

Production and effect of killer toxin by *Saccharomyces cerevisiae* and *Pichia kluyveri* on sensitive yeasts and fungal pathogens

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Killer yeasts from flowers of Indian medicinal plants were isolated and the effect of their killer toxin was determined on sensitive yeast cells as well as fungal pathogens. The toxin of *Saccharomyces cerevisiae* and *Pichia kluyveri* inhibited *Dekkera anomala* accumulating methylene blue cells on Yeast Extract Peptone Dextrose agar (pH 4.2) at 21°C. There was no inhibition of growth or competition between the yeast cells in the mixed population of *S. cerevisiae* isolated from *Acalypha indica*. *S. cerevisiae* and *P. kluyveri* were found to tolerate 50% and 40% glucose, while *D. anomala* tolerated 40% glucose. Both *S. cerevisiae* and *P. kluyveri* did not inhibit the growth of *Aspergillus niger*, *Candida albicans* and *Fusarium* spp.

Keywords: killer yeast, *Saccharomyces cerevisiae*, *Pichia kluyveri*, *Dekkera anomala*, killer toxin, killer phenomenon

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The contamination of wild yeasts and fungi during wine fermentation using *Saccharomyces cerevisiae* is a serious problem in the fermentation industry. In order to reduce contamination in the fermentation broth, killer yeasts may be used as starter cultures or during the fermentation. In the present study, an attempt was made to detect killer yeasts from flowers of medicinal plants and their ability to kill sensitive yeast cells.

The killer yeasts produce polypeptide toxin having specific killing activity towards sensitive yeast belonging either to same genera or other genera¹. Killer yeasts, producing killer toxin, were detected in the species of *Candida*, *Cryptococcus*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Torulopsis*, *Ustilago*, *Williopsis* and *Zygosaccharomyces*².

The killer yeasts such as *S. cerevisiae* are used to initiate wine fermentation and to improve the quality

of wine. But it has been observed that toxicity of the killer toxin of *S. cerevisiae* wine yeast (K2 type) is restricted to sensitive *Saccharomyces* strains and does not affect wild yeasts belonging to the genera *Hanseniaspora*/*Kloeckera*, *Pichia* and *Saccharomycodes*³. Therefore, the present study was carried out to detect the effect of killer toxin of *S. cerevisiae* on 64 yeasts. This is one of the few reports of killer yeasts belonging to genera *S. cerevisiae* and *Pichia kluyveri* to be studied in India.

The common fruit yeast, *P. kluyveri* was isolated from the flowers of *Ixora coccinea*. The apiculate yeast, *Hanseniaspora*/*Kloeckera* occurs as a contaminant on grape surfaces and in freshly pressed juice³. The use of *P. kluyveri* to kill the sensitive yeast cells was investigated.

The flowers (24) of the medicinal plants growing in various locations of Mumbai were collected in clean and dry polythene bags, labelled and brought to the laboratory for enrichment and isolation of yeasts.

All the yeast strains were subcultured at three months interval on Malt Extract Yeast Extract (MEYE) agar and maintained on MEYE agar slant at 4°C. The composition of Yeast Extract Peptone Dextrose (YEPD) agar buffered at pH 4.2 (0.1 M citrate-phosphate buffer) was as follows: yeast extract, 1; peptone, 2; glucose, 2 and methylene blue, 0.005%.

Yeasts from the flowers were enriched in 5 ml sterile broth of MEYE on shaker (120 rpm) at room temperature for 24 hrs. After enrichment, yeasts were isolated on sterile MEYE agar plates and single colony of yeasts was maintained on sterile MEYE agar slants.

One g of middle portion of the flower was suspended in 10 ml sterile saline. The sample was thoroughly mixed on vortex mixer for one min. This sample was then diluted 10 fold till 10⁷ in sterile saline. One tenth of ml of dilution 10⁵, 10⁶ and 10⁷ were surface spread on MEYE agar plates. The inoculated plates were incubated at room temperature for 24 hrs and colonies were counted using colony counter.

Fermentation and assimilation of carbohydrates form principal physiological criteria for the identification and classification of yeasts. All the yeast strains were identified, based on morphological, cultural and physiological characteristics⁴ (Table 1).

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The glucose tolerance of yeasts was carried out in sterile Sabouraud's broth containing glucose in the range of 10-80% at 10% interval. The tubes were inoculated with 0.1 ml of yeast suspension containing 10^6 cells/ml, incubated at room temperature for 24 hrs and the results were recorded. A positive and negative control was kept for the test.

The killer yeasts were detected on YEPD agar. The culture was grown in 200 ml Sabouraud's broth in a 500 ml Erlenmeyer flask for 24 hrs at room temperature on a rotary shaker (120 rpm). After centrifugation ($10,000 \times g$ for 10 min at 5°C), the supernatant was filtered through 0.45- μm -pore-size membrane filter (Millipore). The resulting filtrate was incubated for 4 hrs at 4°C and the precipitate was collected by centrifugation ($10,000 \times g$ for 20 min) and resuspended in equal volume of 0.1 M citrate-phosphate buffer (pH 4.2). This toxin supernatant was stored at 4°C until use⁵.

