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PII: S1049-9644(18)30165-8
DOI: https://doi.org/10.1016/j.biocontrol.2018.03.008
Reference: YBCON 3734

To appear in: *Biological Control*

Received Date: 11 December 2017
Revised Date: 17 March 2018
Accepted Date: 19 March 2018

Please cite this article as: de Ullivarri, M.F., Mendoza, L.M., Raya, R.R., Characterization of the killer toxin KTCf20 from *Wickerhamomyces anomalus*, a potential biocontrol agent against wine spoilage yeasts, *Biological Control* (2018), doi: https://doi.org/10.1016/j.biocontrol.2018.03.008

Characterization of the killer toxin KTCf20 from *Wickerhamomyces anomalus*, a potential biocontrol agent against wine spoilage yeasts

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Abstract

Wickerhamomyces anomalus Cf20 secretes the killer toxin KTCf20 that inhibits several wine spoilage yeasts of the species *Pichia guilliermondii*, *P. membranifaciens*, *Brettanomyces bruxellensis* and *Dekkera anomala*. KTCf20 binds cell wall extracts from the sensitive target *P. guilliermondii* Cd6; however, this capacity was lost when cell wall extracts were pre-treated with fungal β-glucanase. Pustulan and laminarin inhibited killer activity, suggesting that β-1,3 and β-1,6-glucans may be the putative binding sites for KTCf20 on the cell wall of sensitive cells. The toxin was produced and showed to be stable and highly active at physicochemical conditions suitable for winemaking process. In addition, the strain Cf20 is compatible with *Saccharomyces cerevisiae* in co-culture conditions being potential its application in a mixed starter culture. These data suggest that *W. anomalus* Cf20 and/or KTCf20 are promising biocontrol agents against spoilage yeasts during wine-making process.

Keywords: killer toxin; *Wickerhamomyces anomalus*; spoilage yeasts; biocontrol; winemaking

1. Introduction

Some yeast strains can produce killer toxins (KTs), which are antimicrobial proteins or glycoproteins that enable the producing organism to kill competing yeast species. Since their discovery in 1963 in *Saccharomyces cerevisiae* (Bevan and Makower, 1963), KTs have been isolated and studied in a wide number of genera including *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Wickerhamomyces*, *Zygosaccharomyces,* among others (Liu et al., 2013). KTs may be genetically encoded in chromosomal genes (KHR, KHS, PaKT), dsRNA plasmids (K1, K28, Klus) or dsDNA linear plasmids (PaT, zymocin). The modes of action of KTs are highly variable and include the formation of pores in the cytoplasmic membrane; the arrest of the cell cycle of the sensitive yeast in G1 or S phase; rRNA fragmentation and hydrolysis of β-1,3- and/or β-1,6-glucans of the cell wall (Friel et al., 2007; Jijakli and Lepoivre, 1998; Kast et al., 2014; Magliani et al., 1997; Santos et al., 2013). Despite the variety of mechanisms involved, the first step of the killing process of all known KTs is the interaction of the killer toxin with a primary receptor located on the cell wall of the sensitive cells. For example, β-1,3-glucan has emerged as the primary target of some KTs produced by *Wickerhamomyces* and *Williopsis* species (Magliani et al., 2008).

Microbiological contaminations by spoilage moulds and yeasts species such as *Dekkera/Brettanomyces bruxellensis*, *P. membranifaciens* and *P. guilliermondii* are frequent during winemaking (Fugelsang and Edwards, 2007; Garijo et al., 2015). In this context, it is a priority for the wine industry to modulate the growth of these undesirable microorganisms to avoid economic losses. Although killer yeasts have been widely studied as potential biological control agents (Liu et al., 2013; Santos et al., 2009; Villalba et al., 2016), only a few commercial starter cultures with antimicrobial properties are available.

The species *W. anomalus* (formerly *P. anomala*) has the ability to grow under high osmotic pressure, at low pH, and at a broad range of temperatures (Fredlund et al., 2002). Due to its physiological robustness, *W. anomalous* killer strains are commonly found in the most diverse environments, including as resident yeast of wineries. *Wickerhamomyces anomalus* is classified as a biosafety Class-1 organism (Passoth et al., 2006) and many reports suggest their potential as biocontrol agents against plant pathogenic fungi and spoilage yeasts (Druvefors et al., 2005; Friel et al., 2007; Parafati et al., 2016). The aim of this work was to characterize the killer phenotype of *W. anomalus* Cf20, an autochthonous killer strain, previously isolated from wine cellars of the Northwest region of Argentina (Fernández de Ullivarri et al., 2011). The main biochemical properties of KTCf20 and its activity against different spoilage yeasts and *S. cerevisiae* were evaluated in order to validate its application during winemaking.

2. Materials and Methods

2.1 Yeast strains and growth media

The yeast strains used in this study are described in Table 1. *Wickerhamomyces anomalus* Cf20 and all autochthonous yeast strains were isolated from wine cellars from Northwest and Cuyo regions of Argentina. Additional spoilage yeasts were kindly provided by the Yeast Collection of San Juan University (Argentina).

All strains were cultured in YPD (Yeast Peptone Dextrose) broth containing 10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose. If required, the pH was buffered with 0.1 M citric acid/dibasic sodium phosphate. For killer activity assay, YPD-MB (methylene blue) agar (YPD supplemented with 30 mg/l methylene blue and 20 g/l agar) buffered at pH 4.5 was used. All strains were maintained in YPD supplemented with 20 % glycerol at -20 ºC.

