



Biocontrol of *Brettanomyces/Dekkera bruxellensis* in alcoholic fermentations using saccharomycin-overproducing *Saccharomyces cerevisiae* strains

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Abstract

Microbial contamination of alcoholic fermentation processes (e.g. winemaking and fuel-ethanol production) is a serious problem for the industry since it may render the product unacceptable and/or reduce its productivity, leading to large economic losses. *Brettanomyces/Dekkera bruxellensis* is one of the most dangerous microbial contaminant of ethanol industrial fermentations. In the case of wine, this yeast species can produce phenolic compounds that confer off-flavours to the final product. In fuel-ethanol fermentations, *D. bruxellensis* is a persistent contaminant that affects ethanol yields and productivities. We recently found that *Saccharomyces cerevisiae* secretes a biocide, which we named saccharomycin, composed of antimicrobial peptides (AMPs) derived from the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Saccharomycin is active against several wine-related yeast species, namely *D. bruxellensis*. However, the levels of saccharomycin naturally secreted by *S. cerevisiae* during alcoholic fermentation are not sufficient to ensure the complete death of *D. bruxellensis*. Therefore, the aim of the present work was to construct genetically modified *S. cerevisiae* strains to overproduce these GAPDH-derived AMPs. The expression levels of the nucleotides sequences encoding the AMPs were evaluated in the modified *S. cerevisiae* strains by RT-qPCR, confirming the success of the recombinant approach. Furthermore, we confirmed by immunological tests that the modified *S. cerevisiae* strains secreted higher amounts of the AMPs by comparison with the non-modified strain, inducing total death of *D. bruxellensis* during alcoholic fermentations.

Keywords Antimicrobial peptides, glyceraldehyde 3-phosphate dehydrogenase · Microbial contamination · Genetically-modified yeasts · Wine · Preservatives · Fuel-ethanol · Bioethanol

Introduction

Most industrial processes involving alcoholic fermentations (e.g. winemaking, brewing and fuel-ethanol production) are conducted under non-sterile conditions for economic and technical reasons. Consequently, those processes are subjected to chronic microbial contaminations. The main contaminants of wine and fuel-ethanol production processes are lactic acid bacteria and wild yeasts such as *Brettanomyces/Dekkera bruxellensis* (Barros Pita et al. 2011; Fugelsang 1997; Loureiro and Malfeito-Ferreira 2003; Souza Liberal et al. 2007). In wine, *D. bruxellensis* strains produce biogenic amines (Caruso et al. 2002) and phenolic compounds that confer off-flavours to wine described as “barnyard-like” or “horsey” (Fugelsang 1997). In fuel-ethanol fermentations, *D. bruxellensis* strains affect the fermentation efficiency reducing ethanol yields and productivities and leading to

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significant economic losses or even to stuck fermentations (Brexó and Sant’Ana 2018; Souza Liberal et al. 2007).

In wineries, the most common preservative practice is the addition of sulphur dioxide (Ribéreau-Gayon et al. 2006), which is highly toxic to most of the non-*Saccharomyces* yeasts, but not to the majority of *Saccharomyces* strains (Fleet 1992; Romano and Suzzi 1993). However, the use of excessive doses of sulphur dioxide in wine should be avoided since this chemical may have a negative impact on the wine aroma and can cause health problems. In fuel-ethanol fermentations, microbial contaminants are hard to control since disinfectants usually used to combat them (e.g. antibiotics, acid treatments, ammonia, sulphur dioxide, etc.) can either affect the performance of *Saccharomyces* strains or put disposal waste problems (e.g. antibiotics) (Beckner et al. 2011). Besides, given the amounts needed to maintain low contaminating levels, and the potentially corrosive and toxic nature of some of those chemicals (e.g. sulphur dioxide), it is not a viable, long-term solution for bioethanol production facilities (Beckner et al. 2011). Therefore, it is important to find alternatives to the classical chemical preservatives (mainly SO₂ and antibiotics) that might perform a similar role in industrial ethanol fermentations, without its disadvantages (Beckner et al. 2011; Comitini et al. 2004; Mehlomakulu et al. 2014; Santos et al. 2009).

Recently, we found that *S. cerevisiae* secretes antimicrobial peptides (AMPs) during alcoholic fermentations that induce death of several non-*Saccharomyces* wine yeasts, namely of *D. bruxellensis* strains (Albergaria et al. 2010, 2013; Branco et al. 2014, 2017a). This finding opens interesting prospects regarding the possible use of these AMPs as alternative biopreservatives in alcoholic fermentation industrial processes (Albergaria and Arneborg 2016). The natural biocide, which we named saccharomycin, is composed of peptides derived from the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, the GAPDH-derived AMPs levels naturally secreted by *S. cerevisiae* strains during alcoholic fermentations are not sufficient to assure the complete death of some undesirable contaminants, namely of *D. bruxellensis* strains (Branco et al. 2014, 2017a). Besides, the minimal inhibitory concentration of saccharomycin varies considerably amongst wine-related non-*Saccharomyces* yeasts, ranging from 250 µg/ml for *Hanseniaspora guilliermondii*, *Lachancea thermotolerans* and *Kluyveromyces marxianus*, up to 1000–2000 µg/ml for *D. bruxellensis* strains (Branco et al. 2017a).

Lately, AMPs have received increasing attention due to their potential as novel pharmaceutical agents, as well as alternative natural biopreservatives (Hancock and Sahl 2006). As a result, production of large quantities of AMPs, in an economically viable way, is required (Li 2009). AMPs can be reliably prepared by chemical synthesis, but this is extremely expensive. Isolation from natural sources is typically

a complex and time-consuming process that rarely meets the requirements for quantity and cost efficiency. Therefore, none of these processes is an efficient way to obtain AMPs in large amounts (Li 2009; Pyo et al. 2004; Xu et al. 2007). The recombinant approach is relatively low cost and easy to scale up, being a more attractive methodology for large-scale production of AMPs (Li 2009). *Escherichia coli* and yeast are the two major systems used to produce recombinant AMPs. The first yeast employed to produce recombinant proteins was *S. cerevisiae*, since vast genetic techniques are available for this species (Xu et al. 2007).

