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Optimization of killer assays for yeast selection protocols

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ABSTRACT

A new optimized semiquantitative yeast killer assay is reported for the first time. The killer activity of 36 yeast isolates belonging to three species, namely, *Metschnikowia pulcherrima, Wickerhamomyces anomala* and *Torulaspora delbrueckii,* was tested with a view to potentially using these yeasts as biocontrol agents against the wine spoilage species *Pichia guilliermondii* and *Pichia membranifaciens*. The effectiveness of the classical streak-based (qualitative method) and the new semiquantitative techniques was compared. The percentage of yeasts showing killer activity was found to be higher by the semiquantitative technique (60%) than by the qualitative method (45%). In all cases, the addition of 1% NaCl into the medium allowed a better observation of the killer phenomenon. Important differences were observed in the killer capacity of different isolates belonging to a same killer species. The broadest spectrum of action was detected in isolates of *W. anomala* NPCC 1023 and 1025, and *M. pulcherrima* NPCC 1009 and 1013. We also brought experimental evidence supporting the importance of the adequate selection of the sensitive isolate to be used in killer evaluation. The new semiquantitative method proposed in this work enables to visualize the relationship between the number of yeasts tested and the growth of the inhibition halo (specific productivity). Hence, this experimental approach could become an interesting tool to be taken into account for killer yeast selection protocols.

Keywords: killer yeast, killer capacity, biocontrol, semi-quantitative methods, spoilage yeast

RESUMEN

Optimización de la actividad *killer* para protocolos de selección de levaduras. En este trabajo se presenta un nuevo ensayo semicuantitativo que optimiza la detección de actividad *killer* en levaduras. Se evaluó la actividad *killer* de 36 cepas pertenecientes a las especies *Metschnikowia pulcherrima, Wickerhamomyces anomala* y *Torulaspora delbrueckii,* en vista del potencial uso de estas levaduras como agentes de biocontrol frente a las especies contaminantes de vinos *Pichia guilliermondii* y *Pichia membranifaciens*. Se comparó la efectividad de la técnica clásica basada en estrías (método cualitativo) con la del nuevo método semicuantitativo. El porcentaje de levaduras que mostraron actividad *killer* fue más alto cuando se utilizó el método semicuantitativo (60%) que con el método cualitativo (45%). En todos los casos, el agregado de 1% de NaCl en el medio permitió una mejor observación del fenómeno *killer*. Se observaron importantes diferencias en la capacidad *killer* de diferentes cepas dentro de la misma especie. Se detectaron dos cepas de *W. anomala* (NPCC 1023 y 1025) y dos cepas de *M. pulcherrima* (NPCC 1009 y 1013) con un amplio espectro de acción, ya que fueron capaces de inhibir el desarrollo de las tres levaduras sensibles evaluadas. La evidencia experimental demuestra la importancia de una adecuada selección de la cepa sensible al evaluar la actividad *killer*. El nuevo método semicuantitativo propuesto en este trabajo permite visualizar la relación entre el número de levaduras sembradas y el halo de inhibición del crecimiento (productividad específica). En conclusión, este método resulta una herramienta interesante para ser tenida en cuenta en los protocolos de selección de levaduras *killer*.

Palabras clave: levaduras *killer*, capacidad *killer,* biocontrol, método semicuantitativo, levaduras contaminantes

INTRODUCTION

Killer yeasts can produce toxic proteins or glycoproteins (killer toxins) that can cause death in sensitive (killer-sensitive) yeast isolates. The killer phenotype appears to be widely distributed within many yeast genera isolated from different sources (10, 36, 37). The interest in the killer property for industrial and medical applications has recently increased (11, 15, 22); however, the ecological role of killer yeasts has been somewhat overlooked and the conditions governing their behavior in particular habitats are mostly unknown (8).

The killer phenomenon is important in some industrial alcoholic fermentation processes using non-sterile substrates, even in those conducted by selected yeast starters in which wild yeast isolates may interact (17, 26, 28, 36). Killer isolates influence the fermentation process (by provoking changes in the population balance) and cause, in some cases, the unsuccessful implantation of a selected yeast isolate and the subsequent alterations in the final product (40, 43).

