BRETTANOMYCES BRUXELLENSIS AND OFF-ODOURS: GENETIC AND PROTEOMIC APPROACHES TO UNRAVEL THE MOLECULAR MECHANISM OF ETHYL-PHENOL PRODUCTION•

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1. INTRODUCTION

Wine is the result of the activity of various microorganisms on grape juice. However, microorganisms do not always yield positive effects and some can prejudice the wine quality. Particularly *Brettanomyces bruxellensis* is considered to be the main cause of wine spoilage, especially of premium red wines matured in oak casks, often leading to serious economic losses. The development of the yeast *B. bruxellensis,* is nowadays a serious problem for the winemakers due to the production of off-odours, described as phenolic, animal, mousy, wet wool, medicinal, smoky and spicy.

The principal spoilage compounds associated with *Brettanomyces* spp. are reported to be two volatile phenols, 4-ethylphenol and 4-ethylguaiacol, that are produced from the transformation of the hydroxycinnamic precursors, p-coumaric and ferulic acids, naturally present in grapes and must (fig.1).

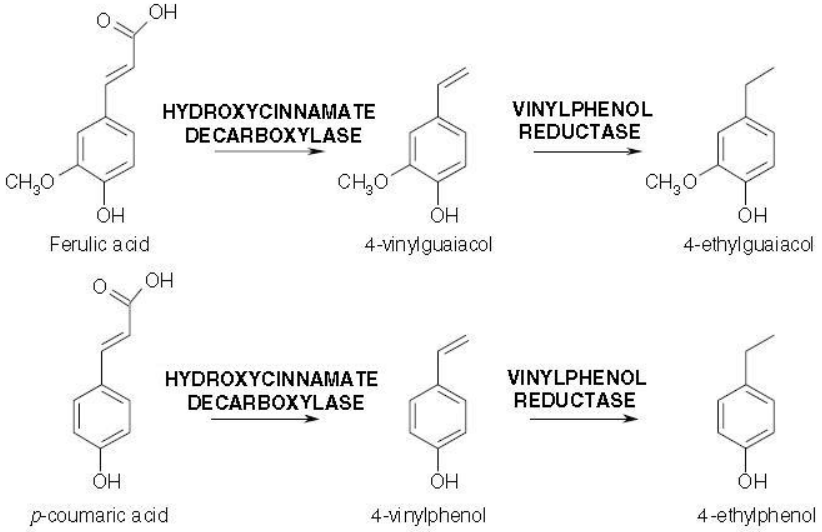

Fig. 1 - Formation of ethylphenols from their hydroxycinnamic precursors.

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The biochemical pathway involves a sequence of two enzymatic reactions. In the first, the enzyme phenolic acid decarboxylase (PAD) decarboxylates the hydroxycinnamic acids into the corresponding vinyl derivative (4-vinylphenol from p-coumaric acid or 4-vinylguaiacol from ferulic acid) and in the second reaction, a vinyl phenol reductase (VPR) converts the vinyl phenols into the corresponding ethyl compound (Edlin *et al*., 1995).

In this work we have applied a molecular biology approach, as well as a proteomic approach, in order to describe the nucleotide and the aminoacid sequences of the enzymes.

2. MATERIALS AND METHODS

2.1. Culture conditions

B. bruxellensis laboratory strain DSM 7001 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was cultivated in YNB media consisting of 6,7 g L^{-1} of Yeast nitrogen base (Sigma Aldrich), 10 g L^{-1} glucose, cloramphenicol (200 mg L^{-1}), cicloheximide (20 mg L^{-1}), p-coumaric acid (100 mg L^{-1}), and vinyl phenol (100 mg L^{-1}) when necessary.

Protein extracts were precipitated with methanol/chloroform according to Wessels, Flugge 1984 and solubilized in rehydration solution (6.5 M urea, 2.2 M thiourea, 4 % w/v CHAPS, 5 mM Tris-HCl, pH 8.8, 0.5 % IPG buffer (GE-Healthcare), 100 mM DTT).

