Food Microbiology 63 (2017) 92–100

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

# Cloning the putative gene of vinyl phenol reductase of *Dekkera bruxellensis* in *Saccharomyces cerevisiae*

Diego Romano [1], Federica Valdetara [1], Paolo Zambelli, Silvia Galafassi, Valerio De Vitis, Francesco Molinari, Concetta Compagno, Roberto Foschino, Ileana Vigentini*

*Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Italy*

ARTICLE INFO

*Article history:*
Received 27 April 2016
Received in revised form
3 October 2016
Accepted 1 November 2016
Available online 3 November 2016

*Keywords:*
Wine
*B. bruxellensis*
Volatile phenols
VPR enzyme
Genetic polymorphism

ABSTRACT

Vinylphenol reductase of *Dekkera bruxellensis*, the characteristic enzyme liable for "Brett" sensory modification of wine, has been recently recognized to belong to the short chain dehydrogenases/reductases family. Indeed, a preliminary biochemical characterisation has conferred to the purified protein a dual significance acting as superoxide dismutase and as a NADH-dependent reductase. The present study aimed for providing a certain identification of the enzyme by cloning the VPR gene in *S. cerevisiae*, a species not producing ethyl phenols. Transformed clones of *S. cerevisiae* resulted capable of expressing a biologically active form of the heterologous protein, proving its role in the conversion of 4-vinyl guaiacol to 4-ethyl guaiacol. A VPR specific protein activity of 9 ± 0.6 mU/mg was found in crude extracts of *S. cerevisiae* recombinant strain. This result was confirmed in activity trials carried out with the protein purified from transformant cells of *S. cerevisiae* by a his-tag purification approach; in particular, VPR-enriched fractions showed a specific activity of 1.83 ± 0.03 U/mg at pH 6.0. Furthermore, in agreement with literature, the purified protein behaves like a SOD, with a calculated specific activity of approximatively 3.41 U/mg. The comparative genetic analysis of the partial VPR gene sequences from 17 different *D. bruxellesis* strains suggested that the observed polymorphism (2.3%) and the allelic heterozygosity state of the gene do not justify the well described strain-dependent character in producing volatile phenols of this species. Actually, no correlation exists between genotype membership of the analysed strains and their capability to release off-flavours. This work adds valuable knowledge to the study of *D. bruxellensis* wine spoilage and prepare the ground for interesting future industrial applications.



## 1. Introduction

Biological origin of wine spoilage can be attributed to the activity of yeasts and bacteria. Although technological advances in oenology and the implementation of Good Manufacturing Practices in winemaking have led to a significant reduction in the risk of wine alteration by bacteria, spoilage linked to yeast contamination is still a potential threat. Most common alterations involve the formation of films or sediments, turbidity, gas production and sensory injuries such as the release of volatile phenols. The responsibility of this latter issue is particularly directed toward the development of yeasts belonging to *Dekkera bruxellensis* species (or its anamorph form, *Brettanomyces bruxellensis*). The negative impact of these compounds on wine has been largely revised in literature (Aguilar-Uscanga et al., 2003; Caruso et al., 2002; Chatonnet et al., 1992, 1995, 1997; Dias et al., 2003a; Fugelsang et al., 1993; Fugelsang, 1997; Joseph and Bisson, 2004; Loureiro and Malfeito-Ferreira, 2003; Mansfield et al., 2002; Romano, 2007; Snowdon et al., 2006) Because of cellar treatments involving the use of sulfur dioxide are not working on resistant strains (Barata et al., 2008; Agnolucci et al., 2010; Curtin et al., 2012a,b; Vigentini et al., 2013), preventing actions against the propagation of *Dekkera bruxellensis* in wine have been proposed, such as the use of ozone (Guzzon et al., 2013) and heat treatments of barrels (Fabrizio et al., 2015) or the application to wine of low electric current (Lustrato et al., 2010) and biocontrol agents (Mehlomakulu et al., 2014). However, the damage caused by *B. bruxellensis* in wines is still persisting and a better understanding of volatile phenol production in this species is required. Volatile phenols originate from

