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CHARACTERIZATION OF THE *SACCHAROMYCES CEREVISIAE* Y500-4L KILLER TOXIN

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ABSTRACT

The strain *Saccharomyces cerevisiae* Y500-4L, selected from the must of alcohol producing plants, liberates a toxin which is lethal to the commercial yeast produced by Fleischmann Royal Nabisco and other strains of yeast. This toxin was characterized, and the maximum production was obtained after 24 hours of incubation at 25ºC in YEPD medium. The maximum activity was achieved between pH 4.1 and 4.5 and between 22 and 25ºC and maximum stability in the pH range 3.8 to 4.5 at –10ºC. The killer toxin was inactivated by heating at 40ºC for 1 hour at pH 4.1. After concentration by ultrafiltration of culture supernatants and purification by gel filtration chromatography, the molecular weight of the purified toxin was estimated by SDS-PAGE to be about 18-20 kDa.

Key words: killer toxin, *Saccharomyces cerevisiae*, killer yeast

INTRODUCTION

Some yeast strains of *Saccharomyces* and other genera secrete into the medium protein compounds also known as killer toxins, which kill sensitive yeasts (1). The capacity to produce killer toxin can confer an advantage over more sensitive competitive strains growing in a fermentative process. Investigations revealed that the occurrence of the killer phenotype in yeast is widespread in alcohol fermentations for beverage production such as in breweries (10), saké (6), wine (3,12,14,22,23) and tequila (8) plants, and more recently in sugarcane producing plants (18).

The killer toxins described are active against a variety of different yeasts often outside the genus and species of the producing strains. However interactions between killer yeasts and pathogenic fungi have also been described, indicating that such killer yeast toxins may have potential as novel antimycotic biocontrol agents for plant fungal pathogens and for treatment of human and animal fungal infections (26).

Killer yeasts and their toxins have found several applications. For example, they have been used as model systems in fundamental research for studying the mechanisms of regulation of eukaryotic polypeptide processing, secretion and receptor binding (5). Killer plasmids of *S. cerevisiae*, which code for killer toxin, have been used as cloning vectors in recombinant DNA technology for the expression of foreign protein. The mouse a-amylase was effectively secreted via the killer toxin signal (24, 25).

Studies on the nature of the killer factor produced by *Saccharomyces* have shown that this protein has a highly specific action spectrum and is dependent on specific pH, temperature and aeration conditions (27). The activity of the toxin is only highly stable in a narrow pH range (4.2-4.7) (13, 16). The stability of the killer toxin decreases with elevation of temperature and is unstable at pH values above 5.0 (19).

There are various distinct killer toxins (29), but the structure and function of only a few have been studied at a molecular level, the K1 killer toxin of *S. cerevisiae* being the best characterized (2). Amino acid composition data for the purified yeast K1 *S. cerevisiae* T158c/S14a killer toxin showed that it is a heterodimeric protein containing a disulfide bond intersubunit with a similar molecular mass, both being about 11 kDa, resulting in a mature ab dimer with 20.658 kDa (30). The molecular weight of the killer toxins differs depending on the strains. The molecular weight of the killer toxin of *S. cerevisiae* strain 28 (KT28) was estimated as 16

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kDa (15). Killer toxins from other yeast genera have molecular weights ranging between 18 and 300 kDa. The *Kluyveromyces lactis* killer toxin was described as a protein dissociated into two subunits with molecular masses of 27 kDa and above 80 kDa (21). The strain *Candida* SW-55 has two toxins, each purified toxin giving a marked band with molecular mass of 36 kDa in SDS polyacrylamide gel electrophoresis (28). The molecular weight of the *Hanseniaspora uvarum* killer toxin was estimated as 18 kDa (17) and that of *Hansenula anomala* as 300 kDa (7).

The strain *S. cerevisiae* Y500-4L, previously selected from the must of alcohol producing plants, showed a high fermentative capacity (11) and considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast and also against the standard killer yeasts K_2 (*S. diastaticus* NCYC 713), K_4 (*Candida glabrata* NCYC 388) and K_{11} (*Torulopsis glabrata* ATCC 15126). However *S. cerevisiae* Y500-4L showed sensitivity to the killer toxin produced by the standard killer yeasts K_8 (*Hansenula anomala* NCYC 435) K_9 (*Hansenula mrakii* NCYC 500), K_{10} (*Kluyveromyces drosophilarum* NCYC 575) and K_{11} (*Torulopsis glabrata* ATCC 15126) (20). The purpose of this investigation was to characterize the *S. cerevisiae* Y500-4L killer toxin.