On the other hand, food spoilage caused by microorganisms is a serious problem for the food industry (20, 38). The exploration of killer yeasts as producers of mycocins

active against these undesired microorganisms seems interesting. Hence, the use of selected killer yeasts as a biocontrol method may be related to the improvement of the food industry by reducing the requirement for chemical preservatives (5, 9, 33). In previous studies carried out in our laboratory, the majority of yeasts isolated from spontaneously fermenting grape must have evidenced the killer character (18, 29, 30, 33). The high variability in the killer phenomenon detected within different producer species provides an exceptional source of antagonist yeasts to be used in biocontrol. In particular, killer yeasts belonging to *Metschnikowia pulcherrima, Wickerhamomyces anomala* (ex *Pichia anomala*) and *Torulaspora delbrueckii* species have shown the broadest killer spectrum against wine spoilage yeasts (19, 33).

As a general rule, most studies on killer behaviour use the seeded-agar-plate technique based on the screening procedure described by Makower and Bevan (23) due to its simplicity and low cost (1, 2, 31, 41). In this traditional qualitative (QLM) experimental approach, yeasts to be tested for sensitivity are seeded as a lawn and the yeasts to be assayed for killer activity are streaked over the surface of the solidified YEPD-MB agar medium. Killer activity is then evidenced by the presence of a growth inhibition halo of the target yeast around the killer yeast streak.

Some slight modifications in the composition of the killer medium were reported, including the addition of glycerol or NaCl as well as variations in pH and temperature (7). However, these modifications were not adopted in general routine killer assays. The use of NaCl in the killer medium was particularly proposed for halotolerant yeasts isolated from olive brines, soy sauce, pickled radish, etc. (11, 16, 39).

The aim of the present work was to develop an alternative rapid and effective semiquantitative killer assay for biocontrol yeast selection. The effect of the addition of different NaCl concentrations as well as the use of serial dilutions of each killer yeast on the detection and comparison of different killer yeasts against spoilage yeasts was evaluated.

MATERIALS AND METHODS

Yeast isolates

Thirty six killer yeasts: 14 *M. pulcherrima*, 13 *W. anomala* and 9 *T. delbrueckii* isolates, from the North Patagonian Culture Collection (NPCC, Neuquén, Argentina) were used (Table 1). All killer yeasts used in this work were selected from screening surveys on yeasts isolated from wine related environments (19, 30, 33). In addition, we analyzed the killer activity of five reference strains including the type isolates of the three species previously mentioned (Table 1).

Three target strains were used: *Pichia guilliermondii* NPCC 1037 and *Pichia membranifaciens* NPCC 1108 (both species classified as potential wine spoilage yeasts) and *Candida glabrata* NCYC 388 (sensitive reference isolate).

Detection of killer activity

Each killer yeast isolate was tested for its ability to kill the three selected target isolates by a classical qualitative (QLM) and by a new semiquantitative (QTM) killer detection method.

In both cases, the seeded-agar-plate technique was used. In this technique, each target yeast isolate is suspended in sterile water (10^6 cells/ml) and 1 ml of this suspension is seeded as a lawn onto 10 ml of molten YEPD-MB agar plates (g/l: yeast extract 1, malt extract 3, glucose 20, peptone 2, agar 20, methylene blue

Table 1. Autochthons and reference killer yeast strains.

Killer species and reference number[1]	Origin
Metschnikowia pulcherrima	
NPCC 1001	Red grape surface
NPCC 1002	Red grape surface
NPCC 1003	Fermenting apple must
NPCC 1004	Fermenting Malbec must
NPCC 1005	Fermenting Malbec must
NPCC 1006	Fermenting Malbec must
NPCC 1007	Fermenting Malbec must
NPCC 1008	Fermenting Trousseau must
NPCC 1009	Fermenting Trousseau must
NPCC 1010	Fermenting Trousseau must
NPCC 1011	Fermenting Trousseau must
NPCC 1012	Fermenting Trousseau must
NPCC 1013	Fermenting Trousseau must
NPCC 1014	Fermenting Trousseau must
NRRL Y711	Grape surface
Wickerhamomyces anomala	
NPCC 1015	Wine vats surfaces
NPCC 1016	Wine vats surfaces
NPCC 1017	Wine vats surfaces
NPCC 1018	Wine vats surfaces
NPCC 1019	Wine vats surfaces
NPCC 1020	Wine vats surfaces
NPCC 1021	Wine vats surfaces
NPCC 1022	Fermenting Semillon must
NPCC 1023	Fermenting Malbec must
NPCC 1024	Fermenting Malbec must
NPCC 1025	Fermenting Malbec must
NPCC 1026	Fermenting Malbec must
NPCC 1027	Fermenting Malbec must
NCYC 434	Unknown (K5 type)
NCYC 435	Unknown (K8 type)
NRRL Y366	Fermenting must
Torulaspora delbrueckii	
NPCC 1028	Red grape surface
NPCC 1029	Wine vats surfaces
NPCC 1030	Wine vats surfaces
NPCC 1031	Wine vats surfaces
NPCC 1032	Fermenting Merlot must
NPCC 1033	Fermenting Merlot must
NPCC 1034	Fermenting Merlot must
NPCC 1035	Fermenting Malbec must
NPCC 1036	Fermenting Malbec must
NRRL Y866	Unknown