2.2 Killer activity

Killer activity (KA) of *W. anomalus* Cf20 was tested against a panel of yeasts by the diffusion plate assay. The plate was seeded with sensitive strain at a final concentration of $1x10^6$ cells/ml (50 µl, OD_{600nm} 2) in YPD-MB agar pH 4.5. An exponential culture of *W. anomalus* Cf20 (30 µl, OD_{600nm} 2) was spotted by triplicated on the agar plates. After 48-72 h of incubation at 20 ºC, strains were designated as sensitive when the spot grew surrounded by a clear zone of inhibition. The assays were repeated twice in triplicate. Also, KA of crude extract was quantified by a diffusion plate method (Fernández de Ullivarri et al., 2014). The diameter of the inhibition zones was measured with a caliber. KA was defined as arbitrary units (aU) per ml and was calculated using the formula: KA (aU/ml) = $10^{(D+5.64)/6.64}$. D is the diameter of the inhibition zone in millimeters and 1 aU is the amount of toxin capable of producing a clear inhibition zone of 1 mm in diameter.

2.3 Production and purification of KTCf20

Wickerhamomyces anomalus Cf20 was inoculated at 1×10^6 cells/ml in YPD broth pH 3.0 and incubated at 25 ºC during 96 h. The culture was centrifuged at 10 000 ×*g* for 10 min and sterilized by filtration with 0.22 µm PVDF filters (Millipore). Glycerol was added to the cell-free supernatant to a final concentration of 10%.

For purification, 1 liter of crude extract was concentrated by ultrafiltration (Amicon YM30, 50 kDa, Millipore) to a final volume of 5 ml. The concentrated fraction was purified by size exclusion chromatography (SEC) through a 10×300 mm Sephadex G-75 column with 0.1 M citric acid/dibasic sodium phosphate pH 3.0 (BCF) at a flow of 0.9 ml/min. Active fractions were pooled and concentrated 10× at 30 ºC for 7 h in a

SpeedVac Savant SPD 121P (Thermo Scientific, Thermo Electron Corporation, Ohio, USA). For experiments, dilutions of the concentrated KT were prepared in BCF pH 3.0. Protein concentration was quantified by the Bradford method.

Purified KTCf20 was subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using silver staining technique (Chevallet et al., 2006). In native electrophoresis, samples were analyzed in a 5% continuous acid polyacrylamide gel soaked in a 0.375 M acetic acid-KOH solution, pH 4.0 (Bollag and Edelstein, 1991).

2.4 Characterization of KTCf20

2.4.1 Sensitivity to proteases

To confirm the protein nature of KTCf20, 50 µl of aqueous solutions of trypsin, chymotrypsin and pepsin (0.1 mg/ml each) were added to 450 µl of KTCf20 (5,000 aU/ml) and incubated at 20 ºC for 4 h. KA was quantified after treatment.

2.4.2 Stability of KTCf20

For pH stability assays, the pH of the KTCf20 dilution was adjusted for 1 h from 1.0 to 7.0 with 1 M HCl or 1 M NaOH. Then, the pH was readjusted to 3.0. To study thermostability, KTCf20 (5,000 aU/ml) was treated for 1 h at -20, 4, 10, 15, 20, 25, 30, 37, 50, 72 and 98 ºC. After treatments, residual KA was measured by the diffusion plate method.

2.4.3 Effect of oenological conditions on KTCF20

At optimal pH and temperature, stability of KTCf20 under conditions present during wine fermentation was evaluated. The effect of different concentrations of ethanol (8, 12, 16%), SO_2 (25, 50, 100 mg/l) and sugars (100, 200, 300 g/l using a ratio 1:1 of glucose:fructose) was studied by treatments during 2 h. Later, residual KA was

measured by the diffusion plate method and cell growth of sensitive strain *P. guillermondii* Cd6. In addition, the activity and stability of KTCf20 was assayed in wine produced by *S. cerevisiae* Cf8.

2.4.4 Binding of KTCf20 to polysaccharides

To study competitive inhibition of KA by cell wall components, 180 µl of KTCf20 (10,000 aU/ml) and 20 µl of different polysaccharides (1% each; >99.5%, Sigma) were mixed as shown in Table S1 and incubated at 20 ºC for 30 min. After incubation, the KA relative to an untreated control was quantified.

2.4.5 Glucanase activity

To investigate the possible mechanism of action of KTCf20, glucanase activity was determined according to the method described by (Bara et al., 2003) using laminarin (0.3%, pH 3.0, Sigma) as substrate. Glucanase from *Trichoderma harzianum* (3 mg/ml, Sigma) was used as a control at dilution that showed the same KA than KTCf20. Samples were incubated at 25 and 37 ºC for KTCf20 and glucanase, respectively, and concentration of released glucose was determined enzymatically (Glicemia kit, Wiener Lab., Argentina, code 1400070).