The main goal of the present work was to construct genetically modified *S. cerevisiae* strains able to overproduce the GAPDH-derived AMPs and to evaluate their biocontrol potential against *D. bruxellensis* during alcoholic fermentations.

Materials and methods

Strains, plasmid and growth conditions

The following yeasts were used in the present work: *S. cerevisiae* CCM1 885 (Culture Collection of Industrial Microorganisms of ex-INETI, Lisbon, Portugal) and *S. cerevisiae* PYCC 5484 (CEN.PK113-5D, *MATa ura3-52 HIS3, LEU2 TRP1 MAL2-8c SUC2*) (Portuguese Yeast Culture Collection, FCT/UNL, Caparica, Portugal); *Pichia pastoris* GS115 (Invitrogen, Karlsruhe, Germany); *D. bruxellensis* ISA 2211 (Instituto Superior de Agronomia, Lisbon, Portugal). All yeasts except *D. bruxellensis* were maintained in YEPD medium (5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose and 20 g/l agar) and stored at 4 °C. *D. bruxellensis* was maintained in YEPD medium with 5 g/l of calcium carbonate (Merck, Darmstadt, Germany) and stored at 4 °C.

Inoculums were prepared by transferring biomass from a YEPD-agar slant (pre-grown at 30 °C for 48 h) into 250-ml flasks with 100 ml of YEPD (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) and incubating flasks at 30 °C and 150 rpm, for 16 h. All media were autoclaved at 120 °C for 20 min.

The centromeric plasmid p416 TEF (Mumberg et al. 1995), containing *TEF* promoter and *CYC1* terminator, was used for cloning. For propagation of this plasmid, *E. coli* DH5α strain was used as host (Hanahan 1983). *E. coli* transformants were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml), at 37 °C. *S. cerevisiae* PYCC 5484 was used as host strain for heterologous expression of the partial sequences *TDH1* (925–963 bp) (codifying region of the *TDH1* gene located between 925 and 963 bp) and *TDH2/3* (925–963 bp) (codifying region of the *TDH2/3* genes located between 925 and 963 bp), previously inserted in p416 TEF. These modified strains are, from now on, called *S. cerevisiae*

pTDH1 and *S. cerevisiae* pTDH2/3, respectively. *S. cerevisiae* PYCC 5484 transformed with empty p416 TEF, from now on called *S. cerevisiae* K1, was used as reference strain and negative control. Transformed yeast strains were grown and maintained in YNB medium without amino acids (DIFCO, Detroit, EUA) with 2% (*w/v*) glucose.

Production and purification of saccharomycin

Saccharomycin (i.e. the naturally secreted GAPDH-derived AMPs) was purified from the cell-free supernatant (7-day-old) of a synthetic grape juice (SGJ) fermentation performed with *S. cerevisiae* CCM1 885. The SGJ, containing 120 g/l of glucose plus 120 g/l of fructose and pH 3.5, was prepared as described in Pérez-Nevado et al. (2006). The fermentation supernatant was ultrafiltrated by centrifugal filter units (Vivaspin 15R, Sartorius, Göttingen, Germany) equipped with 10 kDa cut-off membranes, and the permeate fraction (< 10 kDa) was concentrated (10-fold) using 2 kDa centrifugal filter units. The peptidic fraction (2–10 kDa) was then fractionated by gel filtration chromatography using a Superdex-Peptide column (10/300 GL, GE Healthcare, London, UK) coupled to an HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a UV detector (Merck Hitachi, Darmstadt, Germany). The peptidic supernatant fraction (2–10 kDa) was eluted with 0.1 M ammonium acetate at a flow rate of 0.7 ml/min. The bioactive fraction containing the GAPDH-derived peptides (retention time between 25 and 27 min) was collected and lyophilized.

Sensitivity of yeast species and strains to saccharomycin

To determine the sensitivity of *S. cerevisiae* PYCC 5484, *S. cerevisiae* CCM1 885, *P. pastoris* GS 115 and *D. bruxellensis* 2211 to saccharomycin (i.e. to the naturally secreted GAPDH-derived AMPs), these yeast strains were grown in the presence of different concentrations of the bioactive peptidic fraction (purified as described in the “Production and purification of saccharomycin” section) that contains the GAPDH-derived AMPs (Branco et al. 2014). Growth inhibitory assays were performed in 96-well microplates in triplicate independent assays, with each well containing 100 μ l of YEPD medium without the AMPs (negative control) and with the AMPs at final protein concentrations of 125, 250, 500 and 1000 μ g/ml. Media were inoculated with 10^5 cells/ml of each of the above-mentioned yeast strains, and the microplate was incubated in a Thermo-Shaker (Infors HT, Bottmingen, Switzerland) at 30 °C under strong shaking (700 rpm). Cell growth was followed during 48 h by absorbance measurements (at 590 nm) in a Microplate Reader (Dinex Technologies Inc., Chantilly, USA).

Plasmid DNA manipulations and cloning of the partial nucleotide sequences TDH1 and TDH2/3 in S. cerevisiae strain K1

Plasmid p416 TEF DNA from *E. coli* DH5 α + p416 TEF, previously constructed in our lab, was isolated using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). The nucleotide sequence of *TDH1* (925–963) encoding AMP1 (ISWYDNEYGY SAR) and the nucleotide sequence of *TDH2/3* (925–963) encoding AMP2/3 (VSWYDNEYGYSTR) were used to design primers in order to obtain PCR products containing *TDH1* and *TDH2/3* sequences, resulting from primer dimerization. Forward and reverse primers were designed with the restriction sites for *Xba*I and *Sal*I, respectively (Table 1). PCR amplification of the primer dimer corresponding to the DNA sequence codifying for the AMP1 and AMP2/3 was carried out in an Eppendorf thermocycler with Phusion™ High-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The amplified products were digested with *Xba*I and *Sal*I, purified using the purification kit “GFX PCR DNA and Gel Band Purification” (GE Healthcare, Little Chalfont, UK) and cloned into the corresponding restriction sites of the p416 TEF plasmid, digested by the same restriction enzymes.

