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EXPERIMENTAL ARTICLES

A *Kluyveromyces lactis* Mycocin Active at Neutral pH

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Abstract— A strain of *Kluyveromyces lactis* was found to secrete a fungicidal mycocin active in the pH range from 6 to 9 and exhibiting the highest activity at pH of approximately 7. A few yeast species of the families *Saccharomycetaceae* and *Wickerhamomycetaceae* were sensitive to the mycocin. Some genera and species were heterogeneous in this respect. UV treatment of the mycocinogenic strain resulted in loss of its antifungal activity. Although prokaryotes were not sensitive to the mycocin, the strain under study inhibited growth of some bacteria.

Keywords: yeasts, mycocin, killer toxin, antibacterial activity

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The yeast species *Kluyveromyces lactis* (Dombrowski) van der Walt is widely used in basic and applied studies and in the food industry, being inferior in this respect only to *Saccharomyces*. In particular, they are used in commercial production of β-galactosidase, chymosin, other heterologous proteins, protein–vitamin concentrate, and ethanol from whey [1, 2].

Thirty years ago, some strains of the species were found to secrete a glycoprotein inhibiting growth of other yeasts (killer toxin, mycocin, or zymocin) whose synthesis is governed by linear double-stranded DNA plasmids [3]. Results of the many studies performed ever since are summarized in a number of reviews [4, 5].

Activity of known mycocinogenic *K. lactis* strains is the highest at low pH values and the action spectrum of their mycocins is rather wide [6]. In the present work, a mycocin most active at neutral pH values and characterized by a narrow action spectrum is described.

MATERIALS AND METHODS

In the work, cultures of the All-Russian Collection of Microorganisms (VKM; http://www.vkm.ru) were used. Most species under study are represented by type strains, and the genera, by type species. The *K. lactis* strain VKM Y-1868 was isolated by Tsygankov in 1948 from chal, a Turkmen drink produced from fermented camel milk.

Sensitivity to mycocin of the strain VKM Y-1868 was tested by culture-to-culture technique on the agar medium described below, which was also used to evaluate the mycocin heat stability and stability to proteases (10 mg/mL) by the agar wells technique. When a VKM Y-1868 colony was surrounded by a growth inhibition zone several millimeters in diameter, the tested strain was registered as sensitive; in the absence of such zone, as insensitive. Formation of a narrow (approximately 1 mm) zone indicated that the strain was weakly sensitive. Mycocin-containing culture fluid was obtained by incubation of the mycocinogenic strain in glucose–peptone medium [6]. For approximate evaluation of the molecular mass of the VKM Y-1868 mycocin, the strain was grown on the medium for sensitivity testing covered with a dialysis membrane (Spectrum, United States). After a week of incubation, the membrane, together with the culture growing on it, was removed and the medium was inoculated with a lawn of a mycocin-sensitive strain *Wickerhamomyces subpelliculosa* VKM Y-1088. To eliminate the activity, the plates inoculated with VKM Y-1868 were UV-irradiated (254 nm, ~200 J/m^2) for 3–5 min.

RESULTS

Antifungal activity of *K. lactis* VKM Y-1868 was observed at pH values between 6.0 and 9.0 (citrate–phosphate buffer). In sensitive cultures, the largest growth inhibition zones were observed at pH 7.0–7.5; zone size increased upon addition of 100 mL/L glycerol or 2% NaCl to the medium. Based on these data, sensitivity to the agent secreted by the strain VKM Y-1868 in most cases was tested in buffered glucose–peptone medium [6] at pH 7.0 in the presence of 2% NaCl.

