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Evaluation of Different Assays for the Activity of Yeast Killer Toxin

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ABSTRACT

Four different assays namely methylene blue (MB), thiazolyl blue tetrazolium bromide (MTT), Bromo cresol purple (BCP) and plate count agar were used for the evaluation of killer toxin activity of six isolates of yeast, *Kluyveromyces lactis, Candida silvatica, Candida tropicalis, Rhodotorula mucilaginosa, Rhodotorula lactosa and Lipomyces tetrasporu*s. MTT assay showed more rapid and precise results compared to methyllene blue and plate count agar assays for the assessment of the viability of *Saccharomyces cerevicae* sensitive strain. On the other hand, BCP assay showed incomparable results with the other employed techniques.

Key Words: Yeast; Killer toxin; Fluorescence assay; MTT assay

INTRODUCTION

Killer strains of yeasts secrete proteinaceous compounds, which are active against members of the same species or closely related species. The activities of these toxins are analogous to the activities of bacteriocins in bacterial strains (Lowes *et al*., 2000). Yeast killer toxins were termed mycocins and the killer strain mycogenic in order to emphasize the general nature of the antagonistic interaction (Golubev, 1998).

Mycocins were first observed in a laboratory brewing strain of *Saccharomyces cervisiae* (Bevan & Makower, 1963). Since that time mycocins have been shown to occur in a large number of yeast species of agronomic, environmental, industrial, and clinical interest, including *Candida, Cryptococcus, Debaryomyces, Pichia, Torulopsis*, and *Williopsis* species (Philliskirk & Young, 1975; Young & Yagiu, 1978; Young, 1987; Golubev, 1998).

Biocontrol studies have focused on the potential of mycocins produced by native or transformed yeast (Kimura *et al*., 1995). The killer characteristics have been transferred into starter yeasts in order to combat wild strains during the production of beer (Young, 1987), wine (Boone e*t al.*, 1990) bread (Bortol *et al.*, 1986) and also un-desirable yeast during food preservation (Palpacelli *et al.*, 1991). The killer phenomenon's importance also lies in its potential application as a therapeutic agent (Cailliez *et al*., 1994).

Killer toxin study requires a rapid and reliable assay of toxin activity. Therefore different assay systems were used to measure killer toxin activity, the agar diffusion well bio-assay (Woods & Bevan, 1968), Rhodamine B (Spacek & Vondrejs, 1986), Bromo cresol purple (Kurzweilova & Sigler, 1993), ATP bio-luminescence measurement (Alfenore & Delia, 2003), flowcytometry (Guyard *et al*., 2002) and 2, 3, 5, 6-tetramethyl 1, 4-benzoquinone (Tsukatani *et al.*, 2003). In the present study, the effect of different killer toxins produced by six different species of yeasts was evaluated by the use of four different assays i.e., methylene blue agar diffusion, bromocresol purple, thiazolyl blue tetrazolium MTT and plate count.

MATERIALS AND METHODS

Yeast strains. *Kluyveromyces lactis* ATCC 8585, a reference killer strain carries two linear DNA plasmids, PGK_{11} and PGK_{12} was obtained from MERCIN-CAIRO. *Saccharomyces cerevisiae* ATCC 46427 was used as sensitive indicator strain.

Isolation and Identification of the killer yeast isolates. The species of killer yeast were isolated from agricultural soil samples, which were obtained from fields of Benha City, Elkaliobia governorate, cultivated by orange (cv. Shamouti). They were identified according to conventional yeast identification methods based on the morphology, sporulation and fermentation characteristics, as well as the assimilation of a wide range of nitrogen and carbon sources. The identification was carried out according to Wickerham (1951), Lodder (1971), Ahearn (1974, 1978) and Barnett *et al*. (2000).

