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DOMINANCE OF *SACCHAROMYCES CEREVISIAE* IN WINE FERMENTATIONS: SECRETION OF ANTIMICROBIAL PEPTIDES AND MICROBIAL INTERACTIONS

ALBERGARIA Helena (1)*, BRANCO Patrícia (1), FRANCISCO Diana (1), COUTINHO Rute (2), MONTEIRO Margarida (1), MALFEITO-FERREIRA Manuel (2), ARNEBORG Nils (3), ALMEIDA Maria Gabriela (4,5), CALDEIRA Jorge (4,5)

(1) Unit of Bioenergy, LNEG, Estrada do Paço do Lumiar 22, 1649-038 Lisboa, Portugal; (2) Instituto Superior de Agronomia, Technical University of Lisbon, Tapada da Ajuda. 1349-017 Lisbon, Portugal; (3) Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark; (4) REQUIMTE- Dept. Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (UNL), 2829-516 Monte Caparica, Portugal; (5) Instituto Superior de Saúde Egas Moniz, Campus Universitário, Quinta da Granja, 2829-511 Monte Caparica, Portugal.

*Corresponding author: helena.albergaria@lneg.pt

Abstract

In the present work we investigated the antagonistic effect exerted by *S. cerevisiae* against several wine-related microbial species *vis-à-vis* the secretion of antimicrobial peptides (AMPs). AMPs were partially purified from cell-free supernatants and different *S. cerevisiae* strains were screened for its production. The spectrum of action of the AMPs was determined against a broad range of yeasts and bacteria, at enological growth conditions.

Introduction

Saccharomyces cerevisiae plays a primary role in alcoholic fermentation and has a vast worldwide application in the production of wine, beer, bread and fuel-ethanol. Microbial diversity associated with these industrial fermentative processes is huge and

originates mainly from the natural microflora of musts. Although several yeasts and bacteria species participate in these fermentative processes, *S. cerevisiae* is the dominant microorganism.

The ability of *S. cerevisiae* to out-compete other microbial species during alcoholic fermentation has been traditionally ascribed to its higher fermentative power and capacity to withstand the increasingly adverse conditions established in the medium as the fermentation progresses, i.e. high levels of ethanol and organic acids, low pH values, scarce oxygen availability and depletion of certain nutrients (Bisson, 1999; Bauer and Pretorius, 2000; Hansen et al., 2001). However, recent studies demonstrated that other factors, such as microbial interactions mediated by cell-cell contact

mechanisms (Nissen and Arneborg, 2003; Nissen et al., 2003; Arneborg et al., 2005) and killer-like toxins (Comitini et al., 2005; Pérez-Nevado et al., 2006; Osborn et al., 2007; Albergaria et al., 2010), might play an important role.

Material and methods

In this work we used the following *S. cerevisiae* strains: CCM1 885 (Culture Collection of Industrial Microorganisms, LNEG, Portugal); ISA 1000 (Instituto Superior de Agronomia, UTL, Portugal), ISA 1011, ISA 1200, ISA 1028, ISA 1029, ISA 1053, ISA 1063, ISA 1065, CBS 101 and ATCC 6269; Non-*Saccharomyces* strains used were: *Hanseniaspora guilliermondii* NCYC 2380; *Kluyveromyces marxianus* PYCC 2671 (Portuguese Yeast Culture Collection, UNL, Portugal); *Kluyveromyces thermotolerans* PYCC 2908; *Torulaspota delbrueckii* PYCC 4478; *Dekkera. bruxellensis* ISA 1649, ISA 1700, ISA 1791, ISA 2104, ISA 2116, ISA 2211; *Oenococcus oeni* ISA 4279 and DSM 2529. Inoculums of all yeast strains were prepared in 50 ml of YEPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) incubated at 30°C and 150 rpm for 16 h (72 h for *D. bruxellensis* strains). Inoculums of *O. oeni* were prepared by transferring 1 ml of stock culture into 9 ml of MRS broth and cultures incubated at 25°C without agitation for 48 h.

AMPs were partially purified from *Sc* supernatants of alcoholic fermentations performed in synthetic grape juice (SGJ) (as described in Pérez-Nevado et

al., 2006) at 25°C and 80 rpm of agitation for 5-7 days. Cell-free supernatants were first ultrafiltrated through centrifugal filter units (Vivaspin 15R, Sartorius, DE) equipped with 10 kDa membranes and then concentrated (100-fold) with 2 kDa membranes. These peptidic concentrated fractions (2-10 kDa) were then fractionated by gel filtration chromatography, using a Superdex-Peptide column (10/300 GL, GE-Healthcare, UK). 100 µl of each sample were eluted with ammonium acetate (0.1 M) at 0.7 ml/min and all picks obtained were collected, lyophilised and stored until utilisation. All lyophilised fractions were resuspended in appropriate medium and screened for antimicrobial activity.