The strain exhibited no activity against any of the studied 47 species (42 genera) of basidiomycetous

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Table 1. Yeasts insensitive to the mycocin of *Kluyveromyces lactis* VKM Y-1868 (numbers of studied species and strains)

Aciculoconidium (1, 1)	*Komagataea* (1, 1)	*Sakaguchia* (1, 1)
Agaricostilbum (1, 1)	*Kondoa* (1, 1)	*Saturnispora* (1, 1)
Ambrosiozyma (1, 1)	*Kregervanrija* (1, 1)	*Scheffersomyces* (1, 1)
Babjeviella (1, 2)	*Kuraishia* (1, 1)	*Schizoblastosporion* (1, 1)
Barnettozyma (3, 10)	*Kurtzmanomyces* (1, 1)	*Schwanniomyces* (7, 9)*
Bensingtonia (1, 1)	*Kwoniella* (1, 1)	*Sebacina* (1, 1)
Blastobotrys (1, 2)	*Lachancea* (2, 4)	*Sirobasidium* (1, 1)
Bullera (1, 1)	*Leucosporidiella* (1, 1)	*Sporidiobolus* (1, 1)
Bulleromyces (1, 1)	*Leucosporidium* (1, 1)	*Sporobolomyces* (3, 3)
Candida (5, 5)*	*Lindnera* (10, 12)*	*Sporopachydermia* (1, 1)
Citeromyces (1, 1)	*Lipomyces* (2, 2)	*Starmera* (4, 7)
Clavispora (1, 2)	*Lodderomyces* (1, 1)	*Sterigmatomyces* (1, 1)
Cryptococcus (1, 1)	*Magnusiomyces* (1, 1)	*Sugiyamaella* (1, 1)
Cuniculitrema (1, 1)	*Mastigobasidium* (1, 1)	*Sympodiomycopsis* (1, 1)
Curvibasidium (1, 1)	*Metschnikowia* (9, 11)	*Tausonia* (2, 2)
Cystofilobasidium (1, 1)	*Meyerozyma* (1, 1)	*Tetrapisispora* (2, 2)
Debaryomyces (1, 1)	*Millerozyma* (1, 1)	*Tilletiopsis* (1, 1)
Dekkera (3, 5)	*Mrakia* (1, 1)	*Torulaspora* (4, 4)
Dioszegia (1, 1)	*Mrakiella* (1, 1)	*Tremella* (1, 1)
Dipodascopsis (1, 1)	*Myxozyma* (2, 2)	*Trichomonoascus* (1, 1)
Eremothecium (1, 1)	*Nadsonia* (2, 7)	*Trichosporon* (1, 1)
Erythrobasidium (1, 1)	*Naumovozyma* (1, 1)	*Trigonopsis* (1, 1)
Fellomyces (1, 1)	*Ogatea* (11, 19)*	*Trimorphomyces* (1, 1)
Fibulobasidium (1, 1)	*Pachysolen* (1, 1)	*Udeniomyces* (1, 1)
Filobasidiella (1, 1)	*Peterozyma* (1, 1)	*Wanderwaltozyma* (1, 1)
Filobasidium (1, 1)	*Pichia* (1, 1)	*Wickerhamia* (1, 1)
Geotrichum (1, 1)	*Priceomyces* (1, 1)	*Wickerhamiella* (1, 1)
Hanseniaspora (1, 1)	*Pseudozyma* (1, 1)	*Yamadazyma* (1, 1)
Holtermannia (1, 1)	*Rhodosporidium* (1, 1)	*Yarrowia* (1, 1)
Hyphopichia (2, 2)	*Rhodotorula* (3, 4)	*Zygoascus* (1, 1)
Itersonilia (1, 1)	*Saccharomyces* (5, 54)*	*Zygosaccharomyces* (5, 24)*
Kluyveromyces (4, 27)*	*Saccharomycodes* (1, 3)	*Zygotorulaspora* (2, 2)
Kockovaella (1, 1)	*Saccharomycopsis* (1, 1)	*Xanthophyllomyces* (1, 1)
Kodamaea (1, 1)		