* Corresponding author.
*E-mail address:* ileana.vigentini@unimi.it (I. Vigentini).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.fm.2016.11.003
0740-0020/

hydroxycinnamic acids that are originally esterified with tartaric acid as component of grape cells. Because of their toxicity, in several yeast species, hydroxycinnamic acids are rapidly transformed to less harmful compounds for cells through an enzymatic conversion (Baranowski et al., 1980; Stead, 1995; Suárez et al., 2007). In *D. bruxellensis* this conversion follows two steps: a decarboxylation, that transforms the p-cumaric and ferulic acids in 4-vinyl phenol and 4-vinyl guaiacol (Edlin et al., 1998), and a subsequent reduction to 4-ethyl phenol and 4-ethyl guaiacol, respectively (Dias et al., 2003b). The enzyme that operates the reduction is the vinyl-phenol reductase (VPR) (Chatonnet et al., 1992; Harris et al., 2009; Suárez et al., 2007) is found in a smaller number of species (Chatonnet et al., 1995, 1997; Dias et al., 2003a; Edlin et al., 1995; Saez et al., 2011). Although Godoy et al. (2008) and Tchobanov et al. (2008) isolated a potential VPR protein from *D. bruxellensis*, the full lengths sequence of the enzyme has been only recently released (Granato et al., 2014). While the protein sequence showed a high similarity of the amino acid sequence of the superoxide dismutase SOD1p of *S. cerevisiae*, a deeper bioinformatics investigation reveals that the enzyme is a dehydrogenase/reductase protein hosting a Rossmann fold domain (Lesk, 1995) and a structurally conserved C-terminal region (Oppermann et al., 2003; Jçrnvall et al., 1995), regions that are both required to bind NAD(P)H. In this study, following a cloning approach, the VPR gene is isolated by PCR amplification from *D. bruxellensis* CBS4481 genome and expressed in *S. cerevisiae*, a species not producing volatile phenols. The aim of this work is to demonstrate the biological functionality of the purified protein as the one responsible for the off-flavour production in *D. bruxellensis*. Moreover, in order to expand basic knowledge about the strain-dependent character of this yeast in producing off-flavour, an investigation about the polymorphic status of the gene in different *D. bruxellensis* strains is carried out.

## 2. Materials and methods

### 2.1. Identification of the nucleotide sequence of putative VPR gene

The amino acid sequence of VPR enzyme of *D. bruxellensis* CBS4481 (Granato et al., 2014) was used to obtain the corresponding nucleotide (nt) sequence in the available genomes of *D. bruxellensis* CBS2499 and AWRI1499 strains. The putative VPR gene was identified through the BLASTP algorithm by comparison with the translated whole genome sequences listed in databases (www.ncbi.nlm.nih.gov).

### 2.2. Construction of VPR gene-expression vectors for S. cerevisiae

Commercial plasmid YEplac112 (ATCC® 87590™, Manassas, VA, USA) and pYX012 (R&D System, Wiesbaden, Germany) were used to build the expression vectors for cloning VPR in *E. coli* and in *S. cerevisiae* W303. YEplac112 is a multicopy plasmid (2 μm origin) and it contains the TRP1 gene of *S. cerevisiae* as selective marker. pYX012 is an integrative vector and contains an expression cassette for heterologous protein production in *S. cerevisiae* constituted by the strong promoter of the triose phosphate isomerase gene of *S. cerevisiae* (*TPI1*), a multiple cloning site (MCS) and a polyA tail as terminator (Fig. 1). *Hind*III and *Eco*RI restriction enzymes (Thermo Scientific, Hudson, NH, USA) were used to create the appropriate ends in YEplac112 for inserting the expression cassette (*TPI1* promoter-MCS- polyA) extracted by PCR amplification from pYX012 with primers TPIpromF (5′-AAGCTTGGGAATAAGGGCGACACG-3′) and TPItermR (5′-GAATTCCGCCATTCGCCATTCAGG-3′) carrying the sequences of *Hind*III and *Eco*RI at the 5′end, respectively. The reaction mix contained 10 ng of pYX012 DNA, 0.1 μM of each primers, 200 μM dNTPs, 1X reaction buffer $MgCl_2$ free, 2.5 mM $MgCl_2$ and 1 U Taq polymerase (5 Prime, Hilden, Germany). The amplification was performed in a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). After a denaturation step for 5 min at 95 °C, 35 cycles of amplification (consisting of 1 min at 95 °C, 1.5 min at 68 °C and 1 min at 72 °C) were carried out and were followed by a cycle at 72 °C for 10 min. Amplification fragment was resolved in 1% (w/v) agarose gel (Agarose electrophoresis grade, Invitrogen, Carlsbad, CA, USA) at 100 V for 1 h using 1 × TAE (40 mM Tris pH 8.6, 20 mM acetic acid, 1 mM EDTA) as running buffer and detected by Ethidium Bromide staining (0.5 μg/mL). The band was visualised under UV exposition (GelDOC, Bio-Rad) and the PCR product was submitted for sequencing to verify the correct nucleotide sequence (Eurofins genomics, Ebersberg, Germany). The restricted vector (YEplac112 cut *Hind*III/*Eco*RI) and the amplified fragment containing the *TPI1* promoter were ligated in a reaction mix containing 1× ligase buffer in presence of 1–2 U of T4 DNA ligase (Hoffmann La Roche, Basel, Switzerland). The reaction was incubated overnight at 16 °C. A 1:4 M ratio of vector to insert was maintained to obtain the expression vector YEp112T, according to manufacturer's instructions. Then, YEp112T was linearized to verify the expected final length in base pairs (6494 bps).

