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COMPARATIVE STUDY OF KILLER AND SENSITIVE YEAST STRAINS AND TO ISOLATE KILLER TOXINS

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ABSTRACT Various yeasts were isolated from samples such as rotten, fermented food & fruit juices. Among 19 yeast strains isolated only 4 had killer activity. Isolated yeast was examined for killer activity by using standard sensitive strain of *Candida stellata NCIM 3433* procured from NCIM Pune. The isolate which presented to have highest killer activity was identified as *Candida lipolytica* by VITEK method. The antibacterial activity was highest for *Escherichia coli* and *Pseudomonas aeruginosa* with reduced activity for *Staphylococcus aureus* and *Klebsiella pneumoniae*. The effect of pH, temperature and additives on toxin production was studied. The crude toxin was estimated by Lowry's method & SDS –PAGE study was also carried out. Killer toxin was further purified using ammonium sulfate precipitation & characterized for few properties.

KEYWORDS : killer activity, killer yeast, Candida lipolytica, Candida stellata NCIM 3433.

INTRODUCTION

Killer yeasts are known to secrete a killer protein (mycocin) that is lethal to specific yeasts, but to which they are themselves immune[1]. These yeasts produce toxic proteins or glycoproteins (so-called killer toxins) that can cause death in other sensitive (killer-sensitive) yeast strains. Ecological studies have indicated that killer activity is a mechanism of interference competition with the production of toxic compound from yeast excluding other yeasts from its habitat[2].Yeast strains belonging to species of the genera *Debaryomyces, Hanseniaspora, Kluyveromyces, Pichia, Schwanniomyces, Williopsis, Cryptococcus*, *Metschnikowia* and *Candida* have been found to be toxin-producers[3]. These yeasts have antimicrobial activity inhibiting growth of molds and bacteria. Killer toxin varies among species or strains in the sense of gene structure, molecular size, development and immunity. Each toxin has different recognizing and killing mechanisms on sensitive cells[4]. 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[5].

The control of phytopathogenic molds by yeasts has been studied with great potential usage, mainly inhibiting molds that cause fruit rotting in post harvesting period, because the yeast is a good competitor for nutrient and space[6]. *S. cerevisiae* and its metabolite could control *C.gloeosporioides*, besides, *S. cerevisiae* could colonize grape berry with high quantity[7]. In contrast to filamentous fungi, yeasts do not produce allergenic spores or mycotoxins, reinforcing their safe use for human consumption purposes. In the last decade, several scientific studies have demonstrated the efficacy of antagonistic yeasts as biocontrol agents against many phytopathogenic fungi including species of *Penicillium*[8]. Killer yeast has also been considered useful in biological control of undesirable yeasts in the preservation of food[9]. Furthermore, *W. anomalus* has been granted Qualified Presumption of Safety (QPS) status by European Food Safety Authority (EFSA), which may authorize its use as a novel microorganism in food preservation[10].

MATERIALS AND METHODS

2.1 Isolation and screening yeast for killer character

Samples of soil, fruits, vegetables, fermented foods etc. were enriched in Yeast Extract Powder Dextrose broth under shaking (180 rpm) at 30°C and spread plated on Chloramphenicol Yeast Glucose agar plates which were incubated at 30°C for 24–48 h and colonies appeared were examined microscopically, purified and maintained at 4°C. The yeast isolates were examined for killer character by using the standard sensitive strain of *Candida stellata NCIM 3433* procured from NCIM. *Candida stellata NCIM 3433* was cultivated in YEPD under shaking (180 rpm) at 30°C for 24 h and then the cells were appropriately diluted (10^6 cells/ml) and spread plated (100 µl) on killer assay medium (KAM) [Malt extract 3gms, dextrose 20gms, yeast extract 10gms, peptone 10gms, agar 20gms, methylene blue 0.03ml/L, pH 5]. The yeast isolates to be tested for killer activity were spotted on the lawn of *Candida stellata NCIM 3433 SEN*. The control was set up simultaneously in which standard killer *S. cerevisiae* MTCC 427 (*S. cerevisiae KIL*, procured from NCIM, Pune) was spotted on the lawn of *Candida stellata NCIM 3433 SEN* cells on KAM plate. The plates were incubated at 30°C for 24 h. The plates were observed for killer phenotype. Presence of inhibition zone around the spot of selected isoalte was suggestive of killer phenotype.