[1]**NPCC:** North Patagonian Culture Collection, Neuquén, Argentina; **NRRL**: ARS Culture Collection, Peoria, United States of America; **NCYC**: National Collection of Yeast Cultures, Norwich, United Kingdom

0.003, buffered at pH 4.6 with 0.5 M phosphate-citrate). Different NaCl concentrations (0, 1 or 3% w/v) were added to the killer medium in order to evaluate its effect in the improvement of killer activity detection. Young cultures (48 h in YEPD agar at 26 °C) of both sensitive and killer yeasts were used for killer assays. All experiences were carried out in triplicate.

In QLM, the seeded plates were streaked with thick smears of killer cultures and incubated at 18 ± 2 °C for 48-72 h. The lawn target yeast isolate was designated as sensitive when a clear zone of growth inhibition was observed surrounding the killer cultures streaks (23).

In QTM, 48 h cultures of all isolates to be tested for killer activity were resuspended in sterile water (10^{10} cells/ml) and five serial decimal dilutions were carried out in sterile water. Five microliters of all six consecutive dilutions were spotted on seeded plates and incubated at 18 ± 2 °C for 48-72 h. The diameter of the inhibition halo around the spot was used as a measurement of the killer activity. Halo areas for each sensitive/killer isolates combination were measured on digitalized images (Nikon Image Software (NIS) ver. 3.00), as previously reported (3).

Killer yeasts were evaluated according to two different concepts: (i) effectiveness, defined as the percentage of isolates showing killer activity against a particular sensitive isolate over the total number of yeasts tested, and (ii) specific productivity, defined as a relationship between the production of toxins (size of the inhibition halo) and the number of killer cells assayed (aliquots of known concentration).

The evaluation of dose-response results (killer cell counts vs. inhibition halo in mm) was carried out by lineal regression (Sigma Plot 2000).

RESULTS AND DISCUSSION

According to previous studies carried out in our laboratory, as well as in other laboratories wordwide, *M. pulcherrima, W. anomala* and *T. delbrueckii* killer yeasts species showed a significant inhibitory effect on the growth of different spoilage yeasts (6, 27, 32, 33).

In order to evaluate the effect of NaCl addition on killer behaviour, the three killer species previously described were evaluated against two wine spoilage yeast strains belonging to species *P. guilliermondii* (strain NPCC 1037) and *P. membranifaciens* (strain NPCC 1108) as well as

against the sensitive reference strain *Candida glabrata* NCYC 388. The results using the QLM evidence an increase in halo size in the presence of 1 and 3% (w/v) NaCl with regard to the control (Table 2). Moreover, in some cases killer activity was evidenced only when NaCl was added to the medium (Table 2) (e.g. killer activity of *M. pulcherrima* against *C. glabrata*), indicating that the addition of a NaCl percentage to the killer medium can be an interesting factor to be taken into account for killer yeast screening.

Qualitative methodological procedure (QLM) offers some economy of labor when a large number of yeasts are handled. However, it has been wrongly utilized in many studies for the definition of weak or strong phenotypes according to the extent of the halo produced by the streak (14, 26, 40, 42). We consider that this is an incorrect extrapolation of the methodology for the evident variation in the quantity of cells in the killer yeast streak. Based on this issue, we propose a new semiquantitative method (QTM) to evaluate killer activity which may provide a new and improved tool for selection of yeast isolates to be used as potential biocontrol agents. Killer yeasts in known concentrations were assayed using aliquots of six serial decimal dilutions from 10^{10} to 10^5 cells/ml on YEPD-MB agar plates containing the same sensitive isolates and the same NaCl concentrations previously used in the QLM (Table 3). As a general rule, a gradual increase of the growth inhibition halo was observed proportionally with the increasing amount of killer cells (Table 3 and Figure 1). Again, the addition of NaCl allowed a clearer visualization of the halos; however a delay in the growth of target yeasts in the medium supplemented with 3% NaCl was also exhibited. As shown in Table 3, the highest correlation values between dose (killer cell counts) and response (inhibition halos in mm) were obtained with the addition of 1% NaCl. According to these results, the optimum amount of 1% (w/v) of NaCl was selected to be

Table 2. Killer activity (mm) at different NaCl concentrations of *M. pulcherrima, W. anomala* and *T. delbrueckii* type strains against target strains by QLM.