2.4.6 Location of KTCf20 receptor

2.4.6.1 Cell wall extracts preparation from sensitive yeast strain

Purified cell wall extracts were obtained according to a standardized protocol (Prillinger et al., 1993). A stationary culture of *P. guilliermondii* Cd6 (500 ml) was prepared in YPD pH 5.5 at 30 ºC for 72 h with agitation (100 rpm). It was centrifuged at 8000 ×*g* and washed three times with sterile distilled water until the supernatant had no color. Cells were suspended in 10 ml of sterile distilled water and disrupted in a French press until no whole cells were observed under optic microscope (100×). The cell lysate was centrifuged at 5000 ×g for 5 min and the pellet was washed repeatedly with distilled

water at 0 ºC until the discarded supernatant was clear. In order to remove the cytoplasmic remnants, the pellet was washed repeatedly with 1% sodium deoxycolate (pH 7.8) in constant stirring. Between each wash with sodium deoxycolate, the pellet was washed with distilled water at 0 ºC. The extract was freeze-dried, pulverized and stored at 4 ºC.

2.4.6.2 Interaction of KTCf20 with cell wall extracts

A reaction mix containing 270 µl of purified toxin (10,000 aU/ml) and 30 µl of cell wall suspension (3% w/v) in BCF pH 3.0 was incubated at 20 ºC for 10 min. As control, KTCf20 was incubated with a cell wall suspension previously treated with fungal β-glucanases. Enzymes were inactivated at 100 ºC for 5 min before incubation with KTCf20. After incubation, samples were centrifuged at 13,000 ×*g* for 5 min and the supernatants were separated from the cell wall pellet. To confirm that KTCf20 was bound to the cell wall, we evaluated the toxin desorption as follows: 300 µl of BCF + 1.5 M NaCl were added to the pellet; the suspension was incubated for 5 min at 20 ºC, then centrifuged at 13,000 ×*g* for 5 min and the supernatant was collected. The cell wall pellet was suspended in 300 µl of BCF pH 3.0. The KA of the different fractions was quantified.

2.4.7 *Transmission Electron Microscopy (TEM)*

Mid-exponential-phase cells of *P. guilliermondii* Cd6 were grown in 5 ml of YPD broth and harvested by centrifugation at 10,000 ×*g* for 10 min. Cells were suspended in 1 ml of KTCf20 (10,000 aU/ml) and incubated for 10 h. KTCf20 treated at 100 ºC for 15 min was used as control. Samples were further processed for TEM in CIME (Integral Center of Electron Microscopy, National University of Tucuman, Argentina) using a Zeiss EM109 transmission electron microscope (Oberkochen, Germany).

2.5 Co-culture of W. anomalus Cf20 with S. cerevisiae strains

Pure and mixed cultures of *W. anomalus* Cf20 with *S. cerevisiae* Cf8 or M12 were used as starter cultures for alcoholic fermentation. Flasks containing 100 ml of Malbec red must were aseptically inoculated with the different cultures to obtain an initial cell density of 5 x 10^6 CFU/ml. Mixed fermentation trials were simultaneously inoculated with: I) 10^6 cells/ml of *W. anomalus* Cf20 and 10^6 cells/ml of the *S. cerevisiae* Cf8. II) 10^6 cells/ml of *W. anomalus* Cf20 and 10^6 cells/ml of the *S. cerevisiae* M12.Samples were incubated at 28 °C during 10 days. Enumeration of *W. anomalus* was carried out in YNB added with glycerol (2%) since *S. cerevisiae* is not capable to grow in this medium, whereas yeast total count was performed in YPD medium. Moreover, ethyl acetate concentration in pure and mixed cultures was evaluated by gas chromatography following the protocol described by Mendoza et al. (2011).

2.6 Statistical analyses

One-way analysis of variance (ANOVA) was applied to the experimental data and the Tukey's test was performed for multiple comparisons with the software Infostat. Differences were considered statistically significant for p-value < 0.05.

3. Results and discussion

3.1 Inhibition spectrum of KTCf20

Wickerhamomyces anomalus Cf20 was able to inhibit 27 out of 35 strains assayed (Table 1). Species belonging to the genus *Pichia* were the most sensitive to *W. anomalus* Cf20. Strains of the genera *Cryptococcus*, *Filobasidium*, *Hanseniaspora*, *Metschnikowia* and *Torulaspora* were also sensitive, while *S. cerevisiae* strains were only slightly sensitive or resistant to the presence of *W. anomalus* Cf20. These results

are in agreement with the wide inhibitory spectrum of toxins produced by different strains of *W. anomalus* described in the literature (Friel et al., 2007; Parafati et al., 2016). In contrast to other killer yeasts, this species shows activity against both fungi and bacteria (Druvefors et al., 2005; Guo et al., 2013; Muccilli et al., 2013). In addition, the inhibitory activity of KTCf20 against six spoilage yeasts as well as two *S. cerevisiae* strains (potential starter cultures) isolated from Argentinian wines and wineries was quantified. *B. bruxellensis* Ld1, *D. anomala* BDa15, *P. guilliermondii* Cd6 and *P. membranifaciens* BPm481 showed high sensitivity to the KTCf20 (3,022; 8,552; 24,204 and 34,237 aU/ml, respectively), while strains Ld2 and BZb317 of *Z. bailli* and *Sch. pombe* BSp399 were only slightly inhibit. *S. cerevisiae* Cf8 and M12 were slightly sensitive to Cf20 crude extract (369 and 738 aU/ml, respectively) as compared to the sensitive spoilage yeasts described previously.

3.2 Characterization of KTCf20

3.2.1 Purification

KTCf20 was concentrated by ultrafiltration (cut-off 50 kDa) and purified by SEC. After purification, the specific KA increased 237-fold and the yield was 20% (Table 2). The purified KT was used for further characterization.