2.2 Pathogenic bacteria

The bacterial pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* used in the present investigation were procured.

2.3 Fermentation for killer toxin production

Killer isolate was grown overnight in YEPD under shaking (180 rpm) at 30°C (A600, 0.9–1.0) and then inoculated at 10% (v/v) into killer toxin production (KTP) medium, buffered at pH 5 by 50 mM citrate phosphate buffer, and fermentation was executed at 30°C under shaking (180 rpm) for 24–180 h. The fermented broth (100 ml for routine experiments and 1000 ml for purification experiment) was centrifuged at 10,000 × *g* for 15 min at 4°C. The pellet was discarded and the supernatant (crude toxin preparation) was collected and used for quantification of killer activity and for purification of killer toxin[11].

2.4 Quantification of killer activity

Quantification of killer activity of yeast was done by well plate assay. *S. cerevisiae KIL* or killer yeast isolate was grown in KTP medium (yeast 10gms, dextrose 20gms, peptone 10gms, agar 14.9gms, pH 5 ± 0.2, glycerol 50ml/L) for 24–180 h. Crude toxin was prepared and poured into wells of diameter 7 mm cut on KAM plates pre-spread with *Candida stellata SEN* culture. Plates were incubated at 30°C for 24 h, and zone of inhibition was measured. Killer activity was expressed in terms of arbitrary units (AU), and one AU was defined as the amount of killer toxin preparation which produces an inhibition zone of 10 mm [11].

2.5 Antibacterial activity of killer yeast toxin

The bacterial pathogens such as *E.coli*, *Klebsiella* sp., *S.aureus*, *P. aeruginosa* were grown in nutrient broth for 18 h under shaking (120 rpm) at 37°C (A600, 0.8–0.9) and then spread plated on Muller-Hinton (MH) agar. Wells (7 mm) were cut in MH plates and the killer toxin was poured in the wells and the plates were incubated at 30°C for 72 h, and then observed for zone of inhibition.

2.6 Protein estimation

Protein estimation in toxin preparation was executed by Lowry method using bovine serum albumin as standard.

2.7 Purification of killer toxin from killer yeast strain

Crude toxin preparation was subjected to ammonium sulfate precipitation (20–100%), and each of the precipitated fraction was dissolved in citrate phosphate buffer (50 mM, pH 5), and examined for killer activity and protein content. The fractions collected were examined for killer activity and protein content[11].

2.8 Study of Native and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE)

Native and SDS-PAGE was carried out. Purified sample was loaded (in duplicate) at 50 µl (0.25 mg protein) in native-PAGE gel. Native-PAGE was performed at 100 V for 4–5 h. Then, the gel was cut vertically into two halves so that each of the halves contained the killer toxin protein sample. One half (half I) of the native gel was stained with coomassie brilliant blue (CBB), destained and visualized for protein band[11].

Preparation of SDS PAGE was done by using NATIVE gel with SDS (100µL).The stain was made by using 0.1g of CBB 250 in methanol : acetic acid : water (25 :25:10).The destain is made up of methanol : acetic acid : water (25 :25:10).

2.9 Properties of killer toxin

The purified toxin was put in the wells cut in the KAM plate pre-spread with *Candida stellata NCIM 3433*, and incubation was given at different temperatures (20, 25, 30 and 37 °C), for 24–72 h and activity was determined by measuring the inhibition zone size. For determining thermostability, the purified killer toxin was preincubated at different temperatures (25–70°C) and then assayed for the residual killer activity. Activity of killer toxin without temperature treatment served as control.

The effect of pH on purified killer toxin was examined by conducting the killer activity assay on KAM plates at different pH (4–7). Furthermore, the killer toxin was examined for its pH stability. The toxin preparation was suspended in buffers of different pH (2–7) and pre-incubated for varying time periods (15 min–4 h), and then the residual activity was assayed by well plate assay. The killer activity determined with untreated killer toxin was used as control.