MATERIALS AND METHODS

Yeast strain. The strain *Saccharomyces cerevisiae* Y500-4L (11) was used as killer yeast. The commercial brand of yeast produced by Fleischmann Royal Nabisco was used as the sensitive strain.

Culture media. YEPD broth (1.0% yeast extract, 2.0% peptone, 2.0% glucose, 0.1M citrate-phosphate buffer pH 5.0) was used for killer toxin production. YEPD-MB agar (YEPD containing 0.01% methylene blue and 2.0% agar) was used for killer phenotype determination.

Growth conditions for killer toxin production. The killer strain *S. cerevisiae* Y500-4L was grown in flasks containing YEPD broth at 25°C. Dry mass (cellular growth), pH and killer toxin production were analyzed in triplicates of the medium. Killer activity was determined based on the well test method, where aliquots of 200ml of the supernatant were added to small cylinders (10 mm X 8 mm external diameter) and applied to the YEPD-MB agar, previously sprayed with the sensitive yeast suspension. The plates were incubated at 25°C during 48 h. The killer toxin activity was expressed in arbitrary units (U) (17). Ten U corresponded to that amount of killer toxin that caused an inhibition zone of 10 mm (18 mm measured diameter of inhibition zone minus 8 mm diameter of the cylinder).

Killer toxin concentrate. After 24 h cultivation on YEPD broth at 25°C, the yeast cells were removed by continuous centrifugation at 2800 x g, 4°C. The killer toxin obtained from the supernatant was tested at 8°C with 50% polypropylene glycol (1:1 v/v); ethanol (2:1 v/v); acetone (2:1 v/v); 80% $(NH_4)_2SO_4$ saturation and by ultrafiltration using Amicon YM10 membranes.

Effect of the pH and temperature on killer activity. Killer toxin concentrated 10 times and sterilized using 0.45mm membranes was dissolved in 0.1M citrate-phosphate buffer at different pH values: 3.5, 3.8, 4.1, 4.5, 4.9, 5.4. Aliquots of 200ml of the samples were added to small cylinders and applied to the YEPD-MB agar, previously sprayed with the sensitive yeast suspension, at the same pH as the sample (4). Five plates of each pH were incubated at 8, 16, 22, 25 or 30°C, for 48 h.

Killer toxin stability. The pH stability of the killer toxin was performed using samples which had been concentrated and filter sterilized, and adjusted to different pH values: 2.0, 3.0, 3.5, 3.8, 4.1, 4.5, 4.9, 5.4, 6.0, 7.0 or 8.0. The samples were incubated at -10, 8 and 25°C. After 24 h, the filtrates were adjusted to pH 4.1 and assayed for killer activity.

The temperature stability of the killing activity was determined at pH 4.1. The samples were incubated at 8, 25, 30 and 40°C and assayed from 30 minutes to 4h.

Purification. The killer toxin of *S. cerevisiae* Y500-4L was purified using a two step gel filtration on Sepharose 6B (Pharmacia), described by Zhu *et al.* (30). The sample was eluted from a column (2.5 X 35 cm) equilibrated with 50 mM sodium acetate buffer pH 4.7 at 8°C. Fractions (3 ml) were collected at 0.2 ml/min. Fractions containing the bulk of the 280 nm absorbing material and killer activity were pooled, and the molecular weight of the purified killer toxin was estimated.

Killer toxin molecular weight determination. The molecular weight of the killer protein was estimated by gel filtration chromatography on Sephadex G-100 and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel filtration column was equilibrated with 50 mM Tris-HCl buffer pH 7.5 containing 0.1M KCl. Blue Dextran 2000 and protein molecular weight markers SIGMA MW-GF 200 were used. The sample was eluted at 0.16 ml/min. The purified toxin was identified by SDS-PAGE performed as described by Laemmli (9), on a 12% (w/v) acrylamide monomer, 120 V, 20 mA, 2W for 1 h. Amounts of 57 mg of killer toxin sample and SIGMA SDS6 standard proteins were used. Protein was stained with Coomassie Blue G250.

RESULTS AND DISCUSSION

Growth conditions for killer toxin production. Fig. 1 shows the relation between growth, pH and killer toxin production. Killer toxin activity was maximum (inhibition halo=5 U) after 24 h growth of killer strain *S. cerevisiae* Y500-4L, which corresponds to the end of the rapid growth phase and the beginning of the stationary growth phase. A little decrease of pH medium from 5.0 to 4.7 during fermentation was observed.