Target species	NaCl % (wt/v)	Killer species		
		M. pulcherrima NRRL Y711	*W. anomala* NRRL Y366	*T. delbrueckii* NRRL Y866
P. guilliermondii	0	-	2.3 ± 0.4	2.1 ± 0.5
NPCC 1037	1	-	3.8 ± 0.2	4.3 ± 0.3
	3	-	3.9 ± 1.1	2.5 ± 0.8
P. membranifaciens	0	-	-	-
NPCC 1108	1	-	-	-
	3	-	-	-
C. glabrata	0	-	2.2 ± 0.2	2.3 ± 0.5
NCYC 388	1	3.2 ± 0.2	3.4 ± 0.5	2.8 ± 0.2
	3	-	4.2 ± 1.5	3.5 ± 0.9

Table 3. Growth inhibition halo (mm) of killer strains evaluated at different NaCl concentrations (%) by QTM.

Target species	NaCl % (wt/v)	Killer species (cells/ml)[1]																				
		M. pulcherrima NRRL Y711							W. anomala NRRL Y366							T. delbrueckii NRRL Y866						
		10^{10}	10^{9}	10^{8}	10^{7}	10^{6}	10^{5}	r^2	10^{10}	10^{9}	10^{8}	10^{7}	10^{6}	10^{5}	r^2	10^{10}	10^{9}	10^{8}	10^{7}	10^{6}	10^{5}	r^2
P. guillermondii NPCC 1037	0	-	-	-	-	-	-	-	4.2±0.9	2.8±0.7	2.1±0.4	0.8±0.7	-	-	0.98	1.4±0.2	1.8±0.8	0.8±0.7	-	-	-	0.40
	1	-	-	-	-	-	-	-	5.8±0.6	5.2±1	3.8±0.7	4.1±0.4	3.2±0.5	2.2±0.8	0.99	3.2±0.3	3±1	2.1±0.7	0.9±0.7	0.8±0.8	0.6±0.1	0.92
	3	-	-	-	-	-	-	-	5.7±1.1	5.2±1.1	3.8±0.8	3.2±0.9	1.8±0.7	0.8±0.7	0.98	3.8±0.2	4±0.7	3.2±1	2.2±0.8	1.2±0.8	1.2±0.7	0.91
P. membranifaciens NPCC 1108	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. glabrata NCYC 388	0	2.8±0.7	2.2±0.9	-	-	-	-	0.91	1.5±0.7	1.1±0.7	-	-	-	-	0.95	1±0.5	0.8±0.7	-	-	-	-	0.87
	1	3.8±0.8	1.7±1.1	-	-	-	-	0.99	3±0.5	2.8±0.8	1.8±0.7	0.8±0.5	-	-	0.93	3±0.8	2.1±0.5	1.1±0.5	-	-	-	0.99
	3	-	-	-	-	-	-	-	2.8±0.7	2.7±0.9	1.6±0.7	1.6±0.7	-	-	0.91	3.2±0.8	2.2±0.8	1.8±0.7	0.8±0.5	0.9±0.7	0.8±0.5	0.86

[1] **P. g.**: *P. guillermondii*; **P.m.**: *P. membranifaciens*; **C g.**: *C. glabrata*

[1] r^2: regression coefficients of first-order equations expressing dose-response of killer species against each sensitive strain.

