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Regular paper

The occurrence of killer activity in yeasts isolated from natural habitats*

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Yeast's ability to restrict the growth and kill other yeasts, fungi and bacteria has been known for over 50 years. Killer activity was detected in yeasts deposited in the world collections or isolated from natural habitats. In this study, isolates from the forest environment, leaves of fruit trees, flower petals, cereals and frozen fruit have been screened in terms of their killer activities. Killer activity was tested on strains belonging to six yeast species: *Candida, Rhodotorula, Pichia, Pachysolen, Yarrowia, Trichosporon*. The reference strains were *Kluyveromyces lactis* Y-6682 and *Kluyveromyces marxinanus* Y-8281, well-known to be sensitive to yeast killer toxins. Among one hundred and two tested strains, 24 (23.5% of isolates) showed positive killer action, and 10 (9.8% of the isolates) a weak killer action against at least one sensitive reference strain. The highest killer activity was observed among isolates from forest soil and flowers.

Key words: killer yeast, killer toxin, natural environments

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INTRODUCTION

Many strains of yeasts secrete extracellular proteins or glycoproteins, known as killer toxins. They act by inhibiting the growth of other yeasts, fungi and even bacteria (Dabhole & Joishy, 2005; Bajaj *et al*., 2012). Strains capable of producing toxins may be simultaneously resistant to the killing effect. The various toxins differ in the mechanism of secretion, molecular size, optimum pH and temperature of their activity (Schmitt & Breinig, 2006).

Killer phenotype in yeast was first described in 1963 by Makower and Bevan (Makower & Bevan, 1963). Since then, killer phenomenon has been reported in almost 100 species belonging to more than 20 genera and their number is still increasing (Buzzini & Martini, 2001; Golubev, 2006). Over 11 different killer toxins are known, and they are produced by representatives of such species as *Hanseniaspora, Pichia, Rhodotorula, Williopsis*, *Ustiliago* etc. (Schmitt & Breinig, 2002; Santos *et al*., 2011). The killer phenomenon of yeast cells is widely distributed among strains isolated from the natural habitats: water, wine, soil, fruit, and among yeasts stored in the collections of pure cultures (Vadkertiová & Sláviková, 1995; Vadkertiová & Sláviková, 2007; de Lima *et al*., 2013; de Ullivarri *et al*., 2014). Potential use of killer yeasts and their toxins is intended for various industries. In the brewing industry, winemaking and in production of fermented vegetables, killer yeasts can be used as starter cultures to prevent infection and the development of spoilage strains that might negatively affect the sensory quality of the final products (Antonini *et al*., 2005; Waema *et al*., 2009). Killer yeasts have also been used in biological control of post-harvest diseases and have become an alternative to the use of chemical fungicides (Santos *et al*., 2004). Yeasts producing killer toxins may be used in medicine as novel tools against animal and human fungal infections (Magliani *et al*., 2004). A killer system may be also helpful in bio-typing industrially and clinically interesting yeast cultures (Ochigava *et al*. 2011). In addition, killer yeasts and their toxins have been used as model systems to understand the mechanisms of regulation in eukaryotic polypeptide processing and expression of eukaryotic viruses (Schmitt & Breinig, 2006).

In this study, yeast strains isolated from natural habitats (forest, leaves, fruit, cereals, flowers) were screened for their killer activity against yeast belonging to the species of *Candida*, *Rhodotorula, Pichia Pachysolen*, *Yarrowia*, and *Trichosporon,* in order to find out whether strains from these environments have similar or different spectrum of their killer activity.

MATERIALS AND METHODS

Yeast strains. One hundred and two yeast strains isolated from the forest soil, rotting trees, leaves of fruit trees and bushes, flower petals, cereals, and from frozen fruit were examined for their killer activity (Table 1).

Twelve yeast cultures belonging to six yeast species. *Candida*, *Rhodotorula, Pichia Pachysolen*, *Yarrowia*, *Trichosporon* (Table 2), maintained in the Culture Collection of the Department of Biotechnology, Human Nutrition and Science of Food Commodities University of Life Sciences in Lublin, were used as sensitive strains. The reference strains from NRRL (ARS Culture and Patent Culture Collections, US Department of Agriculture, Illinois) were *Kluyveromyces lactis* Y-6682 and *Kluyveromyces marxianus* Y-8281 well-known to be sensitive to yeast killer toxins (Vaughan-Martini *et al*., 1988).

