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PRODUCTION AND PURIFICATION OF KILLER TOXIN FROM PROBIOTIC YEASTS AND ITS EFFECT ON FOODBORNE PATHOGENS

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ABSTRACT

Probiotic yeast strains viz. *Yarrowia lipolytica* VIT-MN01, *Kluyveromyces lactis* VIT-MN02, *Lipomyces starkeyi* VIT-MN03, *Saccharomycopsis fibuligera* VIT-MN04 and *Brettanomyces custersianus* VIT-MN05 were investigated as producer of killer toxin. Among the five yeasts, three strains showed more killer toxin production. The maximum killer activity (12 aU/mg) against sensitive strain *Saccharomyces cerevisiae* was obtained at pH-3 with 0.5% NaCl at 25°C by using response surface methodology. The purified killer toxin K2 and K3 showed the highest killer activity against foodborne pathogens viz. *Bacillus cereus*, *E. coli, Pseudomonas* spp., *Klebsiella* spp. and *Candida tropicalis*. The purity of the killer toxin was confirmed by HPLC analysis. The molecular mass of killer toxin was 22 kDa (K2), 18 kDa (K3) and 14 kDa (K4). The results of the present study suggest that killer toxins produced by probiotic yeast strains can be used as an antimicrobial agent to control the microbial contamination in the food industry.

Keywords: Antimicrobial activity, foodborne pathogens, killer toxin, probiotic yeasts

INTRODUCTION

Yeasts are capable of producing antimicrobial compounds (killer toxins) that may inhibit the growth of harmful mold or bacteria (**Younis *et al.,* 2017**). Killer yeast and their toxins are having environmental, technological and medical importance. It is possible to screen killer yeast toxins as a potentially novel chemotherapy agent. Killer yeasts have been reported in probiotics, which stimulate immune responses, prevent various enteric diseases, diarrhoea and improve host health (**Kaur and Tiwari, 2016**). The killer toxin production by various genera of yeast species such as *Candida, Saccharomyces cerevisiae, Ustilago, Kluyveromyces, Debaromyces, Pichia, Wiliopsis* and, *Zygosaccharomyces* have been reported (**Parveen and Begum, 2010**). Killer toxins may have potential applications in various industrial sectors such as food, pharmaceutical, agricultural and fermentation due to its antagonism activity against pathogens (**Lopes and Sangorrin, 2010; Chi *et al.,* 2010**). The killer yeast can be used as starter cultures to prevent contamination of wild yeast in alcoholic fermentation industries viz. distilleries, breweries, wineries (**Bajaj *et al.,* 2012**). The effect of killer toxin is dependent on its potency and susceptibility of treated cells under selected conditions. Parameters like pH, temperature and NaCl have an effect on killer toxin production (**Golubev, 2013; Belgacem *et al.,* 2012**).
In the present study, five probiotic yeast strains viz. *Y. lipolytica* VIT-MN01, *K. lactis* VIT-MN02, *L. starkeyi* VIT-MN03, *S. fibuligera* VIT-MN04 and *B. custersianus* VIT-MN05 were investigated for their killer toxin production. The purified killer toxin was tested for antimicrobial activity against foodborne pathogens viz. *Bacillus cereus*, *E. coli, Pseudomonas* spp. and *Klebsiella* spp. and *Candida tropicalis.* The purity and molecular weight of killer toxin was assessed by HPLC and SDS-PAGE, respectively.

MATERIAL AND METHODS

Yeasts strains and media

Probiotic yeasts strains viz. *Y. lipolytica* VIT-MN01, *K. lactis* VIT-MN02, *L. starkeyi* VIT-MN03, *S. fibuligera* VIT-MN04 and *B. custersianus* VIT-MN05 were isolated from different sources and reported as potential probiotics (**Ragavan and Das, 2017**). These strains were grown in YEPD medium and incubated at 30°C for 24-48 h. The pellet was centrifuged at 5000 rpm for 15 min and washed twice with PBS buffer and stored at 4°C for further analysis.