3.2.2 Evaluation of biochemical properties of KTCf20

KTCf20 was sensitive to the activity of the three proteases tested, confirming its proteinaceous state (data not shown). The highest concentration of KTCf20 in cultures was reached at stationary phase (96 h), as is shown in Table 3. Regarding thermostability, the toxin was stable until 35 °C and it lost 90% of its KA after 1 h at 37 ºC. Its thermosensitivity is similar to others toxins such as Kh-II (35 ºC, 30 min) and K5

(35 ºC, 55 min) (Izgü and Altinbay, 2004; Kagiyama et al., 1988). The pH stability was also similar to other KTs produced by *W. anomalus* (Kagiyama et al., 1988; Muccilli et al., 2013) which showed activity and stability at acidic pH (2-4.5).

Incubation of KTCf20 with different concentrations of sugars, ethanol and SO_2 did not affect its KA (Table 4). In addition, this toxin was stable in wine produced by *S. cerevisiae* Cf8 and the high levels of activity persisted after 72 h.

Furthermore, the binding capacity of KTCf20 to polysaccharides with different structural characteristics was studied. Activity of KTCf20 decreased 90 and 100% after the incubation in presence of laminarin (β-1,3-glucan) and pustulan (β-1,6-glucan), respectively, whereas the presence of polysaccharides with other structures (e.g. mannan or quitin) did not affect KA (Fig. 1). These results suggest that KTCf20 binds to β-1,3 and β-1,6-glucans, components in the cell walls of yeast species. The binding of KT produced by *W. anomalus* to these types of polysaccharides has been widely reported (Izgü et al., 2005; Magliani et al., 1997;).

In order to confirm that a possible mechanism of action of KTCf20 is glucanase activity, this toxin was co-incubated with laminarin (β-1,3-glucan) and the glucose concentration was determined. After incubation, 15% (0.46 mg/ml) of the laminarin was hydrolyzed. According to Jijakli and Lepoivre, (1998) who defined 1 unit (1 U) of β-1,3-glucanase as the amount of enzyme releasing 1 µg of glucose per min per ml of enzyme solution, 1 aU of KTCf20 is equivalent to 3.5×10^{-7} U of β-1,3-glucanase.

3.2.3 Location of KTCf20 receptor

In studies of the interaction between KTCf20 and the cell wall fraction of the sensitive strain *P. guilliermondii* Cd6, only 5% of the toxin remained in the supernatant after incubation during 30 min at 20 ºC (Fig. 2). A subsequent washing with BCF (pH 3.0)

was not able to separate the toxin from the cell wall of *P. guilliermondii* Cd6. After increasing the ionic strength of the media using 1.5 M NaCl, 35% of the initial amount of toxin was desorbed. Remnant KA of the cell wall suspension was quantified after treatments. Only 14% (907 aU/ml) of the non-recovered toxin (6421 aU/ml) could be detected. KTCf20 was unable to bind to cell wall pre-treated with fungal β-glucanases. The results shown above point out that KTCf20 is a glucanase that interacts with a receptor located in the cell wall of the sensitive strain *P. guilliermondii* Cd6. In agreement with our results, several KTs (PaKT, K5 and PKT) produced by *W. anomalus* are proteins with glucanase activity that degrade β-1,3- and/or β-1,6-glucans of the cell wall as primary target in sensitive cells (Izgü and Altinbay, 2004; Jijakli and Lepoivre, 1998; Polonelli et al., 2011).

3.2.4. Transmission electron microscopy (TEM)

The effect of the activity of KTCf20 on sensitive cells of *P. guilliermondii* Cd6 was studied by TEM. As compared to the control treated with heat-inactivated KTCf20, cells treated with active KT showed decreased cell wall thickness in many zones, a collapse cytoplasmic membrane, nuclear fragmentation and apparition of electrodense regions and apoptotic bodies (Fig. 3). Also, the plate counts confirmed the loss of viability (decrease of 2.5 log CFU/ml) after treatment with active KT. Cytotoxic effects similar to those observed in cells of *P. guilliermondii* Cd6 have been previously described for *C. albicans* cells treated with killer peptides derived from PaKT (Magliani et al., 2008; Polonelli et al., 2011). Several authors have reported that sensitive cells treated with low doses of KT, unlike when they are treated with high doses, presented typical markers of apoptotic processes (Breinig et al., 2006; Reiter, 2005; Santos et al., 2013). Possibly, the effect of KTCf20 at the low concentration studied (10,000 aU/ml, 3.5×10^{-3} U of β-1,3-

glucanase) on the sensitive cells may be due to an activation of apoptotic mechanisms initiated as a response to cell wall degradation.

3.3 Microfermentations of W. anomalus Cf20 in co-culture with S. cerevisiae strains

In order to evaluate the potential application of the killer strain Cf20 as a starter culture in a mixed formulation with *S. cerevisiae*, microfermentations were performed in Malbec must. Pure cultures of *W. anomalus* Cf20, *S. cerevisiae* Cf8 and M12 showed cell populations of about $5x10^8$ CFU/ml at day 1 of fermentation and they stayed at similar levels (10^8 CFU/ml) during 10 days (Fig. 4A). In fermentations conducted by mixed cultures of *W. anomalus* Cf20-*S. cerevisiae* Cf8 (Fig. 4B) or *W. anomalus* Cf20-*S. cerevisiae* M12 (Fig. 4C), it was observed that *Saccharomyces* strains were not inhibited by presence of killer strain. However, in both mixed fermentations *W. anomalus* showed lower levels of cell population than in pure culture, with a marked decrease of cell counts after 4 days (Fig. 4B and 4C). These results are in agreement with other studies of non-*Saccharomyces* yeasts and *S. cerevisiae* in mixed culture which reported that different non-*Saccharomyces* species only grow during early stages of fermentation (Domizio et al., 2011; Padilla et al., 2017). Additionally, *W. anomalus* is not tolerant to high concentrations of ethanol (Passoth et al., 2006).