The effect of different ions and additives on activity of purified killer toxin was determined. Killer toxin was pre-incubated with either of the ions/additives such as Ca^{2+}, Fe^{2+}, Zn^{2+}, NH_4^+, Mg^{2+}, Mn^{2+}, SDS or EDTA, at final concentration of 6.7 mM for 30 min, and then assayed for the residual killer activity. Killer activity of untreated killer toxin served as control[11].

2.10 Analysis of the killer and sensitive character of the isolated strain

Approximately 10^5 cells/ml of the sensitive test yeast culture *Candida stellata NCIM 3433* must be uniformly suspended in 20 ml malt agar medium containing 0.003 percent methylene blue (w/v) and maintained at 45°C in water bath and immediately poured in sterile petri dishes. Four yeast cultures will be inoculated on to the surface of the surface of the agar medium as streaks of approx. 10-12 mm length and incubated at 25°C for 48 h. Yeast which will inhibit and kill *Candida* spp., would be shown by zones of inhibition encircled by blue margins of killed cells and hence will be designated as killer.

For determining the sensitive character, the cells of isolated yeast would be suspended in the buffered agar medium and the killer strain will be streaked on the solidified agar medium. The formation of an inhibition zone, surrounded by blue margins, representing killed cells would define sensitive yeast strains[12].

The killer phenomenon has potential applications in fermentation processes, biotyping of pathogenic yeasts, and development of novel antibiotic agents for the treatment of human mycoses[13]. 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[10]. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* obtained from laboratory collections, nature, vineyards, clinics and industry, it was found that about 10% carried killer viruses, whereas approximately 25% of the strains were resistant to viral toxins, confirming that killer viruses are a significant factor in the evolution of *Saccharomyces* yeast[14]. The current paper deals with the comparative study of killer and sensitive yeast strains & to isolate killer toxins, in a search for interesting strains for use in biotechnological applications.

RESULTS AND DISCUSSION

3.1 Isolation and identification of killer yeast

Among 19 yeast isolates selected on the basis of their morphological characteristic and Gram staining only 4 isolates exhibited killer activity.

3.2 Analysis of the killer character of the isolated strain by streak method:

As, *Saccharomyces cerevisiae* killer and *Saccharomyces cerevisiae* sensitive reference strains responded only to *Saccharomyces species*, it implies that the killer toxin is highly specific with respect to its ability to recognize the cell wall receptor of sensitive cells[11]. Hence, it can be interpreted that the four killer isolates which presented killer activity with *Candida stellata NCIM 3343(sensitive strain)* also belonged to the *Candida species.*

3.3 Quantification of killer activity:

Clear zone of inhibition were observed for four isolates. The isolate 1, 2, 3, 4 demonstrated zone of inhibition of 30 mm (3AU), 27 mm (2.7 AU), 20 mm (2AU), 10 mm (1AU) respectively. As the sensitive strain used was *Candida stellata NCIM 3343*, the killer isolates obtained would also belong to *Candida spp*. Killer activity varies depending on the origin of the isolate and the sensitive strain that was used.[10]

3.4 Detection of killer character:

The two best isolates were selected & tested for its killer activity by streak method on the malt agar medium which was pour plated by *Candida stellata* & zones of inhibition was observed.

3.5 Identification:

The isolate 1 showing the highest killer activity was identified by biochemicals[15]. It had the ability to ferment D-glucose, glycerol, erythritol and growth in presence of cycloheximide.

Further identification was done by using VITEK method, at Qualilife diagnostics, Mulund. *Candida lipolytica* is an asexual form of *Yarrow lipolytica*, which is white to creamy circular colonies with raised elevation and entire margins were selected and gram stained. Gram stain of the isolate revealed long elongated oval cells.

3.6 Fermentation of killer toxin production:

The killer isolate was fermented for a week at room temperature and then the broth was centrifuged and the supernatant was used as the crude toxin in Lowry's method for protein estimation. The protein was purified by using ammonium sulfate precipitation (26% saturation) at 4°C. The purified toxin was used for protein estimation and SDS PAGE analysis.