Note: * The sensitive species include *C. intermedia* (1), *K. dobzhanskii* (3), *L. fabianii* (6), *L. jadinii* (3), *O. wickerhamii* (2), *Sacch. bayanus* (7), see also Table 3, *S. etchelsii* (1), and *Z. rouxii* (4). The numbers indicated in the table do not include these strains.

yeasts (Table 1), and the spectrum of its activity toward ascomycetous yeasts was rather restricted. Among the latter, out of the tested 160 species (63 genera), only 20 turned out to be sensitive; they were rare representatives of genera (Tables 1 and 2). Moreover, some species also were found to be heterogeneous in this respect. For example, weakly sensitive to the agent produced by *K. lactis* VKM Y-1868 were approximately a quarter of the 53 strains of *Saccharomyces cerevisiae* Meyen ex Hansen and over 40% of the 55 strains of *Wickerhamomyces anomalus* (Hansen) Kurtzman et al. (Tables 3 and 4).

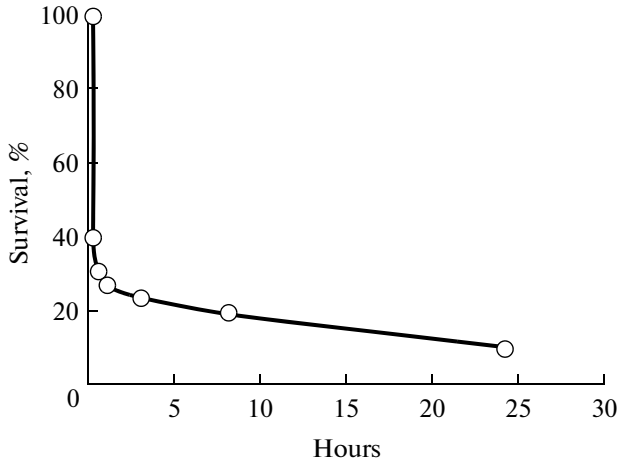
The strain VKM Y-1868 was usually inactive against actinomycetes and bacteria (Table 5), with the exception of strains *Alcaligenes faecalis*, *Paracoccus alcaliphilus*, *Sporosarcina ureae*, and *Stenotrophomonas maltophila*. Weak zones of growth inhibition around the VKM Y-1686 culture in buffered (pH 7.0) medium were also observed in lawns of *Brevibacterium stationis*, *P. denitrificans*, *P. methyluteus*, and *P. solventivorans*.

Table 2. Genera comprising the species both sensitive and insensitive to the mycocin of *Kluyveromyces lactis* VKM Y-1868

Sensitive	Insensitive
	Kazachstania barnettii (1)
	K. exigua (2)
Kazachstania africana (1)	*K. kunashiriensis* (1)
K. bovina (1)	*K. lodderae* (2)
K. viticola (1)	*K. rosinii* (1)
	K. sloofiae (1)
Nakaseomyces delphensis (1)	*K. spencerorum* (2)
	K. transvaalensis (1)
Nakazawaea holstii (5)	*K. unispora* (3)
	K. yakushimaensis (1)
Wickerhamomyces bisporus (1)	
W. bovis (1)	*Wickerhamomyces alni* (2)
W. canadensis (1)	*W. anomalus* (32)*
W. chambardii (1)	*W. ciferrii* (1)
W. lynferdii (1)	*W. mucosus* (1)
W. rabaulensis (1)	*W. pijperi* (1)
W. subpelliculosa (6)	*W. silvicola* (1)
W. sydowiorum (1)	*W. strasburgensis* (1)

Note: In parentheses, the number of studied strains is indicated.
* Also, see Tables 4.

To produce the antifungal agent, the strain VKM Y-1868 was grown under stationary conditions for 5 days. The obtained culture liquid possessed the antifungal activity (see figure). Antifungal agent did not penetrate the dialysis membrane impermeable for compounds of molecular mass greater than 8 kDa.



Cell death of *Wickerhamomyces subpelliculosa* VKM Y-1088 (% to the initial concentration, 2×10^3 cell/mL) upon incubation in toxin-containing culture liquid of *Kluyveromyces lactis* VKM Y-1868.