Moreover, *W. anomalus* is a strong producer of ethyl acetate in pure culture (Suarez-Lepe and Morata, 2012). For this reason the concentration of this volatile compound was determined at the end of fermentation. Figure 5 shows that the strain Cf20 produced high levels of ethyl acetate (434.34 mg/l) in pure culture. However, in mixed cultures with *S. cerevisiae*, the production of ethyl acetate was moderate. Thus, *W. anomalus* Cf20 is a candidate to formulate a mixed culture with *S. cerevisiae* to conduct alcoholic fermentation and control wine spoilage yeasts.

4. Conclusion

In conclusion, KTCf20 of *W. anomalus* is a potential biocontrol agent that is active against a broad spectrum of wine yeasts, mainly spoilage yeast strains. Also, strain *W. anomalus* Cf20 and its killer toxin are compatible with autochthonous starter cultures of *S. cerevisiae*. KTCf20 is a glucanase that interacts with β-1,3 and β-1,6 glucans of the cell wall of the sensitive strain. The optimal activity, stability and production conditions of KTCf20 suggest that *W. anomalus* Cf20 could be used to control undesirable yeasts and to accomplish controlled fermentations during wine production.

Acknowledgments

This work was supported by grants of Agencia Nacional de Promoción Científica y Tecnológica (PICT 2010-0847 and PICT 2013-1930) and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 0320 and PIP 272), Argentina.

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Figure captions

Figure 1. Relative killer activity (rKA) of KTCf20 pre-treated with different polysaccharides.. Values represent the mean of two independent experiments performed in triplicate. Values of each column marked by different letters are significantly different ($p < 0.05$) according to Tukey's test. Linear vertical bars represent standard deviation.

Figure 2. KTCf20 adsorption and desorption from cell wall of *Pichia guilliermondii* Cd6. TC, untreated toxin control; AC, adsorption control; BCF, cell wall with KTCf20 incubated with BCF; BCF+NaCl, cell wall with KTCf20 incubated with BCF+NaCl 1.5 M; CW, remnant cell wall suspension; CW+ZG, cell wall pretreated with fungal β-glucanases and incubated with KTCf20. Values represent the mean of two independent experiments performed in triplicate. Values of each column marked by different letters are significantly different ($p < 0.05$) according to Tukey's test. Linear vertical bars represent standard deviation.

Figure 3. Transmission electron microscopy (TEM) showing the cytotoxic effects of KTCf20 on *Pichia guilliermondii* Cd6. Yeast cells were treated with KTCf20 (**A**) and heat-inactivated KTCf20 (**B**).

Figure 4. Cell counts during microfermentations of *W. anomalus* Cf20, *S. cerevisiae* Cf8 and *S. cerevisiae* M12 in pure (A) and mixed cultures of Cf20 with Cf8 (B) and Cf20 with M12 (C). Values represent the mean of two independent experiments performed in triplicate. Linear vertical bars represent standard deviation.

Figure 5. Ethyl acetate production by *W. anomalus* Cf20 in pure and mixed cultures with *Saccharomyces* strains during microfermentations. Values represent the mean of two independent experiments performed in triplicate. Values of each column marked by

different letters are significantly different ($p < 0.05$) according to Tukey's test. Linear vertical bars represent standard deviation.

Table 1. Killer spectrum of *Wickerhamomyces anomalus* Cf20.

Indicator strain	WaCf20 culture	WaCf20 crude extract (aU/ml)	Characteristics
B. bruxellensis Ld1	1+*	3,022	K-/spoilage
Cryp. albidus Cf16	1+	ND	K-/autochthonous
Cryp. laurentii. Cf12	1+	ND	K-/autochthonous
D. anomala BDa15	2+	8,552	K-/spoilage
F. capsuligenum Cb3	-	ND	K-/autochthonous
F. capsuligenum Cb7	2+	ND	K-/autochthonous
F. capsuligenum Cb11	3+	ND	K-/autochthonous
H. uvarum Cd8	3+	ND	K-/autochthonous
H. uvarum Cd14	1+	ND	K-/autochthonous
H. uvarum Cd17	1+	ND	K-/autochthonous
H. uvarum Cd18	1+	ND	K-/autochthonous
H. uvarum Cd21	1+	ND	K-/autochthonous
Metschnikowia sp. Cf2	2+	ND	K-/autochthonous
P. guillermondii Cd4	4+	ND	K-/spoilage/autochthonous
P. guilliermondii Cd6	5+	24,204	K-/spoilage/ autochthonous
P. kudriavzevii Cd23	2+	ND	K-/autochthonous
P. membranifaciens BPm481	7+	34,237	K-/spoilage
P. mexicana Cf7	3+	ND	K-/autochthonous
S. cerevisiae Cf8	-	369	K+/starter/autochthonous
S. cerevisiae M12	-	738	K+/starter/autochthonous
S. cerevisiae Cf10	-	ND	K-/autochthonous
S. cerevisiae Cf13	1+	ND	K+/autochthonous
S. cerevisiae Cf15	1+	ND	K-/autochthonous
S. cerevisiae Cf17	1+	ND	K-/autochthonous
S. cerevisiae Cf18	1+	ND	K-/autochthonous
S. cerevisiae M7	-	ND	K-/autochthonous
S. cerevisiae M11	1+	ND	K-/autochthonous
Sch. pombe BSp399	-	-	K-/spoilage
T. delbrueckii M1	1+	ND	K-/autochthonous
T. delbrueckii M2	3+	ND	K-/autochthonous
T. delbrueckii M4	1+	ND	K-/autochthonous
T. delbrueckii M9	1+	ND	K-/autochthonous
T. delbrueckii M10	1+	ND	K-/autochthonous
Z. bailli Ld2	-	-	K-/spoilage
Z. bailli BZb317	-	-	K-/spoilage