Cloning was performed according to standard protocols described in Sambrook et al. (1989). The plasmids were cloned into *E. coli* DH5 α strain, propagated, subjected to extraction and restriction analysis. *E. coli* plasmid isolation was performed by alkaline extraction as described in Birnboim and Doly (1979) and modified as described in Sambrook et al. (1989). The correct insertion of *TDH1* (925–963) and *TDH2/3* (925–963) in the plasmid was checked by restriction digestion. Transformation of *S. cerevisiae* PYCC 5484 strain with the plasmids containing the partial sequence of *TDH1* (925–963) (*S. cerevisiae* pTDH1) and *TDH2/3* (925–963) (*S. cerevisiae* pTDH2/3) was performed by the lithium acetate method described in Geitz and Schiestl (1995). Transformants were selected on YNB medium without uracil. Plasmid isolation from yeasts was performed as described in Tillotson et al. (2013) with some modifications. Briefly, one *S. cerevisiae* colony freshly grown on an YNB plate was transferred into 30 μ l of SDS 1% (*w/v*) in deionised water. Afterward, cells were vortexed for 1 min and then frozen at –80 °C for 2 min and subsequently heated at 95 °C for 2 min; the freeze/thaw was repeated once more to ensure cell lyses. Cells were centrifuged at maximal speed for 1 min, and the supernatant was used as a template for PCR reaction. PCR products were loaded in an agarose gel for electrophoresis, and band was extracted from the gel and then purified using the purification kit “GFX PCR DNA and Gel Band Purification” (GE Healthcare, Little Chalfont, UK). The purified PCR products were sequenced by external services

Table 1 Primers used for amplification of the *TDH1* (925–963 bp) and *TDH2/3* (925–963 bp) nucleotides sequences (which codify the AMP1 and the AMP2/3) that were inserted in the p416 TEF plasmid (*Xba*I and *Sal*I restriction sites are underlined), and primers used for quantification by RT-qPCR of the expression levels of the *TDH1* and *TDH2/3* genes

PCR primers	Amplification of the <i>TDH1</i> (925–963) nucleotides sequence inserted in the plasmid
Forward	5'-TGCTCTAGAGCAATGATTTCCCTGGTACGATAACGAATACGGTTACTCC-3'
Reverse	5'-ATACGCGTCTGACTAATCTGGCGGAGTAACCGTATTCGTTATCGTA-3'
PCR primers	Amplification of the <i>TDH2/3</i> (925–963) nucleotides sequence inserted in the plasmid
Forward	5'-TGCTCTAGAGCAATGTTTCCCTGGTACGACAACGAATACGGTTACTCTA-3'
Reverse	5'-ATACGCGTCTGACTAATCTGGCGGAGTAACCGTATTCGTTATCGTA-3'
RT-qPCR primers	Quantification of the expression levels of the <i>TDH1</i> gene
Forward	5'-CAAGAAGGCTGTAAAGGCTG-3'
Reverse	5'-CGGAGGCATCGAAGATGGAA-3'
RT-qPCR primers	Quantification of the expression levels of the <i>TDH2/3</i> genes
Forward	5'-TCACTGCTCCATCTTCCACC-3'
Reverse	5'-TTTGGGTGGCGGTCATGGA-3'

(STAB VIDA, Monte da Caparica, Portugal) in order to confirm the insertion of the sequences of interest.

RNA extraction and cDNA preparation

The modified *S. cerevisiae* strains pTDH1 and pTDH2/3 were pre-grown for 24 h in YNB without uracil (approximately 10^7 cells/ml). RNA extraction from cells was performed with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Small RNAs from the above-mentioned *S. cerevisiae* strains were separated and purified from total RNAs with *mir*Vana™ miRNA Isolation Kit (Applied Biosystems, Foster, CA, USA) according to the manufacturer's protocol. All RNA samples were treated with RNase-free DNase I (Qiagen, Hilden, Germany) and quantified using absorption of UV light at 260 nm. Small cDNAs from modified *S. cerevisiae* strains were synthesised from small mRNAs using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). RT-primers designed by the manufacturer and specific for each small mRNA sequence were used for small cDNAs synthesis (Custom TaqMan® Small RNA Assays, Applied Biosystems, Foster City, CA, USA). Total cDNAs were synthesised from total mRNA of *S. cerevisiae* pTDH1 and *S. cerevisiae* pTDH2/3 using TaqMan® MicroRNA Reverse Transcription Kit and oligo (dT) primer (STAB VIDA, Monte da Caparica, Portugal). All cDNAs synthesis were performed following manufacturer's instructions (Applied Biosystems, Foster City, CA, USA) and used as templates for real-time PCR reactions.

Quantitative reverse transcription PCR (RT-qPCR)

Small and total cDNAs, obtained as described in the “RNA extraction and cDNA preparation” section, were used as templates for two RT-qPCR reactions: one to

analyse the expression levels of the partial *TDH1* (925–963 bp) and *TDH2/3* (925–963 bp) nucleotides sequences from the small cDNA template (plasmid); and another to analyse the expression levels of the same sequences from the total cDNA template (genomic). To determine the best amount of cDNA to be used as template, five dilutions of the cDNA sample were tested. Each dilution was analysed in triplicate.

The specific primers used to quantify the expression levels of the *TDH1* (925–963) and *TDH2/3* (925–963) nucleotide sequences from the small cDNAs and total cDNAs were obtained from Custom TaqMan® Small RNA Assays (Applied Biosystems, Foster City, CA, USA). RT-qPCR amplifications were obtained by using Luminaris Probe qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The RT-qPCR reactions for small cDNAs (2 min, 50 °C; 95 °C 10 min; 40 cycles: 15 s 95 °C, 30 s 60 °C, 30 s 72 °C) and for total cDNA (95 °C 10 min; 40 cycles: 15 s 95 °C, 30 s 60 °C, 30 s 72 °C) were performed in multiplate PCR 96-well clear plates (BIO-RAD, Hercules, CA, USA) in an iCycler iQ™ Real-Time PCR detection system (BIO-RAD, Hercules, CA, USA). RT-qPCR reactions were performed in triplicate. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimers. Additionally, PCR products were resolved on 2% (w/v) agarose gels, run at 4 V/cm in Tris-acetate-EDTA buffer (TAE), along with a 50-bp DNA-standard ladder (Invitrogen GmbH, Karlsruhe, Germany) to confirm the existence of a single product of the desired length. The comparative Ct method was used to quantify gene expression (Livak and Schmittgen 2001). Gene expression of the nucleotide sequences *TDH1* (925–963) and *TDH2/3* (925–963) inserted in the plasmid was normalised with respect to the expression of the corresponding genomic sequences (used as the reference genes).