a-

Pichia guilliermondii

| *Mp* 1008 | *Wa* 1023 | *Td* 1030 |

b-

Pichia membranifaciens

| *Mp* 1008 | *Wa* 1023 | *Td* 1030 |

c-

Candida glabrata
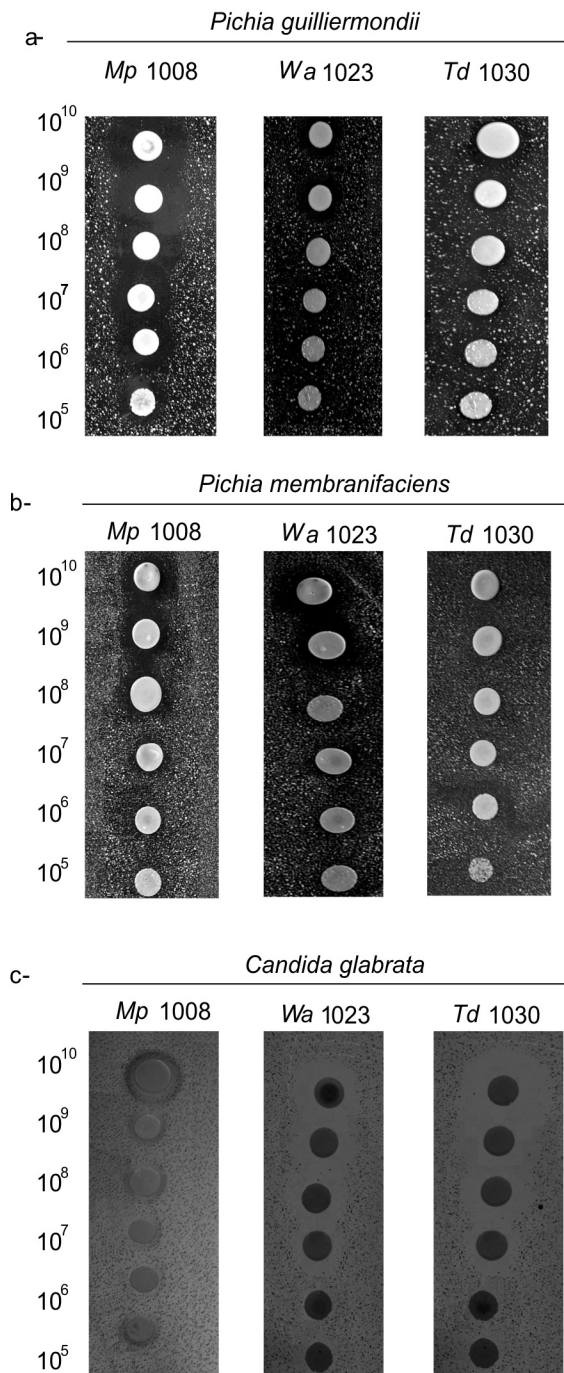
| *Mp* 1008 | *Wa* 1023 | *Td* 1030 |

Figure 1. Growth inhibition halo of killer strains evaluated at different concentrations 10^{10}-10^5 cells/ml; against *P. guilliermondii* NPCC 1037 **a**; *P. membranifaciens* NPCC 1108 **b**; and *C. glabrata* NCYC 388 **c**; by QTM.

added to the YEPD-MB medium used in all subsequent killer assays.

In order to evaluate the effectiveness of this new QTM method in discriminating the best killer yeast to be potentially used in biocontrol protocols, a total of 41 killer yeasts (Table 1) were evaluated against the regional spoilage yeasts previously mentioned. In all the cases, the percen-

tage of yeasts showing killer activity was found to be higher by the semiquantitative method (60% of the total number of crosses considering 10^{10} cells/ml) than by the qualitative method (45% of the total number of crosses) (Table 4). According to our results, it seems to be evident that the relationship between killer and sensitive cells has a great influence on the detection of the killer activity (Figure 1 and Table 4). Moreover, the percentage of yeasts showing killer activity when the QLM method was used resulted similar to the percentage detected at 10^8-10^9 cells/ml in the QTM method (Table 4).

Agar well diffusion bioassay using cell free culture supernatants or concentration disk bioassays have been used by several authors to detect and quantify killer activity (3, 27, 10). In these kinds of methodologies, thorough studies are needed regarding the relative stability of purified killer toxins after long term storage including the establishment of the actual time course of their possible degradation or loss of effectiveness (4). The semiquantitative method is a cost and time saving protocol which makes possible to analyze the cell/toxin ratio without toxin preparations. Our results evidenced that QTM is a useful method to approach the specific productivity of each isolate since it represents a relationship between the number of yeasts tested and the growth of the inhibition halo.

A different spectrum of action and specific productivity were evidenced when the activity of the three killer species against the three target yeasts was evaluated by using the QTM method (Figures 2 and 3). When the killer species were tested against the spoilage isolate *P. guilliermondii* NPCC 1037, killer activity percentages ranging from 100% for the species *W. anomala* and 30% for *T. delbrueckii* were observed (Table 4). Thirty seven percent of *W. anomala* isolates evidenced a maximum inhibition halo diameter over 5 mm when the highest concentration of killer yeasts (10^{10} cells/ml) was evaluated (Figure 2). In particular the *W. anomala* isolates NPCC 1018, 1022 and NRRL Y866 showed killer activity even at the lowest yeast concentrations (10^5 cells/ml), evidencing high specific productivity.