*Number next to "+" represents inhibition zone in mm. "-" no inhibition ND: not determined

Table 2. Purification of KTCf20

	Total Volume (ml)	Total Protein (mg)	Total Activity (10^6aU)	Specific Activity (10^4aU/mg)	Purification (fold)	Yield (%)
Crude extract	1,000	1,550	20.1	1.3	1	100
UF[a]	5	355	15.2	43.0	3.3	76
SEC (G-75)[b]	3	1.3	4.0	307	237	20

[a]Ultrafiltration (50 kDa MWCO)
[b]Size exclusion chromatography

Table 3. Physicochemical features of KTCf20 production, activity and stability.

Production						Activity				Stability	
Time (h)		pH		T (°C)		pH		T (°C)		pH	T (°C)
R	**O**	**R**	**O**	**R**	**O**	**R**	**O**	**R**	**O**	R	R
24-96	96	2.0-4.5	3.0	10-30	25	2.0-5.0	3.0	10-25	20	2.0-4.5	-20-35

R: range

O: optimal

Table 4. Residual activity of KTCf20 under winemaking conditions.

Oenological parameters	Conditions	Residual activity*
Ethanol	8%	100%
	12%	98%
	16%	97%
SO_2	25 mg/l	100%
	50 mg/l	100%
	100 mg/l	99%
Sugars	100 g/l	100%
	200 g/l	99%
	300 g/l	98%
Wine	2h	98%
	24h	95%
	72h	90%

*Values of residual activity represent the mean of two independent experiments performed in triplicate.

