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Killer toxins in new isolates of the yeasts *Hanseniaspora uvarum* and *Pichia kluyveri*

(*Hanseniaspora; Pichia*; yeast; killer toxin)

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1. SUMMARY

From various habitats (plant material, fruits, soil), yeasts belonging to the species of *Pichia kluyveri* and *Hanseniaspora uvarum* were isolated that showed killer activity. According to the activity spectrum against other yeasts these strains belonged to 11 different groups that were distinguishable from the killer strains K_1–K_{10}. The isoelectric points of the killer proteins were in the range of pH 3.5–3.9, the activity optimum was observed at pH 4.2–4.6. Above pH 5 and above a temperature of 25–35°C the killer proteins were inactivated.

2. INTRODUCTION

Since the first description of killer toxin of yeast by Makower and Bevan [1], the genetic and biochemical aspects of this phenomenon have been primarily investigated [2,3]. In *Saccharomyces* the production of the active toxin, an extracellular protein, depends on the presence of virus-like-particles, consisting of two species of protein-encapsidated ds-RNA. Killer strains have been found in yeast culture collections [4,5] and have been isolated from natural habitats [6,7]. So far, killer strains have been reported among strains of the genera *Saccharomyces, Candida, Debaryomyces, Kluyveromyces, Hansenula, Pichia, Torulopsis* [4] and *Cryptococcus* [8]. This paper describes an unexpected seasonal variation when it was attempted to isolate killer yeast strains from natural habitats. Among the strains isolated and identified, the species *Hanseniaspora uvarum* and *Pichia kluyveri* were found, of which the former is so far unknown to produce killer toxin. The killer activity was compared by cross-reaction with known killer strains and the temperature and pH stability as well as the isoelectric point of the toxins were determined.

3. MATERIALS AND METHODS

3.1. Yeast strains and growth conditions

The following yeasts were used as test strains: K_1 (*Saccharomyces cerevisiae*, Fink D 587-2A); K_2 (*S. cerevisiae* NCYC738); K_2 399 (*S. cerevisiae*); K_3 (*S. capensis* NCYC761); K_4 (*Torulopsis glabrata* NCYC388); K_5 (*Hansenula subpelliculosa* NCYC16); K_6 (*Kluyveromyces fragilis* NCYC587); K_7 (*Pichia membranaefaciens*

Dedicated to Prof. Dr. F. Lingens, Stuttgart-Hohenheim, on the occasion of his 60th birthday.

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NCYC333); K_8 (*Hansenula anomala* NCYC435); K_9 (*Hansenula mrakii* NCYC500); K_{10} (*Kluyveromyces drosophilarum* NCYC575); 28 (*S. cerevisiae*); 392–395 (*Pichia kluyveri*); 67 (381) (*S. cerevisiae*, sensitive). The strains K_1 and 67 (381) were from the Wissenschaftliche Station für Brauerei, München. Strains K_2–K_{10} were generously supplied by Dr. T.W. Young. All other strains were from the collection of this institute. The strains were cultured as described previously [9].

3.2. Isolation of yeasts and testing for killer activity

Various samples of leaves, fruit, stems, bark and soil were incubated for 2 days at 25°C in YEP-medium (glucose 2% w/v, peptone 2% w/v, yeast extract 1% w/v) to which 0.02% streptomycin sulphate had been added. Dilutions of this culture were plated on YEP-agar and incubated for 3 days at 25°C. Yeast colonies were streaked for purification and tested for killer activity on methylene blue agar according to the method of Somers and Bevan [10] using the sensitive strain *S. cerevisiae* 67. The same method was used for determining the cross reaction of the strains by using the various test strains indicated.

3.3. Production and concentration of killer toxins

The yeasts were grown in modified B-medium as previously described [9] at pH 3.5 for 3 days at 21°C. The cells were removed by centrifugation and subsequent filtration (0.2 μm filters). The killer toxin was concentrated 100-fold by passing the solution through an Amicon PM-10 membrane. The activity was determined according to the method of Somers and Bevan [10] by pipetting 0.1 ml into wells cut into the agar.

4. RESULTS

From autumn 1982 to summer 1984 various random samples of plant material were collected and used for the isolation of yeast strains that were tested for killer activity (Table 1). The samples were mainly plant material: leaves, fruit, stems, bark and some soil samples, all from the area around Mainz. The collection of samples was purely incidental and no attempt was made to collect samples systematically. Random colonies, preferably different appearing colonies obtained from the same sample, were streaked for purification and used for the further tests. Killer yeast strains were frequently isolated in summer and autumn and no or only few such strains were found in the first half of the year.

The killer strains were found to belong to species of the genera *Pichia* and *Hanseniaspora* (Table 2). To study the strains the methods described by Barnett et al. [11] were used. The strains marked by an asterisk were identified by Dr. Yarrow, CBS, Delft, whose help is greatly acknowledged. All isolated killer yeast strains were tested for killer activity against the killer strains K_1–K_{10}

Table 1

Seasonal variation in the frequency of killer toxin producing yeast strains

Date of sampling		Number of samples	Number of yeast colonies investigated	Number of killer strains
September	1982	1	40	7
November	1982	1	30	2
May	1983	5	47	0
June	1983	15	81	0
July	1983	31	71	15
August	1983	8	11	4
November	1983	10	19	9
February	1984	27	27	1
May	1984	3	40	0
June	1984	4	79	0

Table 2

The newly isolated killer toxin producing yeast strains of *Hanseniaspora uvarum* and *Pichia kluyveri* and their activity against various known killer yeasts