Killer toxin production

Yeasts strains were inoculated on KTP medium and incubated for 72 h at 30°C for killer toxin production. The culture was centrifuged at 10000 rpm for 15 min and the cell-free supernatant was collected. Then supernatant filtered through 0.45 µm membrane filter and precipitated by centrifugation at 10000 rpm for 20 mins and resuspended in 0.1 M citrate buffer (pH-4.2). The crude toxin was stored at 4°C until use (**Parveen and Begum, 2010**).

Experimental design

The effect of temperature (A), pH (B) and NaCl (C) on killer toxin production (response) was studied using Box Behnken design (BBD) by response surface methodology (RSM) (**Belgacem *et al.,* 2012**). The 2D and 3D contour plots were prepared to know the effects of different factors viz. A, B, and C on killer toxin production. Three levels (-1, 0, +1) used in this study were given in Table 1.

Table 1 Three levels used in this study

No.	Parameters	Level -1	Level 0	Level +1
1	pH	2	3	4
2	Temperature (°C)	20	25	30
3	NaCl (%)	0	0.5	1

A quadratic model was performed to calculate the analysis of variance (ANOVA). The experimental designs and regression analysis was done by Design-Expert software (Version 11).

Killer activity assay

The killer assay medium (KAM medium) was prepared and pre spread with sensitive strain *Saccharomyces cerevisiae.* The wells of 7mm diameter were made in which 50µl of crude toxin was pipetted and incubated for 24 h at 30°C. The zone of inhibition was observed, and killer activity was expressed in terms of Arbitrary Units (AU) (**Bajaj and Sharma, 2010**).

Purification of a killer toxin

The crude toxin was precipitated by 30% ammonium sulphate and incubated for overnight at 4°C and protein was harvested by centrifugation at 10000 rpm for 10 min. The residue was dissolved in 50 mM sodium acetate buffer (pH 3.8) and dialyzed with the same for 4 h at 4°C. The solution was applied to pre-equilibrated diethylaminoethyl (DEAE) Sepharose Fast Flow anion exchange column. The bounded protein (killer toxin) was eluted in acetate buffer with 0.5 M NaCl (**Buzdar *et al.*, 2011**).

Antimicrobial activity of the killer toxin

The overnight pathogenic cultures of bacteria viz. *B. cereus*, *E. coli, Pseudomonas* spp., *Klebsiella* spp. and yeast, *C. tropicalis* were swabbed in MHA and YEPD agar plate respectively. Agar well diffusion assay was performed with 50µl of crude toxin and incubated for 24 h at 30°C and the zone of inhibition was observed (**Hernandez *et al.*, 2008; Amin *et al.*, 2013**).

HPLC analysis

The purity of killer toxin was analyzed by reverse-phase C-18 column (250 x 4.60 mm) and UV detector at 280 nm. Acetonitrile and water (80:20) mixture was used as a solvent system at a flow rate of 0.5 ml/min. The killer protein 100 µl was injected on HPLC and chromatogram was obtained over a period of 15 mins (**Santos *et al.*, 2009**).

SDS analysis

The molecular mass of killer toxins was determined by SDS-PAGE analysis. The killer protein (20 µl) was mixed with 5 µl sample loading dye (250 mM Tris. HCl, 10% SDS, 30% (v/v) Glycerol, 10 mM DTT, 0.05% (w/v) bromophenol blue, pH 6.8) and loaded on 12% SDS gel. The protein was stained with coomassie blue R-250 (Sigma) and compared with known marker protein (**Laemmli, 1970**).

RESULTS AND DISCUSSION

Five probiotic yeasts strains showed killer toxin production against sensitive *S. cerevisiae* (Fig. 1a). The KAM plates showed that K2 and K3 indicate highest killer activity than K4 (Fig. 1b). Among the five strains, three yeasts namely, *K. lactis* VIT-MN02 (K2), *L. starkeyi* VIT-MN03 (K3) and *S. fibuligera* VIT-MN04 (K4) exhibited more killer activity (Fig. 1).

