2R* | AACTTAAGCTAAAAGGTAATTGCATCAG |

### Quantification of 4-vinylphenol production

Culture media samples with an optical density of 0.8 (600 nm) were centrifuged at 5000×*g* for 10 min. A total volume of 20 µl was analyzed by the method described by Ross et al. (2009). For quantification purposes, *p*-coumaric acid and 4-vinylphenol were analyzed by HPLC (Shimadzu Corporation, Japan), coupled to a C18 Shimadzu (150 × 4.6 mm) reverse phase column by a gradient elution mix of water/-formic acid (90:10 % v/v) and methanol (Godoy et al. 2014). The calibration curves were prepared to evaluate *p*-coumaric acid and 4-vinylphenol (Sigma Aldrich, EE.UU.) in the 0.5–100 mg $l^{-1}$ range.

### Statistical analysis

All experiments were performed in triplicates and the results were analyzed using Statgraphics Plus v.5.0 (Manugistic, Inc.). Differences between treatment means were compared using the LSD test at $p < 0.05$.

## Results

### Hybrid construction

The promoter *ACT1* and open reading frames *DbPAD* and *DbPAD2* from the *B. bruxellensis* LAMAP2480 genome were amplified using the primers in Table 1. The *Eco*RI (*DbPAD)* and *Sac*I (*DbPAD2)* restriction sites were left intact. As a result, the 1166 bp *ACT1-DbPAD* and 1031 bp *ACT1-DbPAD2* fragments were constructed using the PCR–SOEing PCR technique. The ORFs described in Fig. 1 were obtained from the sequenced fragments.

### Expression of the *DbPAD* and *DbPAD2* reading frames of *B. bruxellensis* LAMAP2480 in *S. cerevisiae* BY4722

The hybrid products *ACT1-DbPAD* and *ACT1-DbPAD2* were cloned in the vector yEPACT4B, containing the *B. bruxellensis* LAMAP 2480 *ACT1* promoter. The expression of the *B. bruxellensis* promoter and gene in *S. cerevisiae* shows the capacity of this yeast to recognize foreign promoters and reading frames, despite the phylogenetic distance between them (Woolfit et al. 2007).

Transformation of *S. cerevisiae* with yEPACT4B vector not including the hybrid sequence was used as control sample. The concentration of 4-vinylphenol produced was quantified once yeast reached an $OD_{(600\ nm)}$ of 0.8 in SD-URA media supplemented with *p*-coumaric acid. The results in Table 2 show that *S. cerevisiae* BY4722/yEPACT4B/*ACT1/DbPAD* and *S. cerevisiae* BY4722/yEPACT4B/*ACT1/DbPAD2* strains produce statistically similar quantities of 4-vinylphenol, whilst the control strains *S. cerevisiae* BY4722, *S. cerevisiae* BY4722/yEPACT4B and *B. bruxellensis* LAMAP2480 produced less 4-vinylphenol.

Analysis of the final concentration of *p*-coumaric acid and 4-vinylphenol in the media showed that yeast containing the *DbPAD* and *DbPAD2* sequences, metabolized most of the *p*-coumaric acid into 4-vinylphenol. In contrast, some *p*-coumaric acid remained in the media of the control yeasts *S. cerevisiae* BY4722 and *S. cerevisiae* BY4722/yEPACT4B.

As expected, the sum of these products was less than 100 mg $l^{-1}$ in the culture media of *B. bruxellensis* since this microorganism is capable of transforming 4-vinylphenol into 4-ethylphenol (Table 2).

