534 C

The production and secretion of killer (K) toxins is a widespread phenomenon in yeasts, although not uniformly distributed within certain genera or species. Several halotolerant yeast strains, displaying a broader *spectrum* of K activity in the presence of salt, were identified in a previous survey. The possibility that these zymocins could be more stable than the ones described so far, lead to the selection of the extremely halotolerant yeast *C. nodaensis* for further work. Preliminary experiments performed to characterize *C. nodaensis* K factor showed that, besides keeping its biological activity at high NaCl concentrations, is stable after incubation in a relatively broad range of temperature and pH values, whether or not in the presence of salt. These results renewed the interest on this zymocin for the development of yeast-based biocontrol strategies in several biotechnological applications. The isolation of this K toxin was already achieved by a experimental protocol involving ethanol precipitation of *C. nodaensis* culture supernatants. Several different approaches are now in progress in order to achieve its purification and further molecular and biochemical characterisation. The identification of the cell wall receptor for this K toxin is also under study, to perform toxin purification by affinity chromatography. The study of this zymocin mode or action is under way and will certainly contribute to evaluate its biotechnological potentialities for several applications.

PARTIAL PURIFICATION AND CHARACTERISATION OF *CANDIDA NODAENSIS* KILLER TOXIN

da Silva S.(1), Aguiar, C. (1), Veríssimo, P. (2), Pires, E. (2) and Lucas, C.

(1) CB-Departamento de Biologia, Universidade do Minho, Braga,Portugal.

(2) CNC- Laboratório de Biologia Molecular e Biotecnologia, Univers. Coimbra, Coimbra, Portugal.

The production of antimycotically active toxins, so-called killer (K) toxins or zymocins, is a widespread phenomenon in various yeast genera, although the most intensively studied killer systems are still those of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* (for reviews see 1-2). During the last two decades, killer toxins and killer yeasts were found to have several potential applications; for instance in the food and fermentation industries, in the bio-typing of medically important microorganisms, in the development of novel antimycotic agents for the treatment of fungal infections and in the field of recombinant DNA technology. This increasingly interest in killer toxins for all the mentioned applications requires undoubtedly a detailed knowledge and understanding of the biology of killer yeasts, which will provide important insights relevant for its use as antimicrobial agents.

In a previous survey, we studied several halotolerant yeasts which killer activity was expressed, even stimulated, under heavy salt-stress conditions (3). From this research, the halotolerant yeast *Candida nodaensis* was identified as one of the strongest salt-stimulated K phenotypes, being selected for further studies. Results obtained so far, in what concerns *C. nodaensis* zymocin activity/stability under temperature, pH and ionic strength, showed that this is in fact a very stable zymocin. Presently, several strategies are under way to achieve the isolation and purification of this zymocin, in order to enable further evaluation of its biotechnological potentialities, namely in the high-salt food products preservation from spoilage by other yeasts.

Partial purification
Molecular Exclusion Chromatography

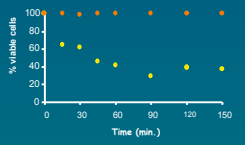


Fig.2. Assay for killer activity quantification (Influence of partially purified K toxin on *P. guilliermondii* viability (fractions with K activity; fractions without K activity).

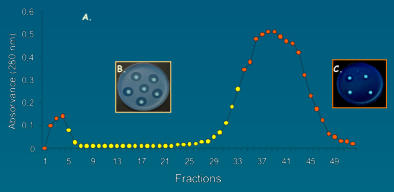


Fig.1. A- Elution profile of K toxin through a Superdex 200 column (Pharmacia) equilibrated and eluted with CPB 200 Mm, pH 4.0 (fractions with K activity; fractions without K activity. B and C -assay for killer activity detection.

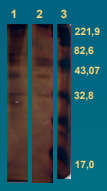
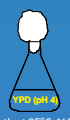


Fig.3. Silver-stained SDS-PAGE (12% gel).
Lane 1 fractions with K activity
Lane 2 fractions without K activity
Lane 3 wt marker polypeptides.