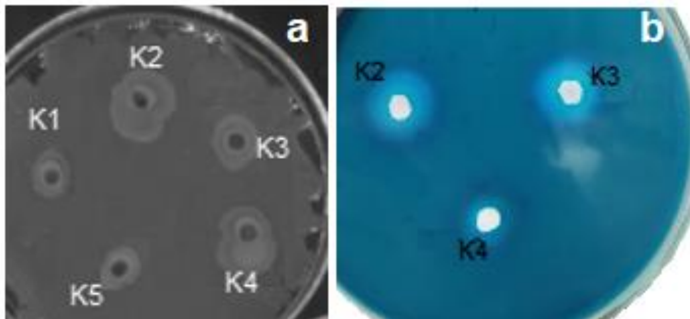

Figure 1 Screening for killer strains against sensitive strain *Saccharomyces cerevisiae*
(a)Screening for killer toxin production in KTP media and (b) Killer activity against sensitive strain *S. cerevisiae* on KAM media.
(K1-*Y. lipolytica* VIT-MN01 toxin, K2- *K. lactis* VIT-MN02 toxin, K3- *L. starkeyi* VIT-MN03 toxin, K4- *S. fibuligera* VIT-MN04 toxin, K5- *B. custersianus* VIT-MN05 toxin)

There are reports on killer toxin production, few yeasts strains of *Kluyveromyces sp.* and other species like *W. saturnus* WC91-2, *P. anomala* YF07b and *S. cerevisiae* showed similar killer activity **(Golubev, 2013; Buzdar *et al.*, 2011; Parveen and Begum, 2010).** The effect of temperature (A), pH (B) and NaCl concentration (C) on killer toxin production was investigated using RSM. The highest killer activity (12 µg/ml) was observed at 25°C with pH-3 and 0.5% NaCl. The lower pH and NaCl increased the killer activity. 2D and 3D plots for the effect of temperature, pH, and NaCl on killer activity were illustrated in Fig. 2

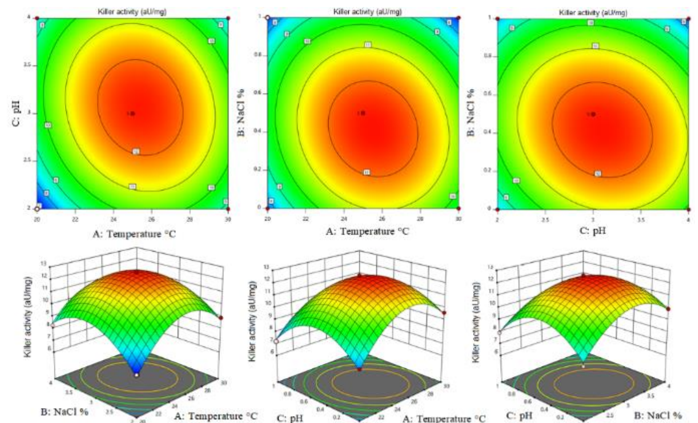

Figure 2 Effects of different parameters on killer toxin production by probiotic yeast
(A) Temperature, (B) pH, (C) NaCl.

Many yeasts strains have been reported for their highest killer activity when the cells were grown at 25°C. Killer toxin showed maximum activity at pH-3, which revealed that protein has a net positive charge because the isoelectric point of the killer toxin was pH-4.5 (**Pfeiffer and Radler, 1928**). These results suggest that killer toxin can efficiently attach on the surface of gram-negative bacteria and inhibit their growth. The actual and predicted values for killer toxin production were shown in Fig. 3. ANOVA for the quadratic model was given in Table 2.