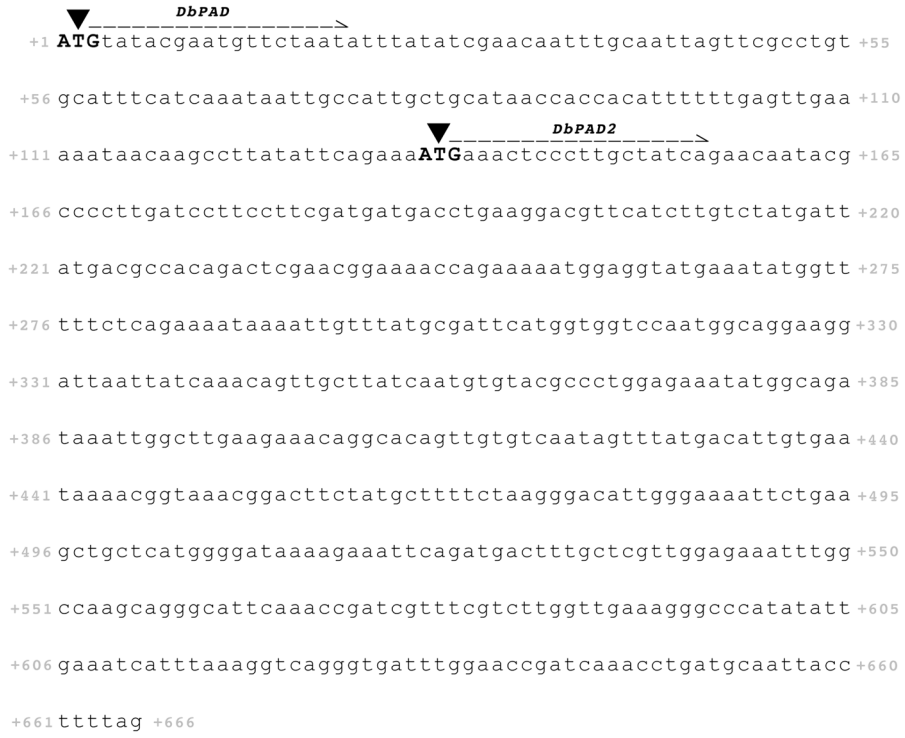


**Fig. 1** Open reading frames *DbPAD* and *DbPAD2*

**Table 2** Quantification of 4-vinylphenol in culture media

| Strain | *p*-Coumaric acid (mg $l^{-1}$) | 4-Vinylphenol (mg $l^{-1}$) |
|---|---|---|
| *S. cerevisiae* BY4722 | 54.8 ± 2.6 | 44.8 ± 2.4 |
| *S. cerevisiae* BY4722/yEPACT4B | 52.9 ± 2.1 | 35.8 ± 4.6 |
| *S. cerevisiae* BY4722/yEPACT4B/*ACT1/DbPAD* | 9.43 ± 1.3 | 106.4 ± 2.3 |
| *S. cerevisiae* BY4722/yEPACT4B/*ACT1/DbPAD2* | 10.6 ± 1.7 | 101.8 ± 3.0 |
| *B. bruxellensis* LAMAP2480 | 18.6 ± 2.3 | 61.6 ± 1.1 |

The experiments were carried out in triplicate

## Discussion

*S. cerevisiae* is recognized for being able to metabolize *p*-coumaric acid to 4-vinylphenol by a decarboxylation reaction using a phenylacrylic acid decarboxylase (Shin et al. 2011). Smit et al. (2003) studied the effect of the *S. cerevisiae PAD1, B. subtilis padC* and *L. plantarum pdc* gene expression on the levels of 4-vinylphenol in *S. cerevisiae* 1278b transformant strains. The results showed there was an increased use of HCA and decarboxylation of *p*-coumaric acid in comparison to the parental strains. Additionally, Mukai et al. (2010) reported that the introduction of the *PAD1* gene in mutants with low *PAD1* activity in *S. cerevisiae* restored the resistance mechanism to HCA, resulting in an increased Pad activity in the modified microorganism.

On the other hand, Godoy et al. (2014) showed that *S. cerevisiae* BY4722 transformed with the *DbPAD* fragment obtained from *B. bruxellensis* produced a greater quantity of 4-vinylphenol than the control strain (not insert included) in laboratory media supplemented with 100 mg $l^{-1}$ of *p*-coumaric acid.