Alcoholic fermentations performed with *D. bruxellensis* and *S. cerevisiae* in single- and mixed-culture

Single- and mixed-culture alcoholic fermentations were performed with the *S. cerevisiae* strains K1, pTDH1 and pTDH2/3 and with the *D. bruxellensis* strain ISA 2211. Mixed-culture fermentations were carried out with *D. bruxellensis* at an initial cell density of 5×10^3 and 1×10^5 cells/ml, respectively, together with 1×10^5 cells/ml of each of the *S. cerevisiae* strains. Two single-culture fermentations were performed with *D. bruxellensis* at an initial cell density of 5×10^3 and 1×10^5 cells/ml, respectively. These *D. bruxellensis* single-culture fermentations were used as negative control of the antagonism exerted by each of the above-mentioned *S. cerevisiae* strains. Single-culture fermentations of *S. cerevisiae* strains pTDH1, pTDH2/3 and K1 were also performed to determine the relative expression levels of the partial nucleotide sequences *TDH1* and *TDH2/3* and the overproduction of the GAPDH-derived AMPs in the modified *S. cerevisiae* strains pTDH1 and pTDH2/3.

All fermentations were carried out in 500-ml flasks containing 300 ml of SGJ (supplemented with 120 mg/l of uracil in fermentations performed with *S. cerevisiae* strain K1) and incubated at 25 °C, under gentle agitation (80 rpm). SGJ fermentations were carried out in duplicates, and samples were taken daily to determine cell growth, sugars consumption and ethanol production. Culturability (CFU/ml) of *S. cerevisiae* and *D. bruxellensis* during the fermentations was determined by the classical plating method. To specifically differentiate colonies forming unit (CFU) counts of *D. bruxellensis* and *S. cerevisiae* in mixed-culture samples, we used YEPD agar medium supplemented with 0.01% of cycloheximide, using the procedure described in Branco et al. (2014) to determine the number of CFUs of *H. guilliermondii* in the mixed-culture fermentations.

Cells of the *S. cerevisiae* strains K1, pTDH1 and pTDH2/3 were collected from the single-culture fermentations after 12, 24, 48 and 196 h and analysed by RT-qPCR (as described in the “Quantitative reverse transcription PCR” section) to determine the partial *TDH1* and *TDH2/3* gene expression levels. Supernatants from those fermentation samples were ultrafiltrated by centrifugal filter units (Vivaspin 15R, Sartorius, Göttingen, Germany) equipped with 10 kDa cut-off membranes, and the respective peptidic fractions were analysed by immunological tests, i.e. by indirect Enzyme-Linked Immunosorbent Assay (ELISA).

Quantification of the GAPDH-derived AMPs excreted by the modified *S. cerevisiae* strains by ELISA

The GAPDH-derived AMPs excreted by the modified *S. cerevisiae* strains during alcoholic fermentations were determined by the indirect ELISA, as described in Branco et al.

(2017b). A specific polyclonal rabbit antiserum raised against the synthetic AMPs (anti-AMP1 and anti-AMP2/3) was obtained from GenScript Inc. Company (GenScript HK Limited, Hong Kong).

Briefly, 100 µl of each of the peptidic fractions (obtained as described in the “Alcoholic fermentations of *D. bruxellensis* and *S. cerevisiae* in single- and mixed-culture” section) from fermentation samples (at time 12, 24, 48 and 192 h) were used for coating each well of the 96-well microplate MICROLON® high binding (Greiner Bio-One, Essen, Germany). Then, the 96-well microplate was incubated overnight at 4 °C. Afterwards, 100 µl of 6 M urea was added to samples in order to denature the proteins and improve their detection by indirect ELISA, as previously described by Hnasko et al. (2011). The microplate was thereafter washed 4 times using a PBS-Tween washing solution (0.05% Tween 20 in 0.01 M PBS). The samples were blocked during 2 h at room temperature by adding 200 µl of blocking solution containing bovine serum albumin (BSA 1% w/v) in PBS and washed 4 times with washing solution. Next, 100 µl of the primary polyclonal antibody specific to GAPDH-derived AMPs (GenScript HK Limited, Hong Kong) diluted in 1% BSA to a final concentration of 10 µg/ml was added to each well and incubated for 2 h at 37 °C. The unbound material was removed by washing the microplate 4 times with PBS-Tween solution. Thereafter, 100 µl of a secondary antibody (anti-rabbit IgG-fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, St. Louis, USA) diluted in 1% BSA to 1.0 µg/ml was added to each well, followed by 2 h of incubation at 37 °C. Subsequently, the microplate was washed 4 times with PBS-Tween solution, followed by the addition of 100 µl/well of alkaline phosphatase substrate (100 mM Tris-HCL, 100 mM NaCl, 5 mM MgCl₂, 1 mg/ml *p*-Nitrophenylphosphate (PnPP) to the microplate and incubation for 10 to 30 min at room temperature in the dark. The enzyme-substrate reaction was stopped by adding 100 µl of 3 N NaOH to each well. The optical density (OD) was measured at 405 nm using a microplate reader (Bio-Rad, Hercules, USA).

A standard curve was constructed using 1 mg/ml of synthetic AMP1 (309–321) (GenScript HK Limited, Hong Kong) diluted to 1:32, 1:64, 1:128, 1:256, 1:512 in a phosphate-buffered saline solution (PBS) at pH 7.2. Three replicates of 100 µl were taken from each diluted standard and transferred to a 96-well microplate MICROLON® high binding (Greiner Bio-One, Essen, Germany) and analysed as above-mentioned. The concentration (µg/ml) of AMPs present in the fermentation supernatants of the *S. cerevisiae* strains above-mentioned was calculated according to the linear regression equation. All samples were analysed in triplicate.