Table 2 ANOVA for Quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	76.11	9	8.46	106.63	0.0001***
A-A	1.46	1	1.46	18.44	0.0036*
B-B	0.6874	1	0.6874	8.67	0.0216*
C-C	3.37	1	3.37	42.54	0.0003*
AB	1.08	1	1.08	13.64	0.0077*
AC	1.10	1	1.10	13.90	0.0074*
BC	1.14	1	1.14	14.37	0.0068*
A^2	25.14	1	25.14	317.03	0.0001***
B^2	15.89	1	15.89	200.31	0.0001***
C^2	19.24	1	19.24	242.55	0.0001***
Residual	0.5551	7	0.0793		
Lack of Fit	0.3661	3	0.1220	2.58	0.1909
Pure Error	0.1891	4	0.0473		
Cor Total	76.66	16			
Std. Dev.	0.2816				
Mean	9.39				
C.V. %	3.00				
R^2	0.9928				
Adjusted R^2	0.9834				
Predicted R^2	0.9197				
Adeq Precision	26.0509				

*** Highly Significant; * Significant.

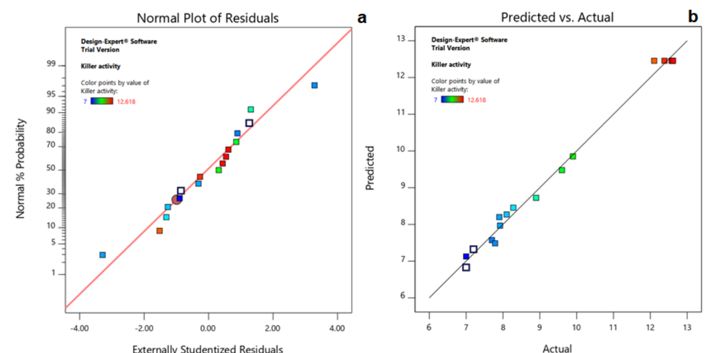

Figure 3 Actual and predicted values for killer toxin production

Antimicrobial activity against *B. cereus, E. Coli, Pseudomonas* spp., *Klebsiella* spp. and *C. tropicalis* were observed in K2 and K3. In case of K4, activity was noted against *E. Coli, Pseudomonas* spp. and *C. tropicalis.* The killer activity was

found to be less in K5 against *B. cereus* (Fig. 4). Protease treated killer toxins served as a control plate for antimicrobial activity against all pathogens either individually (not shown) or as a mixture of all pathogens (Fig. 4f).

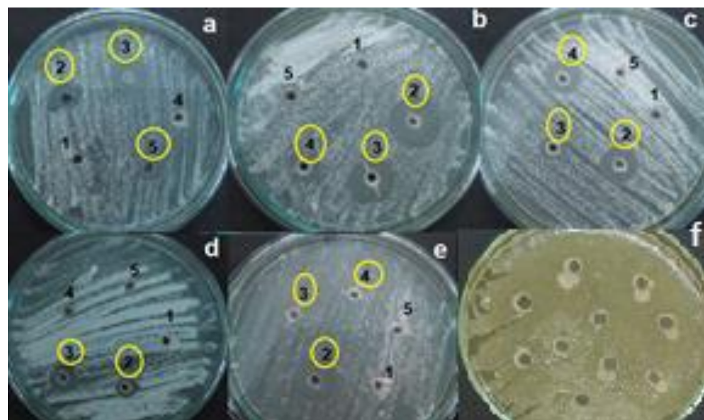

Figure 4. Antimicrobial activity of killer toxin against foodborne pathogens (a) *B. cereus,* (b) *E. coli,* (c) *Pseudomonas spp.,* (d) *Klebsiella spp.,* (e) *C. tropicalis,* (f) Protease treated killer toxin from five probiotics (Control plate)

Moreover, the purified killer protein showed protease activity, which indicated that the inhibition was occurred only by a killer toxin (data not shown). These results suggest that the killer activity of yeasts can be chosen to control bacterial contamination during fermentation (**Meneghin *et al.,* 2010**). Probiotic strain *Lactobacillus plantarum* 2S have been reported for broad antimicrobial activity spectrum against gram positive and gram negative bacteria (**François *et al.,* 2013**). The quantification of purified killer toxin from probiotic yeasts and their killer activities were given in Table 3.