In the present study, *B. bruxellensis* and *S. cerevisiae* transformed with *DbPAD and DbPAD2* were able to produce significant quantities of 4-vinylphenol. The transformed *S. cerevisiae* strains accumulated 4-vinylphenol, while the wild-type *B.*

*bruxellensis* LAMAP2480 strain transforms it into 4-ethylphenol, thereby decreasing the toxicity of 4-vinylphenol and transforming it into a more volatile compound (Dias et al. 2003). Additionally, the concentration of 4-vinylphenol was similar in both *S. cerevisiae* transformants (Table 2).

The results in Table 2 show that the *ACT1/DbPAD2* construct codes for an enzyme with phenylacrylic acid decarboxylase activity, indicating that *DbPAD2* contains the coding sequence for this type of enzymatic activity.

Despite the importance of *B. bruxellensis* in the viticulture industry, its transcriptional regulation mechanisms have not been reported. However, it has been described that mechanisms associated with transcript variants correspond to the activation of alternative promoters or the participation of several terminators during transcription, which allows the production of multiples mRNA from a single coding gene. It also may involve alternative splicing mechanisms, which produce a transcript variant from a single pre-mRNA (Pal et al. 2012). Woolfit et al. (2007) reported the genome sequence of the strain *B. bruxellensis* CBS2499, identifying around 3000 genes, in which it was determined to have 2 % of introns, which is considered low in comparison to *S. cerevisiae* (4 %) or *Schizosaccharomyces pombe* (43 %) (Bon et al. 2003). Likewise, Godoy et al. (2014) did not identify introns in the *DdPAD* gene sequence, suggesting the existence of two ORFs that may allow the activation of alternative starting sites as a mechanism of regulation. It has been described as a mechanism that produce transcripts of different length, which contribute to generate changes in the regulation of gene expression (Dalla Valle et al. 2002).

It has been reported in *Arabidopsis thaliana* that the existence of multiple transcription initiation sites (TSS) within the GSTF8 promoter produces alternate GSTF8 transcriptions, with the potential to generate two proteins that only differ in their N-terminal sequence (Thatcher et al. 2007). The TSS closest to the 3′ region produces GSTF8-S, corresponding to the shortest form of the protein, and the upstream TSS codes for GSTF8-L which is longer. Likewise, it has proteases belonging to the Lon family, a ubiquitous proteolytic machine present in unicellular and multicellular organisms which play a critical role in the elimination of irreversibly damaged proteins. Four genes (*Lon1–4*) have been identified encoding members of the *Arabidopsis Lon* family (Sinvany-Villalobo et al. 2004; Janska et al. 2010; Rigas et al. 2014). Daras et al. (2014) carried out a qualitative expression comparison analysis of *Lon1*. The results strongly suggested the presence of differentially expressed transcripts, *Lon1L* and *Lon1S*, which arise by the conditional selection of TSS. Therefore, the transcription of *Lon1L* or *Lon1S* corresponds to a small fraction of the *Lon1* gene expression, supporting its functional role in specific situations such as growth of the organism under conditions of light and dark. This behavior has also been observed in microorganisms such as *Neurospora crassa,* where the existence of more than one TSS for the *frq* gene, coding for a frequency protein in the circadian clock, forms two transcripts differing in length by 99 amino acids (LFRQ and SFRQ). The initiation sites respond differentially to temperature as well as being regulated by light, resulting in isoforms of FRQ (Colot et al. 2005).

In this context, our results suggest that *B. bruxellensis* generates two alternative *DbPAD* gene transcripts that may be generated by two TSS. Subsequent gene expression and the activity of CD would allow *B. bruxellensis* to detoxify hydroxycinnamic acids and may explain the production of deleterious phenolic aromas in wine.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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