Low effectiveness of QTM at maximum concentration evaluated (10^{10} cell/ml) of killer yeasts species *W. anomala* (13%) and *T. delbrueckii* (0%) against spoilage species *P. membranifaciens* was observed (Figure 3 and Table 4). Seventy seven percent of *M. pulcherrima* killer isolates tested proved to be effective against *P. membranifaciens*, evidencing a higher effectiveness of this species against *P. membranifaciens* than against *P. guilliermondii* spoilage yeasts. Contrarily, *W. anomala* killer yeasts showed a higher effectiveness against *P. guilliermondii* than against *P. membranifaciens* (Figures 2 and 3).

On the other hand, 95% of the panel of killer yeasts was effective against the sensitive reference strain *C. glabrata* NCYC 388. Only isolates *M. pulcherrima* NPCC 1008 and *W. anomala* NPCC 1017 did not show killer activity against the sensitive reference strain *C. glabrata*;

Table 4. Effectiveness of QLM and QTM methods

Killer species	Target species	Effectiveness of killer yeasts[1]						
		QLM	QTM					
(Number of strains)			10^{10}	10^9	10^8	10^7	10^6	10^5
M. pulcherrima	*P. guilliermondii*	26	33	7	7	0	0	0
(15)	*P. membranifaciens*	26	77	47	27	7	0	0
	C. glabrata	88	93	93	73	60	40	20
T. delbrueckii	*P. guilliermondii*	20	30	30	20	0	0	0
(10)	*P. membranifaciens*	0	0	0	0	0	0	0
	C. glabrata	86	100	100	60	30	0	0
W. anomala	*P. guilliermondii*	81	100	100	50	38	31	19
(16)	*P. membranifaciens*	0	13	13	0	0	0	0
	C. glabrata	78	94	94	94	56	25	6
Total effectiveness[2]		**45**	**60**	**54**	**49**	**21**	**10**	**5**

[1]percentage of the killer yeast evaluated against each target yeast
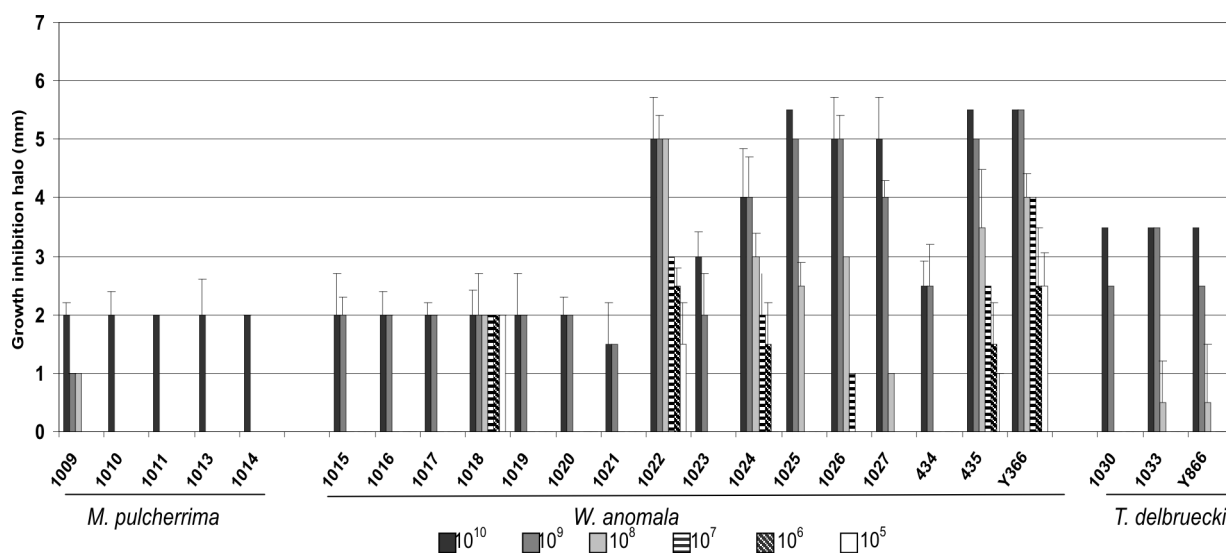[2]percentage of the killer yeast evaluated against all target yeast

Figure 2. Inhibition halo size of killer isolates evaluated at different concentrations against *P. guilliermondii* NPCC 1037. The Arabic numbers indicate the particular isolate number according to Table 1.