The sequence encoding for the VPR protein (465 nt) was obtained by PCR amplification from *D. bruxellensis* CBS4481 genome (Fig. 2). Protocol for DNA extraction was the one described by Vigentini et al. (2012). Primers were designed to allow the insertion of VPR gene in *Bam*HI and *Sma*I restriction sites of YEp112T. Forward primer carries the recognition site for *Bam*HI at 5′end (VPRF: 5′-GTCGGATCCATGGTTAAAGCAGTTGCAG-3′) (Fig. 2). As far the reverse primer, two oligonucletides with the sequence of *Sma*I at 5′end were built: VPRR (5′-ATTCCCGGGTTATGCAGACAAGCCAATG-3′) (Fig. 2) and VPRRTAG (5′-ATTCCCGGGTTAATGATGATGATGATGATGTGCAGACAAGCCAA TG-3′). The latter allows the protein purification because of the presence of a polyhistidine-tag, an amino acid motif that consists of at least six histidine residues, at the C-terminus of the protein. The PCR amplifications were performed in a mixture containing 80–100 ng of genomic DNA from *D. bruxellensis* CBS4481, 50 pmol of each oligonucleotide primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2 U Taq DNA polymerase (5 Prime, Hilden, Germany) in a final volume of 50 μL. The temperature profile used the following cycling parameters: 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 63 °C, 1 min at 72 °C, and final extension 10 min at 72 °C. The amplified fragments were sequenced and nucleotide sequences checked to rule out the occurrence of any mutation during synthesis (Eurofins genomics). Amplicons were then *Bam*HI/*Sma*I double digested and cloned into the same sites of the restricted YEp112T expression vector. Two new vectors named YEp112TVPR (6934 bps PlasMapper Version 2.0) (Fig. 1) and YEp112TVPRT (6952 bps) were generated.

### 2.3. Yeasts and bacteria strains, media and protocols for microbial transformation

One Shot® TOP10 Chemically Competent *Escherichia coli* (Cat. No. C4040-10, Invitrogen Life Technologies, Carlsbad, CA, USA) cells were used as host for VPR gene cloning. Cells were routinely grown in LB broth (10 g/L NaCl, 10 g/L peptone, 5 g/L yeast extract, 1 g/L glucose) at 37 °C. For genetic manipulations, *E. coli* competent cells were transformed with $CaCl_2$/RbCl protocol as described by Kushner (1978). Transformed clones were check on LB medium supplemented with 2% (w/v) agar and 100 μg/mL ampicillin. Plasmid extraction was performed by QIAprep Miniprep Kit according to manufacturing's instructions (Qiagen, Hilden, Germany). *S. cerevisiae* W303 strain (MATα ade2-1 his3-11,15 trp1-1 ura3-1 leu2-3112 can1-100) was genetically modified for expression the VPR gene by lithium acetate method (Hill et al., 1991). Two