3.7 Antibacterial activity of killer yeast toxin against human pathogenic organisms

The isolate with best killer activity was further studied for its activity against human pathogenic organism by well diffusion method (Plate 1). The antibacterial activity by the isolate was highest for *Escherichia coli (32 mm)* and *Pseudomonas aeruginosa (18 mm)* with reduced activity for *Staphylococcus aureus* (23 mm) and *Klebsiella pneumoniae* (25 mm). The antibacterial activity by standard killer yeast was 10 mm for *Escherichia coli* and 25 mm for *Klebsiella pneumoniae*.

Buyuksirit *et.al* demonstrated that fruit yoghurt isolates showed the best antimicrobial activity with 35mm clear zone diameter against *Pseudomonas aeruginosa,* 8mm clear zone diameter against *Staphylococcus aureus* and 10mm clear zone diameter against *Escherichia coli*[4]. Antibacterial activity of purified *P. kudriavzevii* RY55 killer toxin inhibited all the bacterial pathogens tested like *E. coli*, *Enterococcus faecalis*, *Klebsiella* sp., *S. aureus*, *P. aeruginosa* and *P. alcaligenes*, equally well (inhibition zone size 11–12 mm for appropriately diluted toxin)[11]. Hence it can be observed that *Candida lipolytica* presented highest killer activity against *Staphylococcus aureus and Escherichia coli* than compared to *P. kudriavzevii*.

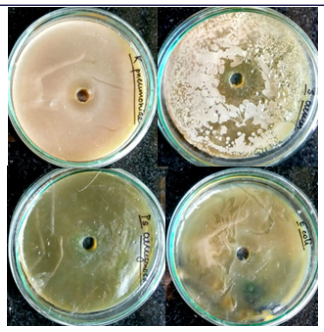

Plate 1: Antagonist activity by killer yeast on human pathogenic organism

3.8 Protein estimation by Lowry method

The concentration of crude and pure protein was found to be 235.44 and 150.9 µg/ml respectively.

3.9 Study of SDS-PAGE:

The SDS PAGE study of yeast killer protein with two reference proteins Bovine Serum Albumin of 67 kDa and lysozyme of 14 kDa was done which revealed that the protein present was of 14 kDa.

3.10 Effect of pH, temperature and additives

The killer toxin was unstable at pH 3 and pH 7 while it was stable at pH 5. At pH 5, as the incubation time increased, a reduction in killer activity was observed (Figure 1B). It has been studied that the killer toxin produced by *P. membranifaciens CYC 1086* was stable only within a narrow pH range (2.5–4.8) which is acidic pH values, with the optimal pH between 3.5 and 4.5 [16]. Wang *et al*. produced killer factor and the proteinaceous killer toxin was found to be unstable without a specific range of temperature and pH (above 30°C and pH 4.0) [17]. It can be observed that killer toxins produced by *Candida lypolytica* was stable at higher pH than *P. membranifaciens* and *W. mrakii*.

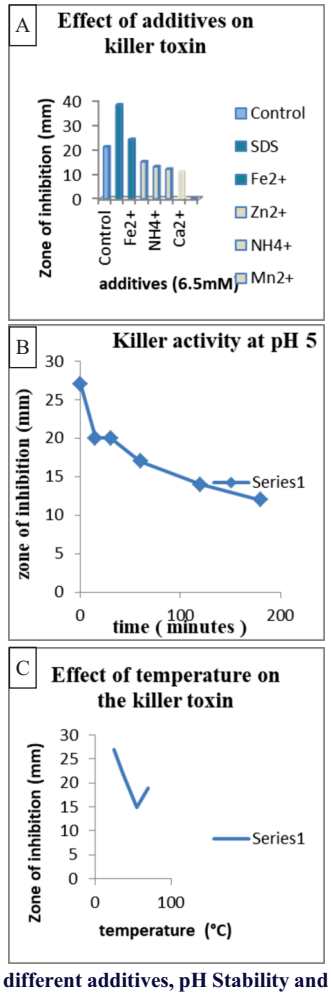

Fig. 1: Effect of different additives, pH Stability and temperature and on the killer toxin

Temperature

The optimum temperature for *Candida lipolytica* was found to be 25°C, while there was no complete inhibition of the activity at 70°C for 15 minutes. *Hansenula* (*Williopsis*) *mrakii* K9 killer toxin has been found to be highly thermostable and retain 100% activity for 10 min even at 100°C [11]. *Pichia membranifaciens* optimal killer activity was observed at temperatures up to 20 °C[16].It can be stated that the isolated toxin was thermostable (Fig.1C).