but these isolates proved to be killer against the spoilage yeasts (Figures 2 and 3). Moreover, in 60% of the cases tested in this work the inhibition halo produced by the killer yeasts was bigger against *C. glabrata* than against the spoilage yeasts. Taking into account that specific productivity and diffusion capacity are the same when the same killer isolate and the same medium are used, the width of growth inhibition halo observed among the tested killer isolate indicates that sensitive yeasts do not interact in the same way with the same killer toxin. In agreement with the current literature (6, 34), which seems to indicate

a mechanism of possible toxin binding until saturation of free receptors, the rationale of this behavior could be found in the different number of free receptors available on sensitive cell walls for interaction. As a matter of fact, several authors suggested that the type and number of free active sites differ among different sensitive isolates (3, 12). Because of its high sensitivity, *C. glabrata* NCYC 388 has been used as reference sensitivity strain for killer yeast screening since the early studies of Young (44). We confirmed these observations and we are in agreement with the use of this isolate for general killer yeast
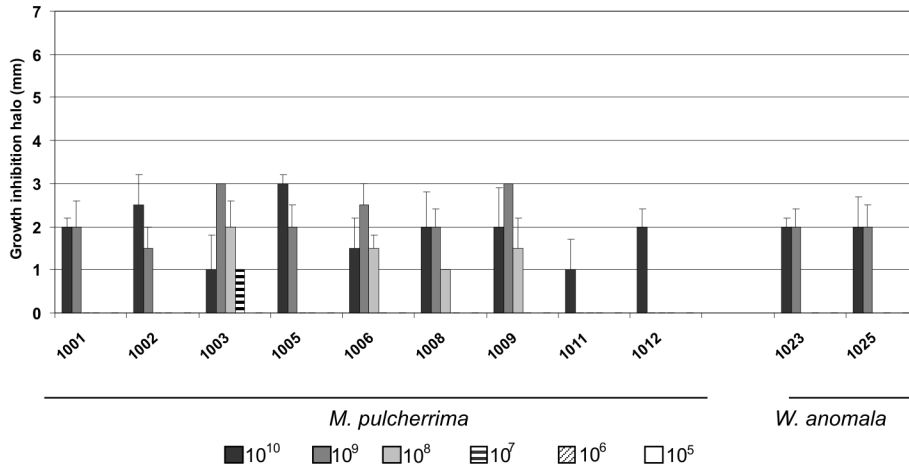
Figure 3. Inhibition halo size of killer isolates evaluated at different concentrations against *Pichia membranifaciens* NPCC 1038. The Arabic numbers indicate the particular isolate number according to Table 1.
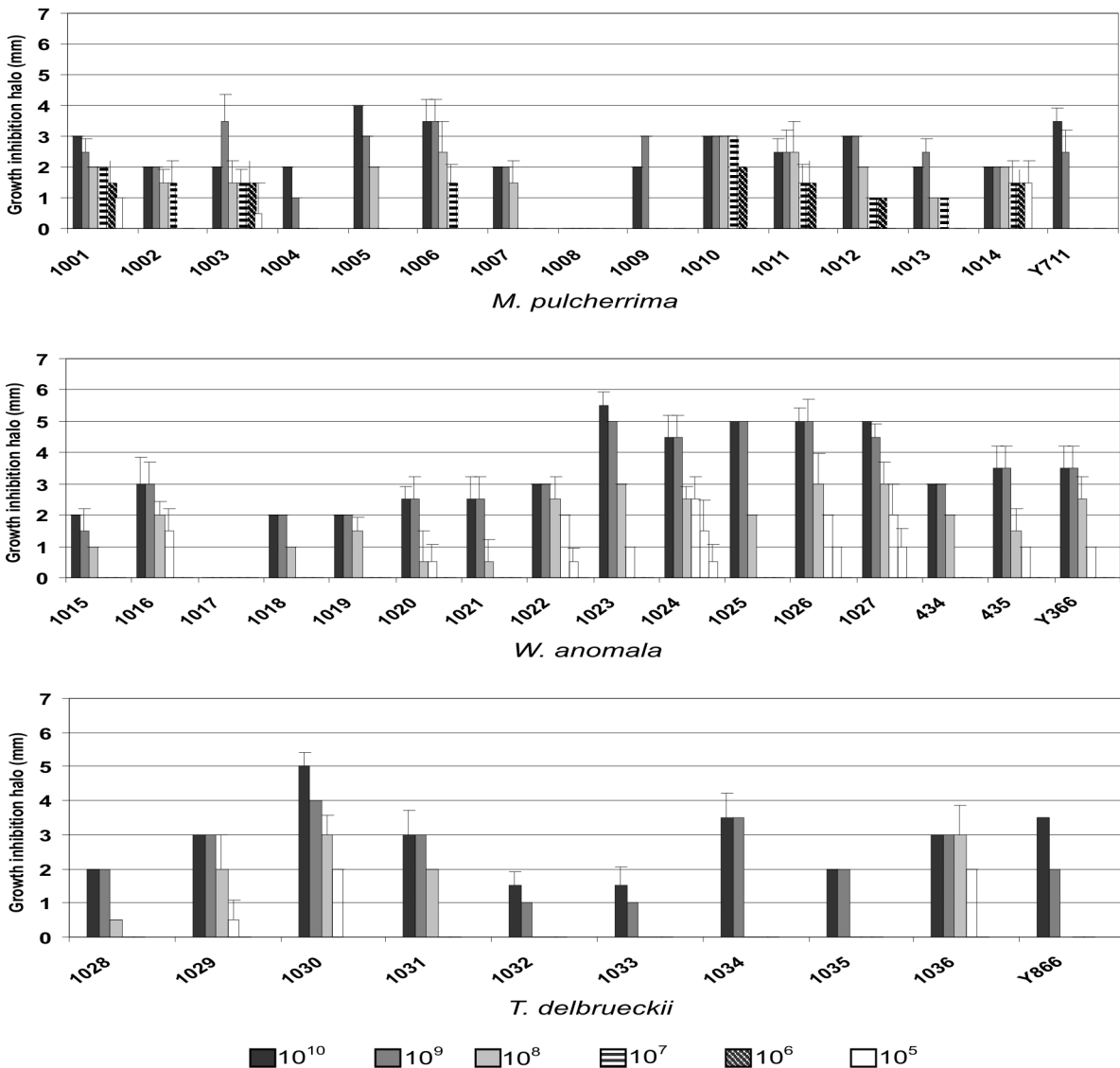