Culture media. All yeasts were cultivated in a YMB medium containing 1% (w/v) glucose, 0.3% (w/v) malt extract, 0.3% (w/v) yeast extract and 0.5% (w/v) peptone. The medium was buffered to pH 4.5 with 0.5 M sodium citrate/ phosphate, and YMA-MB (YM containing 0.003% (w/v) methylene blue, 2% (w/v) NaCl and 2% (w/v) agar) was used in assays for the killer phenotype (Llorente *et al*., 1997).

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Table 1. The origin of yeast isolates used in this study.

Origin	Strain No.
Forest environment	
forest mulch	1, 1a
forest soil	2, 4, 7, 8
rotting tree	3, 3a, 5, 9, 9a,
tree bark	6
Leaves of fruit trees or bushes	
cherry tree	10
chestnut tree	11, 11a
gean tree	12, 12a
apricot tree	13
pear tree	14, 14a, 14b
raspberry	15, 15a, 15b, 15c
Flowers petals	
dandelion	16, 16a
cherry tree	17, 17a, 17b, 17c
apple tree	18, 18a, 18b, 18c, 18d
chestnut tree	19, 19a
viburnum	20, 20a, 20b,
colza	21, 21a
daisy	22, 22a
catnip	23
currant	24
rose	25, 25a, 25b, 25c, 25d, 25e, 25f
jasmine	26, 26a, 26b, 26c, 26d
floxglove	27, 27a, 27b, 27c, 27d, 27e, 27f
poppy	28, 28a, 28b
Cereals	
ears of wheat	29, 29a, 29b, 29c, 29d, 30, 30a, 30b, 31, 31a, 32, 32a, 32b, 33, 34, 34a, 34b, 34c, 34d, 35, 35a, 35b, 35c
wheat germ	36, 37, 37a, 37b, 37c
Frozen fruit	
strawberry	38,38a
raspberry	39
plum	40,40a

Assay for killer phenotypes. The killer activity was investigated by a modified method described previously (Woods & Bevan, 1968; Vadkertiová & Sláviková, 1995; Santos *et al*., 2009). Sensitive strains were grown in YMB medium at 20ºC for 48 h. Each culture was mixed with YMA-MB containing 0.9% (w/v) agar, and the mixture (A_{600} 0.9–1.0) was poured as a lawn onto the surface of a Petri dish containing the assay medium. The plates were incubated for 2–3 h until the agar hardened. Then, wells (8 mm) were sterilely cut in the YMA-MB agar and the potential killer strains were seeded in the wells at 100µl of yeast inoculum per well. The plates were incubated at three different temperatures: 18, 22, 25ºC, for 7 days, and checked daily. A killer effect was recorded when the zone of inhibition around the tested isolates appeared on the plate. Killer activity was measured by subtracting diameter of the well from diameter of the inhibition zone. If the strain inoculated into the well was surrounded by bluish colored cells of a potentially sensitive strain, and a clear zone <1mm or was only surrounded by a blue zone, the reaction was recorded as "w" (weak killer reaction). If the inoculated strain was surrounded by bluish colored cells and a clear zone ≥1mm, it was designated as "+", "++", "+++" (positive killer reaction). The experiments were performed in triplicate.

Table 2. Sensitive strains for testing yeast killer activity.

Species	Strain No.
Candida fluviatilis CBS 6776	C14
Candida freyschussi 3562	C16
Candida pseudotropicalis JPF-Lp 65	C23
Candida parapsilosis	C29
Candida parapsilosis	C30
Rhodotorula pallida CBS 3a	Rh1
Rhodotorula rubra	Rh5
Pichia stipitis CBS 5773	P4
Pichia stipitis CCY 39-50-1	P5
Pachysolen tannophilus Y2462	P6
Yarrowia lipolytica	Y1
Trichosporon cutaneum	T7

RESULTS AND DISCUSSION

The optimal temperature for killer activity of the yeast isolates was 22ºC (Fig. 1), and it is similar to what was reported previously by others (Santos *et al*., 2009). At this temperature, among all tested strains, twenty four showed a positive killer reaction, and ten showed a weak killer reaction against reference strains and stains used as sensitive (Table 2). This corresponded to 23.5% and 9.8% of the isolates, respectively. Results of positive and weak killer activity at 22ºC are presented in Table 3.