Results

Sensitivity of yeasts to saccharomycin and selection of the best strain for cloning the partial genes *TDH1* and *TDH2/3*

In order to select the most adequate host for cloning the genes encoding the GAPDH-derived AMPs, we first tested the sensitivity of *S. cerevisiae* CCM1 885, *S. cerevisiae* PYCC 5484, *P. pastoris* GS115 and *D. bruxellensis* ISA 2211 to saccharomycin. The wine strain *S. cerevisiae* CCM1 885 was chosen since it produces high amounts of the GAPDH-derived AMPs (Branco et al. 2017a). The wine strain *S. cerevisiae* PYCC 5484 was chosen because it contains auxotrophic marks, and thus has been used in our lab for genetic manipulation studies. As for *P. pastoris* GS115, it is a commonly used strain for high expression levels of recombinant proteins. *D. bruxellensis* ISA 2211 sensitivity to saccharomycin was evaluated for comparative purposes.

Sensitivity results (Table 2) revealed that *S. cerevisiae* PYCC 5484 is the most resistant strain to these AMPs, with no growth inhibition observed as compared to the control assay. All the other yeast strains showed growth inhibition by saccharomycin although at different levels (Table 2). Given these results, *S. cerevisiae* PYCC 5484 was selected to be the host strain for heterologous expression of the partial gene sequences *TDH1* (925–963) (codifying for the AMP1) and *TDH2/3* (925–963) (codifying for the AMP2/3).

Overproduction of the GAPDH-derived AMPs by the modified *S. cerevisiae* strains during alcoholic fermentations

In order to check the success of the recombinant approach, single-culture alcoholic fermentations were performed with the *S. cerevisiae* strain K1 (empty plasmid) and the modified *S. cerevisiae* strains pTDH1 and pTDH2/3. The cell viability

Table 2 Sensitivity of some yeasts species and strains to saccharomycin (i.e. the naturally secreted GAPDH-derived AMPs)

Yeast strains	Growth inhibition (%)			
	Saccharomycin concentration ($\mu\text{g/ml}$)			
	1000	500	250	125
<i>S. cerevisiae</i> PYCC 5484	0	0	0	0
<i>S. cerevisiae</i> CCM1 885	43	33	14	12
<i>P. pastoris</i> GS115	32	13	11	9
<i>D. bruxellensis</i> ISA 2211	81	40	0	0

Growth inhibition was calculated as the percentage of growth (measured by absorbance) in the presence of saccharomycin relatively to the control assay (in the absence of saccharomycin)

(CFU/ml) profiles during those fermentations are shown in Fig. 1, showing that the modified strains *S. cerevisiae* pTDH1 and *S. cerevisiae* pTDH2/3 exhibited similar growth rates and profiles than the non-modified *S. cerevisiae* strain K1. The relative expression levels of the partial genes *TDH1* (925–963) and *TDH2/3* (925–963) inserted in the modified *S. cerevisiae* strains pTDH1 and pTDH2/3 were evaluated by RT-qPCR and the respective AMPs production was determined by indirect ELISA.

Evaluation by RT-qPCR of the relative expression levels of the partial genes *TDH1* and *TDH2/3* in the modified *S. cerevisiae* strains

The expression levels of the nucleotide sequences *TDH1* and *TDH2/3* inserted in the plasmid p416 TEF of the modified *S. cerevisiae* strains were normalised with the expression levels of the corresponding genomic sequences (Fig. 2). Both modified *S. cerevisiae* strains (i.e. pTDH1 and pTDH2/3) expressed higher levels of the partial *TDH1* (1.5 fold at

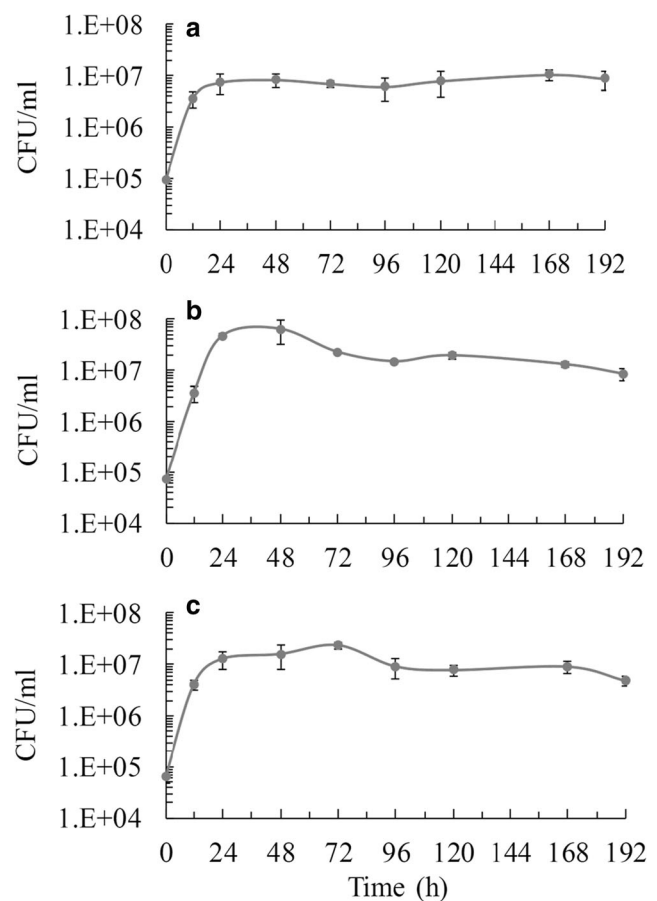


Fig. 1 Cell viability profiles of *S. cerevisiae* (grey circles) during single-culture fermentations performed with the strains K1 (a), pTDH1 (b) and pTDH2/3 (c). Data represented correspond to means \pm SD (error bars) of two independent biological assays