Killer toxin concentrate. The best method to obtain killer toxin from the supernatant was by ultrafiltration at 8ºC, which conferred more stability on the toxin molecule than the other methods tested. Fig. 2 illustrates the killer activity of *S. cerevisiae* Y500-4L killer toxin against the sensitive yeast strain. The killer toxin obtained with polypropylene glycol, $(NH_4)_2SO_4$ and ethanol precipitation showed high killer activity. However, after freezing and gel filtration, the fractions lost the killer activity, showing a higher molecular instability than concentration by ultrafiltration. When precipitated with acetone the toxin showed no killer activity.

Effect of pH and temperature on killer activity. The killer toxin of *S. cerevisiae* showed greatest activity between pH 4.1-4.5 and 22-25ºC. Fig. 3 shows that the optimal activity of the *S. cerevisiae* killer toxin was obtained at pH 4.5 and 22ºC. At 25ºC, the best activity was at pH 4.1. At 30ºC, the activity was low in agar medium, being high at pH 3.5. At 16ºC, the killer toxin showed activity between pH 3.8-4.9. However at this temperature, the sensitive strain showed lower growth than at 22ºC and 25ºC. At 8ºC the sensitive strain did not grow enough and the test for killer activity could not be performed.

Killer toxin stability. Fig. 4 illustrates the pH effect on killer toxin stability. The toxin was more stable in a range from pH 3.8 to 4.5 and retained 80% of its activity in the range from pH 2.0 to 3.5, after incubation for 24 h at –10ºC. At 8ºC, the killer toxin retained 50-60% of its activity after incubation for 24 h, in a pH range from 2.0 to 5.4 and at 25ºC, 35% of its activity in a pH range from 2.0 to 4.5. In the pH range from 6.0 to 8.0, the killer toxin was inactivated at 8ºC and at –10ºC; and was inactivated at pH 5.4 after incubation for 24 h at 25ºC. Shimizu *et al.* (19) observed that killer toxins of eight killer strains, classified as belonging to the genus *Saccharomyces*, were inactivated at pH values above 5.0.