Activity of the cultural fluid was lost after 3 min of heating at 100°C or after treatment with protease XIV of *Streptomyces griseus* (Sigma) and pronases P and E (Serva), although it was retained upon treatment with pepsin, lysoamidase, and protease XIII of *Aspergillus saitoi* (Sigma).

Irradiation of the VKM Y-1868 culture with UV light resulted in loss of the activity in over a half (56%) of the randomly selected colonies. They were insensitive to the agent secreted by the parent strain. The inactive clones obtained, as well as the parent strain, formed zones of growth inhibition of the prokaryotes listed above.

DISCUSSION

Thermolability, sensitivity to proteolysis, and considerable molecular mass of the fungicidal agent secreted by *K. lactis* VKM Y-1868 evidence its proteinaceous nature. These characteristics and its activity against yeast fungi alone support its classification as a mycocin [7]. Successful elimination of antifungal activity by UV irradiation implies that in strain VKM Y-1868, as in other *K. lactis* mycocinogenic isolates, mycocin synthesis is governed by the extrachromosomal genetic elements. Similar to the previously studied strains of the species, activity of VKM Y-1868 was efficiently eliminated by UV treatment.

However, the strain VKM Y-1868 is different from other similar strains in several respects. Mycocins of the known strains act under pH ranging from 5 to 7 with the optimum at pH 6 [6, 8], while pH range of activity of the VKM Y-1868 mycocin is shifted to the alkaline values (up to pH 9) and it is most active at pH values close to neutral. Even greater differences are associated with the action spectra. Over 60 yeast species of the families *Saccharomycetaceae* and *Wickerhamomycetaceae* (*Saccharomycetales*) [6, 8], together with other *Kluyveromyces* species, are sensitive to mycocins previously described in *K. lactis*, while only about 20 species were found to be sensitive to the mycocin of the strain VKM Y-1868 (Tables 1–4).

At the same time, certain similarity may be noted. In particular, the lists of *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* strains sensitive to the mycocin of VKM Y-1868 (Tables 3 and 4) and to the known *K. lactis* mycocins [6] are almost the same. Heterogeneity of *S. cerevisiae* with respect to sensitivity to *K. lactis* mycocins of depends on mating type and ploidy of cultures. It is lower in diploid and polyploid strains than in haploid ones [5, 9]. The same reasons probably cause heterogeneity in mycocin sensitivity among *W. anomalus* strains. The noted differences and similarities apparently indicate that the VKM Y-1868 mycocin and the known mycocins differ considerably at the first stage of their interaction with a cell, adsorption onto its surface, while the mechanisms of lethal effect as such are probably similar.

Table 3. *Saccharomyces cerevisiae* strains sensitive to the mycocin of *Kluyveromyces lactis* VKM Y-1868

Species, strains (original names)
VKM Y-388 (*S. anamensis* Will et Heinrich, T)
VKM Y-390 (*S. ellipsoideus* Reess var. *fulliensis* Steiner, T)
VKM Y-391 (*S. cerevisiae* Hansen subsp. *orsati* Steiner, T)
VKM Y-402 (*S. festinans* Ward et Baker, T)
VKM Y-403 (*S. cerevisiae* Hansen var. *marchalianus* (Kufferath) Dekker, T)
VKM Y-406 (*S. chevalieri* Guillermond, T)
VKM Y-407 (*S. chevalieri* Guillermond var. *lindneri* (Guillermond) Dekker, T)
VKM Y-408 (*S. chodati* Steiner, T)
VKM Y-424 (*S. ellipsoideus* Reess var. *umbra* Castelli, T)
VKM Y-441 (*S. hutensis* Kufferath *ex* Stelling-Dekker, T)
VKM Y-444 (*S. italicus* Castelli, T)
VKM Y-520 (*S. tokyo* Nakazawa, T)
VKM T-1139 (*S. annulatus* Negroni, T)
VKM Y-1144 (*S. pulmonalis* Redaelli, T)
VKM Y-1234 (*S. oleaginosus* Santa Maria, T)
VKM Y-2119 (*S. gaditensis* Santa Maria, T)
VKM Y-2123 (*S. onubensis* Santa Maria, T)

Note: T indicates the type strain.