Methods Applied for Killer Toxins Assay

a) Thiazolyl blue tetrazolium bromide (MTT) assay. The method of Hodgson *et al.* (1994) was used where aliquots of 1.0 mL, containing approximately 1 x 10^8 cells mL^{-1} of the sensitive strain were pipetted into Eppendorf tubes to which 250 µL of crude toxin was added, or toxin-free YEPG medium (1% yeast extract, 1% peptone, 2% glucose). The suspensions were incubated at 22°C for 90 min and the cells (500 µL) were mixed with 50 µL of 5 mg mL^{-1} MTT and incubated at 30°C ±2 for 2 h**.** To each tube 500 µL of propan-2-ol containing 0.04 M HCl was added and the mixture was vigorously vortexed to remove MTT-formazon

from the cells, then centrifuged at 11600 x g for 2 min. The absorbance of the supernatants was measured at 570 nm against cell-free control.

b) Plate count agar (PCA) assay. Cells in exponential phase of the sensitive yeast strain were grown and incubated with the killer toxin as described in the MTT assay. Following the same period of toxin-incubation, cell suspensions were then serially diluted to a final cell concentration of 1 x 10^8 mL. Samples, each of 0.1 mL was plated in duplicate on YEPG agar medium and the number of colonies were counted following an incubation period of 48 h at 30°C ±2.

c) Bromo cresol purple (BCP) assay. The method of Kurzweilova and Sigler (1993) was used. Aliquots of 1.0 mL. containing approximately 1 x 10^8 cells of the sensitive yeast strain were suspended in 5 mM citric acid-Phosphate buffer adjusted to pH 4.6. The buffer was supplemented with glucose to a final concentration of 220 mM and the different killer toxins preparations were added so that the final volume reached was 1.0 mL. The mixture was then incubated with the killer toxin at 25°C±2 on a shaker for 120 min at 100 rpm. Subsequently, 50 µL 10 mM-BCP in citric acid-phosphate buffer was added to the samples and incubation continued for 60 min. The samples were then centrifuged, thoroughly washed with buffer, completed to the same volume and fluourescence was measured using luminescence spectrometer (Perkin Elmer LS 50 B) with the following parameters: emission 600-700 nm, scan speed 200, exit slit 10 and emission slit 2.5.

d) Metlylene blue (MB) agar diffusion assay. 1.0 mL of *S. cerevisiae* broth culture containing 1 x 10^8 cells was mixed in a Petri dish with 20 mL Wickerham agar medium buffered to pH 5.0 (0.1 M citric acid-Phosphate) and containing 0.003% methylene blue. Wells of 7 mm were made in the agar plates and each one was filled with 100 µL of the filtrates containing toxins, and incubated for 48 h at 25°C. Killer activity was recognized by a region of bluish-colored cells or by a clear zone of inhibition bounded by blue colored cells surrounding the wells.

Preparation of killer toxins. Yeast strains inocula were grown for 48 h in 25 mL 0.1 M citric acid-phosphate buffered YEPG broth medium in 250 mL conical flasks under shacked condition of 200 rpm, at 25°C for 48 h. After centrifugation (10000 rpm for 5 min at 5°C ±2) culture filtrates were sterilized through Millipore membrane (filters 0.22 µm pore diameter). Killing efficiency was determined using a concentrated toxin-containing filtrate.

RESULTS

On Wickerham's agar media, the isolated yeasts from collected soil samples, were purified and maintained on slants for further identification.

The isolated yeasts were identified as *Candida tropicalis, Candida silvatica, Rhodotorula lactosa, Rhodotorula muciluginosa* and *Lipomyces tetrasporus.*

The percentage of reduction in *S. cervisieae* cell viability as measured by MTT reduction was comparable to that obtained by colony counting on agar plate and also with methylene blue agar diffusion method (Table I, Fig. 1).

For MTT assay, the increased number of killed cells was correlates with the decrease in measured absorbance. Control sample with *K. lactis* toxin showed the lowest absorbance value of "0.315"; whereas, control sample without toxin showed extensive reduction of MTT dye, which corresponded to an absorbance value of "2.5".

Candida silvatica, Candida tropicalis produced the highest activity of killer toxin with low absorbance values of "0.665" and "0.74", respectively. This was followed by *Rhodotorula muciluginosa* and *Rhodotorula lactosa* with absorbance values of "1.17" and "1.33", respectively.