Fig. 1

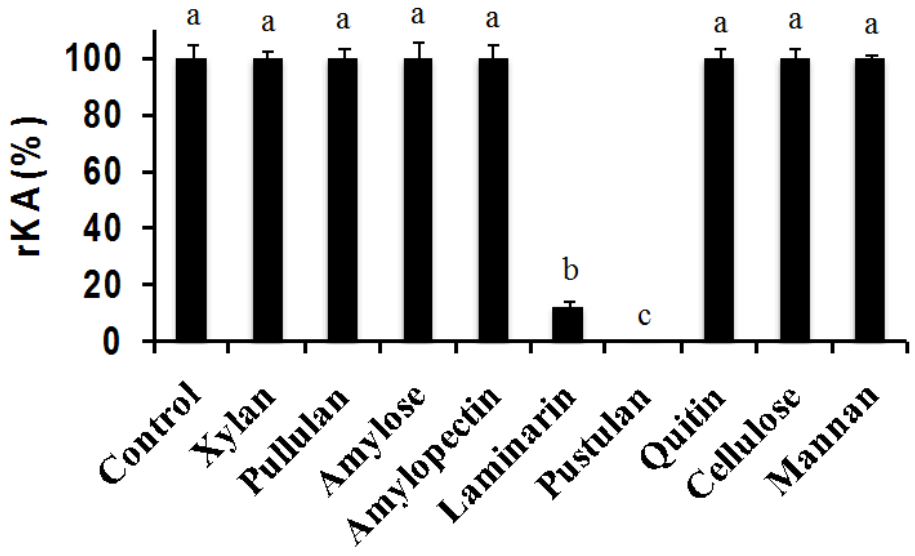


Fig. 2

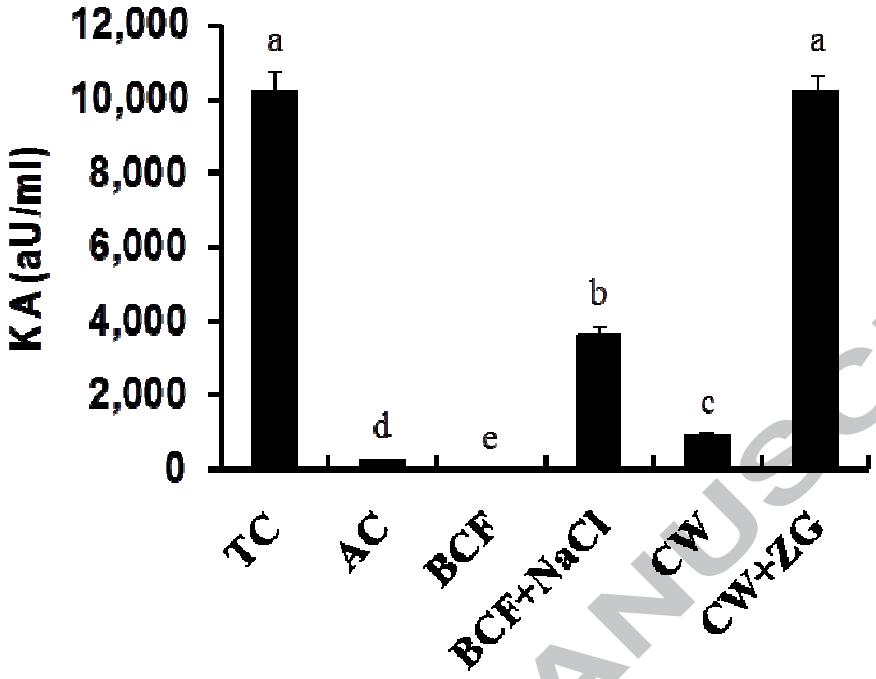


Fig. 3

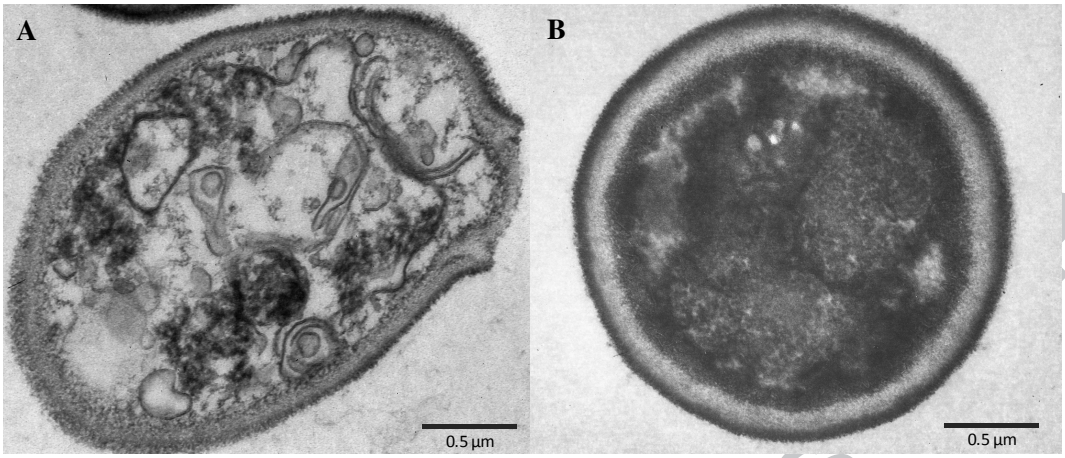
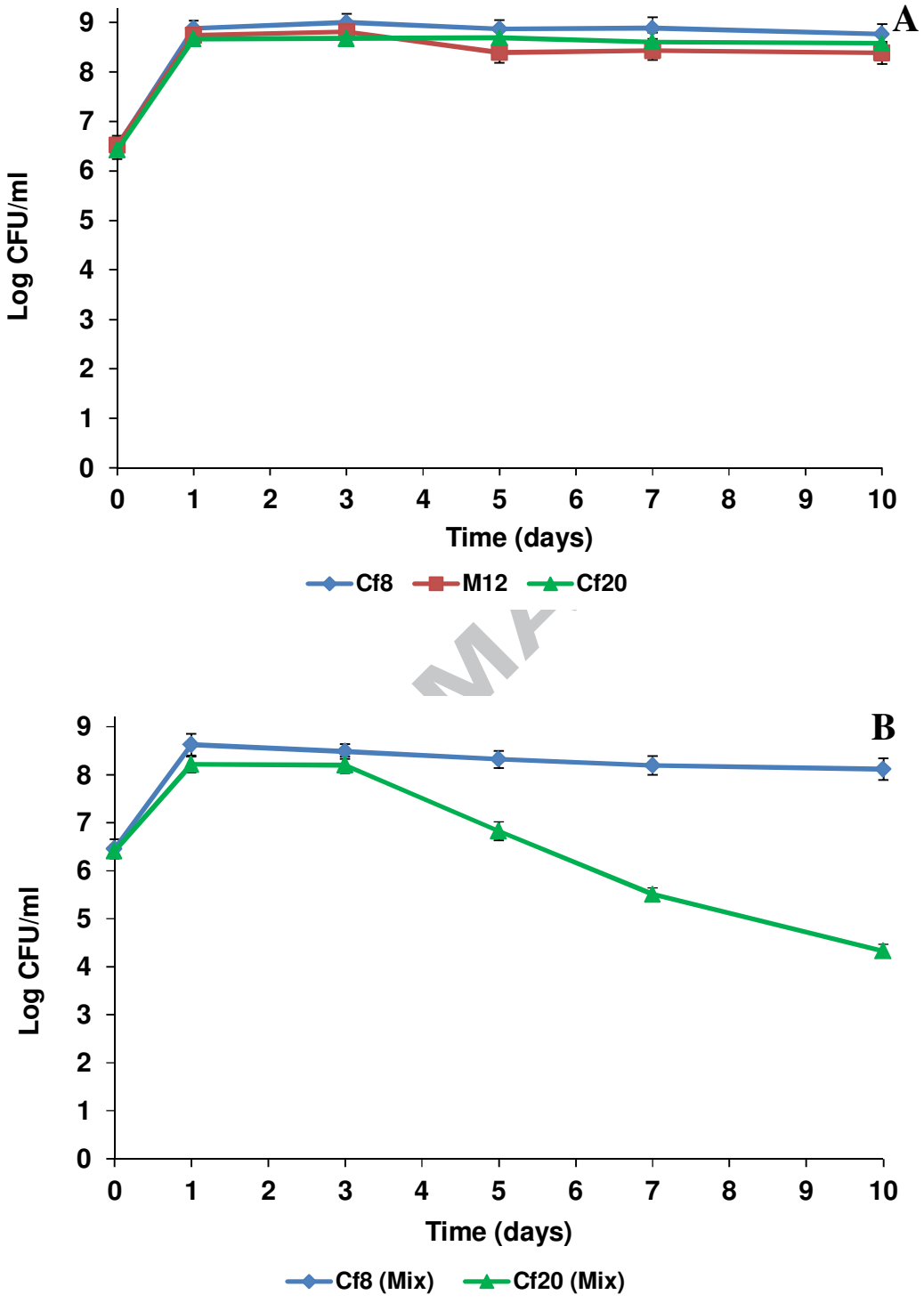