Effect of additives

Ionic species due to their charge interfere with electrostatic ionization status of the molecule and cause structural and Conformational alterations which may be inhibitory or stimulatory for bioactivity. SDS is strong anionic detergent and protein denaturant and interferes with protein structure and function, killer toxin of *P. anomala YF07b* was activated by Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, Na^{+}, and Co^{2+}, however, Fe^{2+}, Fe^{3+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+} and Ag^{+} acted as inhibitors as these decreased the activity[18]. Activity of purified *P. kudriavzevii* RY55 killer toxin was analyzed against the effect of different additives on the activity of purified killer toxin showed that Mg^{2+} and Ca^{2+} did not affect the activity, while, Mn^{2+}, Fe^{2+} and Zn^{2+} caused severe reduction in the activity. NH_4^{+} caused moderate inhibition but EDTA and SDS completely inhibited the killer activity[11]. The killer toxin from *Candida lypolytica* was activated by SDS and Fe^{2+} when the concentration of the additives was 6.5mM while Ca^{2+}, Mn^{2+}, Zn^{2+}, NH_4^{+} caused severe reduction in the activity but Mg^{2+} inhibited the killer activity (Fig. 1A). Addtitives affected *Candida lypolytica* killer activity differently.

CONCLUSION

Yeast constitutes a large group of microorganisms characterized by a strong ability to compete with other microorganisms for niche colonization. The competitive mechanisms have been extensively studied, and among them, killer toxins seem to play a primary role. Killer yeast species have a large biodiversity, in terms of molecular characteristic, genetic determinants, spectra of action and mechanisms of toxin action. Yeast killer toxins, also named mycocins, were initially defined as extracellular proteins, glycoproteins or glycolipids that disrupt the cell membrane function in susceptible yeast bearing receptors for the compound, whose activity is directed primarily against yeast closely related to the producer strain, which has a protective factor. The first mycocins were identified in association with *S. cerevisiae* in the brewing industry. It is widely recognized that the overall product quality in industries, such as winemaking, sausage and dairy production, baking, *etc.*, is mainly correlated with the development of spoilage microorganisms.

Killer yeasts are usually present in spoiled dairy products, citrus fruits, rotten vegetables, sour dough, spoiled juices. Killer activity was checked by using sensitive yeast strain *Candida stellata NCIM 3433* collected from National Collection of Industrial Microorganisms, Pune. From 18 isolates, four isolates were identified to have killer activity against the *Candida stellata NCIM 3433* by streak method and the quantification of the toxin was done by agar well diffusion method. Quantification was done by measuring the zone of inhibition. As the sensitive strain of yeast belonged to *Candida* species the isolates which showed killer activity also belonged to *Candida* species. The best isolate having highest activity was further identified as *Candida lypolytica* by using VITEK.

The isolate was enriched, centrifuged, purified and was tested for its antibacterial activity against human pathogenic organisms (*Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa*). The antibacterial activity was highest for *Escherichia coli* and the activity was further reduced for *Staphylococcus aureus* and *Pseudomonas aeruginosa* and minimum activity was for *Klebsiella pneumoniae* .These studies may provide help for the strong need for new antimicrobial drugs, killer toxin antibodies and derived peptides thereof, which can be easily produced and engineered, are emerging as an important class of therapeutic agents for the treatment of various human diseases.

Protein estimation of the toxin was done by Lowry method by using Bovine Serum albumin as the standard. The SDS PAGE study was also performed for the killer toxin. Optimization of the isolate was done at different pH and was preincubated for different time periods. The toxin was stable at pH 5 and the stability decreased with the increasing time of incubation.

The effect of temperature for the isolate was also studied and the

residual activity was checked. The killer toxin was stable at 25°C and was even stable at 70˚C.

SDS is strong anionic detergent and protein denaturant and interferes with protein structure and function. SDS and Fe^{2+} increased the activity of the killer toxin while reduction in activity was found with Ca^{2+}, Mn^{2}, Zn^{2+}, NH_4^+, Mn^{2+} and activity was inhibited by Mg^{2+}.

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