Newly isolated yeast (streak)		Test strains (lawn)														Arbitrary group designation
		K1	K2 379	K2 399	K3	K4	K5	K6	K7	K8	K9	K10	28	396	67	
H. uvarum	469	−	−	−	−	+	−	(+)	−	−	−	−	−	−	+	G 1
	470 [a]	+	−	−	−	+	−	+	−	−	−	−	−	−	+	G 2
	478	−	+	+	−	+	+	−	−	−	−	−	(+)	−	+	G 3
	471,472 [a],473,474	−	−	−	−	+	−	−	−	−	−	−	−	−	+	G 4
P. kluyveri	476 [a],479,480	+	+	+	+	+	+	−	−	−	−	+	+	−	+	G 5
	484	+	−	−	+	+	(+)	−	−	−	−	+	−	−	+	G 6
	487,490 [a]	+	+	+	+	+	(+)	−	−	+	(+)	+	+	+	+	G 7
	475,477,481,482	+	(+)	−	+	+	+	−	−	−	−	+	(+)	−	+	G 8
	468	(+)	+	+	−	+	−	+	−	−	−	+	−	+	+	G 9
	485 [a]	+	+	−	+	+	(+)	−	−	−	(+)	+	+	+	+	G10
	488 [a]	+	+	−	+	+	(+)	−	−	+	(+)	+	+	+	+	G11

[a] Identified by Dr. Yarrow.

+ killer activity, distinct inhibition or blue zone on lawn of test strain, (+) weak killer activity, − no killer activity.

from Young [7] and some other killer strains. All new strains inhibited *Torulopsis glabrata* K_4; none of these strains inhibited the killer strain *Pichia membranaefaciens* K_7 or *Pichia kluyveri* (392–395). A varying activity was observed against the test strains used. According to the activity spectrum the yeast strains could be arranged in 11 arbitrary groups that were designated G1–G11. The strains of *Hanseniaspora uvarum* belonged to 4, and the strains of *Pichia kluyveri* to 7 groups. All strains of *Hanseniaspora* were inactive whereas all strains of *Pichia* were active against the test strain K_{10}. All new killer strains were tested against each other. However, none of these was sensitive to any of the toxins.

Killer toxins of yeasts are known to be inactivated by high pH and by elevated temperatures [12]. As the toxin production by the species of *Hanseniaspora uvarum* is reported here for the first time, these toxins and those of *Pichia* were further investigated (Table 3). The toxins of the 4 investigated strains were very pH-labile. When kept at pH 2, 3 or 4 the activity remained unchanged, but at pH 5 the toxin was almost completely inactivated within 24 h. The toxins were also temperature-labile; they were rapidly inactivated above 25 to 35°C as indicated. Some differences exist between the toxins of the yeasts investigated. The isoelectric point of the various toxins was determined by isoelectric focussing.

Table 3

Comparison of the killer toxins of *Hanseniaspora uvarum* and *Pichia kluyveri* (pH of inactivation, temperature stability, isoelectric point and pH of optimum activity)

Yeast		pH [a]	°C [b]	Ip	pH [c] optimum
P. kluyveri strain	468	5	30	3.5–3.8	4.2
	485	5	35	3.7–3.9	4.4
H. uvarum strain	470	5	25	3.7–3.9	4.2
	471	5	25	3.4–3.7	4.6

[a] pH value leading to almost complete inactivation within 24 h at 4°C.

[b] Stable at this temperature when incubated for 1 h at pH 4.2.

[c] Tested against the sensitive strain *S. cerevisiae* 67 (381).

The activity was found in the pH range 3.4 to 3.9, with only minor differences between the strains. The optimum activity was observed with the agar diffusion test in the pH range 4.2 to 4.6.

5. DISCUSSION

So far the biological significance of the killer toxins of yeast is unknown. Of course, it can be speculated that killer strains might have an advantage when competing with sensitive strains. It has been shown that in certain fermentations killer strains become dominant when inoculated together with other strains [13]. This is the rationale for attempts to introduce the killer factor into commercially used yeasts [14]. This paper confirms the observation of several authors that killer yeasts are quite frequent. However, the results indicate that a seasonal variation of this frequency might exist. Of course, the available figures are by no means sufficient for a general statement. But among the incidental and arbitrary samples no, or very few killer strains were found early in the year, whereas in the second half of the year killer yeasts were quite frequent. Naturally the samples were different during the seasons, for no leaves are available in winter and no ripe fruit in spring. If this observed seasonal variation of the frequency of killer yeast is not due to the different samples, the following hypothesis might be used for an explanation. If killer yeasts have an ecological advantage, they should be able to compete successfully. Perhaps these killer strains are less persistent than non-killers, thus they might tend to disappear in the 'off-season'. This assumption might be supported by the observation of Rosini [5] who investigated the killer activity of the strains of the yeast collection of Perugia. No killer yeasts were found among the strains isolated before 1955, and the frequency of the killer activity decreased with the age of the strains. However, a later investigation of this laboratory showed that killer yeasts can be isolated from the same soil sample all year round.

To our knowledge the occurrence of killer strains has been observed in 8 different genera. Therefore it is not surprising that killer strains have been found that belong to a further genus (*Hanseniaspora*). Probably other yeast genera will be found that harbor killer strains if more strains will be tested.

So far at least two more killer types have been observed in *Saccharomyces* than the three (K_1–K_3) described by Young and Yagiu [7]. None of the killer strains investigated in this paper is identical with any of the types K_1–K_{10}. Obviously a great number of yeast strains exists that produce somewhat different toxins. On the basis of the cross-reaction with the strains K_1–K_{10}, the new killer strains were designated arbitrary group numbers, only to demonstrate that differences are detectable. However, it is not known if these spectra of activity are influenced by the amount of toxin produced by the tested strains. It is hoped that when more toxin-producing strains become available some sort of reasonable classification will be developed. A useful approach might be the determination of the nature of the nucleic acid coding the protein component of the various toxins.

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