Table 3 Purified killer protein from probiotic yeast strains

Probiotic yeasts	Total protein (mg/100 ml)	Specific activity (10^4 aU/mg)
Y. lipolytica VIT-MN01	0.88±0.21	0.3±0.08
K. lactis VIT-MN02	48±1.23	12±0.86
L. starkeyi VIT-MN03	46±2.32	10±0.54
S. fibuligera VIT-MN04	32±1.76	6±1.76
B. custersianus VIT-MN05	0.56±0.33	0.1±0.06

Average values in (±) SD

Few killer toxin producing strains (bacteria and yeasts) have been reported to show killer activity towards specific strains. The results of the present study suggest that the selected probiotic yeasts strain may have a wide range of antimicrobial activity.

HPLC and SDS-PAGE analysis confirmed the purity of killer toxin. The HPLC chromatogram of K2, K3 and K4 showed a single peak at 2.867, 4.031, and 3.698 min, respectively (Fig. 5).

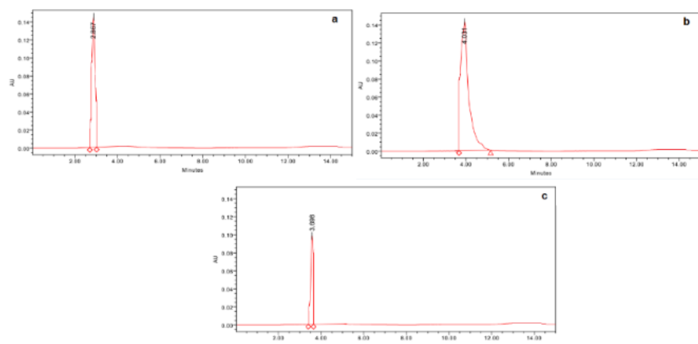

Figure 5 HPLC analysis for a purified killer toxin. (a)K2, (b) K3 and (c) K4

The SDS PAGE showed protein bands at 22kDa, 18kDa, and 14kDa for K2, K3, and K4, respectively (Fig. 6).

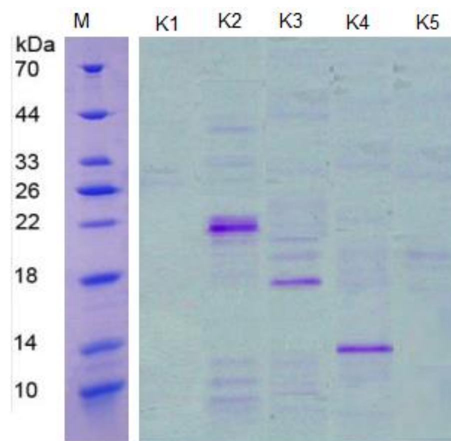

Figure 6 SDS-PAGE of purified killer toxin from probiotic yeasts.
M- Protein marker; K1-*Y. lipolytica* VIT-MN01 toxin, K2- *K. lactis* VIT-MN02 toxin, K3- *L. starkeyi* VIT-MN03 toxin, K4- *S. fibuligera* VIT-MN04 toxin, K5- *B. custersianus* VIT-MN05 toxin

The purified killer toxin was found to be a monomer and showed strong killer activity against foodborne pathogens. Similar results were reported by **Buzdar *et al.,* (2011).**

CONCLUSION

This study suggests that killer toxin produced from probiotic yeast can be a promising approach to maintain a good health of host by enhancing its antagonistic activity against some of the foodborne pathogens. The killer toxin was active and stable under acidic and NaCl conditions which may improve the quality of food during the fermentation process. The results could suggest that killer toxin producer probiotic yeasts *K. lactis* VIT-MN02 and *L. starkeyi* VIT-MN03 may have potential applications in food and pharmaceutical industries.

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