

PRODUCTION AND EFFECT OF KILLER TOXIN BY *SACCHAROMYCES CEREVISIAE* ON SENSITIVE YEAST AND FUNGAL PATHOGENS.

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ABSTRACT

Killer toxins are the proteinaceous toxins produced by some group of yeast designated- Killer yeasts. These toxins have activity against microorganisms other than yeast and the activity is readily detectable only when a suitable sensitive strain is tested. The aim of this study was to extract the killer toxin from yeast cells, to purify by thin layer chromatography and to find out the action of killer toxin on different fungal pathogens.

Keywords: Killer toxins, Yeasts, Thin layer chromatography.

INTRODUCTION

Yeasts are unicellular, non-filamentous fungi; reproduce either by budding or fission. Yeasts may be useful or harmful in food. Yeast fermentation are involved in the manufacture of foods such as bread, beer, vinegar, cheese etc., and are undesirable in causing spoilage of fruit juices, syrups, honey, meats etc. The yeast species useful in food are *Saccharomyces*, *Zygosaccharomyces* etc. The yeast species harmful are *Rhodotorula*, *Pichia*, *Hansenula* etc., Yeasts are mainly grown for their enzymes¹.

Saccharomyces are primitive and morphologically very simple ascomycetes, in which the mycelium is totally absent or poorly developed. *Saccharomyces* is represented by about 40 species. Of which *S.cerevisiae* is the best known; because of its wide spread use in food industries for leavening of bread, production of alcohol, glycerol and invertase. Hence, they are known as “Bakers and Brewers Yeast”. There are two types of fermentative yeast in the genus *Saccharomyces*, top and bottom yeast. Top yeast is very active fermenters and grows rapidly at 20°C. Bottom yeast grows slowly and is best fermenter at low temperature (10-15°C). The contamination with undesirable wild yeast is a major problem in brewing since it results in slow fermentation and low product quality. In order to reduce contamination in the fermentation broth or industries, killer yeast may be used as starter culture for fermentation².

The production of yeast killer toxin is a well established phenomenon in yeast genera and species. Killer yeast secretes a pore forming, proteinaceous killer toxin lethal to susceptible yeast strains¹. The killer activity yeast readily detectable only when a suitable sensitive strain is tested. The killer strain is immune to the effect of its own toxin. The effect of killer toxin is dependent both on its own potency and susceptibility of treated cells under selected conditions. The susceptibility of sensitive yeast to killer toxin is known to depend on various factors such as selected killer toxin, the exposed yeast strain, its growth phase and the state of culture under given experimental condition³.

There exists many classes of killer yeast strains differing particularly in their spectrum of their activity against sensitive strains, in their cross reactivity, genetic determination of killer toxin, killer toxin immunity and the mechanisms of killer toxin action. Killer yeast secretes different killer toxins with activities specific for different yeast target cells⁴. Killer system in yeast was first and best described in *S.cerevisiae* by Bevan and Makower⁵.

In *Saccharomyces* three different killer toxin K1, K2 and K28 have been clearly identified, genetically encoded by double stranded M-dsRNA encapsulated in virus like particles localized in the cytoplasm. Killer system were studied in various genera of yeast and yeast like-organisms such as *Saccharomyces*, *Ustilago*, *Kluyveromyces*, *Pichia*, *Wiliopsis*, *Candida*, *Debaromyces*, *Hansenia spora*, *Sporidiolobus*, *Tilletiopsis*, *Zygosaccharomyces*⁶.

Recently, two new killer types which are encoded on the chromosomal DNA of *S.cerevisiae* have been described. These killer activities were weaker than those of known killer strains⁷. K1 and K2 have virtually identical toxin activities; both bind to β -1-6-D-Glucan components in the walls of sensitive cells and then disrupt their ion exclusion barrier of the target cell plasma membrane¹¹. The third killer K28 binds to α -1-3 linked mannose residues in the mono protein of walls of sensitive strain and the reversible inhibition of DNA synthesis. All three killer toxin coding M-ds RNA differs in their sizes (K1 1.8 Kb, K2 1.5Kb, K28 2Kb) and show little sequence homology⁸.

The lethal effect of K1 toxin involves the disruption of electrochemical ion gradient across the plasma membrane, which results from the increased permeability for H⁺ and an uncontrolled leakage of K⁺ ions and which is followed by a leakage of small molecules from the cell⁵. K1 toxin interacts with the susceptible cell in two step process involving interaction with receptors in the cell wall and in the plasma membrane. The lethal effect of K2 toxin is to the action of K1 toxin. Killer toxin K28 causes blockage of both DNA synthesis and budding cycle, thus causing a loss of cell viability⁹.

The killer toxin is protease sensitive, heat labile macromolecule, stable and act only at acidic pH value. These killer toxin have very important therapeutic value such as an inhibitory effect in *in-vivo* on *Pneumocystis carinii*¹⁰.

MATERIALS AND METHODS

Source of the samples: Samples were collected from various sources like bakery in Vellore and from Microbial type culture collection centre, Chandigarh for the isolation of killer toxin produced by killer strains of *Saccharomyces cerevisiae*.

Media: The media used for the isolation of *S.cerevisiae* was Yeast extract peptone dextrose agar (YEPD), Malt extract yeast extract agar (MEYE) and Sabourauds dextrose agar (SDA).

Processing of the sample: The killer toxins were isolated from the samples and inoculated in MEYE agar and incubated at 25°C for 48 hours. Normal *S.cerevisiae* strain (without the activity of killer toxin production) was also isolated from bakery products (Bakery Yeast). The inoculated in MEYE agar and incubated at 25°C for 48 hours. Cultures were maintained on MEYE agar, YEPD agar slopes. Fresh slant cultures (24hrs old) were used for obtaining yeast biomass in YEPD broth, incubated on shaker at 1500rpm at 25°C for 48 hours. The colony morphology such as colour, size, texture, margin etc was observed.