Figure 4. Inhibition halo size of killer isolates evaluated at different concentrations against *C. glabrata* NCYC 388. The Arabic numbers indicate the particular isolate number according to Table 1.

screenings. However, as each killer yeast has a specific spectrum of action, we strongly recommend the use of those yeasts to be biocontrolled as the target isolate in killer assays.

As mentioned above, important differences were observed in different isolates belonging to a same killer species. These differences, which were not detected by the QLM method, are of relevance for the selection of the best suitable biocontrol agent. Among *M. pulcherrima* killer yeasts, isolates NPCC 1009 and 1013 presented the widest killer spectrum since they are the only ones to inhibit the three yeasts tested. Although pulcherriminic acid synthesized by this species has demonstrated antimicrobial effect (21, 25), no inhibitory activity of this compound was evidenced in the present study. In many cases a brown halo, produced as a consequence of the diffusion of pulcherriminic acid, was observed surrounding the killer colonies with no inhibitory effect against the target yeasts (Figure 1 c).

Among *W. anomala* killer yeasts, isolates NPCC 1023 and 1025 killed the three yeasts tested. An interesting observation is the fact that *W. anomala* isolates from fermentation musts (isolates NPCC 1022 to 1027) showed bigger diameters in the inhibition halos than those produced by the isolates isolated from grapes (isolates NPCC 1015 to 1021). This behaviour was particularly evident when *P. guilliermondii* (Figure 2) and *C. glabrata* (Figure 4) were used as target yeasts. This difference in killer phenotype regarding the origin of the isolates is in agreement with previous studies evidencing that *P. guilliermondii* yeasts isolated from grapes were found to be more sensitive to killer toxins than yeasts of the same species isolated from fermenting musts (19). This result seems to indicate that in an environment enriched with killer yeasts (fermenting must) the surviving isolates are mostly resistant to killer toxins.

Finally, the three *T. delbrueckii* isolates (NPCC 1030, 1034 and NRRL Y866) which were effective against *P. guilliermondii* also exhibited the highest specific productivity against *C. glabrata*. All these differences at intra-specific level detected in the three killer species tested may be due to the production of different amount of toxins or different toxins by each particular isolate (24).

The QTM method proposed in this work enables to visualize the relationship between the number of yeasts tested and the growth of the inhibition halo (specific productivity) in most of the cases. We conclude that, as a primary screening, the QLM method may be used to determine killer activity in a high number of yeasts although its effectiveness may vary depending on the target yeast used, the addition of salts or the number of killer yeasts (Table 3). After this first screening, the QTM method may be used to evaluate the yeast isolates evidencing killer activity in order to select the one showing the best performance.

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