Fig. 4



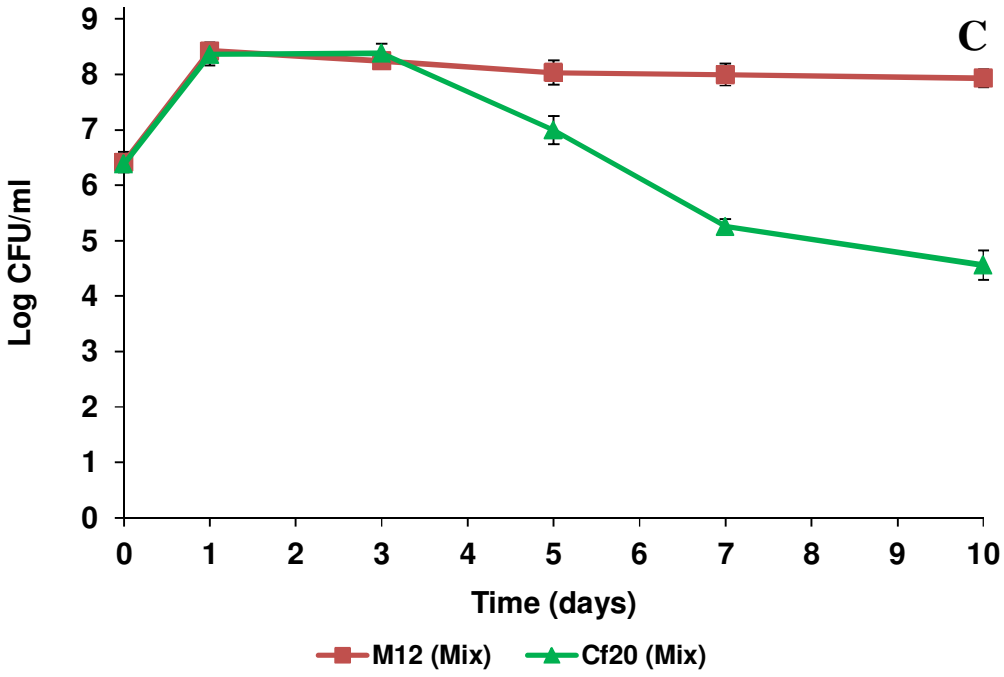
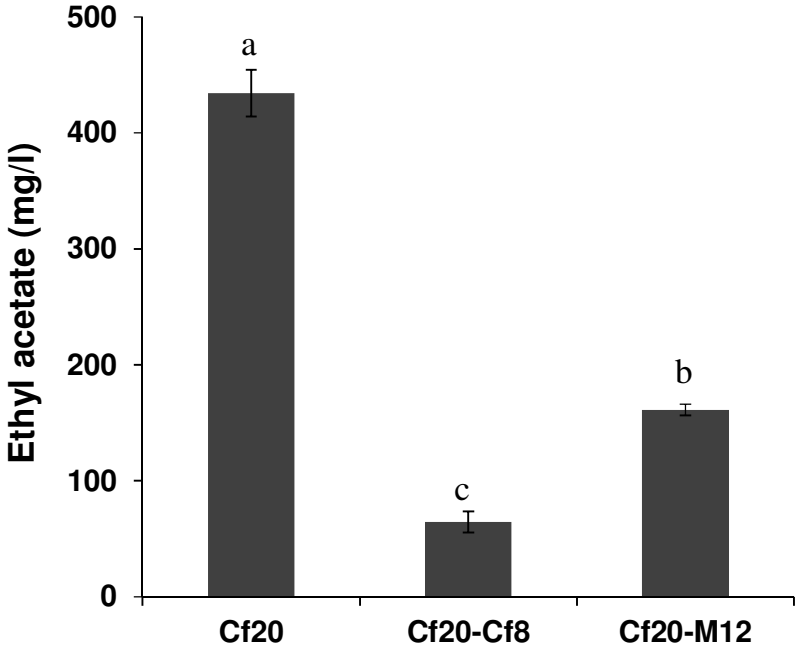


Fig. 5



Highlights

W. anomalus Cf20 produces the toxin KTCf20 active against wine spoilage yeasts

KTCf20 binds to β-1,3 and β-1,6-glucans on the cell wall of sensitive cells

KTCf20 shows optimal activity, stability and production under wine conditions

W. anomalus Cf20 and/or KTCf20 are promising biocontrol agents for wine production