A smear of the selected colonies were prepared and stained with lacto phenol cotton blue and observed under microscope. Biochemical tests such as glucose, maltose, lactose, xylose, sucrose fermentation, nitrate reduction, urease production were done

Toxin detection: The culture was grown in Sabourauds broth for 24 hours at room temperature on a rotary shaker with 120 rpm. After centrifugation at 10000Xg for 10 minutes at 5°C, the supernatant was filtered through 0.45um pore size membrane filter (Millipore). The resulting filtrate was incubated for 4 hours at 4°C and the precipitate was collected by centrifugation at 10000X g for 20 minutes and re-suspended in equal volume of 0.1M citrate phosphate buffer pH 4.2. This toxin supernatant was stores at 4°C until use.

Killer activity assay: 10ml of YEPD agar containing 0.005% methylene blue buffered at pH4.2 was melted, cooled and 1ml of yeast suspension was added and poured on sterile petriplates in which 10ml of basal medium of YEPD was overlaid. Wells were punched in agar (about 6mm). 0.1ml of the toxin supernatant was added and incubated at 21°C for 48 hours. After incubation the plates were observed for the zone of inhibition.

Thin layer chromatography (TLC): The purity of the compound was assessed by TLC. The slurry was prepared in the applicator and applied evenly to a required thickness using a metal scale. The thin layer on the plates was dried for 30 minutes in air and then in a hot an oven at 100°C for another 30 min, this drying made the absorbent layer active. Capillary tubers were used for transferring the sample solution to the thin layer for qualitative and

quantitative work. Solvents used for sample solution were volatile and non-polar as possible. The development tank used in the paper chromatography was used in TLC for the study. Ascending chromatography is the most common technique in TLC. In TLC, the plate was placed in a development chamber at an angle of 450. The bottom of the chamber is covered up to nearly 1mm by the solvent. Three sides of the tank were lined with solvent impregnated paper while top was covered tightly with the lid. The TLC development chamber was perfectly saturated with solvent vapour by closing it by a lid. The plates were developed in a solvent mixture of n-butanol, acetic acid and water. Ascending technique was used for the development method in which the solvents were allowed to rise to the height of about 15-18cm on the normal 20cm tall plate. The plate was removed from the developing tank. Then the solvent front was marked and the plate was finally allowed to dry. Finally, the components were detected by spraying the Ninhydrin reagent.

RESULTS AND DISCUSSION

Isolation of Killer Yeasts: The samples were plated on yeast extract peptone dextrose medium and the growth showed visible, creamy white, mucoid colonies with raised surface.

Cultural characteristics of the yeast isolate: The isolate showed active growth on screening for its ability to grow in yeast extract peptone dextrose medium. The isolated colonies stained by Lacto phenol cotton blue showed budding yeast cells. The killer strains and the normal bakery yeast cells were identified by the standard carbohydrate fermentation tests. The yeast isolates were able to ferment sucrose, maltose, lactose and glucose.

Two killer yeasts and one bakery yeast were isolated and maintained by plating it on YEPD agar. All the three isolates were found to show similar morphological features but they differ from each other in their biochemical and killer characteristics.

The killer strain K1 fermented all the above four carbohydrates, while the killer strain K2 was able to ferment in glucose, maltose, sucrose but not in lactose as shown in table 1. The normal bakery yeast *S.cerevisiae* fermented in sucrose and glucose but not in maltose and lactose. The yeasts were also found to be urease and nitrate negative.

Table 1: Bio-chemical characterization

Carbohydrates	Strains		
	K1	K2	Bakery Yeast
Sucrose	+	+	+
Maltose	+	+	-
Lactose	+	-	-
glucose	+	+	+

+fermented; - not-fermented

Killer toxin assay: The isolated killer toxins showed a varied degree of results, when the sensitive fungal strains were tested. The killer character causes a distinctive halo of growth inhibition around the colonies of a killer strain

when they were grown against a background of sensitive strain. *Aspergillus niger*, *Candida albicans*, *Fusarium* spp were found to be resistance to the action of both K1 and K2 killer toxins shown in table 2.

Table 2: Killer Toxin- assay

Fungi	Killer Toxin	
	K1	K2
<i>Aspergillus flavus</i>	+	+
<i>Aspergillus fumigates</i>	+	-
<i>Rhizopus</i>	+	+
<i>Saccharomyces cerevisiae</i>	+	+
<i>Candida tropicalis</i>	-	+
<i>Penicillium</i>	+	+

+ indicates zone of inhibition of growth

- indicates no zone of inhibition of growth

Curing of the killer character: Killer yeast lack their killer character when they were incubated at 37°C on YEPD agar and on exposure to ultraviolet light for 10 minutes. When these yeast cells were tested for killer toxin assay against a background of sensitive strain, it was found that the yeast had lost their killer activity.

Determination of molecular weight: The molecular weight of the two different killer toxins was analyzed by SDS-PAGE and was found to be 11470Kda. The killer toxins from two different killer strains of *S.cerevisiae* migrated as a single protein band on SDS-PAGE with a molecular weight of 11470 Kda. Thin layer chromatography was also performed to separate and identify the proteins present in the killer toxin.

CONCLUSION

The isolated killer strains can be used in fermentation industries to reduce the contamination with undesirable yeast or fungal species in which in turn increases the quality of the product. They can also be used in wine and beer industries to reduce the contamination. The killer toxins in future might find application in the treatment of fungal infections.

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