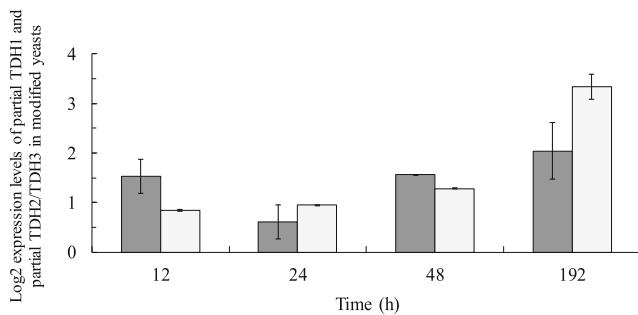


Fig. 2 Relative expression levels of the nucleotide partial sequences *TDH1* (925–963) and *TDH2/3* (925–963) in the modified *S. cerevisiae* strains pTDH1 (dark grey bars) and pTDH2/3 (light grey bars) during single-culture fermentations. The expression levels of the partial sequences *TDH1* and *TDH2/3* in the modified strains was normalised against the respective genomic partial sequences *TDH1* and *TDH2/3*. Values represented are means \pm SD (error bars) of the log₂ expression values of two independent biological experiments analysed in triplicate by RT-qPCR. The cut-off value was set to 1.5

12 h) and partial *TDH2/3* (0.8 fold at 12 h) sequences, relatively to the respective genomic expression levels (Fig. 2). As expected, the amplification cycle threshold (Ct) of both plasmidic partial sequences (i.e. *TDH1* and *TDH2/3*) did not change significantly along the fermentation (data not shown). Conversely, the relative expression of the corresponding genomic sequences *TDH1* and *TDH2/3* varied along the fermentation (data not shown). Consequently, it is not surprising that at 24 h, when the *TDH1* gene starts to be expressed (i.e. at the beginning of the stationary growth phase), the relative expression level of the plasmidic partial sequence *TDH1* in the modified strain pTDH1 was lower than the relative expression level at 12 h (Fig. 2), when the genomic *TDH1* gene is not expressed (Boucherie 1995). As for the partial *TDH2/3* sequence, relative expression levels increased at the end of the fermentation (i.e. at 192 h) (Fig. 2), reflecting the decrease in genomic expression of *TDH2/3*. Our results agree well with those reported by Varela et al. (2005) who found that, under winemaking conditions, *S. cerevisiae* expressed lower levels of *TDH1* during the exponential growth phase than during the stationary growth phase, while the opposite occurred for the *TDH2/3* expression levels.

Determination by indirect ELISA of the AMPs production levels in the modified *S. cerevisiae* strains

The total amount (both genomic and plasmidic) of the GAPDH-derived AMPs excreted by the modified *S. cerevisiae* strains pTDH1 and pTDH2/3 during single-culture alcoholic fermentations was evaluated by indirect ELISA and normalised with the AMPs amount excreted by the non-modified strain *S. cerevisiae* K1 (empty plasmid). Results (Fig. 3) show that there was an increase in the total amount of the GAPDH-derived AMPs excreted by each of the modified *S. cerevisiae* strains relatively to the reference strain

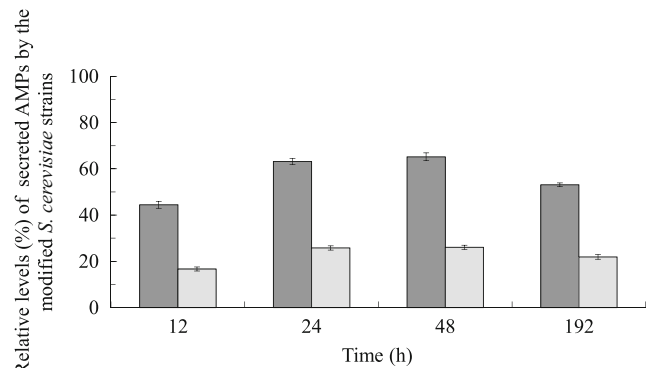


Fig. 3 Relative levels (%) of the GAPDH-derived AMPs secreted by the modified *S. cerevisiae* strains pTDH1 (dark grey bars) and pTDH2/3 (light grey bars) in respect to those secreted by the non-modified *S. cerevisiae* reference strain K1. Values represented are means \pm SD (error bars) of two independent biological assays analysed in triplicate by indirect ELISA

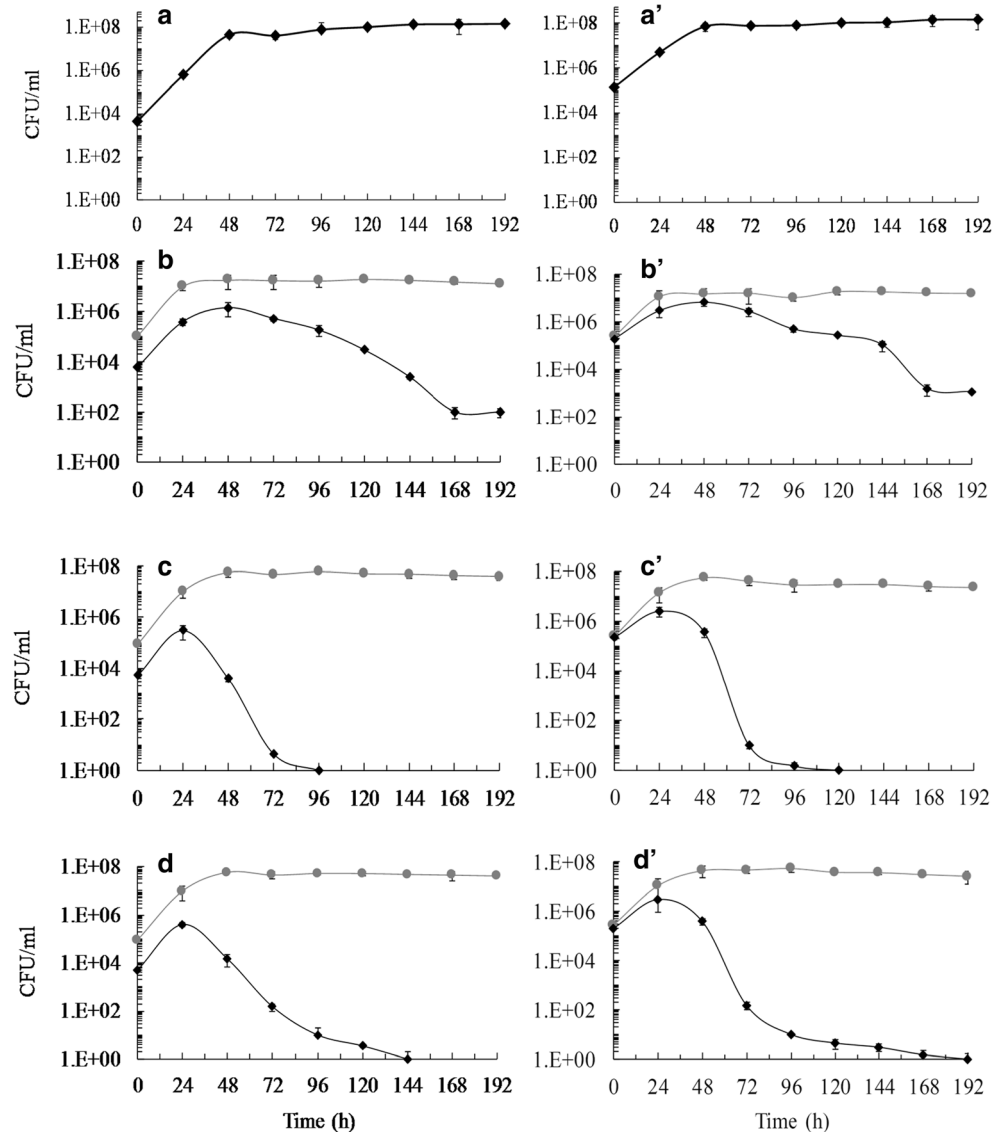
K1. At 12 h, strain pTDH1 produced 44% more AMPs than strain K1, and strain pTDH2/3 17% more (Fig. 3), in agreement with the higher expression levels observed by RT-qPCR. At the beginning of the stationary growth phase (24–48 h), both *S. cerevisiae* modified strains (i.e. pTDH1 and pTDH2/3) reached the maximum overproduction of AMPs. Strain pTDH1 produced 63% more AMPs than strain K1 at 24 h and 65% more at 48 h, while strain pTDH2/3 produced 26% more AMPs than strain K1 at 24–48 h (Fig. 3).