Isolation of *C. nodaensis* killer factor



Growth at 25°C, 160 rpm
Early stationary phase

↓

Centrifugation

2', 7000 rpm, 4°C

↓

Filtration of culture supernatant

0.45 µm

↓

Precipitation of culture filtrate

Adition of 3/4 vol. ethanol

↓

Centrifugation

10', 7000 rpm, 0°C

↓

Liophilization

↓

Ressuspension in
10 mM CPB,200mM NaCl pH 4.0
Ethanol precipitation

Strategies towards further purification of *C. nodaensis* K toxin (A, B, C)

A - Ion Exchange Chromatography

Table 1. Killer activity assay after Ion Exchanger Chromatography.

Ion Exchange Chromatography	Sp Sepharose Fast Flow	Q Sepharose Fast Flow
Type of Exchanger	Strong cation	Strong anion
Killer activity		

C- Cell wall receptor for Killer toxin

Table 2. Fractionation of *P. guillermondii* cell walls

Fraction	Wt (mg)	%
P-1[4]	640	12,8
P-2[4]	84	1,6
S-1[4]	450	9
S-2[4]	1150	28
Chitin[5]	800	29,8

4) Glucans were extracted and partially purified according to *Manners et al.*
5) Chitin were extracted and partially purified as described by Fleet.

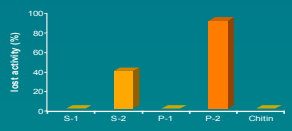


Fig.5. Toxin K activity after adsorption to *P. guillermondii* cell walls components.

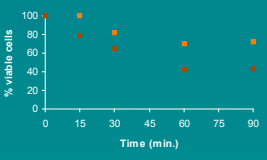


Fig.6. Influence of P-2 fraction on toxin mediated cell death (cells with K toxin ; cells with K toxin after previous incubation with P-2 fraction).

B- Affinity Chromatography with Concanavalin A

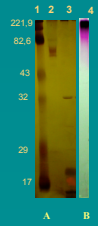


Fig. 4. Silver stained (A) and Schiff-stained (B) SDS-PAGE (12% gel) after affinity chromatography with concanavalin A.
Lane 1, wt marker polypeptides;
Lane 2, fraction with K activity;
Lane 3, fraction which displayed k activity after affinity chromatography;
Lane 4, fraction with k activity after Schiff stained.

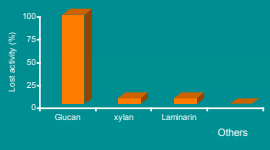


Fig. 7. Toxin K activity after adsorption to different commercial carbohydrates. (others: Amylopectin, Amylose, Chitin, Polygalacturonic acid and Pullan).

Table 3. Different commercial carbohydrates.

Carbohydrate	*Main glucosidic linkage*
Amylopectin	(1-4)-α
Amylose	(1-4)-α
Chitin	(1-4)-β
Laminarin	(1-3)-β
Polygalacturonic acid	(1-4)-α
Pullan	(1-6)- α / (1-4)-α
Xylan	(1-4)-β
Glucan by yeast	

Ionic (A) and/or affinity (B,C) chromatography will be further explored to achieve the purification of *C. nodaensis* K toxin.

The main purpose is to identify, in SDS-PAGE, protein band(s) associated with K activity phenotype.

(1) Magliani, W. *et al.* (1997) Yeast killer systems. *Clin. Microbiol. Rev.*, 10 (3): 369-400.
(2) Schmitt, M. And Breinig, F. (2002) The viral killer system in yeast: from molecular biology to application. FEMS Microbiol. Rev. 26: 257-276.
(3) Aguiar, C. and Lucas, C. (2000) Killer/sensitivity phenotypes and halotolerance, *Food Technol. and Biotechnol.*, 38: 39-46.
(4) Manners, D.J. *et al.* (1973) Biochem. J.135, 19-30.
(5) Fleet, G. H.. (1991). Cell walls, p. 200-277. In A. H. Rose and J. S. Harrison (ed.). The ,yeast vol. 4. Yeast organelles.