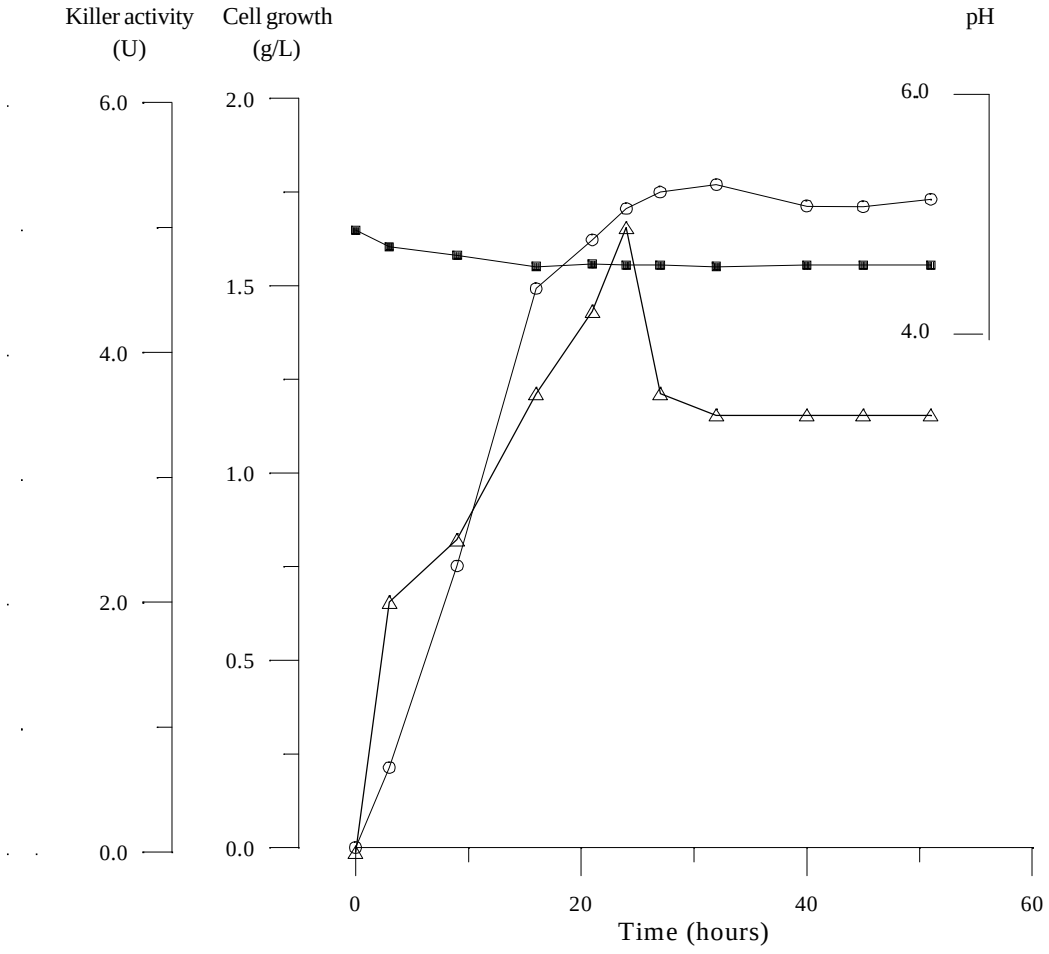

Figure 1. Growth of *Saccharomyces cerevisiae* Y500-4L and production of killer toxin. Cell growth (O), toxin activity (Δ) and the pH of the broth (■) were monitored.

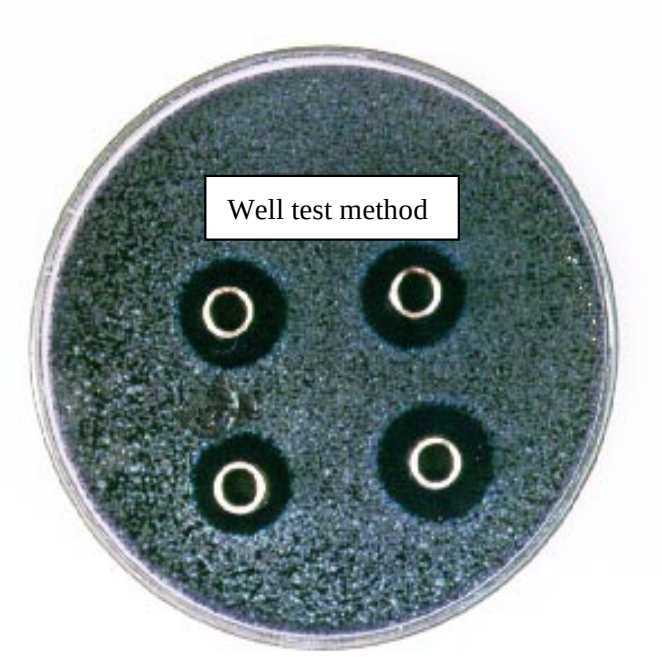

Figure 2. Inhibition zone caused by *S. cerevisiae* Y500-L killer toxin in YEPD-MB agar previously sprayed with a Fleischmann suspension.

Fig. 5 shows the effect of temperature on *S. cerevisiae* Y500-4L killer toxin stability. The toxin was stable after 4 h of incubation at 8°C and 25°C at pH 4.1. At 30°C, the toxin retained killer activity after 1 h of incubation; and lost 50% of this activity after 2 h and 30 min. incubation. The killer toxin was completely inactivated by heating at 40°C for 1 h at pH 4.1, showing instability like other *Saccharomyces cerevisiae* killer toxins, to temperatures above 38°C, after the first hour of incubation (19,27).

Purification and killer toxin molecular weight determination. Killer toxin of *S. cerevisiae* Y500-4L was purified 224 fold from the culture broth of yeast by ultrafiltration and using a two step gel filtration on a Sepharose 6B column (Table 1). The molecular weight of the purified killer toxin protein was estimated by gel filtration chromatography on Sephadex G-100 and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fig. 6 shows the relation between Ve/Vo and log MW of the standard proteins and the killer toxin sample eluted on Sephadex G-100. The molecular weight of the purified toxin was estimated at 43 kDa on gel filtration Sephadex G-100 and one single band was obtained with about 18 to 20 kDa by SDS-PAGE, suggesting the possibility of the killer toxin from *S. cerevisiae* Y500-4L as being a dimeric molecule.