Killer activity varied depending on the origin of the isolate and the sensitive strain that was used. Similar killer activity was observed among isolates derived from the forest, flowers and leaves; these isolates act on all strains of the *Candida* genus: *C. fluviatilis, C. freyschussi, C. pseudotropicalis, C. parapsilosis*; furthermore, strains no. 2, 17, 17a, 17b, 17c, 18 and 20b also limited the growth

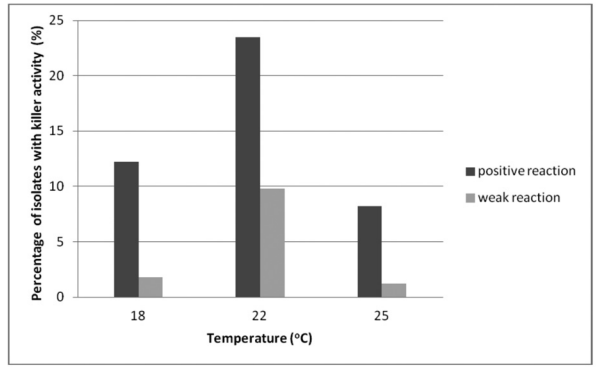

Figure 1. Killer activity of tested isolates at different temperatures.

Figure 2. Killer activity of some strains isolated from cereals against *Rhodotorula pallida*.

of *P. tannophilus*. Isolates 2, 17, 20b, derived from the forest soil, cherry tree, and flowers of vibrunum, respectively, were the only ones of all tested strains that acted lethally on *Y. lipolytica*. Yeasts derived from grains had a much more diverse activity against susceptible strains. Most of them worked by growth inhibition of *C. fluviatilis, C. parapsilosis* and *Rh. pallida*. Two strains derived from frozen strawberry fruits, no. 39 and 39a, were characterized by identical killer profile against C14, C16, Rh1, Rh5, P5, P6 and T7. The broadest and identical killer spectrum was observed for two isolates (17, 20b) from flower petals, and they displayed such activity against eight of the twelve tested strains. Nine of isolated yeasts derived from forest soil, cherry and apple trees, colza, jasmine, frozen strawberry also showed a broad spectrum of activity, but only against seven strains. The strain number 31a, isolated from cereals, limited growth of only one strain, *Rh. pallida,* and furthermore it only demonstrated a weak killer action. The strongest sensitivity was displayed by the *Candida freyschussi* strain (C16).

Precent, 31, of all isolates showed positive or weak action against C16, the largest zones of inhibition (≥ 10mm) were observed when isolates 2, 21a, 29b, 32a, 39 were used as killer strains. The highest number of wide and clear zones was observed among isolates from cereals against Rh1 (Fig. 2). None of the tested strains had the ability to kill the *P. stipitis* yeast (P4).

Killer potential of yeast isolates was also shown by Vadkertiová & Sláviková (2007). Yeast from water, soil and leaves were able to kill the yeast from the *Candida* genus*: C. parapsilosis*, *C. albicans*, *C. krusei*, *C. tropicalis*. Two strains from sediments of a fresh water lake were characterized by the highest activity, and they worked on all the examined test strains. However, the greatest number of killer strains came from leafy materials. On the other hand, yeast isolates from Amazon soil tended to kill yeasts belonging to *Candida, Pichia, and Debaryomyces* species from the same habitat, and did not have any activity against *Rhodotorula* or *Trichosporon* (Vital *et al*., 2002). Studies on the activity of strains isolated from natural environments were also carried out by Mushtaq *et al*. (2015). Strains isolated from dairy products, flowers' nectar, slime fluxes of trees and soil were tested on

Table 3. Yeast isolates that showed a positive and/or weak killer action against sensitive strains at 22°C.