The effect of different pH (4.0, 4.2, 4.4, 4.6 4.8 and 5.0) to detect the killer activity was carried out using toxin supernatant of yeast cells. In order to determine the temperature range for killer activity assay,

Table 1—Characteristics and identification of killer and sensitive yeasts

Characteristics	Yeasts		
	<i>S. cerevisiae</i>	<i>P. kluyveri</i>	<i>D. anomala</i>
Vegetative reproduction	Budding	Budding	Budding
Ascospores	1-4	3-4	1-4
Growth in liquid media	turbidity with islet formation	Sediment formation	Sediment formation
Yeast count	124×10^5 cfu	89×10^5 cfu	136×10^5 cfu
Urease	—	—	—
Diazonium blue B	—	—	—
Nitrate	—	—	—
Fermentation			
Glucose	+	+	+
Maltose	+	—	+
Lactose	—	—	—
Xylose	—	—	—
Assimilation			
Glucose	+	+	—
Maltose	—	—	—
Xylose	—	+	—
Sucrose	+	—	—
Mannitol	—	—	+

The above experiments were carried out in triplicates

samples of killer toxin were tested by the well test assay using YEPD agar plates at temperatures 21, 24, 28 and 30°C . The killer activity assay was performed as given below:

A 10 ml of YEPD agar containing 0.005% methylene blue buffered to pH at 4.2 was melted, cooled and 10^5 cells/ml of 1 ml of yeast suspension was added and poured on the sterile Petri plates in which 10 ml of basal medium of YEPD was overlaid. After the overlay was set, the wells were punched in agar with sterile cork borer (6 mm internal diam), ensuring that the bottom of the wells retained a thin layer of agar. A 0.1 ml of the toxin supernatant was added to the corresponding labelled wells and the plates were incubated at 21°C for 48 hrs. The YEPD agar plates were observed for the zone of inhibition and measured⁶.

S. cerevisiae and *P. kluyveri* showing killer activity were streak inoculated on sterile YEPD agar plates (pH 4.2). The inoculated plates were incubated at room temperature for 24 hrs. The three fungal pathogens, *Aspergillus niger*, *Candida albicans* and *Fusarium* spp. were streaked perpendicular to the streak of the killer yeast. The plates were incubated at room temperature for 48 hrs and observed for the inhibition of fungal pathogens.

Killer yeasts (64 strains) belonging to *S. cerevisiae* and *P. kluyveri* were isolated from the flowers known for their medicinal properties and certain anti-metabolites, which tend to restrict the growth of yeast. Therefore, the enumeration of yeasts in the medicinal flowers was carried out.

The average load of yeasts in 90% of the flowers was found to be $60-95 \times 10^5$ CFU. *S. cerevisiae* and *P. kluyveri* were found to possess all the morphological, cultural and physiological characteristics similar to that of the natural strain as identified according to the standard procedure. *Dekkera anomala* was found in *Butea monosperma*.

S. cerevisiae tolerated 50% glucose in 24 hrs at room temperature. The high glucose tolerance by *S. cerevisiae* is essential for high quality wine and high productivity of ethanol. The occurrence of *D. anomala* as a contaminant during wine fermentation is common. Further it was observed that *D. anomala* could not assimilate glucose but tolerated 40% glucose in 24 hrs. Hence, it could easily be grown in the fermentation broth. *S. cerevisiae* was found to inhibit *D. anomala* showing a zone of inhibition of 10 mm accumulating in the cells stained with methylene blue.

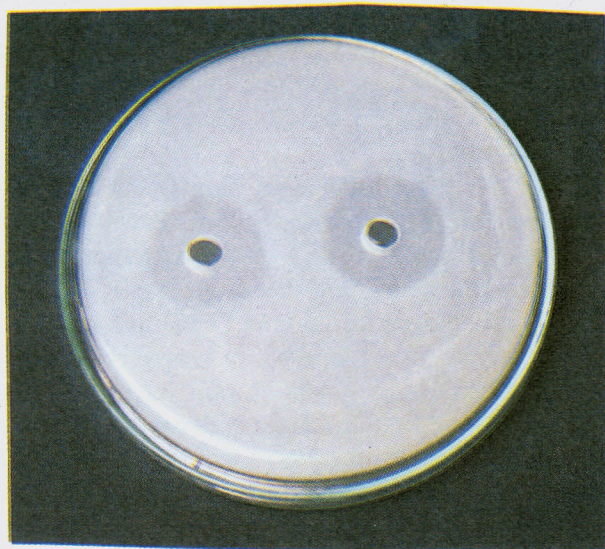


Fig. 1—The toxin supernatant of *S. cerevisiae* (left) & *P. kluyveri* inhibited *D. anomala* showing zone of inhibition

P. kluyveri was found to tolerate 40% glucose and hence, could be grown easily in concentrated fruit juices. *P. kluyveri* also inhibited *D. anomala* showing a zone of inhibition (13 mm). When the killer toxin of *S. cerevisiae* was tested against *P. kluyveri* and vice-versa, no inhibition was found.

All the 64 yeast strains were tested at pH (4.0, 4.2, 4.4, 4.6, 4.8, 5.0) with 0.1 M citrate phosphate buffer on YEPD agar. Both *S. cerevisiae* and *P. kluyveri* were found to exhibit killer activity at pH 4.2. The killer activity was detected at different incubation temperatures (21, 24, 28 and 30°C) and zone of inhibition was clearly seen at 21°C (Fig. 1).

In the fermentation of wine, indigenous yeasts such as *Kloeckera apiculata*, *Hanseniaspora uvarum* and *C. albicans* are used as starter cultures. The starter strains progressively die off with an increase in ethanol concentration, leaving *S. cerevisiae* to complete the fermentation⁷. The killer toxin of *D. anomala* is known to produce a number of

quantifiable effects against sensitive *C. albicans* cells⁸. Therefore, an attempt was made to determine the effect of killer yeasts on fungal pathogens like *A. niger*, *C. albicans* and *Fusarium* spp. None of the fungal pathogens were found to be inhibited by *S. cerevisiae* and *P. kluyveri*. Thus, the killer strains of *S. cerevisiae* and *P. kluyveri* did not inhibit *C. albicans*, which was used as a starter culture during wine fermentation. The present findings suggest the possible use of killer yeasts, *S. cerevisiae* in wine fermentation and *P. kluyveri* as a biopreservative in food industry.

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