On applying the bromocresol purple (BCP) assay, the dye was entered the permeabilized cells of *Saccharomyces cerevisiae* and obviously fluoresced with maximum emission at wave length of 650 nm. Control without toxin has a fluorescence emission value of "41", which was higher than that of *C. tropicalis* "36" and *K. lactis* "27" (Fig. 1).

Lipomyces tetrasporus and *Rhodoturula. lactosa* showed slightly higher fluorescence emission values (higher killer toxin activity) than control without toxin. *C. silvatica* and *R. muciluginosa* showed double the absorbance value of the control sample (80 & 87).

DISCUSSION

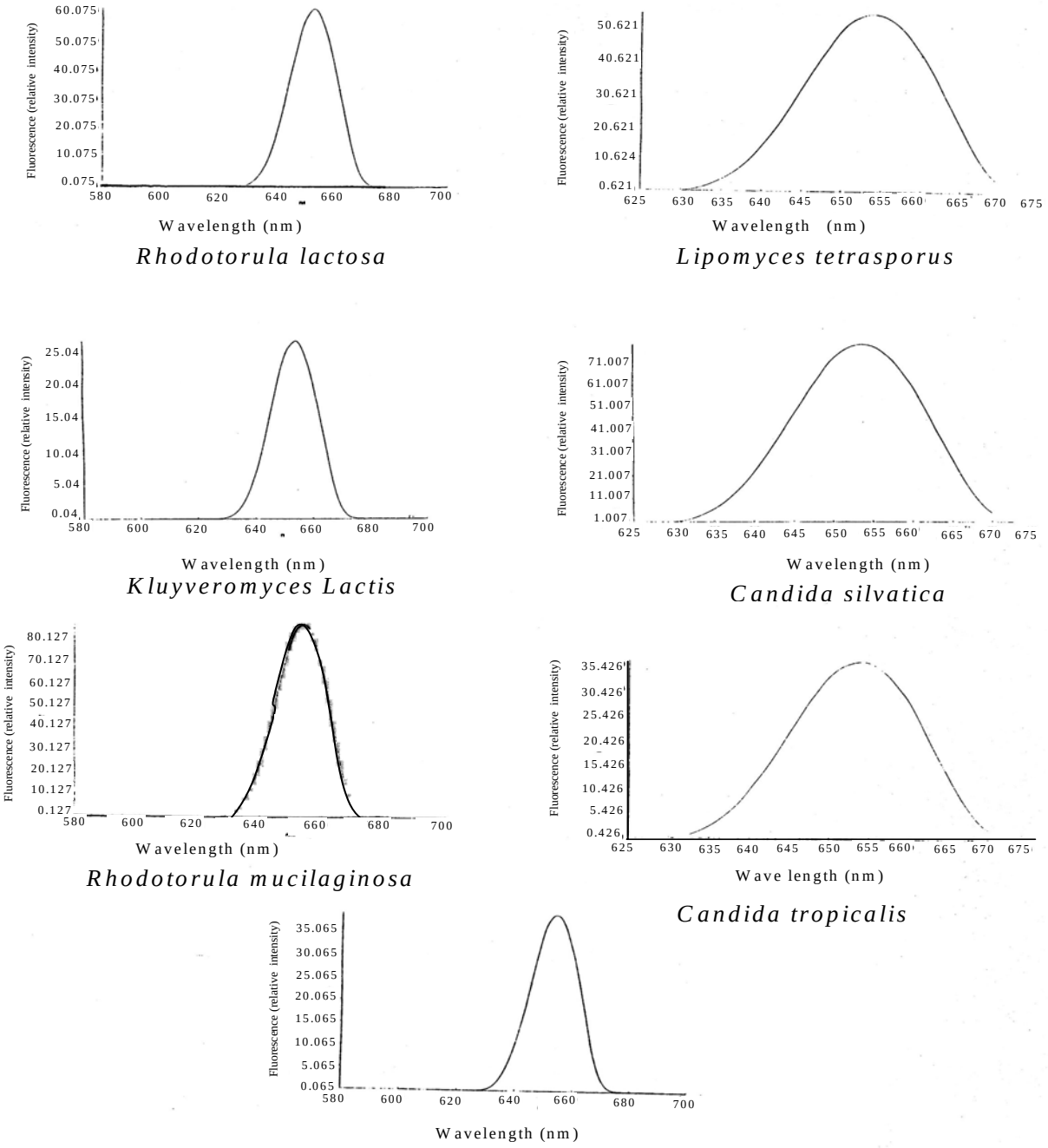
The present study reported that killing activities determined via the three assay (MTT-MB-plate count agar)