Yeast isolates with potential killer activity	Sensitive strain											
	C14	C16	C23	C29	C30	Rh1	Rh5	P4	P5	P6	Y1	T7
2	+	+++	+	+++	++	–	–	–	–	+++	+	–
3	w	w	w	w	w	–	–	–	–	–	–	–
9	w	w	w	w	w	–	–	–	–	–	–	–
9a	+	++	+	++	++	–	–	–	–	–	–	–
14	+	++	++	+++	+	–	–	–	–	–	–	–
14a	w	w	w	w	w	–	–	–	–	–	–	–
17	+	+	++	+	+	–	–	–	+	+	+	–
17a	+	+	+	+	+	–	–	–	+	+	–	–
17b	+	+	+	+	++	–	–	–	+	+	–	–
17c	+	+	+	+	+	–	–	–	+	+	–	–
18	+	+	++	+	+	–	–	–	+	+	–	–
18a	+	+	+	+	+	–	–	–	–	–	–	–
18c	w	w	w	w	w	–	–	–	–	–	–	–
19	w	w	w	w	w	–	–	–	–	–	–	–
20b	+	++	++	+++	+++	–	–	–	+	+	+	–
21a	+	+++	+	+	++	–	–	–	+	++	–	–
23	w	w	w	w	w	–	–	–	–	–	–	–
24	w	w	w	w	w	–	–	–	–	–	–	–
26c	+	+	+	++	+	+	w	–	–	–	–	–
29b	–	+++	–	++	–	+++	–	–	–	+++	–	–
29d	–	+	–	+	–	+++	–	–	–	+++	–	–
30a	–	++	–	+	–	+++	–	–	–	–	–	++
30b	–	–	–	–	–	+++	–	–	–	++	–	–
31a	–	–	–	–	–	w	–	–	–	–	–	–
32a	–	+++	–	++	–	+++	–	–	–	+++	–	–
34a	–	+	–	+	–	+++	–	–	–	–	–	+
34b	–	+	–	+	–	+++	–	–	–	–	–	+
34c	–	++	–	+	–	+++	–	–	–	–	–	+
34d	–	+	–	+	–	+++	–	–	–	–	–	+
35c	–	++	–	+	–	+++	–	–	–	+++	–	–
36	–	w	–	w	–	–	–	–	w	w	–	–
37c	–	w	–	w	–	–	–	–	w	w	–	–
39	+	+++	–	–	–	+++	+	–	+	+	–	+
41	++	++	–	–	–	++	+	–	+	+	–	+

Positive killer action (zone of inhibition in mm): +++ (≥ 10), ++ (9–6), + (5–1); weak killer action (< 1); – no killing action.

sensitive *Pichia* strains. It has been demonstrated that the tested isolates can act as killers, be neutral or sensitive, depending on the environment from which they originate. In another study, yeast isolates from flowers of a medicinal plant have been used. They gave positive reaction against *Debaryomyces anomala* (Dabhole & Joishy, 2005). Sensitivity of yeasts belonging to *Cryptococcus*, *Candida, Debaryomyces, Kluyveromyces, Pichia and Saccharomyces* species was investigated by Keszthelyi *et al.* (2008). *Rhodotorula mucilaginosa*, *C. krusei*, *C. albicans*, and *P. membranifaciens* strains were not sensitive to isolates of *Filobasidium capsuligenum*, ten strains derived from a wine cellar, dried fig, sake, cider, soil and grapefruit. *F. capsuligenum* proved to be active only against *Cryptococcus neoformans* and *C. laurentii* strains. Sensitivity of yeast strains belonging to 21 food spoilage species of 14 genera (*Candida, Debaryomyces, Dekkera, Hanseniaspora, Issatchenkia, Kazachstania, Kluyveromyces, Pichia, Rhodotorula, Saccharomyces, Schizosaccharomyces, Torulaspora, Yarrowia* and *Zygosaccharomyces*) was observed in different studies (Goretti *et al.* 2009). Searching for killer strains among those naturally occurring in different environments is very important, due to their future potential use in plant protection, medicine and industry. In addition, knowledge about the susceptibility of a given strain can be helpful in choosing the best strain for biotyping pathogenic strains or for using as the most resistant starter culture.

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