2.2. Two dimensional electrophoresis

Isoelectrofocusing was performed separating the proteins on 18 cm IPG strip (GE Healthcare) with a linear gradient ranging from 3 to 10. After IEF, strips were sealed at the top of the 1.0 mm vertical second dimensional gels as previously described (Pessione *et al*., 2005). For each sample SDS-PAGE was carried out on 13,5 % T and 3,3 % C acrylamide (Biorad Acrylamide) homogeneous gels. Gels were automatically stained using Processor Plus (Amersham Biosciences) with freshly prepared Colloidal Comassie Blue (Neuhoff *et al*., 1988). Four replicates were performed for each condition. Spot intensities were statistically analyzed by means of the *t*-test: means values were considered significantly different when $p<0.05$. 2-DE gels were digitized with Personal Densitometer SI (Amersham Biosciences). Image analysis and detection were performed with Progenesis PG 200 software (Non Linear Dynamics).

2.4. Primer design

For the DNA sequence of the PAD enzyme, two aminoacidic sequences of *Brettanomyces anomalus* found in the literature were used. The forward primer ANPADF (3'-AAGGTCGCTATTATYATCTAC) was designed using the sequence obtained from Edlin *et al*. 1998 while the reverse primer ANPADR (5'- CATCCAGCACCCCAAGCGG) was designed back-translating the sequence found from Harris *et al.,* 2009.

For the DNA sequence of the VPR enzyme the primers were designed using the putative ORF of the protein described by Tchobanov *et al*. 2008 (VPRF1-3'-TTGACCATCCAGTATGATTCAGG, VPRR1 5'- TGACACCTGGAGT CTGCTTG TAAAGC).

2.6. Nucleic acid extraction and PCR

B.bruxellensis laboratory strain DSM 7001 was grown for 48 hours at 30 °C in YPD medium. Nucleic acid extraction and PCR were performed as described by Cocolin *et al*. 2000.

3. RESULTS

From PCR amplification and sequencing we have identified putative DNA sequences for the two enzymes mainly involved in the ethylphenol production (fig. 2).

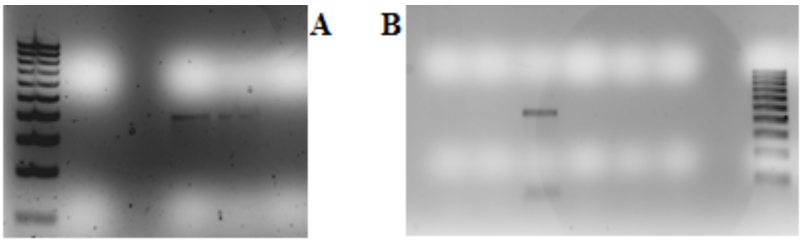

Fig. 2 - PCR amplification results obtained with the specific primers designed as described previously. A) VPR enzyme; B) PAD enzyme.

In order to confirm the correspondence of the two sequences, a proteomic experiment was carried out, where non stimulated (control) cells and cells stimulated with hydroxycinnamic precursors (*p*-coumaric acid and 4-vinyl phenol) were compared. We have found 59 spots corresponding to differentially expressed proteins. These results indicate a significant change in the physiology of the microorganism. It is possible to speculate that the synthesis of proteins involved in transformation of acids is enhanced, but also membrane proteins and proton pumping systems are involved in the hydroxycinnamic acid response. The differentially expressed spots will be identified by MALDI-TOF spectrometry.

From these identifications we expect to:

- obtain the aminoacidic sequence of the two enzymes involved in ethylphenol biosynthesis and compare them with the DNA sequences found with the genomic approach;
- better understand the physiological mechanisms that are induced by the yeast in the presence of hydroxycinnamic acid.

Abstract

Brettanomyces/Dekkera yeasts in wine are able to produce various spoilage compounds that are, at high concentration, detrimental to wine quality. The principal spoiler compounds associated with *Brettanomyces* spp. are vinyl and ethyl-phenols that are responsible for off-odours described as “animal”, “medicinal”, “sweaty leather”, “barnyard”, “spicy” and “clove-like”.

In this paper, exploiting a molecular biology approach, we have identified putative DNA sequences for the two enzymes mainly involved in ethylphenol production.
Furthermore a comparative proteomic study was carried out in order to investigate variations occurring in the proteome of *B. bruxellensis* during growth with and without hydrocinnamic precursors (*p*-coumaric acid and 4-vinyl-phenol). From these results we have found 59 spots, corresponding to differentially expressed proteins. Differences in the protein expression were found especially for low molecular weight spots.

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