There is a considerable number of publications on the activity of mycocinogenic yeasts against bacteria [10–12]. These data generate doubts, since mycocins exert activity at acidic pH values [7] when most bacteria do not grow. Detection of the strain synthesizing mycocin with the activity optimum at neutral pH values allows us to confirm that mycocins as such possess no antibacterial effect.

In rare cases zones of growth inhibition of prokaryote strains were observed around the VKM Y-1868 colonies (pH 7). They, however, were also formed around the colonies obtained from eliminants lacking the mycocinogenic activity. These zones were certainly not caused by the mycocin, but rather were formed due to some other factors secreted by *K. lactis*. Special attention should be drawn to its activity against a frequent causing agent of nosocomial infections *Stenotrophomonas maltophila*, which rapidly gains resistance to many antibacterial preparations [13].

The presence of antifungal and antibacterial activity in *K. lactis*, a constant component of the microbiota of fermented milk product, is probably a factor contributing to their useful properties. In some regions of Turkmenistan, chal is used as a therapeutic treatment [14, 15] for tuberculosis and gastrointestinal disorders.

Table 4. *Wickerhamomyces anomalus* strains sensitive to the mycocin of *Kluyveromyces lactis* VKM Y-1868

Species, strains (original titles)
VKM Y-60 (*Candida pelliculosa* Redaelli, T)
VKM Y-61 (*C. pelliculosa* Redaelli var. *cylindrica* Diddens et Lodder, T)
VKM Y-118 (*Endoblastoderma pulverulentum* Fischer et Brebeck, A)
VKM Y-141, 142, 146, 148–152, 1087, 1907 (*Hansenula anomala* (Hansen) H. et P. Sydow)
VKM Y-154 (*Willia productiva* Berkhout, T)
VKM Y-156 (*Saccharomyces sphericus* von Nägeli)
VKM Y-163 (*S. aceris-sacchari* Fabian et Hall, T)
VKM Y-164 (*Hansenula subpelliculosa* Bedford)
VKM Y-175 (*H. panis* Castelli, T)
VKM Y-2038–2040 (*H. ukrainica* Kvasnikov et al.)
VKM Y-2512, 2513 (*H. anomala* (Hansen) H. et P. Sydow var. *schneggii* (Weber) Wickerham)

Table 5. Prokaryote genera comprising species that were studied with respect to sensitivity to the mycocin of *Kluyveromyces lactis* VKM Y-1868 (number of studied species, strains)

Agromyces (1, 1)	*Haloarcula* (1, 1)	*Paenibacillus* (1, 1)
Alcaligenes (1, 1)	*Haloferax* (1, 1)	*Paracoccus* (8, 8)
Angulomicrobium (1, 1)	*Herbaspirillum* (1, 1)	*Pectobacterium* (1, 1)
Asticcacaulis (1, 1)	*Janthinobacterium* (1, 1)	*Planococcus* (1, 1)
Azospirillum (1, 1)	*Kribbela* (1, 2)	*Promicromonospora* (1, 1)
Brevibacterium (8, 8)	*Kurtia* (1, 1)	*Pseudomonas* (2, 2)
Corynebacterium (2, 2)	*Labrys* (1, 1)	*Rhodococcus* (2, 2)
Enterococcus (1, 1)	*Lactococcus* (1, 1)	*Sporosarcina* (3, 3)
Flavobacterium (2, 2)	*Mycobacterium* (2, 2)	*Stenotrophomonas* (1, 1)
	Oceanospirillum (1, 1)	

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