Spectrum of action of the bioactive fraction was determined against all strains mentioned above by performing antimicrobial tests (AMT) in 96-wells microplates. Lyophilised AMPs were resuspended in 100 µl of YEPD (or MRS for *O. oeni*) medium (with 30 g/l of ethanol and pH 3.5) to a final protein concentration of 1 mg/ml. Control essays were performed for each strain using the respective medium without addition of AMPs. Cultures were inoculated with 10⁵ cells/ml for yeasts and 10⁶ cells/ml for bacteria. Microplates were incubated in a thermo-Shaker (Infors HT, Bottmingen, DE) at 30°C and 700 rpm for yeasts and at 25°C without agitation for *O. oeni*. Growth was followed by absorbance at 590 nm in a microplate reader (Dinex Technologies Inc., USA) and by

colonies forming units (CFU). All AMTs were performed in triplicates.

Results and discussion

In previous work we found that Sc CCMI 885 produces a peptidic fraction (2-10 kDa) during alcoholic fermentation that is active against some wine-related yeast (Albergaria et al., 2010). This peptidic fraction was fractionated by gel filtration chromatography and the picks obtained were tested for antimicrobial activity. Results showed that only one fraction exhibited strong antimicrobial activity and thus this fraction was selected to be further characterised. Here we show

that several other Sc strains exhibiting antagonistic effect against non-*Saccharomyces* strains also secrete these AMPs during alcoholic fermentation.

The spectrum of action of the AMPs present in the bioactive fraction was determined and results demonstrated (Fig. 1) they are active against a wide variety of microorganisms associated with wine fermentations, although the sensitivity of these microbial species towards the AMPs is strain-specific, as shown by the results obtained for different strains of *D. bruxellensis* and *O. oeni* (Figure 1).

Table 1. Death rate of *H. guilliermondii* (Hg) during alcoholic fermentation (AF) performed in co-cultivation with different Sc strains (inoculated with 10^5 cells/ml for each species) and the relative pick intensity (measured by absorbance) of the bioactive fraction detected by gel filtration chromatography in those AF supernatants.

Strain of Sc	AF time (h) of initial Hg death	AF time (h) till total death of Hg	Detection of the bioactive fraction by gel filtration
ISA 1000	48	96	++
ISA 1200	24	168	+
ISA 1063	24	96	++
ISA 1028	24	72	+++
ISA 1029	48	168	+
ISA 1046	24	72	++
ISA 1053	24	72	+++
CBS 101	24	168	+
CCMI 885	24	72	+++

+, ++, +++ is the relative pick intensity (measured by absorbance at 280 nm) of the bioactive fraction in the respective supernatants.

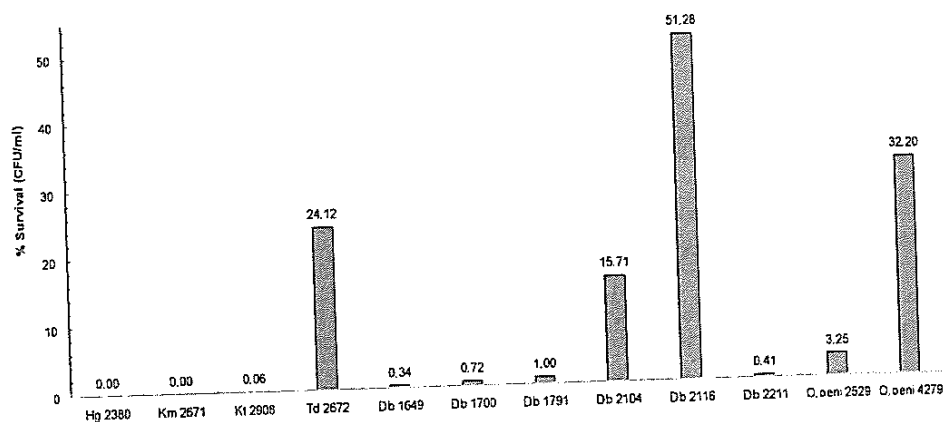


Figure 1: Survival of NS yeasts and *O.oeni* in the AMT performed with YEPD (or MRS) medium (pH 3.5) containing 1 mg/ml of the AMPs. The percentage of viable cells (relative to the initial cell density) in the cultures was determined after 14 h for *H. guilliermondii* (Hg), 48 h for *K. marxianus* (Km), *K. thermotolerans* (Kt) and *T. delbrueckii* (Td), 102 h for all *D. Bruxellensis* (Db) strains and 72 h for *O. oeni* strains.

Conclusions

Our work shows that dominance of the wine habitat by *S. cerevisiae* strains is, at least to some extent, mediated by the production of AMPs.

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