Table I. Comparison between four methods for the determination of killer toxin activity produced by different yeast strains against *Saccharomyces cerevisiae*

Killer toxin producing yeast species	Methods for measuring killer toxin activity			
	Thiazolyl blue tetrazolium bromide (MTT)[1]	Plate count agar. (PCA)[2]	Bromo cresol purple (BCP)[2]	Methylene blue agar diffusion (MB).
Candida tropicalis	0.74	0	36	0
Rhodotorula lactosa	1.3	0	61	+1
Candida silvatica	0.665	0	80	+3
Lipomyces tetrasporus	1.330	80	55	+2
Rhodotorula mucilaginosa	1.17	65	87	+2
Kluyvcromyces lactis ATCC 8585	0.315	0	27	+1
Control (without toxin)	2500	1x10^7	41	0

1-Values corresponds to absorbance at 570nm; 2-Values corresponds to fluorescence emission at 650nm

Fig. 1. Fluorescence emission intensity spectra of bromocresol purple in permeabilized stained cells of the killer toxin producing yeast strains



were higher than the corresponding values obtained from the BCP assay.

Killer toxin of the type K_1 is known to form ion channels in the yeast plasma membrane (Martinac *et al.*, 1990). After a lag period of about 50 min, the killed-exposed cells released into the medium molecules as large as glucose, ATP or leucine (Bussey & Sherman, 1973). Cells with damaged membrane allow the passage of molecules of weight (Mt 200-600) and hence stained by molecules of similar molecular weight e.g. rhodamine B (Mt. 479) and bromo cresol purple (Mt. 540) (Spacek & Vondrejs, 1986).

Plate count assay gave better results than those obtained from bromo cresol purple assay (BCP). This

method determines cells incapable of further division, while BCP test reports on the cells with significant damage to the plasma membrane. There may exist of a small fraction of killer-attacked sensitive cells, whose plasma membrane is only negligibly damaged. These small membrane lesions do not allow the entrance of BCP into the cytoplasm, but are sufficient to prevent further division of such cells (Kurzweilova & Sigler, 1993). Similar results were also obtained by Imai and Ohno (1995).

Considering BCP dye interaction with cytoplasmic proteins, it seems to be advisable to stain the cells only after the killing has been completed (Kurzweilova & Sigler, 1993). This means that BCP has the possibility for entrance into both living and dead cells and this can illustrate the fluorescence emitted from living and un-treated cells of *Saccharomyces cerevisiae*.

The MTT dye, a tetrazolium organic cationic salt (Mt. 414) possesses a low redox potential, which enables it to successfully out compete the cytochromes for electrons in the electron transport system (Burnstove, 1962). The reduction product formazon remains in the cell following loss of cell-wall semi permeability accompanying death. Methylene blue (Mt. 319) was also enzymatically reduced by living cells into leuco-methylene blue. Both MTT and MB assays showed comparable results, on using different killer yeast strains. This was normally predicted because of their similar mode of action. The three dyes (MTT, BCP, MB) pass via cell membrane after then it losses its permeability. Therefore, as the molecular weight of the dye decreases, it was easier to pass through the cell membrane.

Kluyveromyces lactis ATCC 8585 killer toxin producing strain was used as a reference strain it showed a significant result on using MTT assay. On the other hand, a lower killer activity was achieved on using MB and plate count assay, while BCP assay showed a very low activity. Similar results were obtained when testing the killer activity of different yeast isolates which were isolated from rhizosphe.

Although fluorescent staining is recommended as the best way to determine cell viability, a criticism of these dyes is raised in that the activity of intracellular enzyme, the reduction activity of the cell or the membrane permeability to a particular dye is not necessarily related directly to the cell viability as determined by plate count (Imai & Ohno, 1995).

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