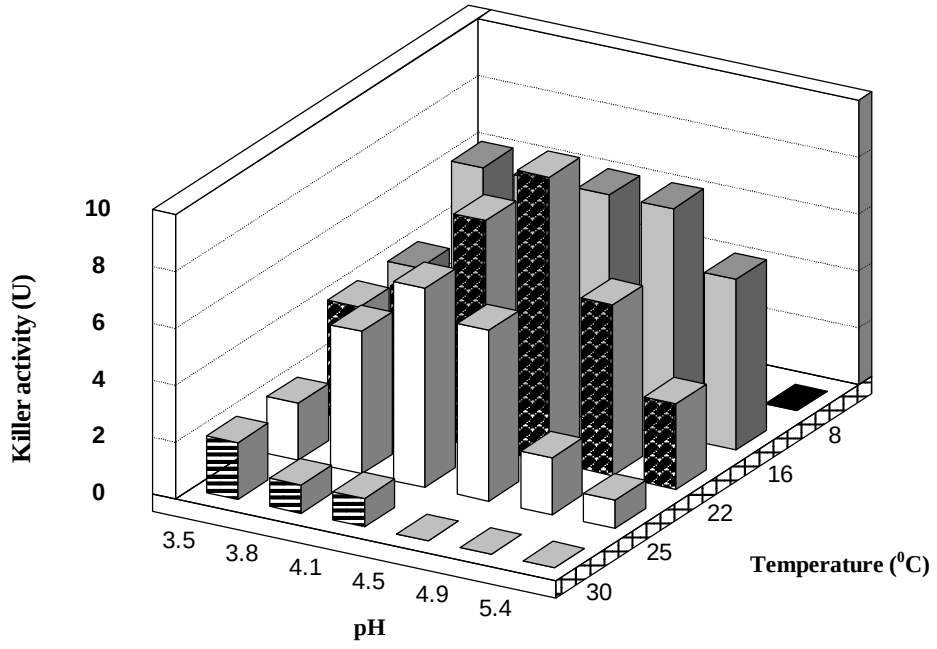

Figure 3. Effect of the pH and temperature on *Saccharomyces cerevisiae* Y500-4L killer toxin activity.

Table 1 Purification of the killer toxin of *Saccharomyces cerevisiae* Y500-4L by ultrafiltration and gel filtration chromatography on Sepharose 6B.

	Volume (ml)	Protein (mg/ml)	Activity of killer toxin (U/ml)	(U/mg)	Factor	Yield (%)
Cell free culture liquid	660	11.15	6	0.54	1	100
Concentrated culture liquid	36	16.81	16	0.95	1.7	15
Gel filtration column	3	0.23	28	121	224	2

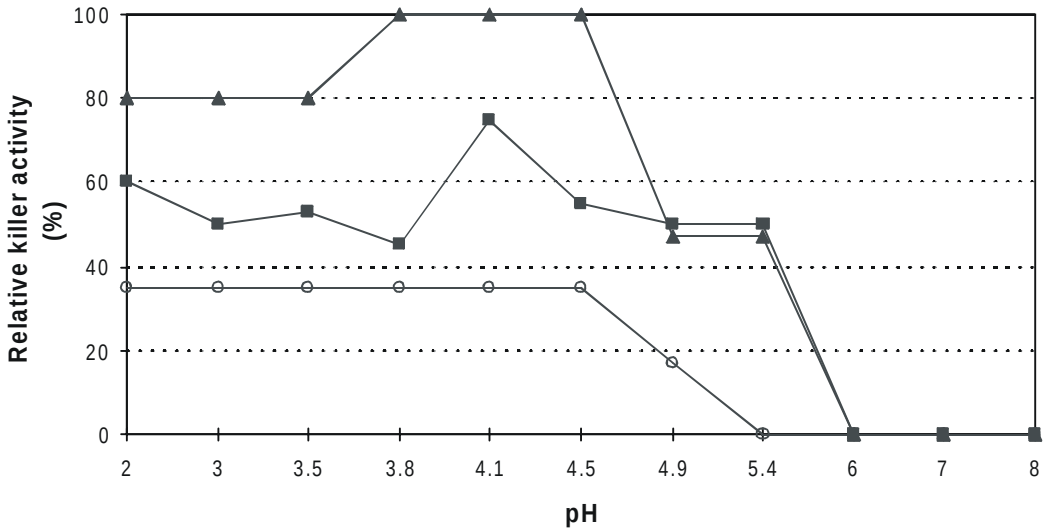

Figure 4. Stability of *Saccharomyces cerevisiae* Y500-4L killer toxin to pH. Toxin activity at 25ºC (O); 8ºC (■) and -10ºC (▲).