Biocontrol of *D. bruxellensis* in alcoholic fermentations by *S. cerevisiae* strains (non-modified strain K1 and modified strains pTDH1 and pTDH2)

The biocontrol potential of *S. cerevisiae* strains against *D. bruxellensis* was evaluated performing mixed-culture alcoholic fermentations with the non-modified strain K1 (containing the empty vector p416 TEF) (Fig. 4b, b') and the modified strains pTDH1 (Fig. 4c, c') and pTDH2/3 (Fig. 4d, d'), respectively. Alcoholic fermentations were also performed with *D. bruxellensis* in single-culture and used as negative control of the antagonistic effect exerted by the *S. cerevisiae* strains (Fig. 4a, a'). The mixed-culture fermentations were performed with different initial cell density ratios of *S. cerevisiae*/*D. bruxellensis* 20:1 of *S. cerevisiae* (10^5 cells/ml) for *D. bruxellensis* (5×10^3 cells/ml) (Fig. 4b–d) and 1:1 for both yeasts (10^5 cells/ml) (Fig. 4b'–d').

In both single-culture fermentations (Fig. 4a, a'), *D. bruxellensis* reached cell density levels of about 10^8 CFU/ml in the first 48 h, keeping high cell viability levels until the end of the process. Conversely, in all mixed-culture fermentations, *D. bruxellensis* grew during the first day but then began to die-off, independently of its initial cell density (Fig. 4b–d and b'–d'). On the contrary, all *S. cerevisiae* strains (i.e. strains K1, pTDH1 and pTDH2/3) grew up to 10^7 –

Fig. 4 Cell viability profiles of *D. bruxellensis* (black diamonds) and *S. cerevisiae* (grey circles) during alcoholic fermentations performed with *D. bruxellensis* in single-culture (**a, a'**) and in mixed-culture with the *S. cerevisiae* strains K1 (**b, b'**), pTDH1 (**c, c'**) and pTDH2/3 (**d, d'**), respectively. **a–d** show fermentations performed at an initial cell density of 5×10^3 cells/ml for *D. bruxellensis* and 1×10^5 cells/ml for *S. cerevisiae*, while **a'–d'** show fermentations performed at an initial cell density of 1×10^5 cells/ml for both species. Data represented correspond to means \pm SD (error bars) of two independent biological assays



10^8 CFU/ml within the first 24–48 h and maintained this cell viability until the end of fermentation, both in the single-culture (Fig. 1 a–c) and mixed-culture fermentations (Fig. 4b–d and b'–d'). Comparing the *D. bruxellensis* growth profiles in the mixed-culture fermentations performed with *S. cerevisiae* K1 (at both initial cell density ratios of 20:1 and 1:1) (Fig. 4b, b') with those performed with the modified strains pTDH1 (Fig. 4c, c') and pTDH2/3 (Fig. 4d, d'), it is clear that both modified strains exerted a much stronger antagonistic effect against *D. bruxellensis* than strain K1. Indeed, the cell viability of *D. bruxellensis* was entirely lost within the first 96 h in the mixed-culture fermentations performed with strain pTDH1 at both initial cell densities (Fig. 4c, c'), while in the mixed-culture fermentations performed with strain pTDH2/3 at initial cell densities of 1×10^5 CFU/ml (Fig. 4d) and 5×10^3 CFU/ml (Fig. 4d'), *D. bruxellensis* viability was lost within 144 and 192 h, respectively. In contrast,

in the mixed-culture fermentations performed with *S. cerevisiae* K1, *D. bruxellensis* viability was never completely lost during 192 h, even when the initial cell density of *D. bruxellensis* was lower than the initial cell density of *S. cerevisiae*, remaining at approximately 10^2 – 10^3 CFU/ml in both cases (Fig. 4b, b'). These results demonstrate that the modified *S. cerevisiae* strains exerted a significantly stronger antagonist effect than the non-modified strain K1. Besides, comparing the effect of initial cell density ratios (20:1 and 1:1) on the viability of *D. bruxellensis* (Fig. 4b–d compared with b'–d'), one can see that it is negligible vis-à-vis the effect induced by overproduction of AMPs by the modified *S. cerevisiae* strains (Fig. 4b, b' compared with c, c' and d, d'). In the present work, we did not use K1 strains with knock-outs in the *TDH1* and *TDH2/3* genes to confirm if deletion of those genomic genes would prevent the growth inhibition of *D. bruxellensis* induced by the overexpressing strains TDH1

and TDH2/3. However, in a previous work (Branco et al. 2014), we confirmed that mutants of the *S. cerevisiae* strain BY4741 deleted in each of the *THD1–3* genes, significantly reduced the antimicrobial effect against *H. guilliermondii* by comparison with that induced by the non-deleted strain (i.e. BY4741). Thus, we are positive that the *TDH1–3* genes are responsible for the growth inhibition of *D. bruxellensis*.

Discussion

Saccharomycin is a natural biocide composed of GAPDH-derived AMPs, which was isolated from *S. cerevisiae* fermentation supernatants and is active against several wine-related non-*Saccharomyces* yeasts and bacteria (Albergaria et al. 2010; Branco et al. 2014, 2017a). This natural biocide inhibits the growth of different *D. bruxellensis* strains, although at different concentrations (Albergaria et al. 2013). However, the GAPDH-derived AMPs levels naturally secreted by *S. cerevisiae* strains during alcoholic fermentation are not sufficient to induce the total death of *D. bruxellensis*. Indeed, the minimal inhibitory concentration (MIC) of saccharomycin against *D. bruxellensis* strains ranges from 1000 to 2000 µg/ml, which is approximately 10-fold the levels found in *S. cerevisiae* fermentation supernatants (Branco et al. 2017a). Thus, the aim of the present work was to construct genetically modified *S. cerevisiae* strains to overproduce the GAPDH-derived AMPs that compose the natural biocide.

The recombinant approach consisted in cloning the plasmid vector p416 TEF in the auxotrophic *S. cerevisiae* strain PYCC 5484, without insertion (strain K1) and with the insertion of the partial gene sequences *TDH1* (925–963) and *TDH2/3* (925–963) that codify for the AMP1 and AMP2/3, respectively. Results showed that both modified *S. cerevisiae* strains (i.e. pTDH1 and pTDH2/3) expressed higher levels of the partial sequences *TDH1* and *TDH2/3*, comparatively with the respective genomic partial sequences (Fig. 2), which means that the plasmidic partial sequences were correctly expressed. In addition, the modified strains were also able to excrete higher amounts of the respective GAPDH-derived AMPs (i.e. AMP1 and AMP2/3) by comparison with the non-modified strain K1 (Fig. 3). These results not only confirm the success of the recombinant approach but also demonstrate that the GAPDH-derived AMPs expressed by the plasmidic vector are excreted to the extracellular medium. Although we do not know how these peptides are transported outside cells, the presence of secretion-signal less-proteins, such as GAPDH, in the cell wall and extracellular media of yeast is now firmly confirmed (Branco et al. 2017b; Delgado et al. 2001; Nombela et al. 2006).

The modified *S. cerevisiae* strains exerted a significantly higher antagonistic effect on *D. bruxellensis* than the non-

modified strain K1 during the mixed-culture alcoholic fermentations (Fig. 4c, c' and d, d' compared with b, b'). More importantly, the modified *S. cerevisiae* strains induced the total death of *D. bruxellensis* during the mixed-culture fermentations, conversely to strain K1 (Fig. 4b, b'), independently of its initial cell density ratios (Fig. 4c, c' and d, d'). Thus, one can conclude that production of higher amounts of the AMPs was the key factor for total death of *D. bruxellensis*. In addition, strain pTDH1 exhibited a relatively higher antagonistic effect than the strain pTDH2/3 (Fig. 4c, c' compared with d, d'), which agrees with our previous finding showing that the AMP1 exerts a stronger antimicrobial effect than the AMP2/3 (Branco et al. 2017a).

The ability of the modified *S. cerevisiae* strains to fully eliminate big populations ($> 10^6$ cells/ml) of *D. bruxellensis* from fermenting broth (Fig. 4c', d') is an important feature for biocontrol of this contaminant both in fuel-ethanol and wine fermentations. In fuel-ethanol fermentations, *D. bruxellensis* often proliferates up to 10^7 – 10^8 cells/ml (Souza Liberal et al. 2007). In the case of wine, *D. bruxellensis* is only regarded as spoilage agent when its population is big enough to produce 4-ethylphenol at levels higher than 620 µg/l, which is the minimal level perceived as negative in red wines (Loureiro and Malfeito-Ferreira 2003). In recent years, several killer toxins (e.g. Kwkt, Pikt, PMTK2, CpKT1, CpKT2) secreted by different yeasts (e.g. *Kluyveromyces wickerhamii*, *Pichia anomala*, *Pichia membranifaciens* and *Candida pyralidae*) have been suggested as biocontrol agents of *D. bruxellensis* under winemaking conditions (Comitini and Ciani 2011; Comitini et al. 2004; Mehloimakulu et al. 2014, 2017; Santos et al. 2009). However, the efficacy of those killer toxins has been demonstrated mainly for low *D. bruxellensis* contamination levels ($< 10^4$ cells/ml) (Comitini and Ciani 2011; Mehloimakulu et al. 2017).

Conclusively, in the present work, we developed a successful genetic approach to overexpressed GAPDH-derived AMPs codifying genes in *S. cerevisiae* strains that were able to induce the total death of *D. bruxellensis* during alcoholic fermentation. Although we tested only one *D. bruxellensis* strain, previous work from our lab demonstrated that saccharomycin is active against other *D. bruxellensis* strains and also against lactic acid bacteria (LAB) (Albergaria et al. 2013; Branco et al. 2014, 2017a). The contamination of bioethanol fermentations with LAB and wild yeasts is a significant industrial problem causing production losses ranging from 2 to 22% (Beckner et al. 2011). The present work gives experimental evidence about the possibility of obtaining saccharomycin-overproducing *S. cerevisiae* strains that could be directly applied to bioethanol fermentations as starter cultures and, simultaneously, act as biocontrol agents of contaminants. Alternatively, recombinant strains could be used to produce saccharomycin at large-scale, thus obtaining a new biopreservative for use in wine and/or in other food

products. This alternative biopreservative might allow reducing the levels of chemical preservatives commonly used in the wine industry, namely of sulphur dioxide, thus preventing economic losses and satisfying consumers health concerns.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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