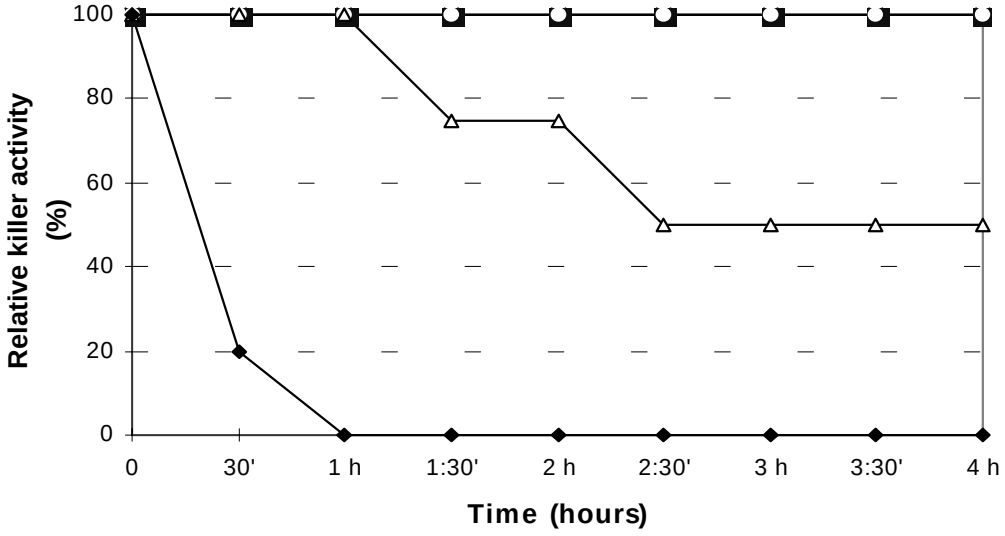

Figure 5. Stability of *Saccharomyces cerevisiae* Y500-4L killer toxin to temperature. Toxin activity at 8ºC (■); 25ºC (O); 30ºC (Δ) and 40ºC (◆).

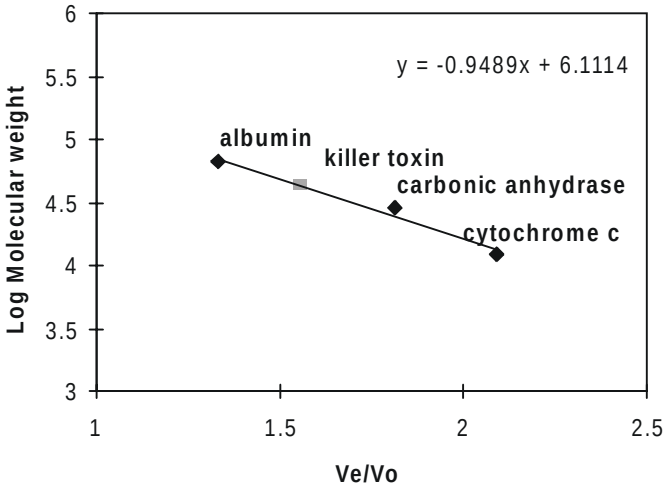

Figure 6. Calibration curve (u) for the determination of molecular weight of the killer toxin (n) of *Saccharomyces cerevisiae* Y500-4L on Sephadex G-100 column.

ACKNOWLEDGMENTS

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RESUMO

Caracterização da toxina "killer" da linhagem de *Saccharomyces cerevisiae* y500-4l

A linhagem de *Saccharomyces cerevisiae* Y500-4L, selecionada de mosto de fermentação de usina de álcool, produz toxina "killer", letal à levedura comercial Fleischmann Royal Nabisco e outras linhagens de leveduras. Esta proteína foi caracterizada, verificando-se que a produção máxima foi obtida após 24 horas de incubação a 25°C em meio YEPD. A toxina "killer" apresentou maior atividade na faixa de pH 4,1-4,5 e temperatura de 22-25°C; e maior estabilidade na faixa de pH 3,8-4,5 a –10°C, sendo totalmente inativada após 1 hora de incubação a 40°C em pH 4,1. Após concentração a partir do sobrenadante do meio de cultura através de ultrafiltração e purificação por cromatografia de filtração em gel, estimou-se, através de SDS-PAGE, que o peso molecular desta toxina é cerca de 18 a 20 kDa.

Palavras-chave: Toxina "killer", *Saccharomyces cerevisiae*, levedura "killer"

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